CAPILLARY ELECTROPHORESIS METHODS FOR PHARMACEUTICAL ANALYSIS

Edited by
Satinder Ahuja
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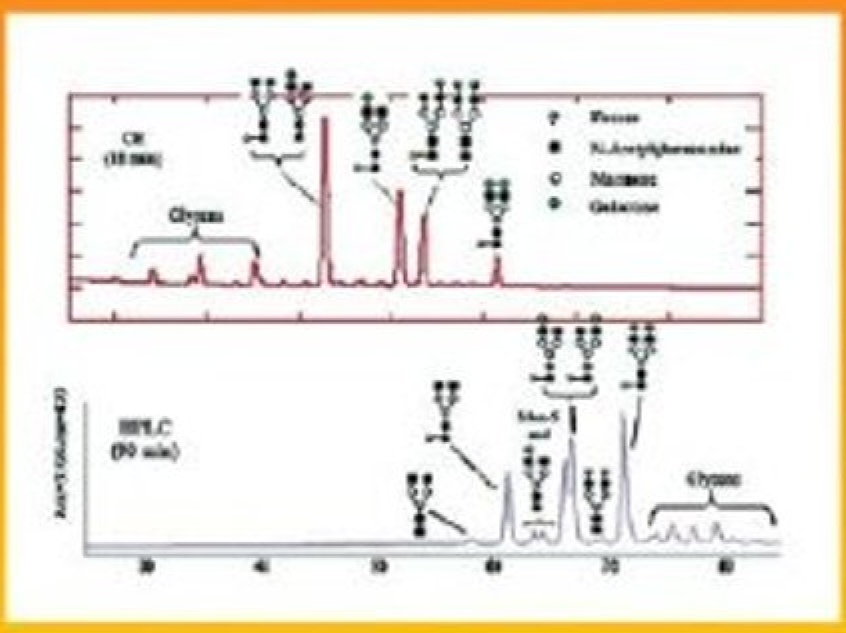

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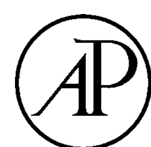

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PREFACE

Capillary electrophoresis (CE) using fused-silica capillaries with internal diameter in the micrometer range was introduced in 1981 and was received with great enthusiasm in the separations world because it promised high separation efficiency, a large degree of flexibility during method development, and a low cost of operation. After a better understanding of the fundamentals was developed, the focus shifted to some real applications. Many papers describing highly efficient separation methods have been published in the last two decades. CE offers several advantages over high-performance liquid chromatography (HPLC), a technique commonly used in the pharmaceutical industry. These include simplicity, smaller sample size, rapid analysis, automation, ruggedness, different mechanisms for selectivity, and low cost. Furthermore, CE offers higher efficiency than HPLC and thus greater resolution power for separating various components. These advantages make CE a very attractive tool in the research and development of pharmaceuticals, quality control, and stability studies.

This book has been planned to provide busy pharmaceutical scientists a complete yet concise reference guide for utilizing the versatility of CE in new drug development and quality control. The text can be broadly classified in five major sections:

- Overview, theory, and instrumentation (Chapters 1−3)
- CE methods and practices (Chapters 4−6)
- Regulatory aspects (Chapters 7−11)
- Applications (Chapters 12−16)
- New developments (Chapters 17 and 18)

Each of the chapters, written by selected experts in their respective fields, is designed to provide the reader with an in-depth understanding of CE theory, hardware, methodologies, regulations, and applications. The text includes state-of-the-art information on CE analysis of

pharmaceuticals and provides the reader a clear and concise understanding of the following important topics:

- How to improve performance of CE methods
- How to develop and validate robust methods in CE
- How to increase precision in CE
- How to make CE method transfers more successful
- How to interpret ICH guidelines relating to CE
- How to perform IQ, OQ, PQ, and CE calibrations

Major applications covered include assays, impurity testing, high-throughput screening, chiral separation, pK_a determination, ion analysis, impurity profiling, orthogonal method, and characterization of proteins, peptides, and nucleotides. Furthermore, the latest developments in capillary electrochromatography (CEC), CE–MS, and coupling chip-based devices to MS are discussed at length.

We would like to thank all of the authors for their valuable efforts in making this book serve as a definitive reference source on CE for laboratory analysts, researchers, managers/executives in industry, academia, and government who are engaged in various phases of analytical research and development or in quality control.

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1 OVERVIEW OF CAPILLARY ELECTROPHORESIS IN PHARMACEUTICAL ANALYSIS

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I. INTRODUCTION

Electrophoresis is a separation technique that is based on the differential migration of charged compounds in a semi-conductive medium under the influence of an electric field. Its origin can be traced back to the 1880s; however, it got major recognition in 1937, when Arne

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Tiselius[1] reported the separation of different serum proteins by a method called moving boundary electrophoresis. In 1948, Tiselius was awarded the Nobel Prize in Chemistry for his contributions. The efficiency of the moving boundary method was enhanced further with the development of techniques such as the paper electrophoresis and gel electrophoresis. Paper electrophoresis is now obsolete; however, gel electrophoresis is still used in biochemistry for the determination of proteins and nucleic acids. Unfortunately, gel electrophoresis is limited by the effect of "joule heating," which gives rise to heat production in the system. This heat cannot be dissipated efficiently and leads to temperature gradients that result in convection and viscosity gradients and finally to an increase of dispersion and, as a result, band broadening. Thus, joule heating induces a decrease in the separation efficiency. Better heat dissipation is possible if narrow bore tubes are used. Hjerten[2] was the first to apply this technology, in 1967, using glass tubes with an internal diameter (I.D.) around 3 mm. Jorgensen and Lukacs[3] are credited for producing the first operational system, in 1981, that used 75 μm I.D. fused silica capillaries and voltages up to 30 kV that could produce separations of proteins and dansylated amino acids, with plate heights of less than 1 μm. Since then, many papers of highly efficient separations have been published.

Capillary electrophoresis (CE) is a powerful separation technique that is widely used in research and development (R&D), quality control (QC), and stability studies of pharmaceuticals. CE offers several advantages over high-performance liquid chromatography (HPLC), a technique commonly used in pharmaceutical analysis. These include simplicity, rapid analysis, automation, ruggedness, different mechanisms for selectivity, and low cost. Furthermore, it offers higher efficiency and thus greater resolution power over HPLC even if only a small sample size is available. These advantages are likely to lead to even greater use of CE in R&D, QC, and stability studies of pharmaceuticals. CE has been found particularly useful for separations of peptides, proteins, carbohydrates, inorganic ions, chiral compounds, and in numerous other pharmaceutical applications.[4–6] The separations of chiral compounds are discussed at length in Chapters 2, 4–7, 9–11, and 16–18.

This book is planned to provide the busy pharmaceutical scientist a complete yet concise reference guide for utilizing the versatility of CE in new drug development and QC.

Each of the chapters has been written by a selected expert in the field to provide the reader with an in-depth understanding of CE theory, instrumentation, methodologies, regulations, applications, and recent developments.

II. VARIOUS MODES OF CE

Various modes of CE can be classified into three main groups:

1. Moving boundary CE
2. Steady state CE
   - Isotachophoresis (ITP)
   - Isoelectric focusing (IEF)
3. Zone CE
   - Capillary gel electrophoresis (CGE)
   - Free solution CE
     - Capillary zone electrophoresis (CZE)
     - Micellar electric capillary chromatography (MECC)
     - Chiral CE (CCE)
   - Capillary electrochromatography (CEC).

These modes as well as theoretical considerations are discussed at length in Chapter 2.

III. INSTRUMENTATION

CE instrumentation is quite simple (see Chapter 3). A core instrument utilizes a high-voltage power supply (capable of voltages in excess of 30,000 V), capillaries (approximately 25–100 μm I.D.), buffers to complete the circuit (e.g., citrate, phosphate, or acetate), and a detector (e.g., UV–visible). CE provides simplicity of method development, reliability, speed, and versatility. It is a valuable technique because it can separate compounds that have traditionally been difficult to handle by HPLC. Furthermore, it can be automated for quantitative analysis. CE can play an important role in process analytical technology (PAT). For example, an on-line CE system can completely automate the sampling, sample preparation, and analysis of proteins or other species that can be separated by CE.

IV. METHOD DEVELOPMENT FOR PHARMACEUTICAL ANALYSIS

CE methods are developed and utilized in pharmaceutical QC for early to late phases of drug development. Chapter 4 covers the approaches for late-phase development for small molecules that can be used in early-phase development, as well as for large-molecular-weight compounds. Late-phase method development in pharmaceutical QC is performed for required stability studies and for release of the drug product or drug substance validation batches, and is intended to be transferred to the operational QC laboratories for release testing of the production batches. Preferably, late-phase methods should be fast, robust, reliable, and transferable. Therefore it is crucial to devote adequate time, thought, and resources to the development of such methods.

The following considerations, when applied during method development, are likely to produce more robust, reliable, and transferable methods: (a) the concerns of the "customer" (user) are considered in advance, (b) key process input variables are identified, (c) critical-to-quality factors are determined, (d) several method verification tests are installed, (e) proactive evaluation of method performance during development is performed, (f) continuous customer involvement and focus are institutionalized, and (g) method capability assessment (suitability to be applied for release testing against specification limits) is established.

V. ANALYSIS OF ACTIVE PHARMACEUTICAL INGREDIENTS AND DRUG PRODUCTS

The development of various modes of CE such as micellar electrokinetic chromatography (MEKC), capillary ITP, capillary IEF, CGE, and fully automated systems in the early 1990s has helped spread the use of CE technology. The human genome project and the great interest in genetics helped boost the development of the technology. Because of its versatility and complexity, CE should be regarded as a family of analytical techniques, rather than a single technique, that are performed on one single instrument. Each mode has its advantages and its limitations. The full potential of CE can be realized only by developing a better understanding of various modes of CE. Successful implementation of CE can make an invaluable contribution to the development of new drugs by increasing the separation speed and resolving new analytical challenges not addressable by other techniques. Chapter 5 focuses on the potential use of CE in the development process of drugs. Various challenges, benefits, and appropriate remediation approaches to overcome the limitations are addressed.

VI. GENERAL CONSIDERATIONS FOR IMPROVING PERFORMANCE OF CE METHODS

Chapter 6 describes the desired improvement of method parameters from the robustness point of view, not from the analyte or the specific analysis point of view. Illustrative examples are provided to help the reader develop methods with the currently available equipment. It is important that the method be described explicitly and unequivocally and that the validation report does not raise expectations that cannot be met in daily use.

VII. CURRENT REGULATORY GUIDANCE

During the last decade, CE has become a mature separation technique for pharmaceutical analysis. Numerous validated methods from pharmaceutical R&D laboratories and academia have been reported in the literature. The information covers identity, API assay, purity determination, enantiomeric separation, and stoichiometry determination (Chapter 7). In addition, CE is frequently applied as an orthogonal technique during the development of stability-indicating liquid chromatographic methods. As a result, CE has been included in various regulatory submissions by different pharmaceutical companies. The growing interest and application of CE as an advanced separation technique in the area of pharmaceutical analysis is gaining recognition by the regulatory authorities. CE has been included as a specific analytical technique in different guidance documents from the United States Food and Drug Administration (FDA) and the International Conference on Harmonisation (ICH). A general monograph on CE has been included in the European Pharmacopoeia (EP), the United States Pharmacopoeia (USP), and the Japanese Pharmacopoeia (JP). In addition, CE is included in a number of specific monographs for several products as, e.g., identity confirmation test or enantiomeric purity test. In order to prevent differences in nomenclature recommendations on the terminology for analytical capillary electromigration, techniques have been published by the International Union of Pure and Applied Chemistry (IUPAC). ICH guidelines should be followed in meeting regulatory approval if CE methods are used in a registration dossier.

VIII. QUALIFICATION OF CAPILLARY ELECTROPHORESIS INSTRUMENTATION

Qualification of CE instrumentation is performed using failure mode and effects analysis as the risk analysis tool (see Chapter 8). The instrument is reviewed in terms of its component modules, and the potential failures of those components are identified. The potential effect of those failures is defined and the risk characterized. Any current evaluation of those failures is identified, and any recommended actions to mitigate the risk are defined. Apart from the qualification dossiers provided by vendors, there seems, at present, to be very little information published on the operational qualification of CE instruments. The latest thinking provided by the FDA in the Guidance for Industry for Quality Risk Management suggests that all qualification activities should be performed using a risk-based approach. Whenever risk is to be considered, the instrument being assessed must be viewed in the context of the "protection of the patient." Analytical instruments may have an impact on the validity of data, determining the safety and efficacy of drug products, or on the product quality of the drug product. They may also impact the identity or potency of the drug product, and therefore it is important to perform risk management throughout the life cycle of the instrument. In some cases, the use of informal risk management processes may also be acceptable.

IX. ROBUSTNESS TESTS OF CE METHODS

In biomedical and pharmaceutical analysis, particularly in the pharmaceutical industry, much attention is paid to the quality of the obtained analytical results because of the strict regulations set by regulatory bodies (Chapter 9). As a result, robustness testing has become increasingly important. The ICH guidelines define robustness as "The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage." A robustness test is the experimental setup applied to evaluate the robustness of the method. The ICH guidelines also state that "One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution tests) is established to ensure that the validity of the analytical procedure is maintained whenever used." The latter definition for robustness has been utilized in Chapter 9 since it is the most widely applied. Although robustness tests are not obligatory yet in the ICH guidelines, they are demanded by the FDA for the registration of drugs in the United States. The setup and treatment of results of such a robustness test are discussed in the same Chapter. Also, a literature review and critique of applications of robustness testing of CE methods has been provided.

X. VALIDATION OF ANALYTICAL METHODS

Validation is the process of proving that a method is acceptable for its intended purpose. It is important to note that it is the method, not the results, that are validated (Chapter 10). The most important aspect of any analytical method is the quality of the data it ultimately produces. The development and validation of a new analytical method may therefore be an iterative process. Results of validation studies may indicate that a change in the procedure is necessary, which may then require revalidation. Before a method is routinely used, it must be validated. There are a number of criteria for validating an analytical method, as different performance characteristics will require different validation criteria.

Various validation characteristics that need to be considered are accuracy, precision, specificity, detection limit, quantitation limit, linearity, and range in accordance with the official guidelines. Validation requirements should be described according to the goal of the method. The validation of analytical methods is generally considered as an inherent requirement of quality assurance systems. There are various approved references (recommendations and guidelines) for realization and interpretation of assay performance and proficiency testing for QC analytical methods. For pharmaceutical methods, guidelines set by the USP, the ICH, and the FDA provide a framework for performing such validations. Thus, when evaluating electrophoretic procedures, the validation must be in stringent compliance with these current guidelines in order to be accepted in all parts of the world. For CE, as a mature but still younger technique, validation studies need to provide stronger evidence of suitability of a given method.

XI. THE NEED FOR CE METHODS IN PHARMACOPOEIAS

Various international pharmacopoeias help assure the quality of drugs worldwide. These pharmacopoeias constantly review and revise their monographs. A different impurity profile can be anticipated if a drug's production process is changed; this results in the development of new analytical methods that need to be incorporated in the pharmacopoeias. In earlier editions, color reactions were performed for identification and purity evaluation purposes.

Nowadays, the pharmacopoeias make use of chromatographic methods and try to replace less sensitive TLC methods with HPLC tests. However, CE methods are rarely used even though they can at times be more useful than HPLC for determining the impurity evaluation of a drug (Chapter 11). CE is currently used, especially in the case of peptides and proteins, in the EP and the USP. These methods and perspectives for new applications are given in the same Chapter.

XII. CE IN IMPURITY PROFILING OF DRUGS

Chapter 12 illustrates possible applications of CE in impurity profiling. Because of the large peak capacity of the technique, CE is extremely well suited to separate the main drug compound from its possible impurities that often have a related chemical structure. Moreover, the high efficiencies obtained, as well as the low reagent consumption, make CE a viable alternative to HPLC in many cases of drug analysis. After a short introduction into the relevance of impurity profiling for regulatory authorities, public health, and the pharmaceutical industry, various applications of CZE, non-aqueous CE, MEKC, microemulsion electrokinetic capillary chromatography, CGE, and CEC are presented.

XIII. ION ANALYSIS USING CAPILLARY ELECTROPHORESIS

Most drugs are charged molecules that are weak bases or acids having a counterion. Basic drugs may have an ionic salt or organic acid as counterion, and acidic drugs a cation. The regulatory agencies (e.g., US FDA and other relevant agencies) require that pharmaceutical products be tested for their composition, strength, quality, and purity. These requirements apply to the excipients as well. This means that the determination of the counterion is an important part of the determination of the purity of the drug. One of the major applications of CE in the pharmaceutical industry is the determination and quantification of drugs counterions. Another application is the impurity determination, to check for the contaminants resulting from the production process. One of the characteristics of many of these ions is that they are UV transparent, while most CE instruments are equipped with a UV detector. For this reason, a special technique called indirect UV detection is often applied. Chapter 13 covers important considerations relating to buffer composition and sample preparation and provides a review of the indirect detection principle, and what is required to make it work.

XIV. ROLE OF CE IN BIOPHARMACEUTICAL DEVELOPMENT AND QC

In the last two decades, CE has advanced significantly as a technique for biomolecular characterization. It has not only passed the transition from a laboratory curiosity to a mature instrument-based method for micro-scale separation, but has also emerged as an indispensable tool in the biotech and pharmaceutical industries (Chapter 14). CE has become a method of choice in R&D for molecular characterization, and in QC for release of therapeutic biomolecules. In the biopharmaceutical industry, more and more CE methods have been validated to meet ICH requirements. To demonstrate the influence of CE in R&D for method development and in manufacturing for the release of therapeutic proteins and antibodies, examples from the pharmaceutical industry are provided in Chapter 14.

XV. CAPILLARY ELECTROPHORESIS AND BIOANALYSIS

The use of CE for the analysis of therapeutic proteins produced by recombinant DNA technology has significantly increased over the past several years. Chapter 15 highlights some of the most important CE applications. The applications are divided into the following areas: (a) capillary sodium-dodecylsulfate as a replacement for traditional SDS-PAGE, (b) CE to monitor charge heterogeneity by CZE and capillary IEF, and (c) oligosaccharide analysis by CE. Finally, an overview of the implementation of CE in the QC of therapeutic proteins is provided.

XVI. CE AS AN ORTHOGONAL TECHNIQUE TO CHROMATOGRAPHY

In the strict mathematical sense, two parameters are orthogonal when the Pearson's correlation coefficient between both is zero (Chapter 16). Considering the comprehensive two-dimensional chromatography, two systems are called orthogonal when the constituent dimensions operate independently and the synentropy across the dimensions is zero. However in one-dimensional chromatography, as considered above, often a less strict definition is applied for orthogonal systems, being "systems that differ significantly in selectivity." As a consequence, some analysts prefer the term "dissimilar" to "orthogonal" in such situations. CE can add important value as an orthogonal technique to chromatography, for instance, in drug impurity profiling. First of all, CE is based on a totally different separation mechanism from partition chromatography and shows selectivity differences from conventional HPLC. This implies that CE should provide additional information about a sample. Moreover CE proved its importance in the critical zones of a chromatogram, i.e., where the co-elution of two components is encountered. As a consequence, it can be very useful to include a CE method as an orthogonal technique in a set of dissimilar chromatographic systems used to screen unknown mixtures.

XVII. CAPILLARY ELECTROCHROMATOGRAPHY OF PHARMACEUTICALS

CEC is a miniaturized separation technique that combines capabilities of both interactive chromatography and CE. In Chapter 17, the theory of CEC and the factors affecting separation, such as the stationary phase and mobile phase, are discussed. The chapter focuses on the preparation of various types of columns used in CEC and describes the progress made in the development of open-tubular, particle-packed, and monolithic columns. The detection techniques in CEC, such as traditional UV detection and improvements made by coupling with more sensitive detectors like mass spectrometry (MS), are also described. Furthermore, some of the applications of CEC in the analysis of pharmaceuticals and biotechnology products are provided.

XVIII. COUPLING CE AND MICROCHIP-BASED DEVICES WITH MASS SPECTROMETRY

Several advantages offered by CE, such as a high efficiency, rapid method development, simple instrumentation, and low sample consumption, are the main reasons for its success in a variety of fields. UV-VIS spectrophotometry is probably the most widely used detection technique with CE because of the simplicity of the on-line configuration. However, its sensitivity, directly related to the optical path length afforded by the I.D. of capillaries, which is in the micrometer range, is low, and it remains the major bottleneck of this technique (see

Chapter 18). UV detection thus requires relatively high analyte concentrations and is often unsuitable for numerous applications, such as the bioanalytical field or impurities peak profiling. Therefore, other detectors are required for the analysis of complex mixtures, and many detection techniques have been already hyphenated to CE, such as laser-induced fluorescence (LIF) and electrochemical (EC) detections. Because pharmaceutical and chemical structures do not always possess a strong chromophore or fluorophore, a derivatization procedure is often mandatory for a sensitive spectroscopic detection, while EC is limited to electroactive substances. In this context, the on-line combination of high-efficiency CE separations with MS provides some major benefits. Among them, it enhances sensitivity and enables determination of co-migrating compounds with different mass-to-charge ratios (m/z). MS provides a higher potential for an unambiguous identification and confirmation of components in complex mixtures and, potentially, gives some information concerning the structure of the separated compounds. Therefore, because of its high sensitivity and specificity, MS is the detector of choice for CE, and CE–MS coupling provides a powerful combination for performing rapid, efficient, and sensitive analyses. Chapter 18 reviews the instrumental aspects for successful coupling of CE with MS regarding interfaces, ionization sources, and analyzers. Practical considerations concerning different CE modes, such as CZE, NACE, MEKC, and CEC coupled with MS, are also discussed and illustrated with a focus on recent pharmaceutical applications. Additionally, quantitative CE–MS is presented, and various methodologies used to achieve sensitive and repeatable analysis are discussed. The final section of Chapter 18 provides an overview on new devices (i.e., microchips) hyphenated to MS, in terms of fabrication methods, microchip designs, MS interfacing, and applications.

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THEORETICAL CONSIDERATIONS IN PERFORMANCE OF VARIOUS MODES OF CE

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ABSTRACT

The origins, principles, methods, and modes of capillary electrophoresis (CE) are discussed. Massive application of electrophoresis methods started after Tiselius's moving boundary method that was optimized by the use of paper or a gel as a semiconducting medium. The applications of paper and gel electrophoresis were situated mostly in the biochemical environment for the analysis of proteins, amino

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acids, and DNA fragments. Further enhancement of the technology led to the replacement of the paper or gel with a capillary column. This resulted in a high performance instrumental separation method known today as capillary electrophoresis (CE). As a result there was a leap in applications over the last two decades, ranging from low-molecular-weight organic, inorganic, charged, neutral compounds and pharmaceuticals to high-molecular-weight biomolecules and polymers. The chemical and biomedical world should know that the human genome project was completed many years earlier than initially planned, thanks to CE. Owing to its potential of performing extremely high efficiency separations, robustness of equipment, automation, ease of use, and flexibility, CE is widely applied in pharmaceutical analyses.

I. INTRODUCTION

With the study of the migration of hydrogenium ions (H^+) in a phenolphthalein gel[1] by Lodge in 1886 and the description of the migration of ions in saline solutions[2] by Kohlraush in 1897, a basis was set for the development of a new separation technique that we know today as "electrophoresis." Indeed, several authors applied the concepts introduced by Lodge and Kohlraush in their methods and when Arne Tiselius[3] reported the separation of different serum proteins in 1937, the approach called electrophoresis was recognized as a potential analytical technique. Tiselius received the Nobel Prize in Chemistry for the introduction of the method called moving boundary electrophoresis.[4]

Electrophoresis is based on the differential migration of charged compounds in a semiconductive medium under the influence of an electric field.[5] The first method of moving boundary electrophoresis required further enhancement of the efficiency. As a result of the developments, well-established techniques such as paper electrophoresis and gel electrophoresis were introduced. Paper electrophoresis became quite successful and was extensively used; however, its application was outnumbered by gel electrophoresis because of its poor resolution power. Gel electrophoresis is still being applied on a routine basis especially in biochemistry, for example for the determination of proteins and nucleic acids. Gel electrophoresis is limited by the effect of "Joule heating," which gives rise to heat production in the system. This heat cannot be dissipated efficiently and leads to temperature gradients that result in convection and viscosity gradients and finally in an increase of dispersion and thus band broadening. The reproducibility of the separations also deteriorates due to these effects as it is strongly affected by the viscosity gradients. Moreover, the analysis times are typically long (order of hours), and the detection methods are tedious, labor intensive, and involves a lot of wet chemistry. Therefore it is generally not possible to develop a fully quantitative assay in conventional electrophoresis. The best that can be achieved is a semi-quantitative analysis, reducing the applicability of the technique further.[6]

Electrophoresis in narrow bore tubes, as performed by Hjerten[7] in 1967, provides a better heat dissipating system. He described an application using glass tubes with an internal diameter (I.D.) of ± 3 mm. The small volume of the narrow bore tube improves the dissipation of heat due to a lower ratio of the inner volume to the wall surface of a tube (Equation (1)). The better the heat dissipation the higher will be the separation efficiency:

$$\text{Ratio} = \frac{\text{volume of the tube}}{\text{surface of internal wall}} \tag{1}$$

In 1974, Virtanen[8] reported the separation of three cations in a 215 μm I.D. glass tube. Five years later, Mikkers et al.[9] reported the application of this technique with 200 μm I.D

Teflon tubes and obtained separations with plate heights less than 10 μm. They also provided a theoretical basis for migration dispersion in free zone electrophoresis.[10]

The potential of free zone electrophoresis as a separation technique was clearly demonstrated and theoretically well covered. Along with the development of fused silica tubing coated with polyimide layer for flexibility, a technology first developed for the communication via optical fibers[8] and used in the seventies for GC columns, electrophoresis in capillaries was ready to advance. The term "capillary electrophoresis (CE)" was introduced in 1981 by Jorgenson and Lukacs.[11−13] They were the first to apply the fused silica capillaries with diameters smaller than 100 μm. Voltages up to 30 kV could be applied using a capillary of 75 μm I.D., resulting in separations with plate heights of less than 1 μm for proteins and for dansylated amino acids.

The next important milestone in CE was achieved in 1984, when Terabe et al.[14] described the method of micellar electrokinetic capillary chromatography (MECC or MEKC). By simply adding a surfactant to the separation buffer electrolyte, it was possible to separate both charged and neutral compounds simultaneously in CE. From this point on, the technique developed rapidly with many applications resulting in a demand for identification information. Coupling of CE to mass spectrometry (MS)[15,16] was a next challenge and the introduction of commercially available CE−MS systems is considered a major achievement toward practical applications. CE−MS couples the CE separation versatility with the detectability, specificity, sensitivity, and possibilities for structural elucidation of MS. A full discussion on CE−MS is provided in Chapter 18.

II. BASIC CE CONFIGURATION

The semiconducting medium in CE consists of a capillary connecting two electrolyte (buffer) containing compartments (Figure 1). Electrodes connected to a power supply are placed in the buffer compartments to generate a voltage difference over the buffer compartments. As a result an electric field is created in the capillary that will allow charged constituents to migrate. A detector is placed downstream toward the end of the capillary and is usually connected with a data processing system. Temperature control system is the key in

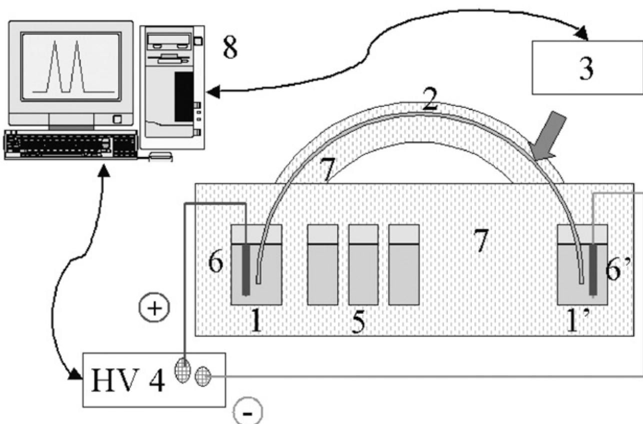


FIGURE 1 Representation of a CE system. 1: electrolyte compartments, 2: capillary, 3: detector, 4: power supply, 5: sample carousel, 6: electrodes, 7: thermostated areas, 8: data workstation.

maintaining consistent performance; therefore, the capillary, electrolyte compartments, detection system (on-column), and sample vials (carousel) are housed in a temperature-controlled environment. This configuration is the basic instrumental setup of CE equipment.

The main challenge during early days, when the first commercial CE instruments were introduced (1988) by Applied Biosystems (Foster City, CA) and Beckman Coulter (Fullerton, CA), was to overcome poor reproducibility and how to improve separation efficiency. Different approaches were proposed resulting in many instrument companies introducing CE systems in the early years of the last decade (e.g., Isco (Lincoln, NE), Bio-Rad (Hercules, CA), Waters (Milford, MA), Applied Biosystems, ThermoQuest (Santa Fe, NM), and Dionex (Sunnyvale, CA)). However, at the end of the decade only Beckman Coulter and Agilent Technologies (Wilmington, DE) remained as major manufacturers of single-capillary instruments. Current CE systems can be categorized into three groups: (i) single-capillary, (ii) capillary-array, and (iii) chip-based instruments[17] as summarized in Table 1.[6] The single-capillary instruments are for general purpose applications, whereas the capillary-array instruments are mainly applied for DNA sequencing because of the high throughput capability. More recently chip-based instruments are introduced for specific applications such as DNA sequencing. It is expected that the use of this type of instruments will grow in the future, covering other specific application methods.

III. CE CHARACTERISTICS

Compared to conventional electrophoresis and other chromatographic techniques, the advantages of CE include the very high separation efficiencies that can be obtained, the low sample volume consumption, on-column detection, the low organic solvent consumption, generally fast analyses, and lower operational costs (Table 2).[18] These advantages have made CE to be applied widely to various challenges in analytical chemistry and is therefore considered as a complementary technique to established chromatographic techniques such as HPLC, GC, and others. In a number of specific application areas (e.g., ion analysis, chiral separations, protein analysis, and DNA sequencing) CE is often considered to be an alternative technique.

The injection volumes in CE are extremely small because of the use of capillaries with very small diameters. Typical injection volumes are in the order of 10–50 nL (a fog droplet is ± 10 nL). Injection of such small volumes of sample into the capillary is very challenging and requires specific approaches including use of rotary-, split- and micro-injectors, electrokinetic and hydrodynamic injection. Although all these injection techniques have shown to be quite appropriate, electrokinetic and hydrodynamic injection methods are mostly applied. Recent commercial instruments are usually equipped with these two injection modes as standard methods.[1,7,19] Chapter 3 provides more details on the different injection modes.

A. Detection

In CE, detection is typically performed on-column with optical detection systems. The on-column detection approach minimizes zone broadening, as it avoids connection devices or fittings. When building CE systems, instrument manufacturers have copied instrument configuration from established separation techniques (especially liquid chromatographic techniques) and slightly modified for adjustment to CE application. The path length of the detection cell is determined by the diameter of the capillary in on-column detection with optical detectors (less than 100 μm). Together with the small injection volume (some nL), the

TABLE 1 Instrumentation for Capillary Electrophoresis

Manufacturer	Instrument	Application	Features
Single-capillary instruments			
Agilent Technologies	Agilent CE	General purpose	Diode array detection
Applied Biosystems	Prism 310 Genetic Analyzer	DNA chemistries	Single capillary with LIF detection
Beckman Coulter	MDQ	General purpose	96-well sampling, numerous detectors
CE Resources (Kent Ridge, Singapore)	CE P1	Portable	Potential gradient detection
	CEL1	General purpose	Many detectors available
Convergent Bioscience (Mississauga, Toronto, Canada)	iCE280	Isoelectric focusing	Whole capillary imaging
Dycor (Edmonton, Canada)	VEGA	Research in bio/chem warfare	Epiillumination fluorescence microscope
Micro-tek Scientific (Sunnyvale, CA)	Ultra-Plus II	CEC, μ-LC, capillary zone electrophoresis (CZE)	Gradient elution
Prince Technologies (Emmen, The Netherlands)	Various models	General purpose	Modular system
Unimicro Technologies (Pleasanton, CA)	Trisep-100	CEC, μLC, CZE	Gradient elution, several detectors
Capillary array instrumentation			
Applied Biosystems	Prism 3100 Avant	Many DNA applications	4 capillaries, upgradeable
	Prism 3100 Genetic Analyzer	Many DNA applications	16 capillaries
	3700 Genetic Analyzer	Many DNA applications	96 capillaries
Beckman	CEQ2000XL	DNA sequencing, fragments	8 capillaries
	Paragon	Serum proteins	7 capillaries
CombiSep (Ames, IA)	MCE 2000	Combinatorial screening amino acids pK_a	96 capillaries, UV detector
Molecular Dynamics (Sunnyvale, CA)	MegaBACE 4000	Sequencing, genetic analysis	384 capillaries
	MegaBACE 1000	Sequencing, genetic analysis	96 capillaries
	MegaBACE 500	Sequencing, genetic analysis	46 capillaries
Spectrumedix (State College, PA)	SCE 9610	High throughput, genetic analysis	96 capillaries
Chip-based instrumentation			
Agilent Technologies	Agilent 2100	DNA, RNA fragments	12 runs/chip sodium dodecyl sulfate (SDS), proteins
Caliper Technologies (Mountain View, CA)	AMS 90 SE	DNA fragments	Reusable separation channel

Source: Adapted from reference 6.

TABLE 2 Characteristics of CE in Comparison with Other Separation Techniques for the Case of Biomolecular Separations

Characteristics	CE	Gel electrophoresis	HPLC
Separation mechanism	Charge-based (polarity)	Size and charge	Polarity partitioning; size, ion-exchange
Analysis times	5–30 min	2 h to several days	10–120 min
Efficiency	Very good to excellent	Very good	Good
Detection	On-column optical/ electrochemical/ conductivity/MS/etc.	Visible and fluorescent stains and dyes/auto-radiography	Optical/electrochemical/ conductivity/MS/etc.
Sample volume	5–50 nL	1–100 μL	10–100 μL
Precision	Good to excellent	Fair	Good to excellent
Accuracy	Good to excellent	Fair to poor	Good to excellent
Absolute detection limit	Zepto[a] to nano moles	Nano to micro moles	Nano to micro moles
Relative detection limit	ppm	ppm	ppb
Organic solvent consumption	Milliliters	–	Liters
Equipment cost	High	Moderate	Moderate to high
Cost of supplies	Low	Moderate	Moderate to high
Manual labor	Low	High	Low
Automation	High	Limited	High
Preparative	No	Yes	Yes

[a] 10^{-21}.

sensitivity of the detection in terms of concentration (Molar) is seriously limited in CE. In terms of mass detection the sensitivity is very good (Table 3).[4]

Photometric detection, especially UV (including diode array and multi-wavelength UV detection) is by far the most frequently applied detection technique. Most compounds can be detected directly as they are able to produce a direct analytical signal (Figure 2). In direct detection using a UV detector, a peak is detected each time a zone containing a UV-absorbing compound passes the detector cell. Besides photometric detection the application of MS in CE is attractive as it provides structural information.[20] Although coupling of a micro column to a mass spectrograph is not as straightforward as coupling to optical detection techniques, recently CE–MS has advanced significantly.[21,22]

CE–MS/MS is a very powerful and promising technique in pharmaceutical analysis. Because of its unique selectivity due to the different separation principle compared to, e.g., HPLC, CE–MS provides novel opportunities to develop orthogonal methods. Especially during impurity profiling of drugs, CE–MS/MS approaches are very beneficial as an orthogonal chromatographic methodology.[23,24] Practical applications have demonstrated that the recent instrumentation generally meets the expectation. Characteristics as sensitivity, repeatability, and reproducibility are excellent and allows detecting impurities at very low levels in pharmaceutical samples.[23] Other detection systems applied include hologram-based refractive index detection,[25] electrochemical detection,[26,27] conductivity[28–30] and amperometric[31,32] detection.

When using the current commercially available CE systems equipped with photometric detectors, it is not possible to detect some compounds that do not show light absorption. For example the detection of low-molecular-weight organic and inorganic ions[57–60] is not

TABLE 3 Detection Systems Applied in CE and Their Limits (for Approximately 10 nL Injection Volumes)

Method	Concentration detection limit (Molar)	Mass detection limit (moles)
UV–VIS	10^{-5}–10^{-8}	10^{-13}–10^{-16}
Fluorescence	10^{-7}–10^{-9}	10^{-15}–10^{-17}
Amperometry	10^{-10}–10^{-11}	10^{-18}–10^{-19}
Indirect UV	10^{-3}–10^{-6}	10^{-11}–10^{-14}
Indirect fluorescence	10^{-5}–10^{-7}	10^{-13}–10^{-15}
Indirect amperometry	10^{-8}–10^{-9}	10^{-16}–10^{-17}
Laser-induced fluorescence (LIF)	10^{-14}–10^{-17}	10^{-18}–10^{-21}
Conductivity	10^{-7}–10^{-8}	10^{-15}–10^{-16}
Mass spectrometry	10^{-8}–10^{-9}	10^{-16}–10^{-17}

Source: Adapted from reference 6.

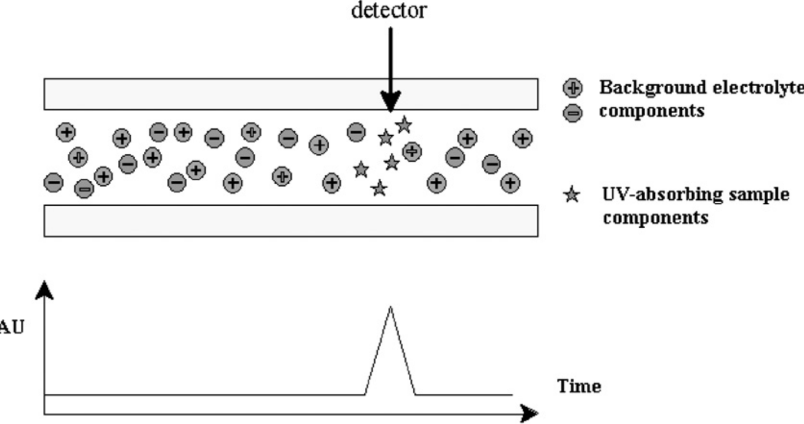


FIGURE 2 Schematic representation of direct on-column detection in CE.

possible (see Chapter 15). In such cases indirect UV detection can be applied by adding a light-absorbing component to the buffer electrolyte. As a result a constant background signal (baseline) is produced that will show a dip each time a zone containing non-UV-absorbing components pass the detector cell (Figure 3).

B. Corrected (Time) Peak Areas

In CE with photometric detectors the recorded peak area changes with the migration time for the same compound at constant concentration,[33,34] a typical characteristic for "flow-dependent" detectors.[35] As can be observed in Figure 4, the peak area for the same compounds is linearly ($r = 0.999$) correlated to the migration times when determined at different run voltages. The migration time in CE decreases when the migration velocity of a compound zone is increased due to, e.g., rising voltages. Although the concentration of the

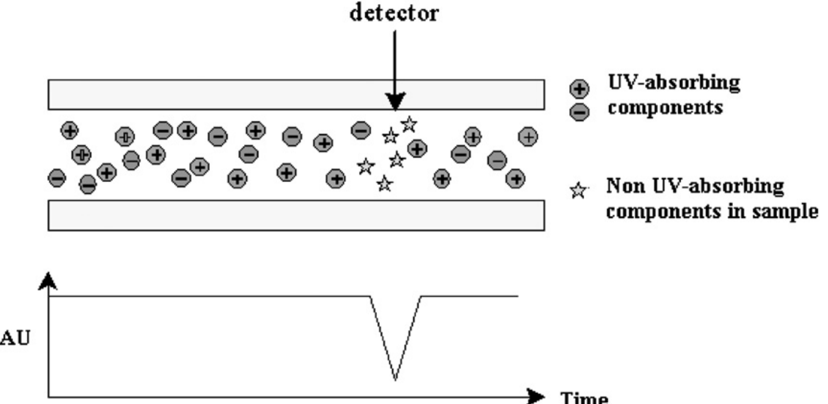


FIGURE 3 **Schematic representation of indirect on-column detection in CE.**

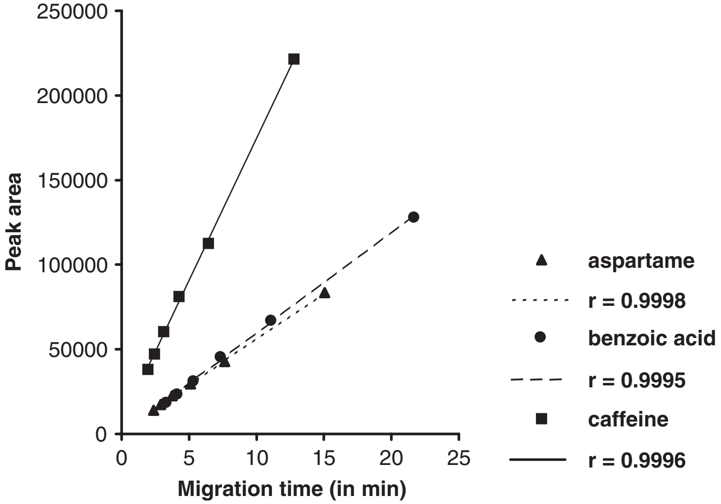


FIGURE 4 **Relationship between peak areas and migration times in CE. The obtained migration times were recorded by the injection of three compounds at different voltages.**

compounds remains constant, the peak area still decreases with the decreasing migration times.[6]

The change in peak area is proportional to the time spent by a zone in the light beam of the detector cell and can be described by the following expression of the area in function of the migration time:[6]

$$\text{Area} = \frac{C \times \text{Abs} \times w_{\text{det}} \times T_{\text{m}}}{l} \tag{2}$$

As can be derived from this expression, the peak area is directly proportional to migration time (and inversely related to the potential).

When the migration velocity (migration time) is not well controlled, the resulting peak area will vary leading to poor reproducibility. For this reason, a time correction is typically applied in CE by dividing the peak areas with the migration time.

C. Temperature Control

As can be seen in Figure 5, despite the use of narrow bore capillaries, the temperature difference between the wall of the capillary and the surrounding air/liquid can rise up to several degrees (exceeding 70°C)[36,37] due to self-heating of the capillary because of the power production within the capillary. The power production (*W*) is directly proportional to the applied potential difference for the generation of the electric field:

$$W = V \times I = I^2 \times R \tag{3}$$

where *V* is the applied voltage (in V), *I* the current flow (in A), and *R* the resistance (in Ω) of the medium. For this reason the possibility of applying high voltages is limited to a maximum of about 30 kV. An efficient temperature control procedure for the capillary is one of the primary prerequisites for obtaining reproducible peak areas and migration times.[36] The difference in temperature at the interface between the wall of the capillary and the surrounding environment can increase significantly and therefore requires an efficient cooling device. In CE systems temperature control is achieved by maximizing the Joule heat dissipation through cooling with either air (forced air convection or with a Peltier device) or liquid (Peltier device) circulation. It was shown that in most cases temperature control systems with liquid circulation perform better than temperature control systems using air.[36,37]

IV. PRINCIPLES OF CE

A semiconducting medium and an electric field are the basic needs of electrophoresis. In the case of CE the semiconducting medium is composed of a capillary filled with an electrolyte or a gel. An electric field is generated by applying a voltage difference across the capillary. As a

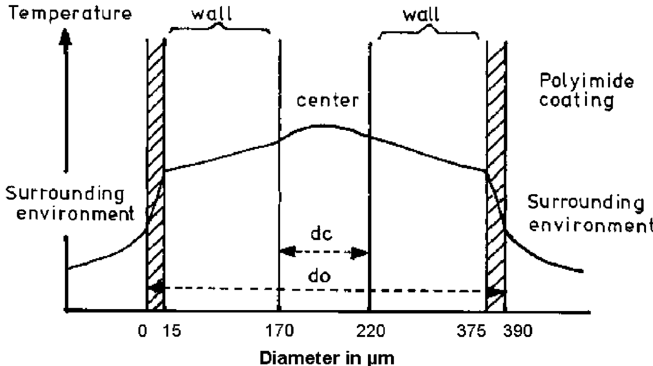


FIGURE 5 Distribution of the temperature in the capillary and the surrounding environment; d_c and d_o represent the inner and outer diameter of the capillary, respectively. Modified with permission from reference 5.

result components in the capillary are affected by physical forces coming from electroosmosis and electrophoresis.

A. Electroosmotic Mobility (μ_{eof})

Electroosmosis or electroendosmosis is the bulk movement of the solvent (electrolyte solution) in the capillary due to the zeta potential at the wall–water interface of the capillary. The resulting flow of the solution in the capillary is known as the electroosmotic flow (EOF). Any solid–liquid interface is surrounded by solvent and solute constituents that are oriented differently compared to the bulk solution. Due to the nature of the surface functional groups like the silanol groups in the typically applied fused silica capillaries, the solid surface may have an excess of negative charge.[38] Counterions from the bulk solution in the capillary are attracted to the wall. A differential charge distribution is created at the surrounding area of the capillary wall compared to the bulk solution within the capillary channel as illustrated in the model represented in Figure 6.[6]

A double layer of charged counterions is formed adjacent to the wall. In the case of fused silica capillaries a fraction of the cations in the double layer adsorb strongly to the wall,

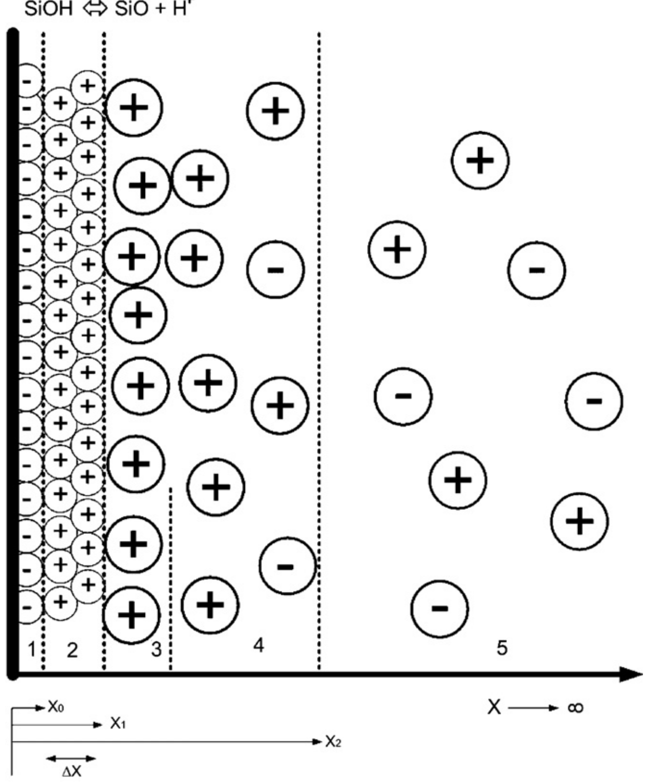


FIGURE 6 Distribution of charges at the internal wall of a silica capillary. *x* is the length in cm from the center of charge of the negative wall to a defined distance, 1 = the capillary wall, 2 = the Stern layer or the inner Helmholtz plane, 3 = the outer Helmholtz plane, 4 = the diffuse layer, and 5 = the bulk charge distribution within the capillary.

resulting in an immobilized compact layer of tightly bound cations. This layer is also called the Stern layer or inner Helmholtz plane containing ions of which the solvation sheath has been stripped off. The second layer, known as the diffuse layer, contains cations that arrange themselves in a mobile, loosely held layer of solvated ions. The initial part of the diffuse layer, at the side of the Stern layer, is known as the outer Helmholtz plane.[39,40] Because of the distribution of charges at the double layer at capillary wall–electrolyte interface a potential field is generated as shown in Figure 7. The potential at the surface of the wall is given by[41]

$$\vec{\psi}_0 = \frac{\vec{Q}_w}{\varepsilon \times x_0} \tag{4}$$

where Q_w represents charge of the wall, ε the dielectric constant of the medium (in Farad m^{-1} or A s V^{-1} cm^{-1}), and x_0 the distance from the surface of the wall to the center of the charge. This surface potential decreases with the distance in the double layer to reach a zero value at the bulk distribution of ions in the capillary. The potential drop is exponential and can be expressed according to Gouy–Chapman as

$$\vec{\psi}_x = \vec{\psi}_0 \exp(-\kappa x) \tag{5}$$

where ψ_x represents the potential at a distance x and κ is the Debye–Huckel constant:

$$\kappa = \frac{8\pi \times e^2 \times n_0 \times z^2}{\varepsilon \times k \times T} \tag{6}$$

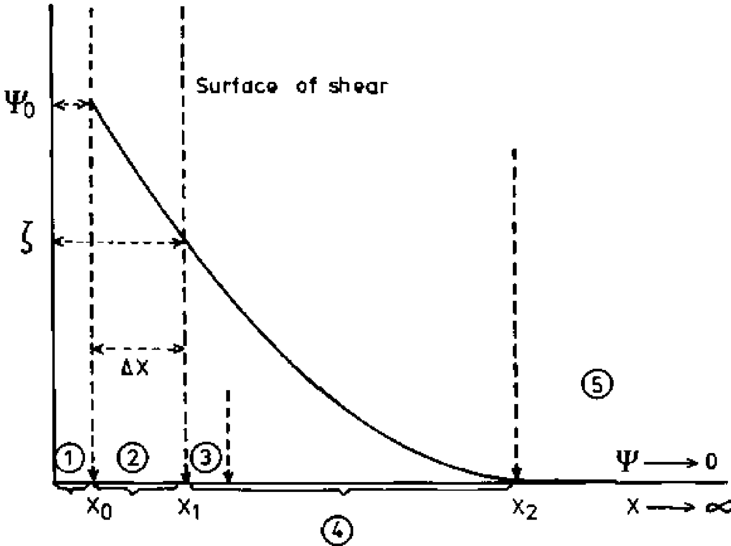


FIGURE 7 **Potential difference (ψ) at the internal wall of a silica capillary due to the distribution of charges. x is the length in cm from the center of charge of the negative wall to a defined distance, ϕ is the zeta potential, 1 = the capillary wall, 2 = the Stern layer or the inner Helmholtz plane, 3 = the outer Helmholtz plane, 4 = the diffuse layer, and 5 = the bulk charge distribution within the capillary.**

Here e is the electronic charge, n_0 the bulk concentration of ionic species (ionic strength), z the valence of the electrolyte, k the Boltzmann constant, and T the absolute temperature.

The electrokinetic or zeta potential (ζ) is the potential at the surface of shear that coincides with the outer Helmholtz plane at the boundary of the Stern layer. In the model represented in Figure 6 the surface of shear is situated at a distance $x_1 = x_0+\Delta x$ from the wall, Δx being the statistical average of the thickness of the hydration (solvation) shell.[41,42] When an electric field is applied, ions in the diffuse layer will start to migrate. The extent of the migration velocity is determined by the zeta potential. Cations in the diffuse layer move toward the cathode. Because these cations are solvated they drag the liquid along with them, resulting in the bulk movement of the solvent through the capillary. This phenomenon is called electroosmosis and is physically observed as the EOF. At the surface of shear (outer Helmholtz plane) the velocity of the EOF rises from a zero value to a limiting value (electroosmotic velocity: $\vec{v}_{\mathrm{eof}}$) at an infinitive distance from the wall where the distribution of the cations equals to that of the bulk.

The Helmholtz–Von Smoluchowski[1,39–44] equation relates the electroosmotic velocity $\vec{v}_{\mathrm{eof}}$ to the zeta potential in the following way:

$$\vec{v}_{\mathrm{eof}} = \frac{\varepsilon\zeta}{4\pi\eta} \times \vec{E} \tag{7}$$

The electroosmotic velocity is characterized by a mobility factor, namely the electroosmotic mobility ($\vec{\mu}_{\mathrm{eof}}$):

$$\vec{\mu}_{\mathrm{eof}} = \frac{\varepsilon \times \zeta}{4\pi \times \eta} \tag{8}$$

As is derived from Equation (8), $\vec{\mu}_{\mathrm{eof}}$ can be adjusted by changing the dielectric constant and/or the viscosity of the medium, but also ζ. As mentioned before, the zeta potential is mainly influenced by the distribution of charges at the capillary wall. All alterations resulting in a change of the charge distribution at the capillary wall like changes in the pH, ionic strength, valence of ions in the buffer electrolyte, etc., can be applied to adjust the velocity of the EOF.

The pH is an important factor that can influence the ionization of the surface silica groups. As a result, ζ is directly dependent on the pH. Therefore, the relationship of μ_{eof} as a function of pH is governed by the behavior of the dissociation of the silanol groups. Different capillary materials result in different profiles of the electroosmotic mobility as a function of the pH (due to differences in ζ). Typically a sigmoid curve behavior resembling the titration curve of the surface active groups is observed.[42,45]

Due to the extremely small size of the double layer (thickness ranges from 3 to 300 nm), the EOF originates close to or almost at the wall of the capillary. As a result, the EOF has a flat plug-like flow profile, compared to the parabolic profile of hydrodynamic flows (Figure 8). Flat profiles in capillaries are expected when the radius of the capillary is greater than seven times the double layer thickness[18,46] and are favorable to avoid peak dispersion. Therefore, the flat profile of the EOF has a major contribution to the high separation efficiency of CE.

The zeta potential and the thickness of the double layer ($1/\kappa$) decrease rapidly with an increase in ionic strength or the valence of the electrolytes in the capillary (Equations (5) and (6)). Therefore, the ionic strength and the nature of the ions in the electrolyte solution are very important parameters determining the strength of the EOF. Careful control of the ionic

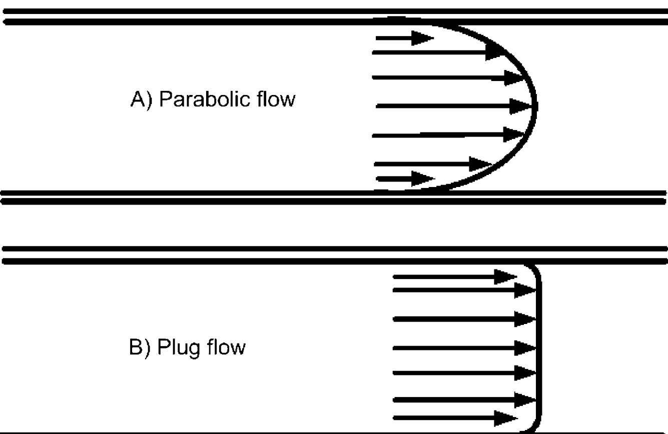


FIGURE 8 Flow profiles of a hydrodynamic flow like in HPLC (A) and of electroosmosis like in CE (B).

strength leads to a better reproducibility of the EOF.[44] The direction of the EOF can even be reversed by changing the charge of the wall. In a silica capillary this can be achieved by adding a cationic surfactant, such as cetyltrimethylammonium bromide (CTAB), to the buffer electrolyte. It is also possible to reduce the EOF or even inhibit it by the addition of specific modifiers. These effects are also obtained by modifying the internal wall of the capillary by derivatization or by applying a radial external field.[1]

B. Electrophoretic Mobility (μ_i)

Charged compounds undergo the influence of the electric force ($\vec{F}_{el}$) and the Stokes' frictional force ($\vec{F}_s$) in an electric field. When these two forces (in kg cm s^{-2}) are in equilibrium at steady-state conditions, the following equations can be written:

$$\vec{F}_{el} = \vec{F}_s \tag{9}$$

$$\vec{q}_i \times \vec{E} = 6\pi \times \eta \times r_i \times \vec{v}_i \tag{10}$$

where $\vec{q}_i$ (in C) is the net ionic charge of a compound, $\vec{E}$ (in V cm^{-1}) the electric field strength, η (in g cm^{-1} s^{-1}) the viscosity of the medium, r_i (in cm) the hydrodynamic radius of the charged compound, and $\vec{v}_i$ (in cm s^{-1}) the migration velocity of the compound. From Equation (10) the migration velocity can be expressed as a function of characteristics of the system and that of specific to the component:

$$\vec{v}_i = \frac{\vec{q}_i}{6\pi \times \eta \times r_i} \times \vec{E} \tag{11}$$

As can be observed in Equation (11) the migration velocity is directly proportional to the ionic charge of a compound and the applied electric field strength. It is inversely proportional to the viscosity of the medium and the hydrodynamic radius of the compound. The electric field strength is determined by the applied voltage difference ($\vec{V}$, in V) and the

total length of the capillary (L, in cm) as described in the following equation:

$$\vec{E} = \frac{\vec{V}}{L} \tag{12}$$

Because the length of the capillary and the applied voltage are typical for a specific system, the migration velocity is a characteristic specific for one system. It is therefore not possible to make comparisons between runs with different applied voltages or obtained with capillaries of different lengths. For this reason one utilizes a normalized velocity characteristic that is called the electrophoretic mobility ($\vec{\mu}_i$, in $cm^2\,V^{-1}\,s^{-1}$):

$$\vec{\mu}_i = \frac{\vec{q}_i}{6\pi \times \eta \times r_i} \tag{13}$$

As can be derived from the characteristics of the electrophoretic mobility, the migration of a compound can be influenced by altering the charge, the viscosity of the medium, and the dynamic radius of the compounds. Introducing Equation (13) in Equation (11), the electrophoretic mobility is the proportionality factor in the linear relationship between the migration velocity and the electric field strength:

$$\vec{v}_i = \vec{\mu}_i \times \vec{E} \tag{14}$$

From Equations (13) and (14) it is derived that the charge of a compound is the major parameter of selectivity adjustment in CE. The charge of a compound can be adjusted through the degree of dissociation (α_i) by altering ionization of acidic or basic functional groups. In this way the migration velocity is also directly proportional to α_i. The proportionality factor in the relationship of the migration velocity and the electric field strength in such a case is called the effective electrophoretic mobility ($\vec{\mu}_{eff}$) and the migration velocity the effective migration velocity ($\vec{v}_{eff}$):

$$\vec{v}_{eff} = \alpha_i \times \vec{\mu}_i \times \vec{E} = \vec{\mu}_{eff} \times \vec{E} \tag{15}$$

The migration time (t_i) of a compound is determined by the migration velocity and the distance it has migrated:

$$t_i = \frac{l}{\vec{v}_{eff}} = \frac{l}{\vec{\mu}_{eff} \times \vec{E}} = \frac{l \times L}{\vec{\mu}_{eff} \times V} \tag{16}$$

The migration distance (l) in CE is equal to the length of the capillary from the inlet (injection site) to the point of detection and L is the total length of the capillary. The migration time increases with increasing capillary length and reduces with increasing mobilities of the compounds and applied voltages.

C. Electrophoretic Migration

The overall migration in CE is determined by the combined effect of the effective and the electroosmotic mobility. The apparent electrophoretic mobility ($\vec{\mu}_{app}$) is therefore used as the proportionality factor in the relationship between the migration velocity and the electric field strength. Similarly the migration velocity is now called the apparent migration velocity ($\vec{v}_{app}$). Since the $\vec{\mu}_{app}$ is equal to the sum of $\vec{\mu}_{eff}$ and $\vec{\mu}_{eof}$, the apparent migration velocity is expressed as

$$\vec{v}_{app} = \vec{\mu}_{app} \times \vec{E} = (\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{E} \tag{17}$$

The final observed migration time is described by

$$t_i = \frac{l}{\vec{v}_{app}} = \frac{l}{(\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{E}} = \frac{l \times L}{(\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{V}} \tag{18}$$

where l equals the length of the capillary from the inlet (injection site) to the point of detection and L is the total length of the capillary.

In CE using fused silica capillaries the EOF is directed toward the cathode; therefore, the apparent migration velocity of cations is positively affected, while the migration of anions is negatively affected. Neutral compounds are also transported through the capillary toward the cathode because of the EOF. When the electroendosmotic mobility is sufficiently high it is even possible to separate both cations and anions in one single run (Figure 9). When $\vec{\mu}_{eof}$ is greater than $\vec{\mu}_{eff}$, anions that originally migrate toward the anode are still carried toward the cathode due to a positive apparent velocity. Neutral compounds migrate with the velocity of the EOF, but are unresolved under one peak in the electropherogram.

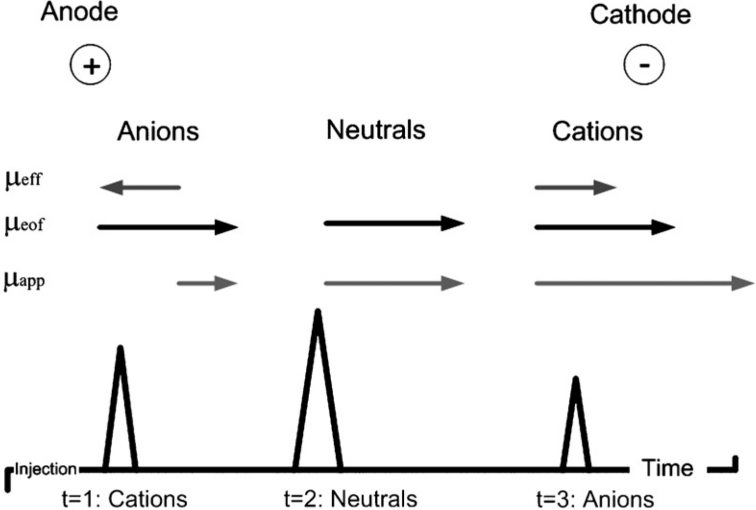


FIGURE 9 Migration of cations, anions, and neutral compounds in capillary zone electrophoresis in an ordinary fused silica capillary.

It is the effective mobility that determines selectivity since the effect of the electroosmotic mobility is equal for all the sample constituents. The effective mobility can be worked out from the apparent mobility if the extent of $\vec{\mu}_{eof}$ is known. Several methods[1] are applied to determine $\vec{\mu}_{eof}$; however, the procedure of applying a neutral marker is commonly used. A marker is a compound that migrates only due to the EOF (neutral). The velocity of a neutral compound represents the velocity of the EOF when no other disturbing processes such as wall adsorption occur. Using the practically obtained migration time of the neutral marker (t_{eof}) the velocity of the EOF can be calculated:

$$\vec{v}_{eof} = \frac{l}{t_{eof}} = \frac{l \times L}{\vec{\mu}_{eof} \times \vec{V}} \tag{19}$$

The effective mobility of a compound can now be calculated from the practically obtained migration time (t_m) of that compound and the migration time of the neutral marker:

$$\vec{\mu}_{eff} = \frac{l \times L}{\vec{V}} \times \left(\frac{1}{t_m} - \frac{1}{t_{eof}}\right) \tag{20}$$

where l equals the length of the capillary from the inlet (injection site) to the point of detection and L is the total length of the capillary.

D. Efficiency

In order to achieve a good separation between two compounds with close mobilities, it is important to have each compound migrating in narrow bands (zones) through the capillary (column). These narrow bands in the separation column are reflected as narrow peaks in the final electropherogram. The broadness of bands in the electropherogram of a CE separation is determined by the dispersion of the migrating solute zones in the capillary (in the background buffer electrolyte). CE is a dynamic process; therefore, dispersion effects are bound to occur.

The extent of the dispersion can be represented by the standard deviation of the "Gaussian" zones and corresponds to the width of the peak at 0.607 of the maximum height[47,48] that can be easily measured in the electropherogram. The total system variance (σ^2_{tot}) is affected by several parameters that lead to dispersion (Equation (21)). According to Lauer and McManigill[49] these include radial thermal (temperature gradient) variance (σ^2_T), electrical field perturbation (electrodispersion) variance (σ^2_E), wall adsorption variance (σ^2_W), EOF variance (σ^2_F), injection variance (σ^2_I), and longitudinal (axial) diffusion variance (σ^2_L). Several authors[10,47,50–52] have described and investigated these individual variances further and have even identified additional sources of variance, such as detection variance (σ^2_D), and others (σ^2_O):

$$\sigma^2_{tot} = \sigma^2_T + \sigma^2_E + \sigma^2_W + \sigma^2_F + \sigma^2_I + \sigma^2_L + \sigma^2_D + \sigma^2_O \tag{21}$$

The individual variances in Equation (21) cannot be suppressed to a zero value as they are inherent to the principle of the technique. It should be possible, however, to control the contributions of these sources of variance by proper instrumental design and selection of optimal working conditions. The extent of the dispersion will affect the efficiency of the separation system, which is usually expressed in terms of the number of theoretical plates (N)

or the height equivalent to a theoretical plate (HETP). The number of theoretical plates is more often used in literature to express the separation efficiency and may be defined as follows:

$$N = \frac{l^2}{\sigma_{\mathrm{tot}}^2} \tag{22}$$

where l is the migration distance of a zone (length of the capillary from injection to the point of detection) and σ_{tot} is the standard deviation of the assumed Gaussian zone distribution. The HETP can be calculated from the number of theoretical plates as shown in the following equation:[2]

$$\mathrm{HETP} = \frac{l}{N} \tag{23}$$

1. Radial Thermal Variance

An increase in Joule heating may result from the power generated by a flow of current through the buffer electrolyte solution in the capillary. The formation of a radial temperature gradient[46] leads to a parabolic zone deformation. A radial temperature gradient will result in a corresponding radial viscosity gradient. (In aqueous media the viscosity shows a strong dependence on the temperature.) Since the electrophoretic mobility is indirectly related to the viscosity of the medium, a radial velocity gradient of the migrating solute is observed. It is established that the mobility of a solute increases by 2% when the temperature is increased by 1°C. The temperature difference (ΔT) between the center of an open capillary and its wall is given by

$$\Delta T = \frac{\vec{E}^2 \times \lambda \times c \times d_{\mathrm{c}}^2}{16\kappa_{\mathrm{t}}} \tag{24}$$

where $\vec{E}$ corresponds to the electric field strength, λ is the equivalent conductivity of the electrolyte, c represents the molar concentration, d_{c} is the inner diameter of the capillary, and κ_{t} represents the thermal conductivity of the medium. Equation (24) clearly illustrates the dependence of the radial distribution of the temperature on the inner diameter of the capillary. It is therefore obvious that d_{c} has to be reduced ($< 100\,\mu\mathrm{m}$) in order to apply the desired high potentials ($\vec{E}$). The use of small diameter capillaries reduces ΔT formation and allows to apply high electric fields that result in high separation efficiencies. In addition, radial zone distribution will be reduced to a controllable level.[45]

2. Electrical Field Perturbation Variance

When the solvent strength of the sample diluent in HPLC does not match well with the solvent strength of the mobile phase at initial conditions, peak deformation is bound to occur. In CE a comparable phenomenon is observed with differences in conductivity between the sample zone and the bulk electrolyte in the capillary.[10,53] The conductivity (γ, $\Omega^{-1}\,\mathrm{m}^{-1}$) of a solution is given by the cumulative effect of the contributions of different ions:

$$\gamma = F \times \sum_i c_i \times \mu_i \times \vec{Z}_i \tag{25}$$

where F is the Faraday constant, c_i the concentration of an ion, μ_i the electrophoretic mobility of an ion, and $\vec{Z}_i$ the charge of an ion. Since the conductivity of an injected sample may differ from that of the bulk electrolyte in the capillary, the electric field strength in the sample zone and the bulk electrolyte is adjusted according to the following equation to maintain a constant current flow:[10]

$$I = \frac{\pi \times d_c^2}{4} \times \vec{E}_i \times \gamma_i = \frac{\pi \times d_c^2}{4} \times \vec{E}_b \times \gamma_b \qquad (26)$$

where I represents the current (in A), $\vec{E}_i$ and γ_i correspond to the electric field strength and the conductivity in the sample zone, respectively, whereas $\vec{E}_b$ and γ_b represent the same for the bulk electrolyte. From Equation (27) it is derived that the conductivity and thus the field strength (Equation (26)) strongly depend on the concentration (ionic strength), the electrophoretic mobility, and the ionic charge differences of the ions in the capillary. These dissimilarities are common in CE analysis; therefore, differences in field strength between the sample zone and the bulk electrolyte can be expected. As a result band distortion, especially at the boundaries of the sample zones, is likely to occur.

Three situations may occur regarding the migration velocities of the ions in the sample zone ($\vec{v}_s$) and in the bulk electrolyte ($\vec{v}_b$), as shown in Figure 10:

(a) $\vec{v}_s < \vec{v}_b$; the conductivity of the sample zone is higher than that of the buffer electrolyte. In other words the electric field strength in the sample zone is smaller than that in the buffer electrolyte. Under these conditions the top of the zone migrates faster than the back flank. A tailing peak with a steep front side is observed.

(b) $\vec{v}_s = \vec{v}_b$; the conductivity in the sample zone is equivalent to that of the buffer electrolyte. This occurs in the ideal situation when there are no electric field perturbations present. The migrating zones do not show electrodispersion and the peaks are symmetrical.

(c) $\vec{v}_s > \vec{v}_b$; the conductivity of the sample zone is lower than that of the buffer electrolyte. The electric field strength in the sample zone is therefore higher than that in the buffer electrolyte. In such a case the front flank of the sample zones migrates faster than the top. A fronting peak with a steep backside is observed.

Electrodispersion can be avoided by increasing the bulk electrolyte concentration (limited by Joule heating) or by diluting the sample (limited by the sensitivity of the detection system). As demonstrated by Mikkers et al.[10] electrodispersion due to concentration differences can be neglected when the sample concentration is smaller than the bulk electrolyte concentration by a factor of 100. Besides differences in concentrations, the electrophoretic mobility and the ionic charge are also important. Proper selection of the bulk electrolyte consisting of ions with matching ionic charge and mobilities with that of the analytes is therefore crucial. The phenomenon of electrodispersion is sometimes advantageously used to enhance the detectability of analytes. By selecting appropriate conditions it is possible to concentrate the analytes in a narrow band by using the stacking effect. For example when the field strength in the sample zone is higher than that in the bulk electrolyte, the ions in the sample zone have a higher migration velocity than those in the bulk electrolyte. The sample constituents migrate fast but are slowed again ("stacked") at the border of the sample zone, close to the bulk electrolyte. Therefore, the distribution of the analyte zone is skewed with a higher density at the flank rather than at the top of the zone (pre-concentration).

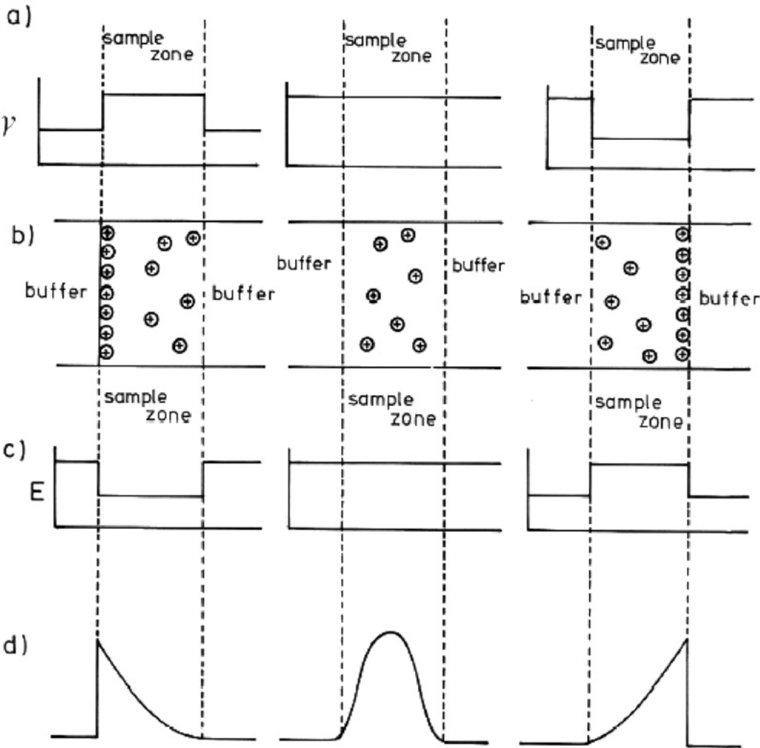


FIGURE 10 **Effect of electric field perturbations due to differences in conductivity between the sample and the buffer electrolyte zone on the shape of the peaks, (a) conductivity distribution, (b) sample ions distribution, (c) electric field strength perturbations, and (d) effect on the peak shapes. Adapted with courtesy of Dr. D. Heiger of Agilent Technologies Inc.[5]**

3. Wall Adsorption Variance

Depending on the characteristics of the wall and the analytes, analyte–wall interactions may occur in fused silica capillaries. Wall adsorption is frequently observed in the analysis of large molecules,[49,54] and it is also observed in separations of small ions.[34] Usually it concerns coulombic interaction mechanisms, but sometimes hydrophobic interactions are also possible. The variance due to wall adsorption is given by the following expression:[51]

$$\sigma_{\mathrm{W}}^2 = \frac{2(1-\theta)^2}{\tau} \times \mu \times l \times \vec{E} \tag{27}$$

where θ represents the fraction of free particles (not adsorbed) and τ represents the mean free lifetime of a particle. The effect of analyte–wall adsorption can be reduced by proper selection of the capillary material, the buffer pH, ionic strength, wall surface deactivation, the use of lower sample concentrations, and capillaries of larger inner radii, because this reduces the surface area to volume ratio (inverse of Equation (1)). However, there is no universal approach to finding a solution for wall adsorption.[49,51,52]

4. Injection Variance

The injection variance is considered to be a component of the extra-column zone broadening effects and is related to the width (w_{inj}) of the ideal rectangular sample plug (volume). The variance is given by

$$\sigma_{\mathrm{I}}^2 = \frac{w_{\mathrm{inj}}^2}{12} \tag{28}$$

In order to avoid column overloading during sample analysis, it is necessary to keep the width of the injection plug smaller than 1% of the total length of the capillary.[50]

5. Detection Variance

Because of on-column detection the variance of the detection process is reduced to the variance related with the width of the detector cell (w_{det}). It is expressed similarly to injection as

$$\sigma_{\mathrm{D}}^2 = \frac{w_{\mathrm{det}}^2}{12} \tag{29}$$

According to the literature,[51] the contribution of the detection variance to the total system variance is usually below 0.01%.

6. Axial Diffusion Variance

The variance due to longitudinal diffusion is expressed by the Einstein equation:[47,50–52]

$$\sigma_{\mathrm{L}}^2 = 2 \times D_i \times t_i \tag{30}$$

where D_i represents the diffusion coefficient of a solute (in $\mathrm{m^2\,s^{-1}}$) and t_i is its migration time. By substituting Equation (18) for t_i in Equation (30), one obtains:

$$\sigma_{\mathrm{L}}^2 = 2 \times D_i \times \frac{l \times L}{(\vec{\mu}_{\mathrm{eff}} + \vec{\mu}_{\mathrm{eof}}) \times \vec{V}} \tag{31}$$

From Equation (31) it is clear that the variance due to longitudinal diffusion is negatively influenced by the length of the capillary and the diffusion coefficient of the solute. However, it is positively affected by the applied potential and the apparent mobility of a solute. According to this equation, fast migrating zones will show less variance due to axial diffusion.

7. Electroosmotic Flow Variance

The EOF flow has a flat profile, almost as a piston; therefore, its contribution to dispersion of the migrating zones is small. The EOF positively contributes to the axial diffusion variance (Equation (31)), when it moves in the same direction as the analytes. However, when the capillary wall is not uniformly charged, local turbulence may occur and cause irreproducible dispersion.[50]

8. Other Sources of Variance

The brand broadening of a zone can also be caused by other sources that are not yet identified or not considered to be important at this stage of knowledge. For example, it has been shown that the coiling of a capillary in a cassette or on a spool also has an effect on zone broadening.

9. Number of Theoretical Plates and Height Equivalent to a Theoretical Plate

At optimal working conditions[1,11,45,55] many of the sources of variation are supposed to be controlled and may be considered negligible. Factors such as σ_T^2 (proper thermostating, small diameter of capillary), σ_F^2 (proper capillary rinsing) and σ_E^2 (matching of sample and buffer electrolyte conductivity), σ_W^2 (proper capillary selection) should be under control. If the influence of σ_O^2 can also be neglected, then the expression of σ_{tot}^2 is simplified to

$$\sigma_{tot}^2 = \sigma_I^2 + \sigma_D^2 + \sigma_L^2 \tag{32}$$

$$= \frac{w_{inj}^2}{12} + \frac{w_{det}^2}{12} + 2 \times D_i \times \frac{l \times L}{(\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{V}} \tag{33}$$

As can be derived that only σ_L^2 influences the broadening of a migrating zone, when the sample volume injected is very small, the detection path length is very narrow and the capillary diameter is very small. In such a case, σ_{tot}^2 is approximated by σ_L^2 and Equation (33) can be simplified further to

$$\sigma_{tot}^2 = \sigma_L^2 = 2D_i \times \frac{l \times L}{(\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{V}} \tag{34}$$

By substituting Equation (22) for σ_{tot}^2 in Equation (34) one obtains an expression for "N," the number of theoretical plates:

$$N = \frac{l^2}{2 \times D_i \times ((l \times L)/((\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{V}))} \tag{35}$$

$$= \frac{(\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{V} \times l}{2 \times D_i \times L} \tag{36}$$

$$= \frac{(\vec{\mu}_{eff} + \vec{\mu}_{eof}) \times \vec{E} \times l}{2 \times D_i} \tag{37}$$

As can be derived, N is directly proportional to the applied field strength, the migration distance from injection to detection, and the apparent mobility of a solute. The diffusion coefficient and the length of the capillary are inversely related to the efficiency.

The HETP can also be calculated by substituting Equation (23) for N in Equation (36):

$$\text{HETP} = \frac{2 \times D_i \times L}{(\vec{\mu}_{\text{eff}} + \vec{\mu}_{\text{eof}}) \times \vec{V}} \tag{38}$$

As can be derived from Equation (38) a higher efficiency will be obtained by applying high voltages and for compounds with a high electrophoretic mobility and low diffusion coefficients. It is thereby important not to use very long capillaries.

E. Resolution and Selectivity

The type of anion and cation, pH, ionic strength, concentration, addition of modifiers such as complexing agents, organic solvents, surfactants, etc., all are parameters of the bulk electrolyte in the capillary that can affect the selectivity of separation. Selectivity may be expressed in terms of mobility differences ($\Delta\mu$) or in mobility ratios (α):

$$\Delta\mu = \mu_1 - \mu_2 \tag{39}$$

$$\alpha = \frac{\mu_1}{\mu_2} \tag{40}$$

In chromatographic and related separation techniques the basic requirements for the resolution (Rs) of two peaks are a column with a high number of plate counts and a factor to induce some selectivity for the separation.[11] Basically resolution is the product of separation efficiency and selectivity:

$$\text{Rs} = \frac{1}{4} \times N^{1/2} \times \frac{\Delta\mu}{\bar{\mu}} \tag{41}$$

where N is the number of theoretical plates, $\Delta\mu$ represents the difference in electrophoretic mobility of two compounds, and $\bar{\mu}$ is the average mobility of the two migrating bands of interest. By considering the effective and electroosmotic mobilities, the following expression is obtained:

$$\text{Rs} = \frac{1}{4} \times N^{1/2} \times \left[\frac{\Delta\mu}{\bar{\mu}_{\text{eff}} + \vec{\mu}_{\text{eof}}}\right] \tag{42}$$

where $\bar{\mu}_{\text{eff}}$ is the average effective mobility of the two migrating bands of interest. By substituting Equation (37) for N in Equation (42), one obtains the following expression:

$$\text{Rs} = \frac{1}{4}\left[\frac{(\vec{\mu}_{\text{eff}} + \vec{\mu}_{\text{eof}}) \times \vec{E} \times l}{2 \times D_i}\right]^{1/2} \times \left[\frac{\Delta\mu}{\bar{\mu}_{\text{eff}} + \vec{\mu}_{\text{eof}}}\right] \tag{43}$$

Equation (43) is worked out in

$$\text{Rs} = 0.177 \times \Delta\mu \times \left[\frac{\vec{E} \times l}{D_i(\bar{\mu}_{\text{eff}} + \vec{\mu}_{\text{eof}})}\right]^{1/2} \tag{44}$$

From Equation (44) it can be derived how to improve the resolution of two peaks:

- Increase selectivity by increasing the difference in mobilities. This can be achieved by using appropriate electrolyte systems and other additives in the background electrolyte (e.g., complexing agents).
- Apply maximal potential difference across the capillary and generate the highest electric field strength.
- Create conditions where the diffusion coefficients of the compounds are low. Typically, larger compounds have slower diffusion coefficients; therefore, formation of molecular complexes is an interesting tool to enhance separation in CE. In addition, the viscosity of the background electrolyte may be increased also to reduce diffusion effects. Another parameter that may be helpful too is lowering the temperature of the separation.
- Generally a slower migration ($\bar{\mu}_{\text{eff}} + \vec{\mu}_{\text{eof}}$) will enhance selectivity, while on its turn a slower migration is not beneficial for efficiency increase (Section IV.D.9). Therefore, migration rates need to be optimized for optimal peak separation on a case-by-case basis. Slower migration can be achieved by minimizing the EOF through lowering the pH or through the use of coated capillaries. Additives that increase viscosity can be added to the buffer electrolyte as well. Lower temperatures for the separation can also decrease migration.

Equation (44) is the expression of the resolution for CE in electrophoretic terms. However, the application of this expression for the calculation of Rs in practice is limited due to D_i. The availability of the diffusion coefficient of different compounds in different media is not always straightforward. The practical calculation of the resolution is frequently done with an expression that uses the width of the peaks obtained in an electropherogram. This way of working results in Rs values that are more realistic as all possible variances are considered (not only longitudinal diffusion in Equation (44)). Assuming Gaussian distribution for the migrating zones, the resolution can be expressed as follows:

$$\text{Rs} = 1.177 \times \left[\frac{T_{\text{m2}} - T_{\text{m1}}}{W_{1/2(1)} + W_{1/2(2)}} \right] \tag{45}$$

where T_{m1}, T_{m2} and $W_{1/2(1)}$, $W_{1/2(2)}$ represent the migration times (in min) and the width of the peaks at half height (in min), respectively. The width of the peaks can be determined at different parts of the full height. In these cases the proportionality factor will change. For example for the width at baseline, this value is equal to 2 instead of 1.177.

V. METHODS AND MODES IN CE

The technique of capillary electrophoresis is classified into three main groups of methods:[6]

(1) Moving boundary CE
(2) Steady-state CE
 - Isotachophoresis (ITP)
 - Isoelectric focusing (IEF)

(3) Zone CE
 - Capillary gel electrophoresis (CGE)
 - Free solution CE

  - ○ Capillary zone electrophoresis (CZE)
  - ○ Micellar electrokinetic capillary chromatography (MEKC)
  - ○ Chiral CE (CCE)
- Capillary electrochromatography (CEC).

The zone capillary electrophoretic methods are applied the most and are therefore discussed in more detail. The others are discussed briefly.

A. Moving Boundary CE

Moving boundary electrophoresis was introduced by Tiselius[3,7] in the late 1930s, but has now become outdated due to its poor separation capabilities. The technique can be performed in a capillary utilizing the same configuration of a recent CE system as described in Section V.C.2. The sample is filled as the only constituent in the electrolyte compartment at the injection side. In the other compartment an electrolyte is used with a mobility that is higher than the mobilities of the sample constituents. The capillary also contains the high-mobility electrolyte and connects the two compartments. By applying an appropriate potential, the ions migrate in the generated electric field. The electrolyte in the capillary, having the highest mobility, migrates first along the capillary, followed by successively slower components of the sample. After a certain time, the fastest compound in the sample zone emerges from the bulk of the sample and is followed by a zone containing a mixture of the next slower compound and the fastest compound. This zone is again followed by a zone containing a mixture of the third slower compound and the first two fastest compounds, etc. In fact, it is only possible to obtain a pure zone of the fastest migrating ion of the sample, which makes the method less attractive for systematic use.[3,41] Moving boundary CE can be interesting for preparative separations of bulk material.

B. Steady-State Capillary Electrophoresis

ITP and IEF are the main representative modes in steady state CE. A certain stage of steady-state in terms of migration is achieved in these methods. Current commercial CE systems with an experimental setup that is similar to the one described in Figure 1 can be used in these methods. Typically, a gradient to be generated is necessary to obtain a separation. By using "leading" and "terminating" electrolyte systems a potential gradient is created in ITP. The injection side compartment is filled with a "terminating electrolyte" which has a mobility much smaller than that of the sample constituents. The capillary and the compartment at the detection side are filled with a "leading electrolyte" with a higher mobility than that of the sample constituents. The sample is injected in a small plug through the inlet of the capillary. When an appropriate voltage is applied, the ions migrate according to their mobility (see Section IV.D.2) – first the leading electrolyte, followed by the sample constituents with successive mobilities, and finally the terminating electrolyte. Separations with high efficiencies may be achieved due to the differential migration rates of the zones that tend to migrate away from each other. Separation of the zones would imply that parts of the capillary would be occupied with water only (with very low conductivity) and the current flow would be cut. In order to maintain the current flow through the capillary, the zones migrate in separate but adjacent to each other. The potential gradient is constant within each zone, but increases in a stepwise fashion from zone to zone.[1–2,7,19] ITP results in very high-resolution separations, but is useful for the separation of ionic compounds only. It usually requires capillaries that are pre-treated in order to avoid the occurrence of the EOF. ITP has shown its benefits as a sample pre-concentration step prior to other CE type of separations.

A pH gradient is used in IEF that is generated by electrolyte systems typically consisting of ampholytes. A coated capillary is used in order to avoid electroosmosis. The IEF mode is especially suitable for the separation of not only zwitter ionic compounds like proteins, peptides, amino acids, but also various drugs. After injection of the sample and application of the potential, the sample constituents migrate to a pH in the capillary where their net-charge is equal to zero. At this point, the isoelectric point, the mobility of a compound is also equal to zero and therefore the migration of the compound is stopped. Band broadening is avoided due to the focusing effect of the pH gradient. In a next step the zones are brought to the detector and removed from the capillary (for example by applying a pressure difference).[1,7,19] Applications of IEF are provided in Chapters 12 and 16.

C. Zone Capillary Electrophoresis

In this group of methods the sample constituents can migrate differentially in zones through the capillary in a medium that can be either a gel (CGE) or an electrolyte (free solution CE). The experimental setup of commercial CE systems for zone electrophoresis is similar to the one presented in Figure 1.

1. Capillary Gel Electrophoresis

In CGE the capillary is filled with a polyacrylamide or agarose gel that forms a polymeric network[56] in the capillary, providing a molecular sieving mechanism for separation of species by molecular size (also determines the charge density) as the analytes migrate through the pores of the gel. In addition, the gel avoids zone broadening due to diffusion and prevents wall adsorption and electroosmosis. Separations of extremely high efficiency (30 million theoretical plates per meter) have been obtained. CGE is especially useful for the separation of large biomolecules, such as proteins, polynucleotides, and DNA fragments, as will be discussed extensively in Chapters 14 and 15. It has considerable advantages over conventional slab-gel electrophoresis: small sample requirement, automation, high sensitivity, high throughput, rapid and efficient molecular mass determination, trace quantitation and is applicable to a wide variety of large biomolecules.[1–2,7,19,35,38,54,57–60] The application of capillary array instruments in this area is very typical for high throughput. CGE is the method of choice in the genomics field and thanks to the introduction of the high throughput instruments, gene sequencing has advanced greatly today.

2. Free Solution Capillary Electrophoresis (FSCE)

CE in free solution mode is currently the most applied technique approach. In FSCE the capillary is filled with only an electrolyte (background electrolyte), usually a buffer to maintain the pH. The same commercially available equipment are applied with an experimental setup that remains similar to the one used for the previous methods and presented in Figure 1. The separation selectivity is based on the difference in effective mobilities between compounds, resulting in differences in migration velocities. FSCE can be applied for the separation of many compounds, including small and large ionic and neutral compounds, both organic and inorganic. FSCE is divided into three important modes due to the addition of specific additives in the electrolyte: CZE, MEKC, and chiral capillary electrophoresis (CCE). These modes are discussed in further sections.

(a) Capillary Zone Electrophoresis:

CZE is the basic technique of the free solution method in CE. Separation of components is achieved due to a differential migration caused by differences in effective mobilities. The

capillary contains a buffer electrolyte that guarantees the current flow and controls the pH, ionic strength, and viscosity. Samples are injected and electrophoretically separated in various individual "pure" zones when an electrical field is applied. Since only ionized compounds can have a differential migration in CZE, neutral compounds are not separated. The neutral compounds are carried by the EOF to the detection site and therefore migrate with the mobility and velocity of the EOF. Several modifiers (complexing agents and organic solvents) are used to enhance the selectivity.[1,7,59]

CZE has been applied for the determination of both organic and inorganic charged compounds. The analysis of low-molecular-weight inorganic and organic ions has developed into a distinct field of interest[34,61] and is therefore fully discussed in Chapter 15. In order to distinguish this field from other areas of application in CE, it is referred to as "small ion capillary electrophoresis (SICE)."[62] Simple CZE methods can be applied for the analysis of charged compounds ranging from small molecules to proteins and DNA fragments. Figure 11 shows the determination of benzalkonium chloride in a nasal pharmaceutical formulation

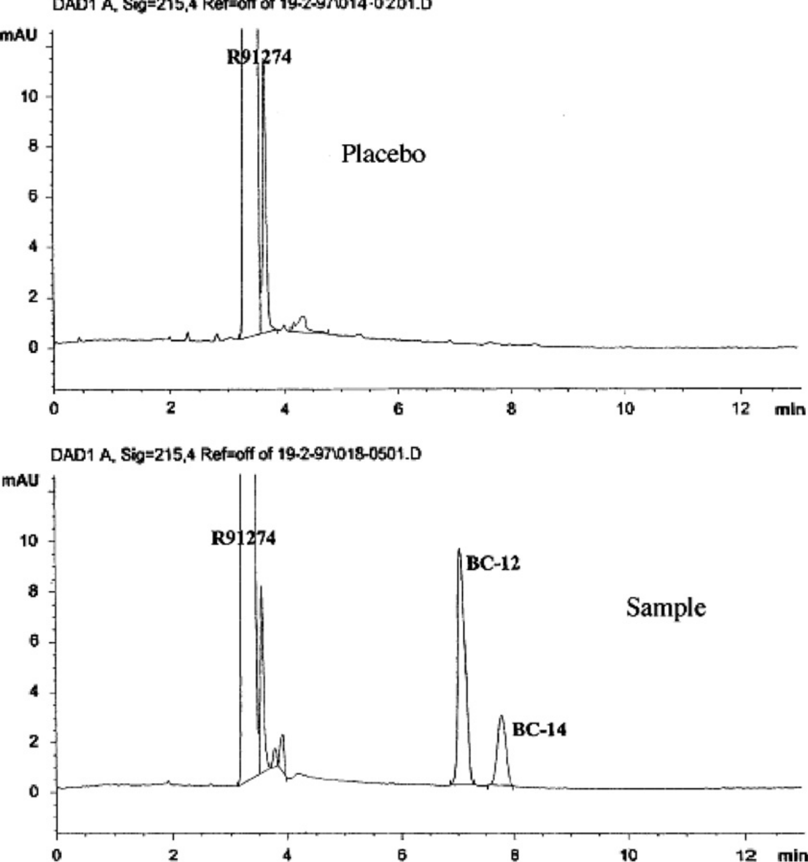


FIGURE 11 Electropherograms obtained for the analysis of a nasal formulation for the determinations of benzalkonium chloride (BC) in the presence of active pharmaceutical ingredient (R91274) and other placebo ingredients. Conditions: 75 mM sodium phosphate buffer, pH = 2.3, 35 cm fused silica capillary (effective length 28.5 cm) × 75 µm I.D., injection 10 s at 35 mbar, 20°C, 15 kV (positive polarity) resulting in a current of approximately 95 µA, detection UV 215 nm.

using a phosphate buffer at pH 2.3 (containing 75 mM phosphoric acid and 42.7 mM triethylamine), with an uncoated fused silica capillary. As can be seen, both benzalkonium derivates with chain lengths C12 and C14 present in the sample are easily separated from each other and from the active pharmaceutical ingredient.[63]

(b) Micellar Electrokinetic Capillary Chromatography:

Terabe et al.[14] added surfactants to the buffer electrolyte as a means to generate a mechanism based on chromatographic polarity to separate neutral compounds with CE. Above the critical micellar concentration (CMC) surfactants form micelles in the aqueous solution of the buffer electrolyte. Micelles are dynamic structures consisting of aggregates of surfactant molecules. Depending on the surfactant applied, they can be highly hydrophobic in their inner structure and hydrophilic at the outer part. The micelles typically applied are positively or negatively charged usually, but can also be neutral. During electrophoresis micelles also show a differential migration due to their effective mobility. Owing to their polarity, neutral compounds are solubilized by the micelles. In case the neutral solutes show no affinity for the micelles, they migrate with their own effective mobility which is equal to the mobility of the EOF. If a solute is highly hydrophobic and therefore has a very strong affinity for the inner part of the micelles, then it will migrate with the mobility of the micelles. In other words, the solutes are partitioned between the micellar and aqueous phase. For this reason the technique is called micellar electrokinetic capillary chromatography, abbreviated as MECC or MEKC. The most applied surfactant in MEKC is sodium dodecyl sulfate (SDS). Figure 12 shows a schematic representation of the mechanisms in MEKC. There are three important zones to be mentioned in a MEKC type of separation once the voltage is applied: the EOF zone, the sample zone, and the micellar zone. Neutral components from the sample are partitioned between the EOF zone and the micellar zone. The partitioning is characterized by the extent of the partitioning coefficients: K_{m} and K_{eof} for the micellar and EOF affinities, respectively.

A neutral compound with high affinity (K_{m}) for the micelles will migrate unseparated together with the micellar zone. Similarly, neutral compounds with high affinity (K_{eof}) for the EOF will migrate unseparated together with the EOF zone. Only the neutrals with an intermediate affinity for both the micellar and the EOF zones will show differential migration and thus a potential separation from each other. It is important to mention that not only neutral compounds are solubilized by the micelles, but also charged molecules as the partition is based on polarity. Therefore, the technique provides a way to resolve both neutral and charged compounds by CE. In fact, MEKC combines electrophoretic and chromatographic principles in one technique.[1,2,7,14,58,64–66]

The migration time (t_{m}) of a neutral compound is situated in a "migration window," between the migration time of the EOF ($\pm 0\%$ solubilization) peak (t_{eof}) and the migration time of the micellar ($\pm 100\%$ solubilization) peak (t_{mc}). The migration time of the micellar zone is experimentally obtained by injecting a micellar marker, e.g., Sudan III with the sample. Sudan III is a highly hydrophobic dye that is solubilized completely by the micelles. The peak of Sudan III in the electropherogram therefore represents the migration of the micelles. The detection of the EOF zone (t_{eof}) is performed by injecting a neutral marker as methanol, mesithyloxide, or formamide in the sample. In chromatographic terms the solubilization of compounds in the micelles can be interpreted as a retention of a solute by the pseudo-stationary phase (since this stationary phase also moves), i.e., the micellar phase. Similarly to conventional chromatography the extent of the retention can be expressed by a capacity factor, k', describing the ratio of the total moles of a solute in the micellar and the aqueous phase:

$$k' = \left[\frac{t_{\mathrm{m}} - t_{\mathrm{eof}}}{t_{\mathrm{eof}} \times (1 - (t_{\mathrm{m}}/t_{\mathrm{mc}}))}\right] \tag{46}$$

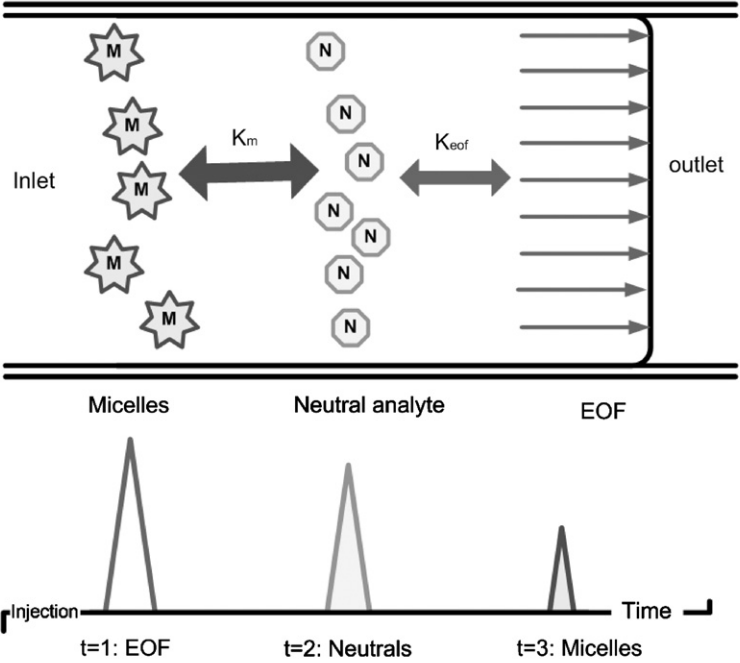


FIGURE 12 Schematic overview of the separation principle in MEKC. Compound "*N*" is partitioned between aqueous phase represented by the EOF that moves toward the cathode in a fused silica capillary and the typical SDS micelles "*M*." Reconstructed typical electropherogram with three peaks, *t* = 1: A neutral compound with no affinity for the micelles migrates with the velocity of the EOF. *t* = 2: A neutral compound with an affinity for both the micellar and the aqueous phase migrates with an intermediate velocity. *t* = 3: A fully solubilized neutral compound migrates with the velocity of the micelles.

Resolution in MEKC is given by

$$\mathrm{Rs} = \frac{N^{1/2}}{4} \times \left[\frac{\alpha - 1}{\alpha}\right] \times \left[\frac{k_2'}{k_2' + 1}\right] \times \left[\frac{1 - (t_{\mathrm{eof}}/t_{\mathrm{mc}})}{1 + (k_1' \times (t_{\mathrm{eof}}/t_{\mathrm{mc}}))}\right] \tag{47}$$

where α represents the separation factor given by k_2'/k_1' and k_1', k_2' are the capacity factors for peaks 1 and 2, respectively. Equation (47) is equal to the expression used in conventional chromatography with the exception of the last part.

As polarity plays an important role in the analysis of small organic compounds such as pharmaceuticals (most organic compounds can be neutral), the introduction of MEKC resulted in a boom of applications in this area. In biomedical analysis with CE, MEKC is the technique of choice for the analysis of drugs in pharmaceutical preparations or in body fluids. Several groups of drugs have been analyzed with considerable success.[1,7,58,67] A typical example of a MEKC separation is shown in Figure 13 for the determination of the organic purity of levocabastine drug substance.[68] For this product it was difficult to separate the ortho-, meta-, and para-fluoro place isomers using HPLC. Therefore, a MEKC method was developed, validated, and filed to the regulatory authorities. Actually, this method is the first CE method to be published in a pharmacopoeia monograph. MEKC applications will be reviewed extensively in Chapters 5, 6, and 11.

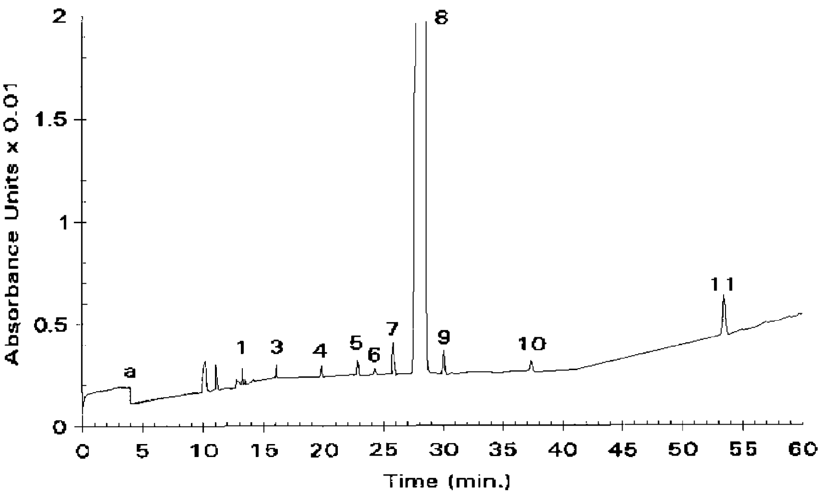


FIGURE 13 Determination of the organic impurities (compounds 1–11) in levocabastine drug substance. Conditions: 225 mM borate buffer, 2.16% w/v SDS + 1.3% w/v Hydroxypropyl-β-cyclodextrin + 10% v/v 2-propanol, pH = 9, 57 cm fused silica capillary (effective length 50 cm) × 75 μm I.D., injection 5 s at 35 mbar, 50°C, detection UV 214 nm, current program: 0–15 min: 75–130 μA, 15–40 min: 130 μA, 40–60 min: 130–200 μA (a = auto zero).

(c) Chiral Capillary Electrophoresis (CCE):

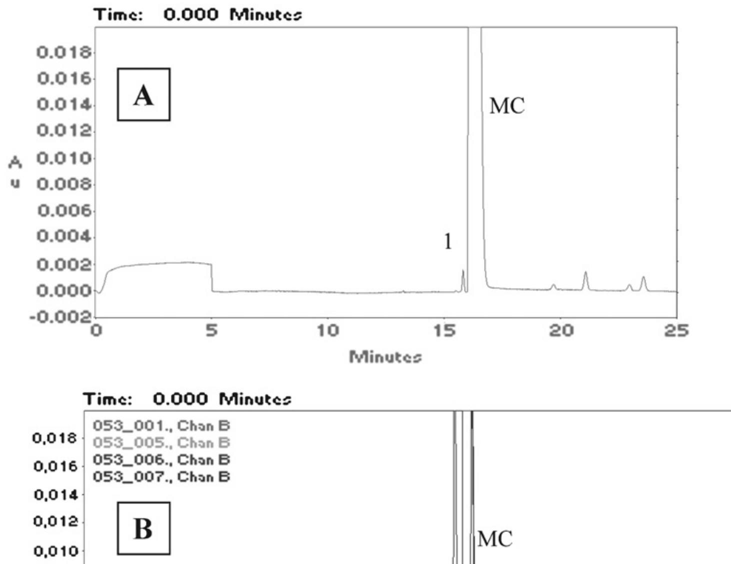
CE has been applied extensively for the separation of chiral compounds in chemical and pharmaceutical analysis.[1,7,58,67–69] First chiral separations were reported by Gozel et al.[70] who separated the enantiomers of some dansylated amino acids by using diastereomeric complex formation with Cu^{2+}-aspartame. Later, Tran et al.[71] demonstrated that such a separation was also possible by derivatization of amino acids with L-Marfey's reagent. Nishi et al.[72] were able to separate some chiral pharmaceutical compounds by using bile salts as chiral selectors and as micellar surfactants. However, it was not until Fanali[73] first showed the utilization of cyclodextrins as chiral selectors that a boom in the number of applications was noted.[1,67,73] Cyclodextrins are added to the buffer electrolyte and a chiral recognition may easily be obtained due to the resulting hydrophobic inclusion, hydrogen bonding, and steric repulsion mechanisms. Owing to their spherical structure the selectors (hosts) can spatially enclose another (guest) or part of another molecule. Therefore, this mechanism is also known as the "host–guest" interaction. Apart from the cyclodextrins, crown ethers have also been applied for the separation of chiral compounds.[58] Enantiomeric separation was also achieved by using proteins as chiral selectors. An example described in literature is the chiral separation of Leucovorin with bovine serum albumin (BSA) as the chiral selector.[74] Other proteins like transferrin, ovalbumin, etc., can also be applied.

Cyclodextrins are the most frequently applied chiral selectors nowadays. Both the native as well as the derivatized cyclodextrins are massively applied in CCE. The major challenge, however, is finding the right cyclodextrin and at its optimal concentration. Up till now it has not been possible to predict based on theoretical grounds which cyclodextrin will be the suitable chiral selector. The selection is based on trial and error approaches, leading to complex, laborious, and time-consuming efforts. Strategies have been proposed to speed up and simplify this selection.[75,76] After selecting the right chiral selector, the test method has to be optimized for robustness and transferability. With respect to this, an optimization of the cyclodextrin concentration, the buffer concentration, pH, and the concentration of, e.g., an

organic modifier is very critical. In order to reduce the experimental work, design of experiments methodology is a preferred approach.[77,78] As a result robust, reliable, and transferable CE methods can be developed that are accepted in new drug applications to regulatory authorities.[79] Figure 14 shows a typical CCE separation for the determination of the chiral purity of a pharmaceutical ingredient. As can be seen, the unwanted enantiomeric impurity can be detected down to the necessary low levels in the samples, just by adding an optimized amount of cyclodextrin to a simple phosphate buffer.

3. Capillary Electrochromatography

Like in MEKC, CEC combines the principles of CE and chromatography, with the major difference that the micelles are replaced by very small, i.e., less than 3 μm, solid or semi-solid



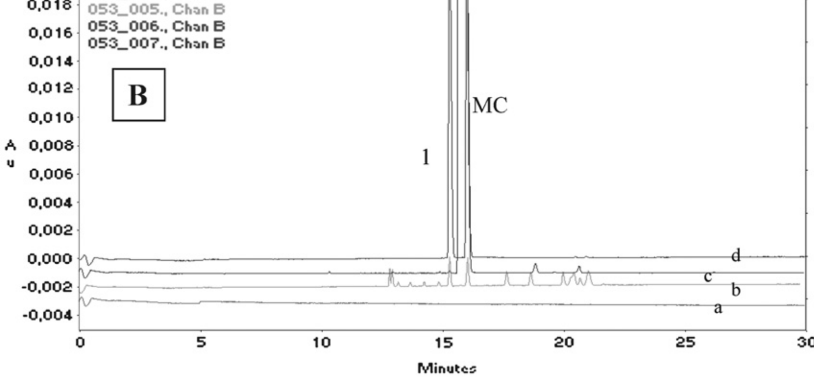


FIGURE 14 Determination of the enantiomeric purity of active pharmaceutical ingredient (main compound = MC, peak 1 is the enantiomeric impurity). Conditions: 100 mM sodium phosphate buffer pH = 3.0, 10 mM trimethyl β-cyclodextrin, 60 cm fused silica capillary (effective length 50 cm) × 75 μm I.D., injection 10 s at 35 mbar, 25°C, 20 kV (positive polarity) resulting in a current of approximately 100 μA, detection UV 230 nm. The sample solution is dissolved in a mixture of 55% (v/v) ethanol in water. (A) Typical electropherogram of an API batch spiked with all chiral impurities, (B) overlay electropherograms showing the selectivity of method toward chiral and achiral impurities, a = blank, b = selectivity solution mixture containing all known chiral and achiral compounds, c = API batch, d = racemic mixture of the main compound and the enantiomeric impurity. (See color plate 1.)

particles in a packed or open column. The particles form a typical stationary phase as we know from ordinary HPLC. The mobile phase is obtained through the electrically driven flow resulting from the EOF. In CEC, separation of solutes is achieved by differential migration resulting from chromatographic partitioning, electromigration, or a combination of both. The separations are performed using packed columns or in open tubular mode.[80] In open tubular mode the stationary phase is coated on the inner surface of the capillary column. CEC is almost similar to HPLC, but results in a higher separation efficiency due to the flat profile of the EOF (mobile phase) and probably the stacking effect due to electrodispersion. Additionally, in CEC small particle sized phases can be applied since the EOF does not generate back pressure. The particles can be sol–gel,[80] molecularly imprinted polymers,[81] continuous monolithic beds,[82] and polymer/layered silicate nanocomposites[78] with reverse phase (e.g., ODS C18), normal phase, ion exchange (SCX), or size exclusion[79] properties.

Typically CEC is performed at high pH to ensure a fast EOF, resulting in short analysis times. In combination with high voltage applications and short capillary lengths the analysis speed is increased further. A lower pH may be needed sometimes for separation of acidic compounds in order to reduce dissociation for better partitioning. CEC at lower pH is performed with ion exchange stationary phases containing sulfonic acid groups on, e.g., a SCX phase. The sulfonic acid groups remain ionized at low pH and thereby generate a sufficiently high EOF. Applications in CEC range from small organic neutral,[81] basic,[82] and acidic[83] drugs (including chiral compounds[84]) to peptides,[85,86] proteins,[87] DNA fragments.[87]

CEC theoretically has the potential to become the method of choice in separation science; however, its development depends on the state of the art of the column manufacturing processes and the robustness of the columns/instrumentation. Furthermore, evidence to demonstrate reproducibility of separations from column to column still has to be established. In addition, the formation of bubbles in the capillary due to Joule heating and variations in EOF rates on passing from the stationary phase through the frit and into the open tube is still very challenging in packed column CEC. Apparently, the use of monolithic columns or applications in open tubular CEC[85] is supposed to overcome many of these issues. Currently, many efforts are placed in improving column technology and in the development of chip-CEC[88] as an attractive option for "lab-on-a-chip" separations. Chapter 18 is devoted to this promising technology in CE.

VI. SUMMARY AND CONCLUSIONS

The contribution of electrophoretic methods to analytical chemistry and biopharmaceutical science has been very significant over the decades. Especially in the biochemical area for the analysis of proteins, amino acids, and DNA fragments, electrophoresis is still the first choice method. With the introduction of the capillary column, the technique has evolved into a high performance instrumental method. Capillary electrophoretic applications are widespread from large biomolecules to even small organic, inorganic, charged, neutralcompounds, and pharmaceuticals. Currently, CE is considered to be an established tool in pharmaceutical analysis and has demonstrated to solve many analytical problems. The major application areas still are in the field of DNA sequencing and protein analysis, and also for the determination of low-molecular-weight pharmaceuticals and other compounds. Indeed, it was thanks to CE that the human genome project was completed many years earlier than initially planned.

Electromigration and chromatographic methods developed as separate techniques over many decades. Today both methods have converged into a single approach: capillary electrochromatography. The approach is still under development, but has already demonstrated to be very promising. From the current findings and the overall CE expertise that was

built up since the start of the last decade, it can be concluded that CE technology has grown to a certain maturity that allows to develop and apply robust analysis methods. CE is already described as general monographs in European Pharmacopoeia[89] and the USP.[90] The number of monographs of drugs is increasing gradually. In order for CE to grow further, a firm understanding of CE-related issues and skillful CE analysts are needed. Applying general chromatographic know-how, e.g., from HPLC to CE, is not the right approach. Usually this is the major reason for failures in CE applications. Dedicated and trained CE analysts are necessary to be successful.

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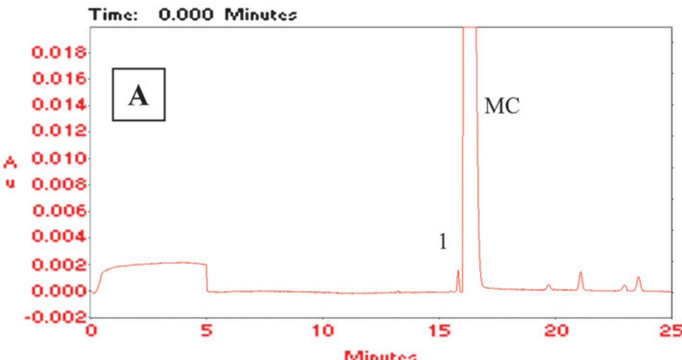

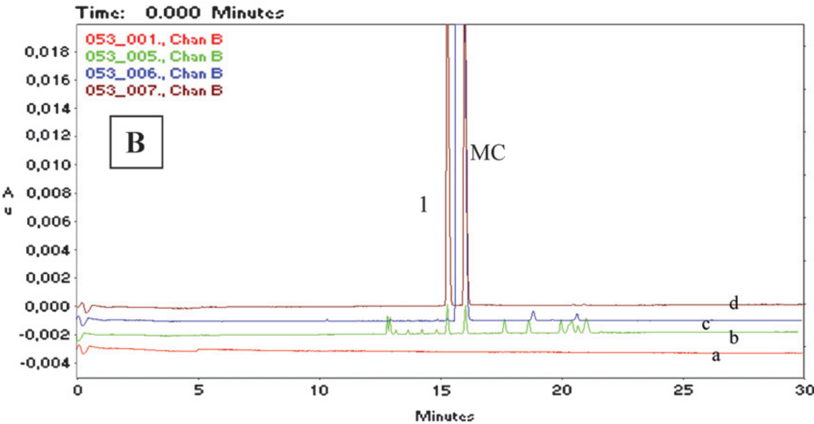

PLATE 1 **Determination of the enantiomeric purity of active pharmaceutical ingredient (main compound = MC, peak 1 is the enantiomeric impurity). Conditions: 100 mM sodium phosphate buffer pH = 3.0, 10 mM trimethyl *β*-cyclodextrin, 60 cm fused silica capillary (effective length 50 cm) × 75 μm I.D., injection 10 s at 35 mbar, 25°C, 20 kV (positive polarity) resulting in a current of approximately 100 μA, detection UV 230 nm. The sample solution is dissolved in a mixture of 55% (v/v) ethanol in water. (A) Typical electropherogram of an API batch spiked with all chiral impurities, (B) overlay electropherograms showing the selectivity of method toward chiral and achiral impurities, a = blank, b = selectivity solution mixture containing all known chiral and achiral compounds, c = API batch, d = racemic mixture of the main compound and the enantiomeric impurity.**

3

EQUIPMENT CONSIDERATIONS FOR CAPILLARY ELECTROPHORESIS

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ABSTRACT

In this chapter, it will be shown how to build the capillary electrophoresis instrument from its components into a robust, functioning analytical instrument. The various components are presented, including a wide range of detection techniques. Some basic troubleshooting along with advice on how to qualify the system is presented along with future prospects for the technique.

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I. CAPILLARY ELECTROPHORESIS INSTRUMENTATION

Capillary electrophoresis (CE) instrument is quite simple. A CE, at its core, is merely a high-voltage power supply (capable of voltages in excess of 30,000 V), capillary (approximately 25 to 100 μm inner diameter), buffers to complete the circuit (e.g., citrate, phosphate, acetate, etc.), and a detector (e.g., UV-Vis). There are additional complexities, of course, but at its heart, the CE is a simple instrument.

For the pharmaceutical scientist, understanding the theory and application of the equipment is usually not sufficient; there is the matter of compliance. The qualification of CE is similar to that of other instruments. Installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) apply in much the same way as high-performance liquid chromatography (HPLC). This chapter details the different parts of the modern CE instrument, equipment-related issues and troubleshooting, instrument qualification, and the future of the CE instrument.

A. Historical Perspective

As mentioned in earlier chapters the foundations of electrophoresis are traceable to over a century of work via different researchers and CE began to emerge once researchers started to use capillaries. In 1965, Tiselius and Hjerten[1] were the first to investigate the feasibility of electrophoresis in capillary tubes. Using 3 mm inside diameter capillaries, it was necessary to rotate the capillary to remove heat and improve theoretical plates. The modern era for CE began in 1981 with the pioneering work of Jim Jorgenson and K.D. Lukacs at the University of North Carolina, Chapel Hill,[2] whereby they demonstrated electrophoresis in narrow glass capillaries of 75 μm. CE has since gained an acceptance on a wealth of applications, and by 1990, five companies were marketing CE systems. In the early days, CE developed a reputation for variability issues. As a technique in its infancy, this should not be surprising. The early instruments lacked the necessary technology needed to ensure reproducible migration times, including control of the temperature variations across the capillary, precise injection control, and stable power supplies. Modern instruments and end users have addressed each of these sources of variability such that, today, CE can have precision and accuracy near HPLC technology.

CE has been touted as a replacement for HPLC in the pharmaceutical industry. This was a shame, since the techniques are so different. For many measurements, it is an orthogonal technique to HPLC. Whereas HPLC separates based on interaction with the stationary phase, CE separates based on the ratio of charge to mass. There are numerous examples of where CE exceeds the resolving power of HPLC (e.g., ion analysis, chiral analysis, DNA quantification, separation, large molecule analysis, etc.).

B. Components of Instrumentation

A diagram of a typical CE instrument showing the various components is shown in Figure 1.

1. Power Supply and Electrodes

CE applications require a reversible high-voltage power supply, ±30 kV and current approximately ±200 μA. Stable regulation of the voltage is necessary (±0.1%) to maintain high migration-time reproducibility. Switchable polarity is required when injecting at both anode and cathode. Constant voltage operation is the most common CE approach; however, some applications utilize constant current, e.g., capillary isotachophoresis. Controlled voltage,

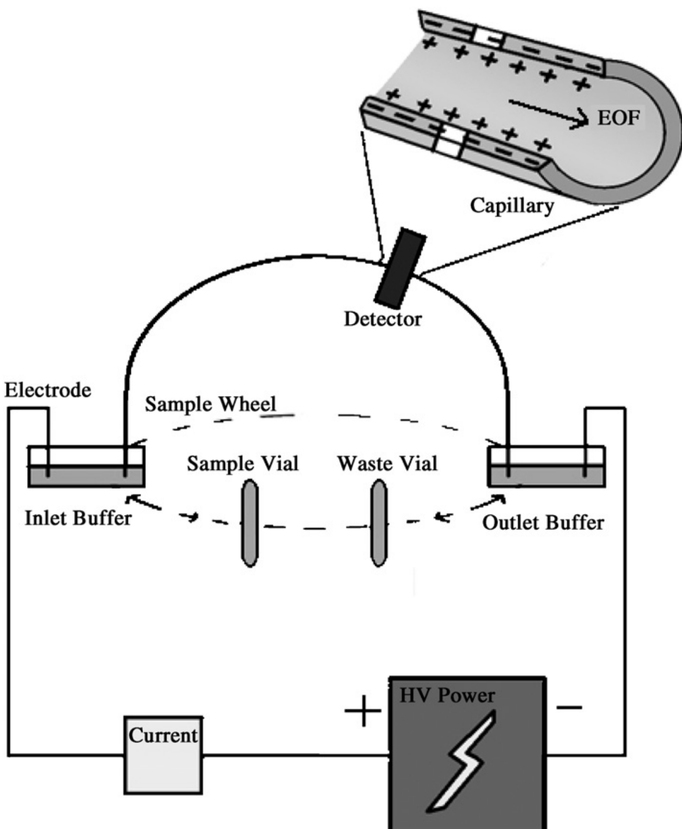

FIGURE 1 **Schematic of a CE instrument. Drawing is courtesy of Kevin Daniel O'Flaherty.**

current, and pressure ramping instead of stepwise application improve accuracy and precision of both migration time and injection amount.

The standard CE separation runs at positive high voltage, whereby the inlet electrode is the anode and the outlet electrode is the cathode. Typically, the field strength or voltage across the capillary length, V/cm, is about 300 V/cm. Thus, if an end user has a 100 cm long capillary, then the voltage required to meet a 300 V/cm field strength is 30 kV. To obtain the same field strength on a 30 cm long capillary, the voltage needs to be 10 kV. Although efficiencies can be improved at higher field strength, excessive field strengths will cause the capillary to overheat and produce reduced resolution. Under standard Capillary Zone Electrophoresis (CZE) conditions, positive effective field strength generates an electroosmotic flow (EOF) toward the cathodic or detection end of the capillary. This EOF is generated by the negative-charge surface silanol groups on the inside wall of the glass capillary. Positive counterions attracted to the wall then move to the outlet or cathodic electrode during initiation of the positive high voltage creating the EOF flow.

2. Autosampler

Sampling systems can be anything from a 4–48-position carousel to a 96-well plate. CE systems vary widely in the autosampler whereby a variety of vials in a multitude of shapes and

volumes exist. Sample and buffer vial volumes can range from 100 μL to a few mL. In all cases, the inlet and outlet vials and solutions are at the same height to minimize any siphoning. When one vial is higher than another, siphoning affects reproducibility and efficiency. The majority of instrument designs use a large number of run buffer vials so that "fresh" solutions can be exchanged for contaminated run buffers when the instrument has performed a number of runs, typically 5−10. The larger the volume in the run buffer, the greater the number of runs can be completed before the run buffers need replacement. There are a wide variety of reasons why the run buffers need replacement. The inlet buffer can be altered via sample or solvent contamination caused by a film on the outer edges of the capillary rinsing into the inlet vial. The outlet buffer can have contamination from ions that pass from the inlet to the outlet of the capillary. Both buffers are susceptible to buffer evaporation and bacterial growth. It is known that electrolysis of solution can alter the running buffer pH and subsequently change EOF. These occurrences tend to cause buffer depletion, and it is important to select an autosampler that has the ability to quickly change out the inlet and outlet buffer vial during long sample sequences, which will ensure long-term performance stability. In general, an autosampler that can handle multiple volume solutions is preferred. Typically, inlet and outlet buffers have sample volumes in milliliter while sample vials are in the microliter to milliliter volume range. A microliter-size sample vial can be advantageous when sample size is limited, while a large sample vial is preferred when the same sample is injected multiple times. An autosampler with many positions for buffers allows the user to perform multiple separations under a variety of different buffers, which aids the scientist in method development. With a sophisticated autosampler, unattended method operation for parameters such as buffer type and concentration, gel type and concentration, surfactant concentration, and pH can be explored.

A key requirement is that the autosampler needs to be temperature stabilized so that the viscosity of the buffer remains consistent over time. Changes in sample or buffer temperature will directly affect the sample injected along with the migration time. Temperature stabilization can be accomplished in a variety of ways including oven and a peltier cooler.

3. Injection Modes

There are two main injection strategies for CE, electrokinetic and pressure. Each type of injection mode has its advantages and disadvantages. Depending on the application, the appropriate mode must be chosen.

Electrokinetic injection involves using voltage to inject sample onto the capillary. The sample serves as a buffer reservoir (Figure 1). A voltage is applied and the analyte(s) migrate onto the capillary. The amount of material injected is dependent on the mobility of the analyte(s). The quantity injected is given by

$$q = \frac{(\mu_e + \mu_{eof}) \cdot V \cdot \pi r^2 \cdot C \cdot t}{L_t}, \tag{1}$$

where q is the quantity injected (in the same units as concentration), μ_e is the mobility of the analyte, μ_{eof} is the EOF mobility, V is the voltage, r is the capillary radius, C is the concentration (in g or moles), t is the time, and L_t is the total capillary length.

Electrokinetic injection is useful when the analyte is in the presence of interfering species (with different mobilities), qualitative applications, or when viscous buffers or gels are being used. It is usually not suitable for quantitative applications since the variability caused by conductivity, microenvironments, and matrix differences significantly reduces the reproducibility. Since sample depletion can be a significant issue, it is recommended that different samples are used when repeated injections are needed.

Pressure (or hydrodynamic) injection involves using pressure to "push" the sample onto the capillary. The sample loaded onto the column is independent of mobility and is indiscriminant with regard to what is loaded onto the capillary. The quantity injected is given by Hagen–Poiseuille equation:

$$q = \frac{\Delta P \cdot r^4 \cdot \pi \cdot C \cdot t}{8 \cdot \eta \cdot L_t}, \tag{2}$$

where q is the quantity injected (in the same units as Concentration), ΔP is the applied pressure (or pressure difference), r is the capillary radius, C is the concentration (in g or moles), t is the time, η is the sample viscosity, and L_t is the total capillary length.

Pressure injection is useful for quantitative applications or when non-viscous buffers and gels are used. The length of time will affect the band broadening, so this should be minimized, although variability can result if the time is too short. Modern instrumentation allows for reproducibility better than 1% using pressure injection (keep in mind, the typical injection volumes are 10–100 nL).

4. Capillary

Until Jorgenson's and Lukacs' paper in 1981,[2] the major obstacle in free-solution electrophoresis was the generation of Joule heat caused by the passage of electricity through the buffer medium. Heat generation occurs uniformly throughout the medium and removal is only along the walls of the capillary, thus a temperature gradient results between the center of the capillary and the buffer–wall interface. The warmer fluid in the center will be less viscous, causing electrophoretic mobilities to be greater in the central regions. Thus, the electrophoretic plug flow can develop a bowed shape with the zone center migrating faster than the edges. Decreasing the capillary internal diameter serves to increase the surface-to-volume ratio and increase heat dissipation. Once the plug flow was maintained by using capillaries of less than 100 μm inside diameter, the major obstacle was overcome to allow the survival of the technique. The next challenge became manufacturing instrumentation at the microscale.

Thus, it can be said that the capillary itself is the most important CE equipment consideration when considering CE performance. Considerations must be given to capillary ID, OD, and length. Other considerations include the cleanliness of the window and the cutting of the capillary ends. A poorly selected or prepared capillary will cause poor results and much valuable time lost.

Consideration must be given to capillary cutting whereby the methodology of cutting can have dramatic implications in the instrument performance. Typically, capillary ends are cut with a ceramic cleaving stone. When a capillary cut is performed, the cleave leaves minimal chips or cracks, and the polyimide is flushed with the capillary end face. When making a capillary cut, the capillary is secured while the ceramic stone scores the capillary at a 45-degree angle. The user can then gently pull apart each side of the capillary.[3]

When making a capillary window, the polyimide coating on the capillary needs to be removed, typically 2–3 mm. Longer capillary windows become more prone to breakage when the capillary is bent near the window. A variety of tools can be used to create a window, including a UV laser, a resistively heated hot-wire device, sulfuric acid heated to 130°C, a plasma pen, and a butane lighter flame.[4] When making an optical window, the capillary must have the polyimide completely removed but still retain sufficient mechanical strength for routine handling and installation into the system detector. The simplest tool to make a reproducible window is to use a heated window burner (e.g., MicroSolv), which can make perfect 1 or 2 mm in length windows. Once the window is made, the window must be carefully cleaned. The window needs to be cleaned thoroughly with high-quality methanol to ensure the

highest optical transmission properties of the fused silica, which allows for on-column absorbance detection down to 200 nm.

Optimization of the window length and window aperture provides a means to optimize sensitivity and resolution. For maximum UV sensitivity, the goal is to illuminate the ID of the capillary with the maximum amount of light. For fluorescence detection a key is to illuminate the ID while minimally illuminating the OD or capillary wall. Slits or apertures have been used to direct light to the capillary window region.

The biggest issue with CE is the concentration sensitivity. As researchers continue to use smaller ID capillaries to provide higher resolution, the detection limit loss becomes a huge issue. Because of the indirect relationship between detection limit and resolution, the typical ID capillary is either 50 or 75 μm. Compared to HPLC detection, the capillary has a pathlength about 12−20 times smaller and concentration detection limit has been found to be as much as 100-fold less. To improve detection limits, a "bubble cell" capillary can be used which increases the capillary internal diameter at the point of detection, increasing the sensitivity by a factor of 3 to 5 without a significant loss of resolution. Before taking the time to set up CE equipment, the user must first determine if a sufficient concentration detection limit exists for the application at hand. It is recommended to test out the sensitivity by filling the capillary with the UV absorber and to determine if there is a signal change versus the buffer background. Figure 2 shows an example of the effect of a capillary bubble cell on the separation of proteins using UV detection.

Capillary dimensions are important equipment considerations. Short capillaries can be beneficial when the user has a lower voltage power supply. Separations are dependent on V/cm and smaller capillary lengths can work with smaller voltage power supplies. Lower voltages are in turn less likely to cause electrical arcing and baseline noise issues. The main obstacle with short capillaries is to have a condensed optical system, which provides a good L_d/L_t ratio. L_d is the length in centimeter from the inlet side of the capillary to the detection window, while L_t is the total length of the capillary. In general, the goal is to have the highest possible L_d/L_t ratio or optical capillary working length, which in turn provides the greatest resolution.

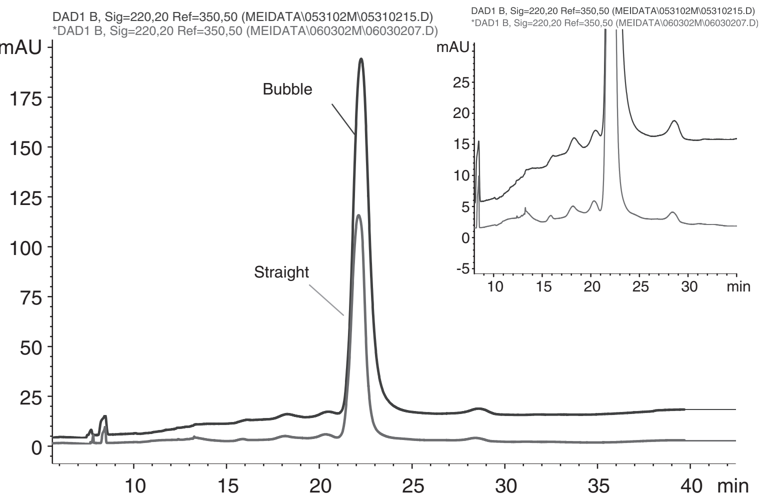

FIGURE 2 Comparison of extended light path capillary versus a standard capillary. An improvement in signal is noted with the addition of the bubble cell capillary. Data is courtesy of Amy Guo (Amgen, Inc., Seattle, WA).

Longer capillaries are advantageous for interfacing to other instrumentation, e.g., mass spectrometers or microscopes. Longer capillaries can also benefit from utilizing multiple windows and detectors. For example, one capillary could have an optical window for UV, fluorescence, and mass spectrometry (MS). When using capillary isoelectric focusing (IEF), sample loading can be improved by using long capillaries (e.g., 150 cm) and relatively large inner diameter capillaries (e.g., 100 μm ID) without loss of separation efficiency.[5]

The capillary requires temperature regulation to $\pm 0.1°C$ due to a strong viscosity dependence on sample injection and also migration time. Instruments implement two common approaches, one is to use oven/fan qto control the capillary temperature and the other is thermostatic control by liquid circulating around the capillary. Both approaches will work fine for the quantity of heat generated by CE. However, if the system is being operated at high V/cm or under high concentration of salts in the run buffer, then the liquid cooling system will become more effective. When the run buffer ionic strength (e.g., sample stacking or to help with solute−wall interactions) is increased, even small ID capillaries may generate excessive heat; therefore, capillary liquid cooling can be advantageous.

Capillary formats in manufacturer's equipment can range from 1−8 to 96 capillaries. A 96-capillary system will be advantageous for high-throughput applications whereby nearly a 100-fold is possible over conventional methods. Capillaries will have their own inlet and outlet buffers, and they will provide 96 simultaneous separations. All the capillaries are densely packed over a linear UV diode array of 1024 pixels.[6] Figure 3 shows a picture of the capillary array from Combisep, Ames, IA. This technology has been applied to a variety of applications including pK_a[7] and log P[8,9] analysis, protein analysis,[10] and small molecule analysis[11] (e.g., lovastatin production monitoring). A review of this technology from *Drug Discovery Today* magazine is available.[12]

Similar to HPLC columns, the conditioning of the CE column plays a key role in maintaining reproducibility. It is important to flush the capillary thoroughly after every run to maintain consistent precision of migration time and area. This can be a common user problem in CE whereby efficient purging and re-conditioning of the capillary is not routinely accomplished. Fused silica capillary silanol groups are typically activated with sodium hydroxide, water, and then buffer, which ensures the largest double layer, zeta potential, and EOF. A typical protocol includes a capillary purging of base for 5 min, then water for 2 min,

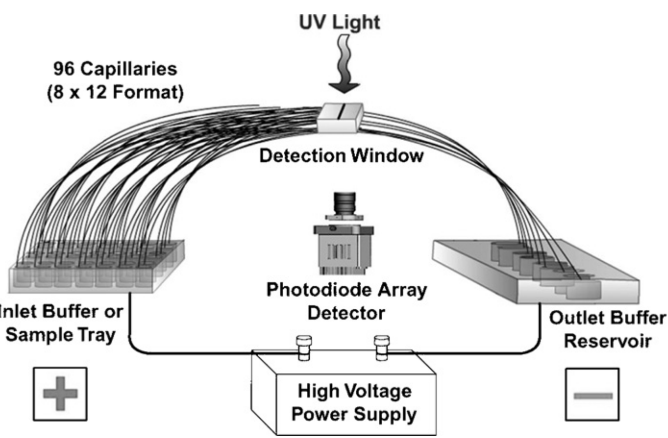

FIGURE 3 A schematic of a 96-capillary array instrument. The picture is courtesy of Combisep (Ames, IA).

and finally buffer purge for 10 min. By following this procedure, the operator will ensure an optimum EOF, shortest migration times, and no carryover from injection to injection.

5. Detector

After optimization of the correct capillary parameters (ID, OD, L_t), detection at the microscale level became the next major challenge for the survival of CE. Despite the challenges, many of the common HPLC detectors have a CE complement, e.g., absorbance, fluorescence, conductivity, photodiode array, and mass spectroscopy. Small dimensions mean universal detectors such as refractive index cannot be used. A sample of detectors will be discussed. The technical aspects of each detector will not be covered except in relation to the CE instrument. Readers are advised to consult an instrumentation textbook for more details on theory of operation.

The most common type of detector utilized in CE is the UV-Vis (absorbance) detector. The sources for the absorbance detector are tungsten/deuterium lamp combinations. This robust detection system detects the percentage transmittance and quantitates by comparing the percentage transmittance of a set of known concentrations in a standard curve. In the past, fixed wavelength detectors offered increased sensitivity when compared to variable wavelength detectors (e.g., photodiode arrays). However, improvements in electronics have made this difference negligible. The use of a variable wavelength detector allows for qualitative as well as quantitative information. The choice of buffer is important for UV-Vis measurements, whereby it is important to pick a buffer with UV transparency at the wavelength of interest. Detection limits for these detectors are usually in the micromolar range. Detection limits can be improved through the use of unique geometry capillaries [e.g., bubble cells (Figure 2) or "Z"-cells]. These detectors are inappropriate for molecules without a chromophore; many pharmaceutical compounds cannot be analyzed with these detectors.

Fluorescence detectors are useful for many quantitative applications (fluorescence is of limited utility for qualitative applications). It is rare for pharmaceutical compounds to be fluorescent, so derivatization is nearly always needed. The primary difference between fluorescence detectors is the source of the excitation. Lamps, such as xenon arc lamps, or lasers can be used as the excitation source. Continuous lamps are advantageous in that multiple wavelengths can be used, allowing for a variety of fluorophores to be selected. Lasers are more limited in their wavelength choices (the main laser choice for purchase with a CE system is the argon ion laser, with excitation wavelengths of 488 or 514 nm), but offer high power at those wavelengths. The signal achieved in fluorescence is directly related to the power of the excitation source, so more power means better detection limits. Detection limits can be 10−100 times better than absorbance. When laser-induced fluorescence (LIF) is used, the detection limits can be sub-picomolar! A number of excellent fluorophores are available with groups used for conjugating to a wide variety of different reactive groups (e.g., amino, carboxyl, etc.). Schematics of an LIF (Figure 4) and its performance for the detection of IgG antibody (Figure 5) are shown below.

Conductivity detectors are useful for their universal nature (e.g., no derivatization is required) and micromolar detection limits. These detectors are especially useful for inorganic and small organic ions. Small charged analytes in biological matrixes with a high equivalent conductivity can be detected in a ppb and sub-ppb range as long as low-conductivity background electrolytes are used.[13] A significant advantage is the ability to use the detector anywhere along the capillary since a detection window is not required. An example of a contactless conductivity detection is shown in Figure 6.

Electrochemical (EC) detectors have been used for detection in CE. EC methods offer an advantage over the spectroscopic detection methods because electrochemistry that occurs directly at an electrode surface is not limited by the small dimensions inherent in CE.[14,15] The

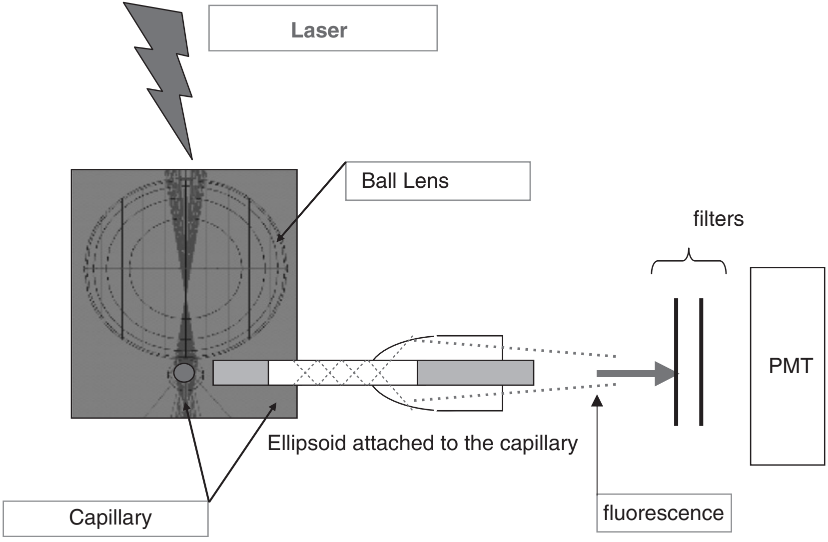

FIGURE 4 **A schematic of an LIF detector. Schematic courtesy of Picometrics.**

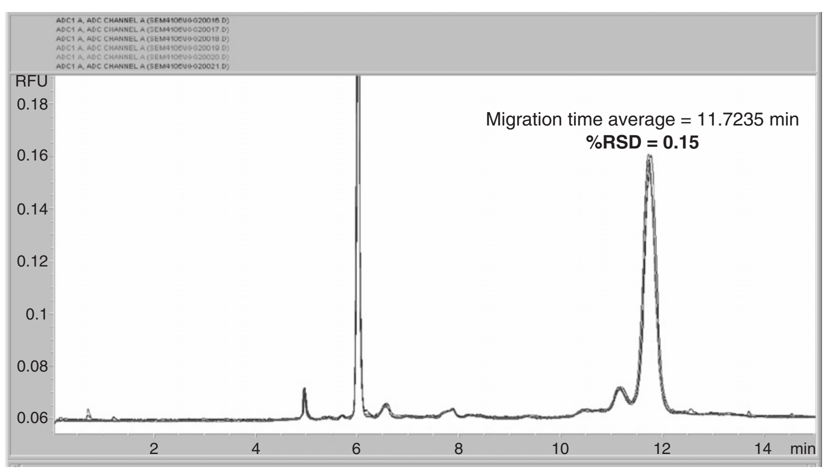

FIGURE 5 **Six replicates with a migration time %RSD of 0.15% for a 100 ng/mL solution of IgG antibody. Data courtesy of Picometrics.**

difficulty in conducting electrochemistry in the presence of an electrophoretic current has probably limited the widespread application of Electrochemical detector (ECD).[8] Pulsed amperometric detection (PAD) has shown utility for the analysis of carbohydrates.

Convergent Bioscience has an interesting approach to detection in CE by using a CCD (charge-coupled device) camera to image the entire capillary (Figure 7). A UV-transparent capillary is needed and the entire capillary is excited with a xenon lamp. This is useful in cIEF

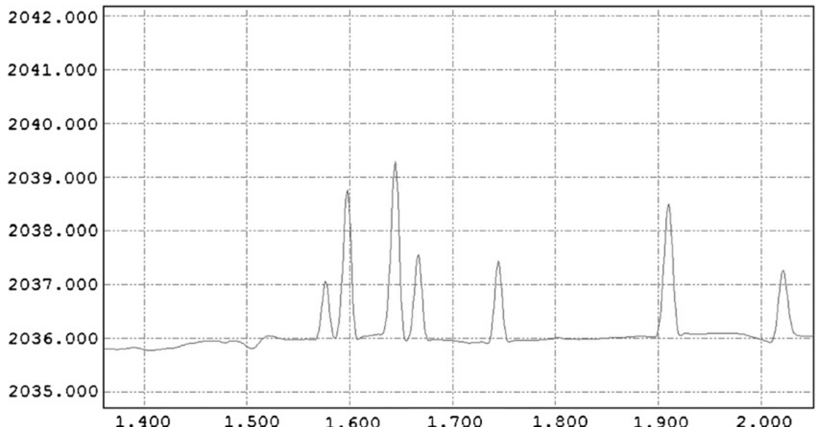

FIGURE 6 **An electropherogram using detection by contactless conductivity of anions with an injection of 20 mbar for 5 s. Sample concentration (in order) 0.5 ppm bromide, chloride, nitrite, nitrate, sulfate, and fluoride and 1.0 ppm phosphate. Data courtesy of TraceDec.**

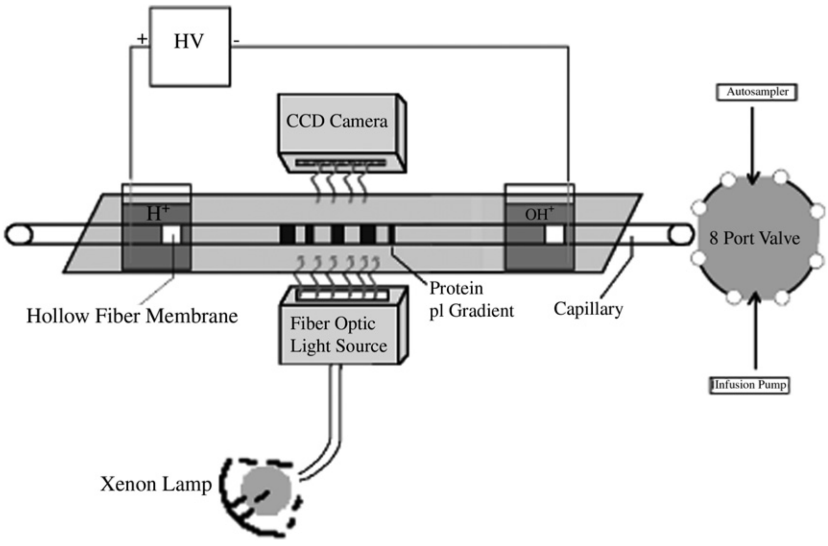

FIGURE 7 **Schematic of a whole capillary-imaged CE instrument. Drawing courtesy of Kevin Daniel O'Flaherty and Christopher James O'Flaherty (adapted from the Convergent Bioscience Web site, www.convergentbiosci.com).**

as mobilization is not required. Conventional cIEF technology uses on-column UV-Vis absorption. After the IEF process is complete (e.g., sample proteins have been separated according to their pIs), the focused protein zones are migrated past the detector. The mobilization step introduces several sources of variability such as uneven separation resolution and result in increased analysis time relative to the imaged capillary. The iCE_{280} instrument is capable of up to eight samples per hour with resolution of up to 0.03 pI units over a pH range of 2−11 and results comparable to gel IEF.

6. Interfacing

MS has been successfully interfaced to both gas and liquid chromatography and the interface to CE has also been successfully developed. CE−MS is serving an analytical role in the area of small sample sizes commonly found in biological, biomarker, or cellular samples. Liquid chromatography is ideally suited for trace analysis when large amounts of sample are available. Compared to HPLC, CE offers different selectivity, higher efficiency, fast method development, and shorter analysis times.

With the work of Fenn and co-workers, liquid chromatography−electrospray interfaces for mass spectrometers were developed in 1984.[16] Subsequently, the Pacific Northwest Laboratory began work in the area of CE−ESI−MS under the direction of Richard Smith and published the initial paper describing on-line CE−MS in 1987.[17] Initial interface designs involved removing the polyimide at the end of the capillary in favor of a layer of silver for electrical contact. This interface was limited due to below optimum flow rates and limited lifetime of the metallized capillary. The introduction of the sheath flow design[18] dramatically improved the CE−MS results. In lieu of being connected to a standard outlet buffer, the CE−MS interface used the outlet end of electrophoretic capillary connected directly to the electrospray mass spectrometer.

A CE-to-MS hardware adapter is needed to position the end of the capillary near the MS entrance port. The adapter typically includes alignment screws to adjust the capillary for maximum performance. The CE−ESI−MS sprayer consists of the capillary holder with a port to provide sheath liquid bathing the outer wall of the capillary end. The outer sheath flow provides an electrical contact with the CE system and facilitates the electrospraying of buffers at ideal flow rates and eluant solution properties. Typically, the small coaxial sheath flow is in the flow rate range of 1−5 μL/min. Optionally, there is nebulizing gas to produce smaller droplets and to help ionize the liquid before it is delivered to the entrance of the MS. The capillary can have orthogonal spray geometry to improve system cleanliness and reduce solvent background. A pictorial of a CE−MS interface is shown in Figure 8.

The capillary inlet should be at a similar height as the capillary outlet to avoid siphoning. Capillary outlet ends are commonly etched conical capillary tips to minimize the mixing and to stabilize the electrospray. When using the electrospray source, typical CE buffers are not volatile and can cause instability with the electrospray source. Additionally, the performance of CE−MS improves with running buffers of lower ionic strength and surfactant concentration. High salts can reduce ion intensity (through ion pair formation) and it is possible that salt

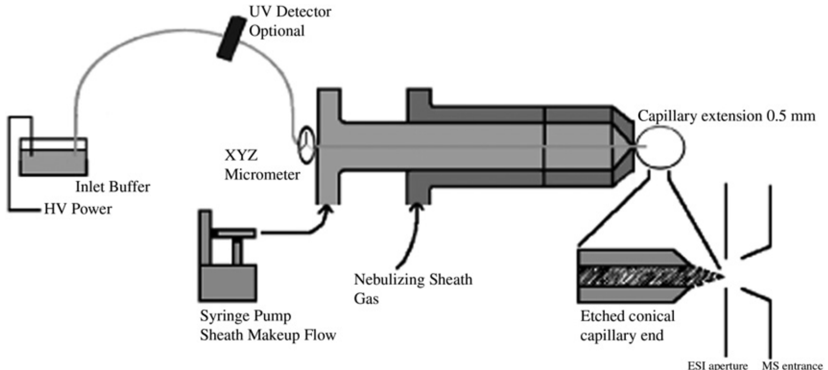

FIGURE 8 Schematic of a CE−MS interface. Drawing courtesy of Kevin Daniel O'Flaherty and Christopher James O'Flaherty.

FIGURE 9 A Prince Technologies CE (Groton Biosystems, Boxborough, MA) interfaced to a Thermo Finnigan mass spectrometer.

crystals can contaminate or plug the MS entrance. Volatile reagents based on 5−100 mM ammonium acetate (pH 3.5−5.5) or acetic or formic acid in aqueous or organic solvents have proven well suited for CE−ESI−MS. It is possible to use running buffers as high as 0.1 M salt as long as there is a suitable sheath flow. Performance of CE−MS improves as the ID of the capillary is reduced, approximately 25 μm or less is optimum; however, plugging is a greater issue at smaller capillary internal diameters.[19]

Commercial interfaces to allow CE−MS upgrades are available. As an example, Thermo Finnigan offers an Electrospray Interface (ESI) upgrade kit and instructions for their ESI mass spectrometer. The CE adapter includes a capillary micrometer adapter, and auxiliary liquid and gas sheaths. An example of a CE interfaced to an MS is shown in Figure 9. To prevent an unstable electrospray, it is recommended that the sheath liquids are fully degassed prior to using. The manufacturer recommends sheath liquids to be Isopropanol (IPA)/H_2O, 1% acetic acid or H_2O/MeOH, 1% acetic acid. The flow rate of the sheath liquid can range from 1 to 3 μL/min and will vary depending on the position of the CE capillary relative to the sheath tube and the viscosity of the sheath liquid. Ideally, the capillary should be slight protruding from the sheath liquid, but it has been found that the optimum capillary protrusion will depend on the viscosity of the run buffer. Higher viscosity of liquids are optimized when the capillary is protruding, while lower viscosity liquids are optimized when the capillary is recessed with respect to the end of the ESI nozzle. The use of a sheath gas can increase the stability of the spray, but it is user and application dependent.[20,21]

An example qualification test for CE−MS involves the simple separation of two small pentapeptides; Leu-enkephalin (*m*/*z* 556) and Met-enkephalin (*m*/*z* 589). The sample mixture is composed of 100 pmol/μL of each component in 100% H_2O, 1% acetic acid. The CE column is a 50 μm ID × 65−80 cm bare fused silica pre-washed with 0.1 M NaOH and H_2O, and then pre-rinsed with the running buffer solution of 100% water, 1% acetic acid. The injection method is pressure at 50 mbar for 10 s and an ESI sheath liquid in the ratio of 60/40 IPA/water, 1% acetic acid at 2 μL/min. The spectra are collected every 3 s over a 350−3000 *m*/*z* scan.

A CE run can also be spotted on a matrix assisted laser desorption ionization (MALDI) plate by using a probot microfraction collector by LC Packings. The matrix solution, 2 mg/mL

of alpha-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile and 0.1% Trifluoroacetic acid (TFA), is added at a flow rate of 4 μL/min. One spot is deposited every 15 s and in turn is analyzed by the mass spectrometer. Calculation of an entire CE−MS run can consist of 1000−1500 single spectra collected within 5 min.

7. Capillary Electrochromatography

Capillary electrochromatography (CEC) is one of the most recent developed methods in CE. CEC is a hybrid technique between CE and HPLC. The first thorough study of CEC appeared in 1987.[22] In CEC, there are two major separation mechanisms, namely solute/bonded phase interactions and electrophoretic mobility. For neutral species, only the bonded phase interaction results in differences in migration rates while charge species movement through the capillary depends on both separation mechanisms. Columns used in CEC include open tubes, packed columns, monoliths and microfabricated structures. One of the first successful open tubular CEC separations was described by Tsuda and co-workers using an octadecyl-modified 30 μm ID capillary.[23] With open tubular, the stationary phase is affixed to the inner wall. To reduce diffusion, it is recommended to use capillary ID of <20 μm[24] and IDs near 50 μm if the coating has long chemical bonds radiating toward the interior of the capillary. On-capillary windows can be used with this technique, but the window needs to be gently prepared so as not to damage the interior coating. Since the capillary internal diameter needs to be relatively small to reduce diffusion, unsatisfactory results occur due to limits on sample size as well as path length in optical systems.

Another option is packed columns, whereby capillary IDs of less than 100 μm can be used. Packing can be reverse-phase HPLC spherical particles with diameters of 1.5−5 μm. Retaining frits are used to hold the chromatographic material in place. Optical detection is not typically done on capillary due to light scattering from the particles. With this method you need an open segment of open capillary butted against the packed section. Connections can be secured by polytetrafluoroethylene (PTFE) shrinking. Frits or sintered silica are prepared on both ends of the packing material which are mechanically strong and can resist pressures used to pack/rinse the column. The frits are sintered silica-based packing using heat at typically 430°C for 12−15 s. The hope of improved separations with packed CEC columns has not currently been realized and some researchers believe that the silanol-based frit causes peak tailing.

Current research in CEC involves the use of monolith capillaries, which are fritless, packed capillaries having stationary phase bound to the capillary wall. Using porous polymer monoliths, the retention of a packed column can be found in an open tubular capillary. In general, CEC remains unsettled. Frit technology is unreliable and research into monolithic capillaries is still a work in progress. Recent progress in CEC can be found in the reviews by Colon and co-workers.[25,26]

8. Software

Like with most analytical instrumentation, the software is a key to increase the power of the instrument performance. Similar to HPLC, CE requires software to perform fast, reliable quantification of the electropherogram peaks. General features include a good chromatogram software package. Special features include the ability to calculate EOF and mobility. The software should also have control over the CE system for features such as fluid handling (e.g., vials), detector wavelength, pressure, high voltage, and limits on current. The software should allow sequence run tables with methods and also allow collection from single or multichannel data acquisition boards.

II. EQUIPMENT-RELATED ISSUES

CE has suffered from an assortment of common operator errors, which in turn have characterized the technique as not being robust. Like any analytical piece of equipment, there can be hardware and chemistry/operator issues. CE requires a keen background and user knowledge of the technique so as to avoid common problems that may initially be diagnosed as instrumental issues. For the early user of CE, the table below lists some common problems followed by their root causes and corrective actions. Following the suggested corrective actions should help the beginner get the maximum performance out of the CE instrumentation.

Problem	Root cause	Corrective action
Poor resolution	Overinjection of sample	Injection plug should be 1−2% of total capillary length
	Capillary end not square	Re-cut capillary, laser cuts are best. Follow procedure in reference 3
	Poor stacking between run and sample buffer	Sample dissolved in buffer that is 10 times more dilute than run buffer. If sample buffer cannot be diluted, increase run buffer ionic strength, however, not beyond point whereby excessive heat or current results (>100−200 μA)
	Poor selection of column ID	Reduce column ID
	Sample residue on outer capillary wall	Dip capillary in water rinse prior to high voltage (HV) start
	Capillary overheating	Improve temperature regulation
Poor sensitivity or no signal	Detector lamp either is not on or is weak. Also, poor selected optical wavelength	Replace detector lamp if necessary. Switch lamp on and check wavelength
	Light intensity through cell is low	Confirm window alignment and cleanliness. Confirm polyimide layer of capillary is properly removed from detector region and that light beam is in focus
	Broken or plugged capillary	Replace capillary or remove a few millimeter on inlet side of capillary and retry
	Sample is old or diluted	Make fresh standards or increase concentration
No current	No voltage established	Apply voltage
	Air bubble in capillary	Remove air bubble and source, reduce field strength, V/cm. Degas liquids
Current fluctuations	Voltage is high	Lower voltage
	Small air bubbles or capillary not properly flushed	Flush capillary with filtered (0.22 μm) degassed buffers
Poor precision of migration time and area. Poor accuracy of sample	Capillary inner surface is not clean	Clean capillary with methanol-water, 0.1 N HCL and then 0.1 N NaOH

(Cont.)

Problem	Root cause	Corrective action
	Contamination of run and sample vials or buffer depletion/evaporation	Replace buffer. Implement cooling to reduce buffer evaporation. Especially important when organic solvents are used
	Sample-to-sample viscosity differences	Use internal standards
	Electrokinetic injection and dissimilar ionic strength of samples − matrix effects	Control ionic strength of sample buffer
	Siphoning from one buffer to another	Balance fluid levels between inlet and outlet side
	Voltage and temperature not stable	Improve control of voltage and temperature

III. VALIDATION AND COMPLIANCE REQUIREMENTS

For the purposes of this chapter, software validation and qualification are ignored. A separate chapter in this volume (Chapter 8) focuses on CFR 211, Part 11 requirements.

Instrument qualification or validation refers to the process of demonstrating an instrument's suitability for its intended use. The first step is to write protocols to cover the four main qualification elements: design qualification, installation qualification, operational qualification, and performance qualification. Another qualification element, re-qualification, can be written as a separate protocol or included as part of the PQ protocol. These protocols must be performed in sequence. If the protocol meets its acceptance criteria, then the next protocol in the sequence can be executed.

A. Design Qualification

Design qualification (DQ) is the process used to determine a system that will function within the intended purpose. It can be compared to a user's requirements document for a piece of software. For example, if a CE is being purchased to run DNA sequencing samples, then the system purchased will need to include a fluorescence detector. The main vendors for CEs have similar options for their CE instruments reducing the utility of DQ protocols. Their main utility is to define the specific equipment needed for the purchase order. This only needs to be performed before the system is purchased initially. If desired, this can also be done when additional features need to be purchased (i.e., new detectors, etc.).

B. Installation Qualification

IQ is the process of ensuring that the instrument is installed correctly, the documentation have been created (e.g., logbooks) or stored (e.g., instrument manuals, original software disks, etc.), and inventories have been updated (i.e., serial numbers are input into the appropriate computer systems).

IQ is usually performed upon initial setup in the laboratory. IQs should be repeated when new components are added (e.g., adding an MS detector) or the system is moved to a new

location where the move could affect the instrument's operational status. IQs do not need to be performed for replacing consumables (e.g., capillaries, filters, etc.) or when the instrument is moved slightly (e.g., different location on the same bench). If a replacement component is added to the system, the IQ can be amended to show the replacement of the component.

Another important part of IQ is the generation and approval of an instrument-operating procedure, if applicable. This procedure defines the use and care for a CE instrument. This generic procedure is useful to train new analysts on the technique, define the requirements for routine and corrective maintenance, and other relevant information.

C. Operational Qualification

Operational qualification (OQ) is the process where each component of the system is demonstrated to work properly with the appropriate accuracy and precision. OQ demonstrates that the system works as the *manufacturer* intended it to work. Example tests for each component of the CE system are listed below.

Power supply

High voltage accuracy
High voltage stability
Stable voltage control of $\pm 0.1\%$ is necessary to maintain acceptable migration-time reproducibility.

Autosampler

Temperature accuracy
Temperature stability
The temperature control of the autosampler should be similar to the temperature control of a refrigerator. The unit should be able to maintain a temperature of 5 ± 3°C with accuracy within 0.1°C.

Injector

Precision (hydrostatic and electrokinetic)
Injection time linearity (hydrostatic and electrokinetic)
The Groton Biosystems protein analyzer tests injection reproducibility at three pressures (e.g., 25 mbar at 0.2 min, 50 mbar at 0.1 min, and 100 mbar at 0.05 min). Each injection pressure is injected five times and the complete injection linearity relative standard deviation (RSD) needs to be less than 5%. In addition, the carryover from successive injections is investigated.

Capillary compartment

Temperature accuracy
Temperature stability
The protein analyzer tests temperature stability and accuracy at three set points over 10 min (e.g., 30−40−50°C). The temperature linearity is collected on the stability data as well.

Detector

Source intensity
Wavelength accuracy
Noise

Drift
Linearity
The protein analyzer tests response linearity (absorbance/fluorescence), wavelength accuracy and UV linearity, dynamic noise, drift, and the zero offset.

OQ is performed after completion of the IQ. An OQ is also performed after major maintenance (only on the specific hardware).

D. Performance Qualification

PQ is the demonstration that the entire system functions as one unit. PQ is also the demonstration that the system works as *you* intend to use it. This is done by running an assay and ensuring all system suitability parameters are met. If a number of different methods are going to be run on the system, then the "worst-case" method should be selected. It is not necessary to run every method on the system, but this can be done.

For the CE analyzer, a sample method is run to demonstrate that the entire system is functioning properly. A cystosine molecule, 100 ppm, is injected and separated at 30 kV. Reproducibility of migration time and area is tested. The reproducibility for nine consecutive runs is expected to have an RSD of less than 1% for migration time, peak area, and corrected area. The linearity is tested by injecting sample at three different pressures and adjusting the time to arrive at equal amounts injected [Equation (2)]. The three settings and three consecutive runs should have an RSD of less than 5% for migration time, peak area, and corrected area.

E. Re-qualification

Re-qualification (RQ) is a combination of OQ and PQ. This can be written as a separate protocol or can be included as part of the PQ protocol. This protocol can be executed anytime to demonstrate that maintenance has been performed, or periodically to demonstrate that the system is within tolerances. The RQ protocol should be able to be executed as needed without getting new signatures. Suppose the power supply has been corrected. An RQ protocol could include the high voltage accuracy and stability tests along with system suitability for one of the methods run on the system. This would demonstrate that the system is performing appropriately after the maintenance, as the *manufacturer* and *you* intend.

F. Finalizing the Package

Once the qualification protocols are complete, a qualification report is written. The qualification report should summarize the results from each of the protocols, explain any deviations or non-conforming data, and declare that the instrument is suitable for use (or unsuitable if the acceptance criteria were not met or the non-conforming data could not be justified).

IV. FUTURE DEVELOPMENTS

Early on in CE, the literature was flooded with applications that drove the technology beyond graduate school curiosity. Since Microphoretics introduced the first commercial CE instrument in 1988, equipment suppliers have come and gone. Many of the large equipment suppliers entered the CE market and learned the market cannot support too many instrument

companies. The decision to purchase CE equipment has not been as rapid as first thought. During early instrument introductions, CE has had criticism in the areas of injection, separation, and detection. To help end users gain acceptance, CE must first overcome a number of obstacles such as (1) technical and training issues with the end customer, (2) competition against LC instruments, (3) CE instrument validation issues, and (4) concentration limit on sensitivity issues. In the past, validation of methods using CE has been difficult since CE has had specific problems such as injection repeatability, capillary-to-capillary variation, and instrument-to-instrument differences. Over the years, CE instruments have improved injection precision and reliability, and standard CE detectors have matured, making the utilization commonplace. However, due to these early obstacles, the technology still suffers from a stigma on being difficult to use and is not as robust as HPLC. Nonetheless, CE is still alive and well, and has gained popularity in a variety of applications in which HPLC has difficulty, i.e., small and large molecules. Capillary gel electrophoresis with pumpable, user replaceable gels is a logical replacement for slab-gel electrophoresis due to providing on-column detection and quantitation.

Many of the problems of CE have been solved, which included operator expertise and robust capillaries. It was found that the EOF in CE was not constant from run to run, day to day, or capillary to capillary. Coated capillaries were not found to be reliable enough to ensure consistent EOF. It was found that via use of dynamic coatings, the EOF can be controlled to allow migration time precision to approach LC values on a run-to-run, day-to-day, or capillary-to-capillary basis. The quantitative precision of CE is similar to LC, provided an internal standard is employed. For instance, a collaboration exercise between eight biopharmaceutical companies and a regulatory authority showed less than 2% RSD for the determination of bovine serum albumin (BSA) molecular weight and less than 9% RSD for the quantitation of the three main components of IgG (IgG light chain, IgG heavy chain, and IgG non-glycosylated heavy chain).[27] Similarly, a group of seven pharmaceutical companies found agreement within 0.6% for the chiral analysis of Clenbuterol.[28] Migration time reproducibility can approach 0.4%, even across many companies, when the internal standard is used.[27]

Today, CE features include simplicity of method development, reliability, speed, and versatility, which continue to provide popularity to the technique. CE is valuable because it is quantitative, can be automated, and can separate compounds that have traditionally been difficult to handle by HPLC. CE is currently used in many industries and has developed niche applications in the areas of biological science, pharmaceutical, biotechnology, forensic science, and environmental safety. Biological science applications include genetic analysis via introduction of polymer gels into the separation buffer and analyzing complex proteins, glycoproteins, and protein–protein interactions. Pharmaceutical applications include providing efficient chiral analysis using sulfated cyclodextrins, and forensic applications include separation of highly polar basic drugs used in forensic toxicology.

CE is also finding a place in the world of Process Analytical Technology (PAT). PAT is an FDA initiative to use various technologies to improve the efficiencies in the pharmaceutical and biopharmaceutical industry. By automating the collection of data in near real time, analytical data can be used to control the process versus doing "postmortem" analysis. Greater process understanding is possible due to increased data point collection. By understanding the manufacturing process in more detail, improvement in manufacturing precision is possible along with improved product quality. Groton Biosystems currently offers an on-line CE system which can completely automate the sampling, sample preparation, and analysis of proteins or other species that can be separated by CE. Sampling and analysis systems can be attached to bioreactors, process pipes, downstream purification column, etc. It is expected that in the future more bench scale analytical systems (e.g., CE, HPLC, and GC) will be used in the areas of process, pilot, and manufacturing departments.

It is expected that CE instrumentation of the future will be dominated by electrophoretically microfabricated chips which include plastic separation channels and integrated detection systems. Specific chips can be designed for specific modes of CE such as sodium dodecyl sulfate (SDS) gel, micellular electrokinetic chromatography (MEKC), and capillary IEF. With these chips, the sample preparation is done in the microfluidic channels before separation and detection. It can be said that the future of single capillary and capillary array electrophoresis is in microfabricated systems. These lab-on-a-chip devices enable are a network of channels and wells that are etched onto glass or polymer chips to build mini-labs. Pressure of electrokinetic forces move picoliter volumes in finely controlled manner through the channels. Lab-on-a-chip device enables sample handling, mixing, dilution, electrophoresis, and chromatographic separation and detection on single integrated systems. Currently, applications include DNA, RNA, and protein sizing assays.[29] The proteinassay-optimized sizing ranges up to 80 kDa and is ideal for analyzing reduced antibodies. Electrophoretic microfabricated chips are currently offered by Agilent Technologies and Caliper Technologies. Currently, CE is the basis for virtually all microfluidics for a lab-on-a-chip device.

V. SUMMARY

CE instrumentation has come a long way from its rather humble origins. Today's CE instrument is a robust, user-friendly instrument capable of analyzing a wide range of analytes. CE has established itself as an important instrument in the analytical laboratory and an indispensable one in many laboratories. The improvements in the equipment over the years have allowed this to occur. The future for CE is bright and further improvements, including additional robustness and miniaturization, are sure to set the stage for even brighter prospects.

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4 METHOD DEVELOPMENT FOR PHARMACEUTICAL ANALYSIS

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ABSTRACT

Development of capillary electrophoresis (CE) methods to be applied in late phase of pharmaceutical development for low-molecular-weight compounds is discussed in great detail. The important message is that all stakeholders of the analytical methods, i.e., the development lab and the receiving quality control (QC) labs, need to collaborate closely to generate a test method description that is well written, easy to apprehend, simple to perform, and that will result in a robust and reliable performance within a QC environment. Indeed, the late phase methods are developed for transference to the QC labs and are supposed to last for the entire product lifetime. Especially for CE methods, there are additional demands to be satisfied. Thorough knowledge and skills in CE-specific applications, which may be unlike typical chromatographic expertise, are required. Development strategies specifically applicable to CE are discussed with practical examples. Although the approaches proposed are intended for late phase method development of low-molecular-weight pharmaceutical products, they can be easily applied in the early phase of development and also for high-molecular-weight compounds.

I. INTRODUCTION

Capillary electrophoresis (CE) methods for pharmaceutical quality control (QC) analysis are developed and applied in early to late phase of development. Although there are many application areas in early development as will be covered in other chapters of this book, this chapter will emphasize the late phase development of low-molecular-weight compounds. Nevertheless, the approaches that are discussed for late phase development may also be applied for early methods and to high-molecular-weight compounds.

Late phase method development in pharmaceutical QC is performed for registration stability studies and for release of the drug substance (DS) and drug product (DP) validation batches. Late phase methods are intended to be transferred to the operational QC laboratories for release testing of the production batches. Preferably, late phase methods are developed as such that they are fast, robust, reliable, and transferable methods. Therefore, it is crucial to devote adequate time, thoughts, and resources to the development of such methods. Precise, accurate, robust, reliable, and transferable methods are required for QC testing.[1] In the classical approach of method development (still followed by many pharmaceutical companies), the methods are constructed in an analytical development (AD) lab. The AD lab develops and then transfers the method to the QC or the stability lab. In their effort to develop the best method as they can, the development lab typically merely has the opportunity to talk to the application labs. The application labs on their turn are focused on testing samples and talking to the development lab may even be considered as a waste of time. As a result the needs of the customers are not accounted for during development of methods, which potentially may give rise to issues during transfer and even to complaints during application. In addition, the performance of the method during real analysis at the customer side is also not known to the development lab. Therefore, complaints are generally perceived as subjective and not real. The real challenge of the method is performed during transfer studies. Many method-related surprises are frequently observed at this stage. In cases with more severe issues the transfer fails and the method has to be redeveloped, resulting in delays that may heavily impact the product development time lines and even jeopardize the filing date.

Frequently CE methods tend to fail due to inadequate development and lack of expertise in the receiving labs. Although CE has been available for decades, the technique is still considered new. Skilled CE analysts are not readily available in the receiving labs. The same is mostly justified for the development lab too, since sometimes an HPLC analyst is asked to develop a CE method. After a short (1 week) training the HPLC analyst pretends to be a CE expert. Indeed, due to the apparent simplicity of CE a method can be easily developed and established. It is at

this stage that many system failures are observed. When CE machines and methods are being cursed as if they are processed by a ghost, statements like "CE methods are lousy," "CE is not reproducible," "CE is not sensitive," or "CE methods are not robust" are frequently uttered.

The approaches and strategies presented in this chapter are intended to overcome these issues for CE methods. Recently a more advanced approach toward chromatographic method development was introduced[1,2] in pharmaceutical product development that also is beneficial for CE methods. In the advanced approach (i) the voice of the "customer" is captured, (ii) key process input variables are identified, (iii) critical to quality (CTQ) factors are determined, (iv) several method verification tests are installed, (v) proactive evaluation of method performance during development is performed, (vi) continuous customer involvement and focus is institutionalized, and (vii) method capability assessment (suitability to be applied for release testing against specification limits) is established.

The aim of the advanced approach in method development is a first time right development process for late phase methods, with targeted customer focus, robust, reliable, and transferable methods. These methods result in reduced customer complaints, less rework, improved quality of the methods, objectively monitored method performance, improved partnership with the customers, significantly reduced OOS, and a high probability for success during method transfer. In contrast to the classical approach, a redesigned process is needed that starts with the generation of a method definition requirement (MDR). This form contains target values that are set for many CTQ's prior to the start of method development process. During course of the method development process, Design of Experiment (DOE) approaches and Measurement System Analyses (MSA)[3] studies are systematically performed. The performance of the process is continuously monitored by a formal feedback round.

A schematic representation of the advanced development process is shown in Figure 1. As can be observed, method development is a continuous practice in which all stakeholders are involved (no departments groups or labs included). Improving the quality attributes of methods is the goal, resulting in a reduction of complaints at the customers. This is achieved through partnering (continuous involvement) with the application labs early in the method development process. The customer's voice is captured early and accounted for during

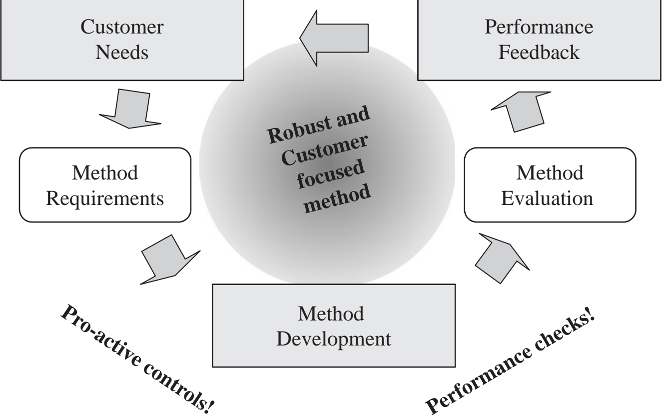


FIGURE 1 Schematic overview of an advanced method development process. Method development is a continuous process in which all stakeholders collaborate intensively to design the final method. Reprinted with permission from reference 1.

development of the method. General needs are translated into CTQ attributes and treated as such during development. Product-specific customer needs are captured in the MDR.

All requirements described in the MDR are considered during method development. Either an intuitive or an experimental design approach may be applied when optimizing methods, resulting in an optimal separation of the main compound and all relevant impurities in a reasonable analysis time using typical CE conditions. The development activities are mainly performed by the AD lab (Method Development Phase). Before submitting the method it should be evaluated for robustness and daily lab-to-lab application performance (Method Evaluation Phase). Is the method doable in all the application labs? Is the method description acceptable? Are all customer needs accounted for?

The final method description is drafted following development and evaluation phases, fully validated (Method Validation Phase) and transferred (Method Transfer Phase) to the application labs. After the thorough evaluation built in the development process, the transfer activities are expected to be carried out seamlessly. It is almost guaranteed that method transfer will be successful since by then the application labs would already be familiar with the method. Each time samples are analyzed by the application labs, method performance is monitored (Method Performance Monitoring Phase) and evaluated. The information gathered is provided to the development labs, thereby they are continuously informed about both the good and poor method performance (Performance Feedback). Potential issues are discussed based on objective data and are resolved in close collaboration with each other. As can be derived from Figure 1, the advanced method development approach has proactive controls (method requirement), performance checks (method evaluation), and reactive controls built within the process, allowing to reduce customer complaints, avoid rework, improve method quality, track method performance, involve the customers, and promote partnership.

II. CE METHOD TYPES USED IN QC TESTING

A. Identification Methods

Identification of compounds of interest is performed using relative migration times. The relative migration time of the peaks of interest are compared with the relative migration time of the peaks for the compounds of interest in the selectivity solution: a qualitative reference solution containing the compounds of interest. If the relative migration time is within a range of, e.g., 5%, the peak recognition can be considered as positive. A suggested sample injection sequence can be

1. Blank
2. Selectivity sample solution injection
3. Sample solution injections
4. Selectivity sample injection.

In order to show that the selectivity of the separation remained constant throughout the entire sample sequence, the selectivity solution is re-injected after each 10 sample injections and at the end of the sequence.

B. Purity Methods

1. Chiral Methods

Enantiomeric determinations are typically done according to a normalized peak area (area%) calculation procedure. A reporting threshold solution containing the lowest level to be

reported by specification of the product is injected to demonstrate the detectability of the method. This is typically in the order of 0.10% compared to the main compound concentration. The selectivity solution is utilized to demonstrate the separation capability in the method and to allow peak identification. A suggested sample injection sequence can be

1. Blank
2. Reporting threshold solution injection
3. Selectivity sample injection
4. Sample solution injections
5. Selectivity sample injection (after each 10 sample injections and at the end of the sequence).

2. Achiral Purity Methods

Achiral purity determinations are typically done according to an external calibration calculation procedure, either with or without internal standardization. The detectability of low-level impurities is generally considered to be poor in CE, due to the inherently low sensitivity of on-column, most frequently applied UV detector. In order to allow optimal detectability of low-level impurities, the calibration is performed against a 10% w/w (compared to the nominal concentration of the sample solution at 100% w/w) reference standard solution. There are many different variations on external calibration procedures (e.g., fixed or moving average), however, the approach discussed here is frequently applied in industry. Two independent reference solutions (separate weighing) are typically used. The first is applied to check the accuracy of the second reference solution weighing, since the calibration is done on the second reference solution. The second reference solution is injected 5 × to generate the calibration average response value and is re-injected after every 10th sample solution injection and at the end of the sequence to show that the initially determined average calibration response value is still valid throughout the entire sample sequence (demonstrate that there is no system drift). The sample solution remains at 100% w/w level. A suggested sample injection sequence can be

1. Blank
2. Reporting threshold sample injection
3. Selectivity sample injection
4. Check reference solution 1 injection (1 ×)
5. Reference solution 2 injection (5 ×)
6. Sample solution injections
7. Reference solution 2 re-injection (after each 10 sample injections and at the end of the sequence)
8. Selectivity sample injection (after the last reference solution 2 at the end of the sequence).

C. Assay of Main Compound and Determination of Impurities Methods

Chiral or achiral assay and purity determinations are done according to an external calibration calculation procedure, either with or without internal standardization. The calibration is performed against a 10% w/w (compared to the nominal concentration of the sample solution at 100% w/w) reference standard solution. The sample solution for the purity determination remains at the 100% w/w level, while that of the assay determination is diluted 10 times. The reason for the difference in concentration levels is similar to the purity method. A suggested sample injection sequence can be

1. Blank
2. Reporting threshold sample injection
3. Selectivity sample injection
4. Check reference solution 1 injection (1 ×)
5. Reference solution 2 injection (5 ×)
6. Sample solution injections at 100% w/w level for purity determinations
7. Sample solutions injections at 10% w/w level for assay determinations
8. Reference solution 2 re-injection (1 × , after each 10 sample injections and at the end of the sequence)
9. Selectivity sample injection (after the last reference solution 2 at the end of the sequence).

D. Assay Methods

1. Chiral Assay Methods

Enantiomeric assay determinations are typically applied to characterize racemic mixtures using the normalized peak area (area%) calculation procedure. The selectivity solution is utilized to demonstrate the separation capability in the method and to allow peak identification. A suggested sample injection sequence can be

1. Blank
2. Selectivity sample injection
3. Sample solution injections
4. Selectivity sample injection (after each 10 sample injections and at the end of the sequence).

2. Achiral Assay Methods

Achiral assay determinations are done according to an external calibration calculation procedure, either with or without internal standardization. The calibration is performed against a 100% w/w (compared to the nominal concentration of the sample solution at 100% w/w) reference standard solution. The sample solution for the assay determination remains at the 100% w/w level. A suggested sample injection sequence can be

1. Blank
2. Reporting threshold sample injection
3. Selectivity sample injection
4. Check reference solution 1 injection (1 ×)
5. Reference solution 2 injection (5 ×)
6. Sample solution injections at 100% w/w level for assay determinations
7. Reference solution 2 re-injection (1 × , after each 10 sample injections and at the end of the sequence)
8. Selectivity sample injection (after the last reference solution at the end of the sequence).

E. Limit Test Methods

A limit test is a semi-quantitative test that allows the determination of the presence of a specific compound above a well-defined concentration level. The determinations are done according to an external calibration calculation procedure, either with or without internal standardization. The calibration is performed against a reference solution at threshold level (e.g., 0.10% w/w compared to the nominal concentration of the sample solution at 100% w/w).

The sample solution for the limit test determination remains at the 100% w/w level. A suggested sample injection sequence can be

1. Blank
2. Selectivity sample injection
3. Reference solution at threshold injection (5 ×)
4. Sample solution injections at 100% w/w level
5. Selectivity sample injection (after each 10 sample injections and at the end of the sequence).

III. METHOD DEVELOPMENT PROCESS

As discussed in reference 1, as with HPLC methods, late phase CE methods are developed in close collaboration with the customers (QC labs). The diagram presented in Figure 2

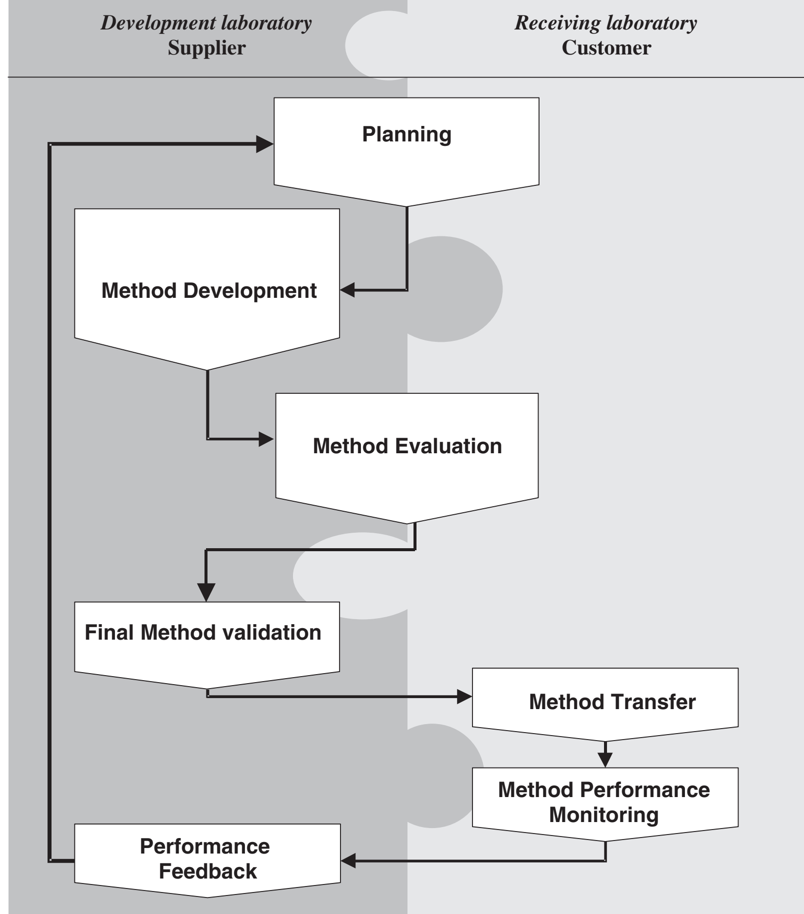


FIGURE 2 Diagram illustrating the full method development process for late phase methods. Taken with permission from reference 1.

demonstrates the late phase development process in more detail[1,2,4] and clearly shows the interlink between the development and the receiving laboratory. The collaboration of the development lab (supplier) and the receiving lab (customer) should be like two pieces of a puzzle that closely fit each other to complete the overall picture. Both labs work together in a joint effort to develop their product, i.e., the analytical method. In this way, the quality of the delivered method is guaranteed. The late phase development process is performed in different steps and can easily require a couple of months to complete. Therefore, an early start with a thorough planning is a must. The following steps are included[1]: Planning, Development, Evaluation, Validation, Transfer, and Performance Monitoring.

IV. PLANNING PHASE

A. Method Definition

Before starting method development the requirements for the late phase method are set in an MDR. The MDR is a formal document in which all parties involved agree upon the method development requirements and is generated in close collaboration and in agreement with all stakeholders including Quality Assurance (QA), QC sourcing sites, Stability, Development, and Regulatory. The development process starts with a thorough development plan. Time lines are set and preferably a communication plan is made available. The same method development team will evaluate the delivered method for attainment of the set requirements. In order to allow a smooth development of final QC methods for testing DS and DP in commercial production, a couple of pre-requisites are preferentially fulfilled and supportive information as described in reference 1 is made available.

B. Development Plan

A development plan with an acceptable time line is generated. The plan comprises time lines, requirements of laboratory staff, instrumentation and equipment, materials/consumables, guidelines, development samples, and standards, and is communicated to all stakeholders.

C. Method Requirements

1. General Requirements

There may be many considerations that are targeted to meet the criteria for late phase methods since they are considered to deliver product characterization data of both the DS and DP. For example, it may be very interesting to have the same electrophoresis system for the DS and DP method, allowing easy comparison of impurity profiles. As per ICH guidance, DP methods do not require quantification of synthesis impurities, but these impurities are labeled when they occur in the electropherogram. Although the guidelines[5,6] generally require to report unknown impurities above 0.10% in DS and 0.20% in DP, it is recommended to develop methods that are able to detect impurities at lower levels. Currently, it is very important to develop methods with short run times. For CE, typical run times of 30 min are considered to be acceptable. The separation of the peaks of interest should have target resolutions of >1.5.

2. Technical Requirements

Maximal detector response:

- The maximal detector response for the nominal concentration of the API (100%) is 75% of the qualified detector linear dynamic range.

Temperature:

- Methods are performed at temperature-controlled conditions (preferably 25°C).

Background electrolytes:

- MS compatibility is not a must, but preferred. For achiral purity methods an alternative MS compatible, supportive method is available to support identification purposes.
- Buffers are prepared by accurately weighing or pipetting the components of the buffer system. Adjusting the pH through titration is avoided. It is recommended to check the pH of the buffer solution.
- THF should not be used because of high viscosity and reactivity.

Electrophoresis mode:

- Constant voltage/current runs are preferred for final methods.
- When constant voltage/current conditions cannot be used, linear gradients are preferred. When justified, step gradients of voltage or current can be used for analysis time reduction.
- The analysis time is adjusted according to the worst case results during robustness testing.
- A rinsing step at the start of each run is needed.
- A diluted buffer solution (typically more than 10 times) is preferred as sample dilution solvent, but when necessary up to 40% v/v organic solvents can be added.
- Conditioning of a new bare fused silica capillary is a must do! Different approaches are described, however, the following may be applicable and generally results in optimal results:
  - 30 min 1 M NaOH solution,
  - 15 min water,
  - 5 min drying by purging air or nitrogen gas through the capillary.
- Daily rinsing of the capillary is recommended prior to analysis. The following approach is proposed:
  - 15 min (dependent on capillary length, equivalent to 10 × capillary volume) with 0.1 M NaOH or 10% v/v phosphoric acid depending on the pH of the background electrolyte,
  - 10 min with water,
  - 5 min drying by purging air or nitrogen gas through the capillary.

3. Pre-run Rinsing

Prior to every injection of a sample solution, the capillary has to be regenerated to obtain initial wall surface properties. An immaculate capillary wall is primordial for a reproducible endosmotic flow, which is critical for reproducible migration times and therefore separations. Assuring fresh capillary wall conditions is one of the most critical requisites of reproducible CE separations. Different groups have tried to overcome the wall regeneration through applications of either stationary[7,8] or dynamic[8] wall coatings. These approaches require complex chemistries and conditioning approaches. A simple pre-run rinsing approach, schematically represented in Figure 3, has been demonstrated to be readily applicable and results in adequate reproducibility in many industrial applications.

Step 1: Rinse with a suitable organic solvent (e.g., methanol, acetonitrile, etc.).

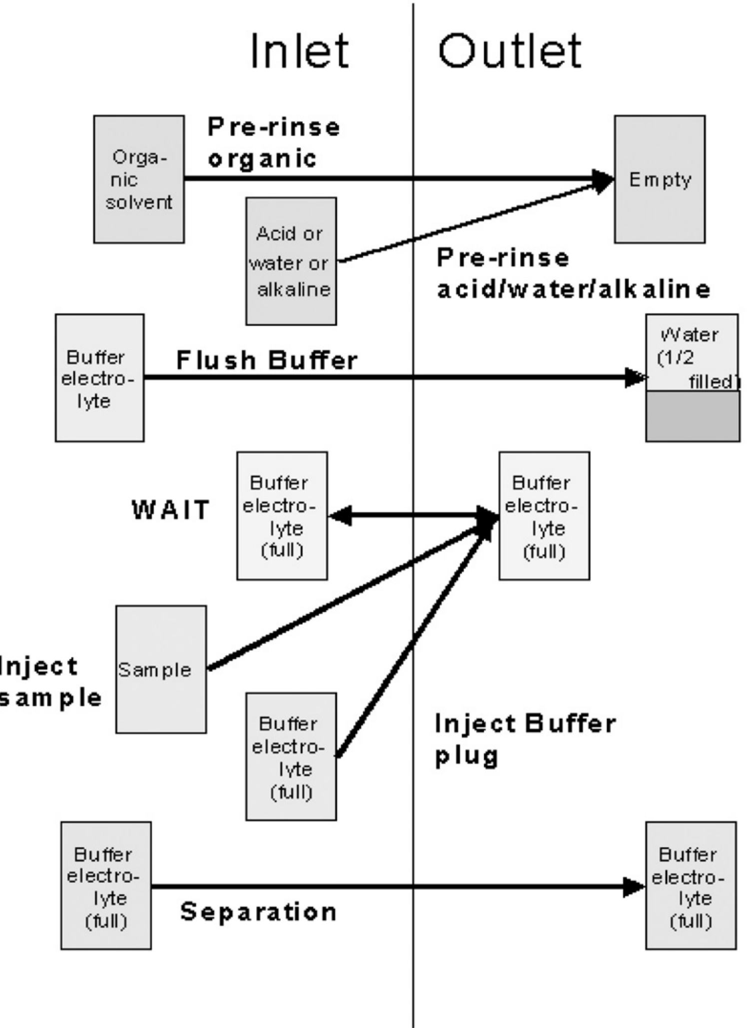


FIGURE 3 **Schematic representation of a capillary conditioning routine performed prior to each injection of a sample solution in CE (pre-run rinsing) to guarantee optimal performance.**

Step 2: Rinse with acid (e.g., 10% v/v phosphoric acid), water or alkaline solution (e.g., 0.1 M NaOH) depending on the running pH region.

Step 3: Condition the capillary with the background electrolyte. Use a separate inlet vial (than the running buffer vials) and purge the electrolyte to a waste vial that is half filled with water.

Step 4: After rinsing, dip both ends of the capillary and the electrodes for approximately 5 s in two separate vials fully filled with background electrolyte. (This is needed to wash out potential crystalline salt/ particles in order to avoid spikes and current cuts due to arching.) The same set of dip-vials are used throughout a sequence.

Step 5: In order to improve the reproducibility of migration times further, in some cases it is recommended to apply the running voltage for 1 min during this “wait” step.

4. Sample Injection

Step 6: The sample is preferentially injected by hydrodynamic mode since in this way a representative amount of sample solution is introduced in the capillary inlet. The outlet of the capillary is maintained in the dip-vial.

Step 7: After sample injection a small plug of background electrolyte is injected. The outlet of the capillary is still maintained in the dip-vial.

5. Separation Run

Step 8: Use separate set of vials filled with background electrolyte for the separation run. It is, however, important to determine the maximum number of runs that can be performed using one set of run vials. This will be discussed further in the durability section.

6. Capillary

A suitable, globally available uncoated fused silica capillary is preferred for simplicity. The capillary is tested for durability together with buffer capacity under the specified method conditions. To avoid reproducibility difficulties of migration times due to joule heating, the maximum current flow in conventional CE is preferentially kept below 150 μA. The target run time is preferentially below 30 min. Taking into consideration all the above recommendations, reproducible separations and stable migration times are easily obtained. As can be observed in Figure 4 compared to the typical approach of capillary conditioning, reproducible results can be obtained.

7. Glassware

Small volume pipettes are avoided since they may lead to significant errors in the final results. Similarly, typical sizes for volumetric flasks should be between 25 and 500 ml if possible.

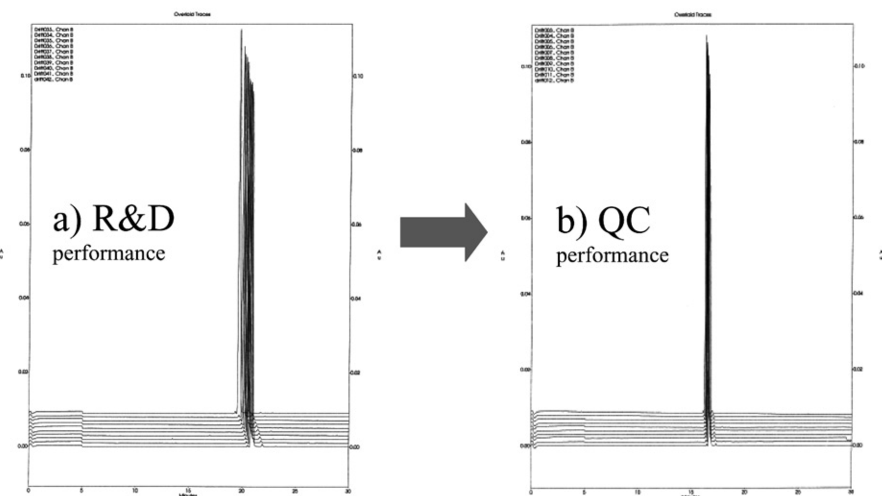


FIGURE 4 Effect of proper capillary pre-injection conditioning routines (pre-run rinsing). Injections of 10 consecutive sample solutions: (a) conventional rinsing, i.e., 5 min with NaOH 0.1N followed by 5 min with water or buffer electrolyte. The conventional rinsing approach is suitable in R&D applications (b) preconditioning according to the protocol described in Figure 3. Reproducible migration times are primordial in QC applications.

8. Sample Preparation

As is true for other chromatographic techniques,[1] in assay of main compound methods of DS, weigh reference standard and sample material >100 mg. For purity and DP analysis methods, reference standard and sample material >50 mg are weighed. In chiral methods where the concentration of the enantiomer is determined as normalized %areas, there is no specific minimum amount targeted. However, where it is possible, efforts should be made to weigh more than 25 mg. For DP analysis, at least 20 solid units are used in the preparation of the sample solutions. Alternatively, the sample may be prepared by taking an aliquot from a grind of at least 20 solid units. The number of manipulation steps during sample preparation should be kept to a minimum. Parallel dilutions are preferred over serial dilutions (dilutions are made directly from initial stock solutions with the lowest number of dilution steps).

In order to select the optimal sample diluent that allows adequate detection at low level and good peak shape, investigate the influence of pH, type of organic solvent, percentage of organic solvent, addition of additives (cyclodextrin, surfactants, etc.), injection volume, different counter ions, etc.

V. METHOD DEVELOPMENT AND OPTIMIZATION

Method development and optimization are started with review of the currently available methods within the company or in literature. Available methods are used as a starting point and evaluated against the method requirements set in the method definition. If necessary the method is optimized or redeveloped in order to fulfill the requirements. DOE tools (response surface design) are preferentially applied to obtain the best optimal conditions in terms of robustness. Application of DOE methodology is not new in chromatography[9,10] and CE.[11,12] DOE is frequently applied also for enantiomeric separations in CE.[13–16] Especially in development and optimization of separation methods for chiral applications, DOE approaches have demonstrated to be very beneficial. In chiral separations with CE, a chiral selector is added to the buffer electrolyte. This selector forms a stereo-specific complex with the compound of interest, leading to a potential separation (Figure 5). Finding the right chiral selector for a separation is not straightforward. For this reason, many strategies have been developed to facilitate an efficient screening of many potentially suitable chiral selectors.[13] Recently, a broadly applicable[15] simple, straightforward, rapid, and efficient screening approach that utilizes DOEs methodology was reported.

This approach includes first a screening-in function of the pH to determine the optimal migration conditions, followed by a selection of the right chiral selector by means of Taguchi designs. In this approach, several variables such as the type and concentration of cyclodextrin, the concentration of buffer electrolyte, and the percentage of organic modifier are varied simultaneously to find initial separation conditions rapidly (Figure 6). After obtaining the initial separation conditions by applying the screening approach, the strategy is continued with a further optimization of the selected system. It is highly recommended to do this optimization using a response surface design[16] in order to select the most optimal *and* robust separation conditions. All the more so since the separation in CE originates within the solution in an open tube (capillary) without any supportive media. Any change in one of the many parameters that influence the separation may jeopardize the outcome of the method. When methods are developed not knowing the robustness of the optimum (local optimum), unforeseen issues and troubles may be obtained during routine analysis. The advantage of applying an experimental design is that it allows to find the optimal separation conditions with a limited amount of experimental effort.

As a typical example, the separation of the enantiomeric separation for an experimental drug R209130 (Figure 7), a compound with three chiral centers, resulting in eight

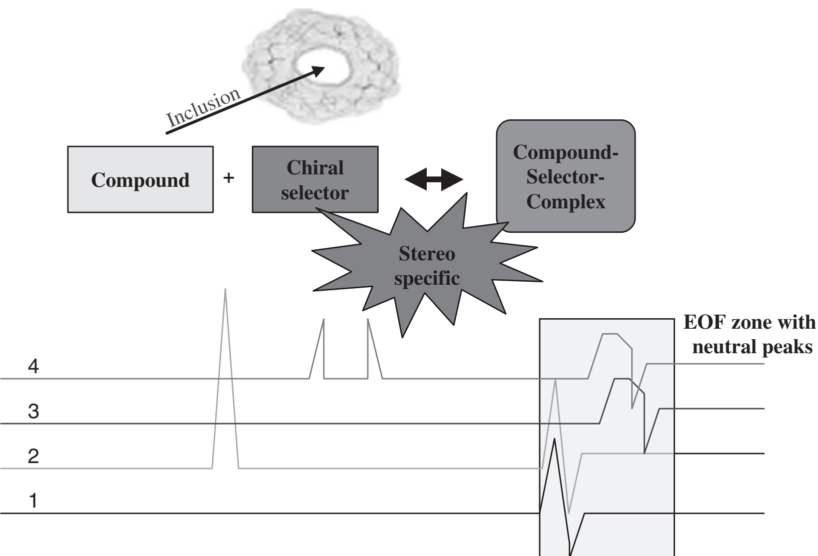


FIGURE 5 **Schematic representation of the mechanism for enantiomeric separation in chiral CE of basic compounds with cyclodextrin type selectors. The model electropherograms represent: 1: blank run with buffer electrolyte at acidic pH; 2: sample run with buffer electrolyte at acidic pH, no enantiomeric separation is observed; 3: blank run with background electrolyte including a selector, e.g., cyclodextrin. Note a small delay in the EOF zone; and 4: sample run with background electrolyte containing a selector, e.g., cyclodextrin, resulting in enantiomeric separation of the peaks.**

stereoisomers is discussed.[16] The diastereomeric impurities are R268670, R287539, R167155, R167152, R287537, and R287536, while R209130 is the main compound and R167153 the enantiomeric impurity. Since the test compound is a basic structure, Steps A−C screening methods for basic compounds were applied according to the approach described in reference 15 and resulted in initial separation conditions that were further optimized using a Box−Behnken design for four factors, selected and set up in the Minitab™ Statistical Software package (Minitab Inc., www.minitab.com) to optimize the method. The factors studied were α-cyclodextrin (α-CD) concentration, sulfated-*β*-cyclodextrin (S-*β*-CD) concentration, buffer electrolyte concentration, and the applied run voltage. The resolution factor between each neighboring peak pair was calculated (R1−R7 for all eight peaks) and the migration time of the last peak (analysis time) was monitored as response factors.

The DOE allows generating response surfaces for each response factor and then determining the optimal conditions by overlaying all the response surfaces. Since there are many response surfaces to be considered for the separation of all eight stereoisomers, the overlaying approach is complex. Using the multiple response optimizer tool available with the Minitab software (and with other commercial software programs), a "separation dashboard" is generated as shown in Figure 8. The individual influences of each factor on the separation of each peak pair and on the analysis time are transferred through a desirability function that ranges from 0 (undesired conditions) to 1.0 for a highly desired separation condition.[17] The separation of each peak pair is predicted in function of the studied factors. In Figure 8, the vertical straight lines indicate the selected factor level, while the horizontal dotted lines predict the expected resolution value. By moving the vertical lines and thereby changing the separation conditions, one can instantly observe the impact of the selected separation condition on all the

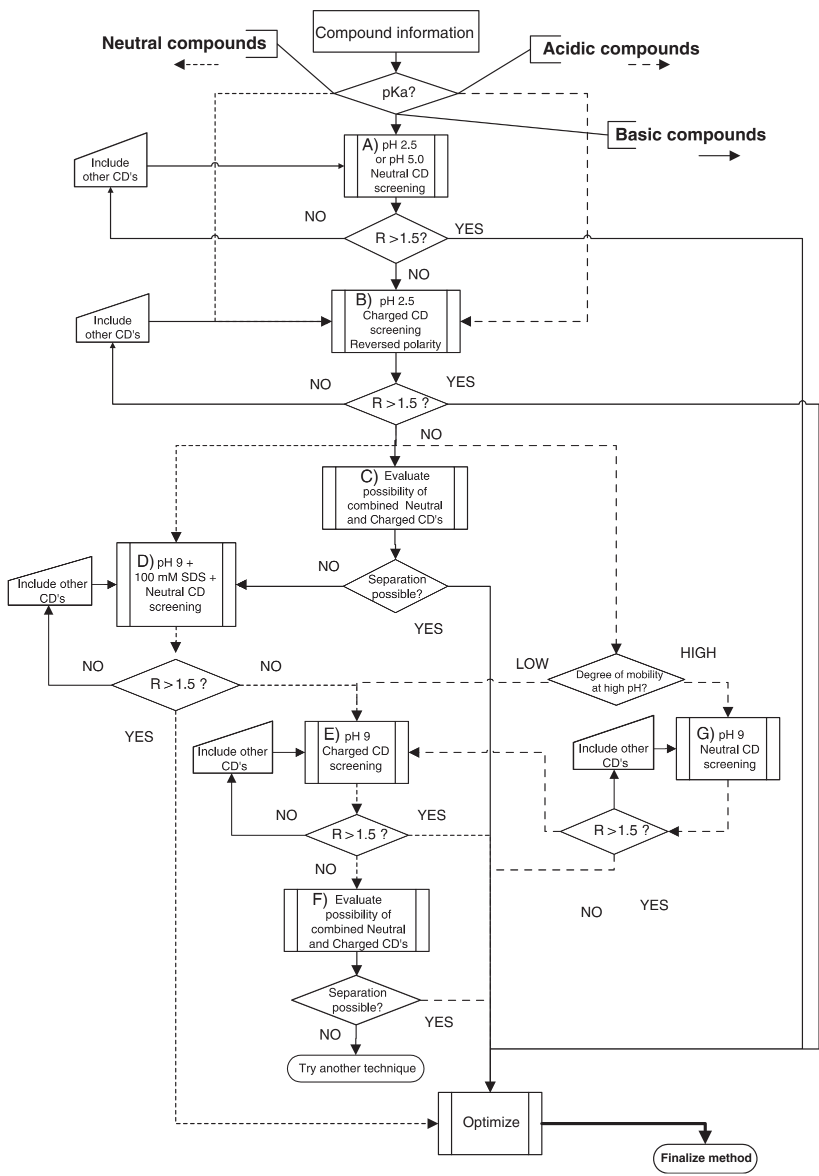


FIGURE 6 Overall road map of the chiral screening strategy for pharmaceutical compounds. Solid lines are for basic compounds, dashed lines for acidic compounds, and highly dashed lines are for neutral compounds.

studied responses. In this way, the "separation dashboard" helps in the search for optimal conditions simply by dragging the vertical lines.

For the current example, optimal conditions were selected at 5 mM α-CD, 2% w/v S-β-CD, a buffer electrolyte concentration of 10 mM, and a run voltage of 10 kV. The resulting electropherogram obtained at the predicted optimal conditions is shown in Figure 9. These separation conditions were included in the draft test method description.

FIGURE 7 Structure of the main compound R209130. This compound has three asymmetric carbon atoms (depicted by ∗), resulting in eight stereoisomers.

A. Capillary Durability and Buffer Capacity Check

The maximum number of injections for a selectivity batch solution is determined on one capillary using one set of background electrolyte vials. Additionally, a durability test is performed on one capillary by making >50 repetitive injections of a worst case sample solution (e.g., a crude DS batch or an old sample taken from a pending stability study). The number 50 is chosen as such to reflect the worst case situation toward a typical analysis sequence. Figure 10 shows the results of a chiral separation of a racemic mixture for an intermediate of nebivolol (beta-blocking agent) injected 50 times consecutively. As can be observed there is hardly any shift in migration time due to the capillary pre-run rinsing conditioning routine performed as described in Figure 3.

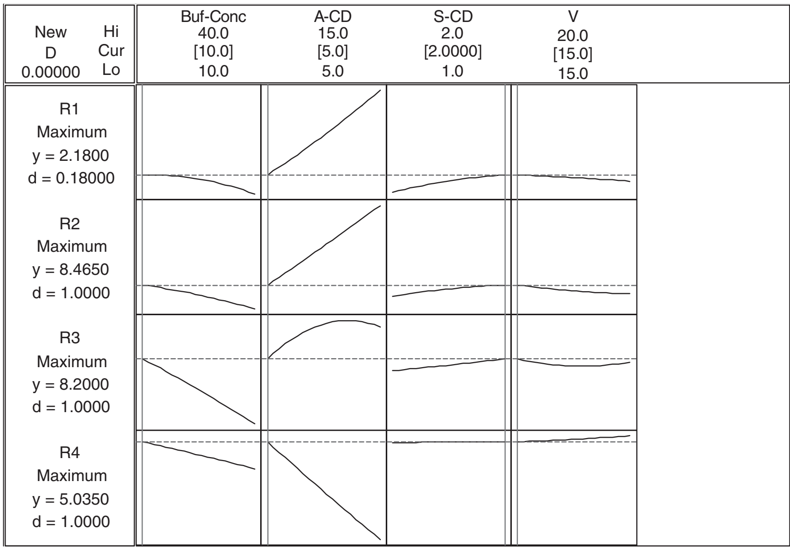
The sequence is started and ended with the injection of a selectivity batch solution. After each 10-sample solution injection the selectivity batch solution is re-injected. The result is qualitatively evaluated. In order to demonstrate the run-to-run variability, the selectivity is checked on at least three different capillaries by at least triplicate injections of a selectivity batch solution on each capillary. The result is qualitatively evaluated. As can be observed in Figure 11, the separation is nicely reproducible on three different capillary columns for the same intermediate of nebivolol.

When 50 consecutive injections are not possible with one set of buffer electrolyte vials, it should be investigated at approximately which injection the buffer electrolyte starts being depleted and thereby results into migration time shifts. As can be observed in Figure 12a, the enantiomeric separation of Levaquin oral solution starts showing a drift after approximately 30 injections due to buffer depletion. Therefore, the buffer electrode vials are replaced after every 25 injections. As can be observed in Figure 12b, the migration times hold very well up to the targeted 50 injections.

B. Pre-validation Evaluation

Following method optimization a number of important method validation characteristics are checked in a brief pre-validation study. If the method would still show significant deficiencies, method optimization may need to be repeated. Suggested parameters to be checked may include

- Specificity/selectivity against placebo, stressed placebo, stressed samples, relevant impurities (including enantiomers for chiral methods), and degradation products
- Specificity for assay of main compound: peak purity of the API is checked (e.g., using CE−PDA, CE−MS, etc.)
- Verification that the reporting threshold can be attained



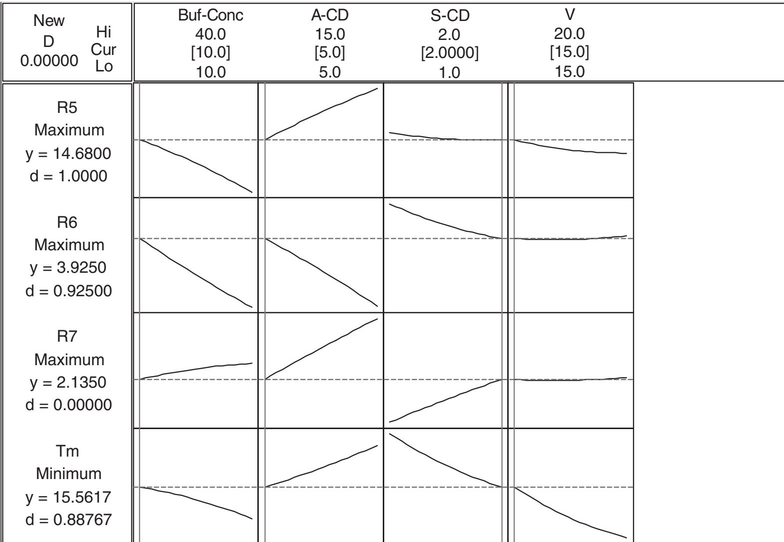


FIGURE 8 **Separation dashboard describing the influence of the factors on each of the studied responses based on the results of the Box−Behnken design. The closeness to a target response that is aimed for each response in this study is represented by a desirability value (*d*). Reproduced with permission from reference 16.**

- the linearity of the API
- Filtration studies
- Stability of solutions if not previously determined
- Accuracy by comparison of the analysis results of the new method and the previous method
- Precision is evaluated by analyzing a batch of DP or DS in triplicate.

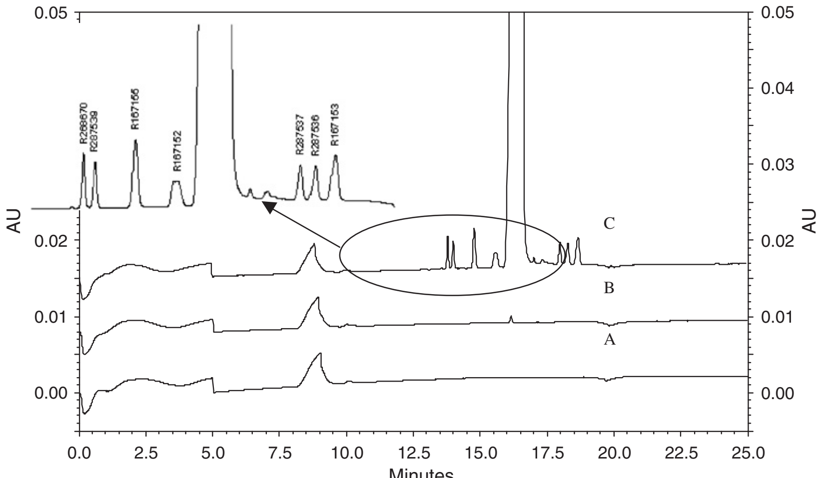


FIGURE 9 **Electropherograms of a blank solution (A), a solution at RT level (B), and a solution of a production batch spiked with all stereoisomers of R209130 (C). Conditions: uncoated fused silica capillary of 60 cm total length, 50 cm length to the detector, internal diameter of 75 μm, and detection wavelength of 200 nm. Rinse for 1 min with methanol and 1 min with 10% v/v H_3PO_4 to a waste vial different from that of the background electrolyte. Change the rinse vials after each 10 separation runs. Buffer: 5 mM α-CD, 2% w/v S-β-CD, a buffer electrolyte concentration of 10 mM. Runs are performed at a voltage of 10 kV. Reproduced with permission from reference 16.**

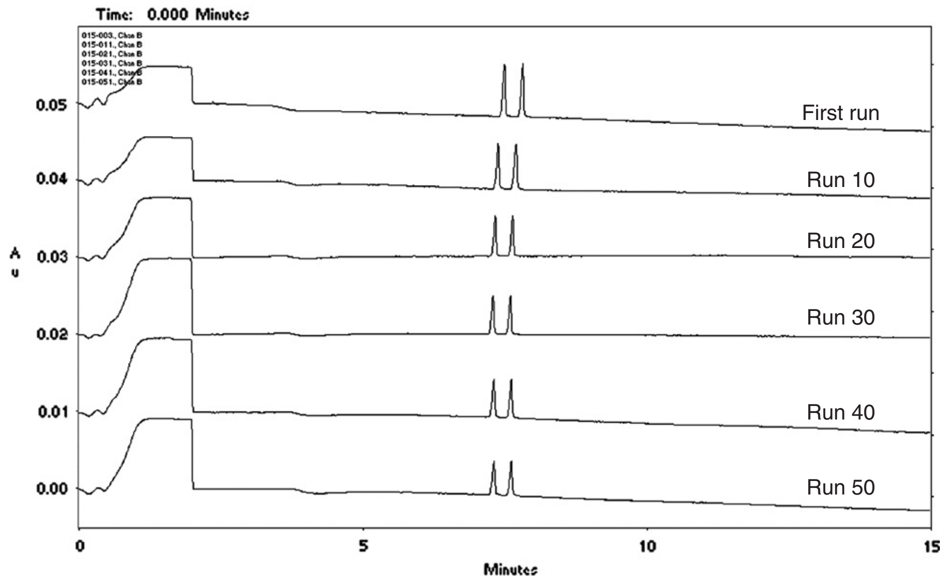


FIGURE 10 **Capillary durability check for an enantiomeric separation in CE. An overlay of the 1st, 10th, 20th, 30th, 40th, and 50th electropherograms of consecutive injections of a racemic mixture for an intermediate product of nebivolol is presented.**

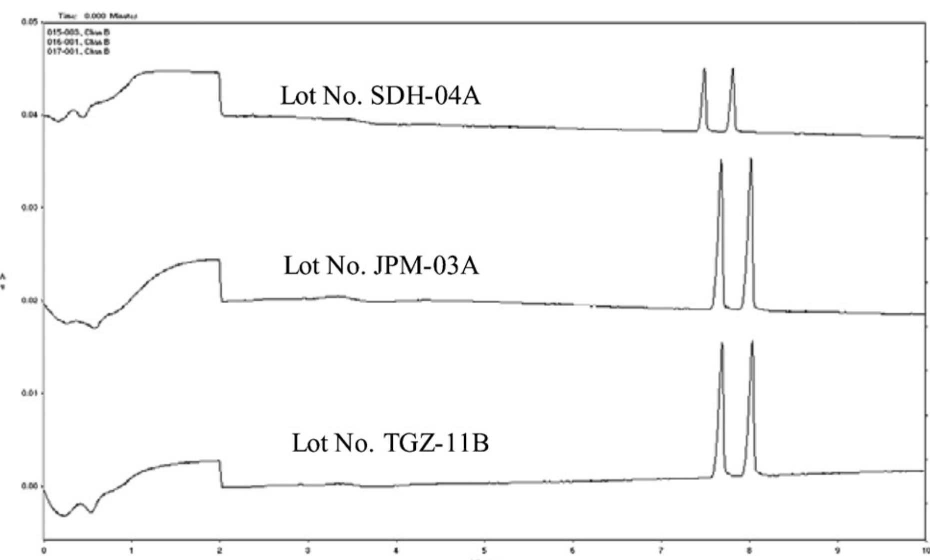


FIGURE 11 **Capillary-to-capillary reproducibility check for the same enantiomeric separation for the racemic mixture of an intermediate product of nebivolol. An overlay of three electropherograms performed on three different capillary lots is presented.**

C. Robustness Testing

Robustness testing of the electrophoretic method is conducted in order to identify possible critical method parameters that may impact optimal performance during real time applications. Additional evidence of robustness can be derived indirectly from tests in a further stage of the method development process, i.e., from intermediate precision assessment and inter-laboratory evaluation (AMERT: Analytical Method Evaluation Ring Test). In the robustness testing either an experimental design (preferred) or a step-by-step approach where each variables is evaluated sequentially may be used. The outcome of robustness testing is the identification of critical parameters and the determination of System Suitability Test limits. Detail procedures for Robustness testing are discussed fully in Chapter 9.

As a practical example the robustness testing performed for galantamine (Figure 13), a drug against Alzheimer's disease, is discussed.[18] Figure 14 shows the typical separation that is obtained for the product. As can be observed both the chiral and achiral impurities are nicely separated from the main compound and from each other. The robustness test was done by applying a Plackett−Burman design in which 11 factors (eight method variables+three dummies) were examined (Table 1). The influence of the method factors was investigated on the resolution of a critical peak pair, tailing factor of the main compound and on the analysis time. The main effect plot (Figure 15) shows the estimated response as a function of each experimental factor. In each plot, the factor of interest is varied from its low level to its high level, while all other factors are held constant at their central values.

VI. SYSTEM SUITABILITY TESTS AND LIMITS

System suitability tests are an integral part of analytical methods. They determine the suitability and effectiveness of the operating system.[1] Analytical confidence in the obtained

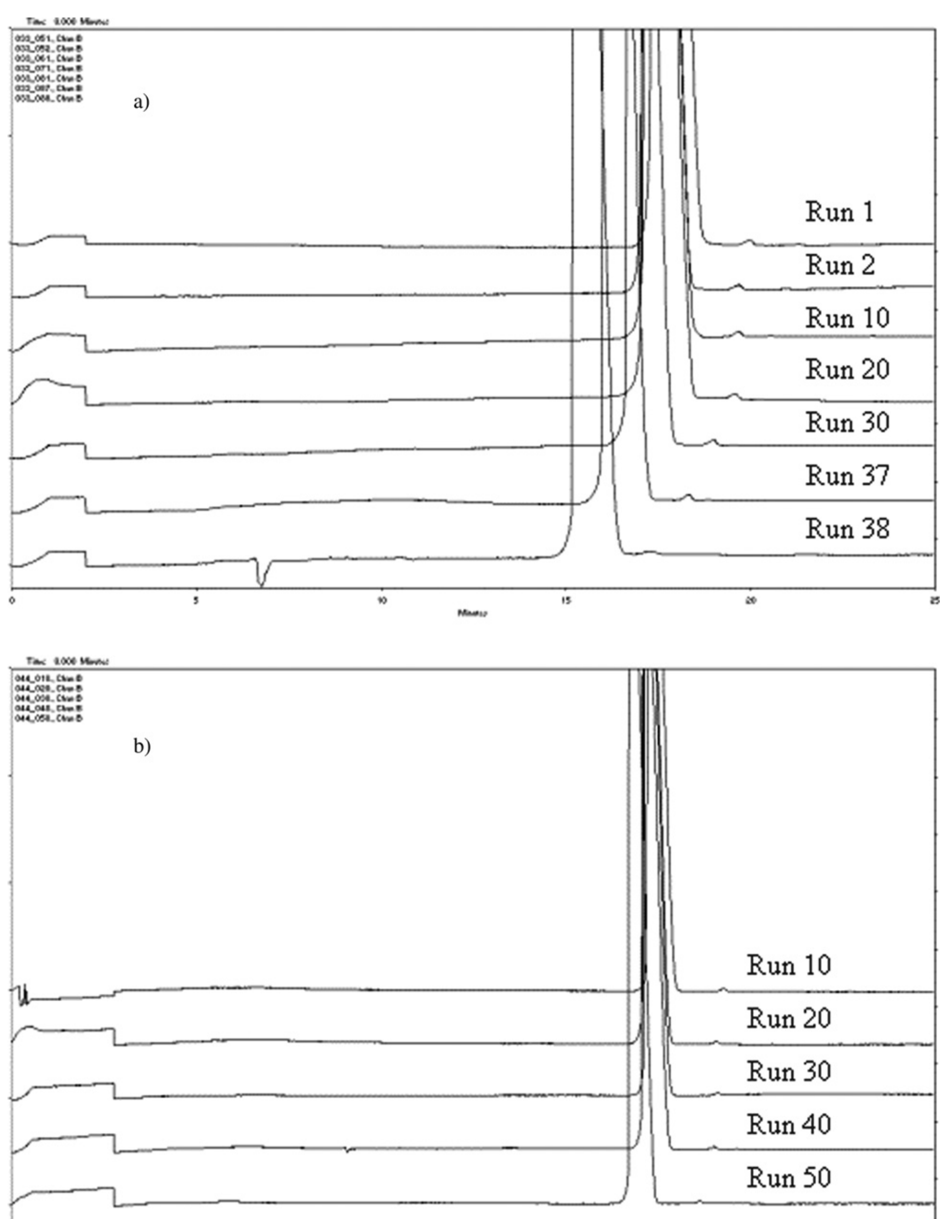


FIGURE 12 **Enantiomeric separation of Levaquin oral solution. (a) An overlay of the 1st, 2nd, 10th, 20th, 30th, 37th, and 38th electropherograms of consecutive injections of Levaquin oral solution samples is presented. Starting from the 30th injection a shift in the migration time is observed. (b) Replacement of the separation run vials after 25 consecutive injections allows to have acceptable migration time reproducibility up to the 50th injection.**

FIGURE 13 **Structural formula of the main compound galantamine hydrobromide.**

data for samples is increased when it is supported by examination of the separation and system performance prior to analysis of samples. The SST-limits for the different parameters usually are established based on the experimental results obtained during the optimization of a method and on the experience of the analyst. As mentioned before, SST-limits are preferentially set based on the outcome of robustness studies. SSTs should include tests to examine both separation and system performance.

A. Separation Performance

The selectivity is demonstrated by analysis of a mixture of the relevant compounds, e.g., a selectivity batch/sample solution. The resulting electropherogram is compared with the selectivity electropherogram provided in the method description. The resolution between the critical peak pair(s) is determined. The target resolution should be >2.0 (the calculation expression is mentioned in the method description). In case there is no critical peak pair in the chromatogram, a parameter describing the peak shape, e.g., tailing factor of the main compound is used.

B. System Performance

Depending on the type of calculation procedure, a set of SST-parameters is selected including system precision, reporting threshold, and system drift checks. If normalized peak area reporting is applied, the evaluation of the system precision is obviously not necessary. Frequently applied parameters and tentative limits comparable to HPLC methods are compiled in the Table 2.[1]

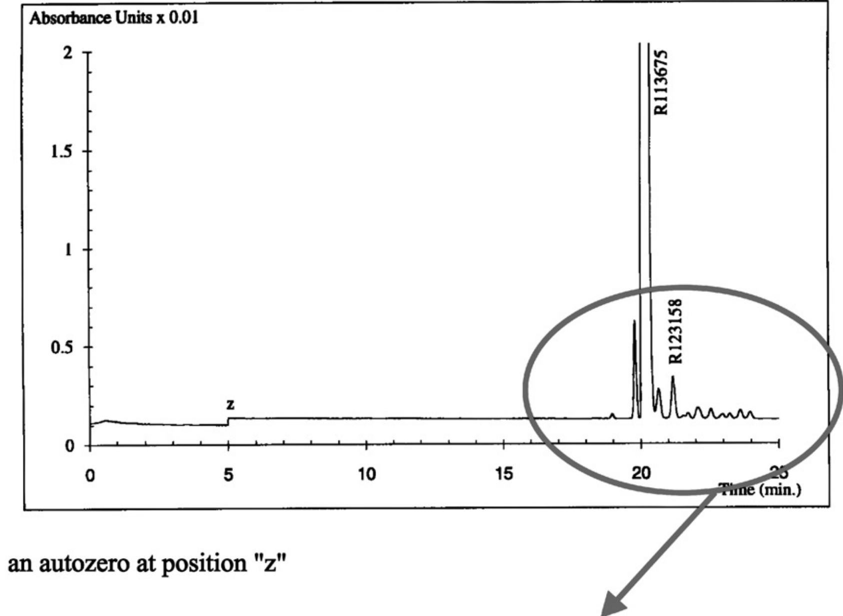
C. Practical Difficulties in System Performance

The current status and characteristics of the technology in CE may lead to difficulties that are frequently encountered during daily application in a QC environment. There are four important characteristics of CE technology that requires our attention when developing robust methods:

1. Nanotechnology
2. High speed and efficiency analysis
3. New technology.

1. Nanotechnology

An important characteristic is that in CE we are working in a nano-environment. This aspect is generally overlooked since the instrumentation is very basic and appears to be very



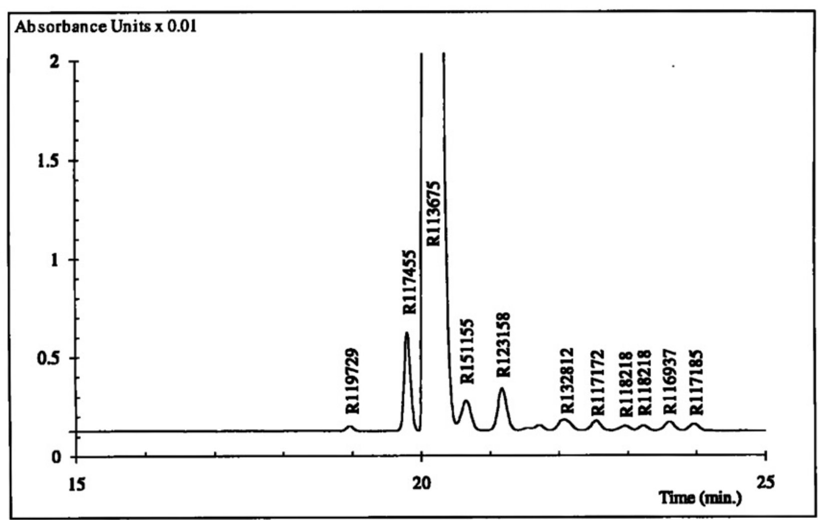


FIGURE 14 Specimen electropherogram showing the specificity of the method toward the separation of the main compound (R113675) and the enantiomeric impurity R123158. Reprinted with permission from reference 18.

simple in setup and easy to miniaturize. The basic configuration of CE does not require mechanical systems. Sample size in CE is very small (nanoliters), leading to excellent mass sensitivity, peak capacity, and low reagent consumption. On the other hand, the same features lead to important drawbacks in CE. First of all the small injection volume places high

TABLE 1 Method Factors that are Studied in the Robustness Evaluation

Factor	Units	Limits	Level (−1)	Level (+1)	Nominal
Concentration of cyclodextrin in the buffer electrolyte (Conc_CD)	mg/25 ml	±10 mg	476	496	486 mg/25 ml
Concentration of the buffer (Conc_buf)	mg/100 ml	±20 mg	870	910	890 mg/100 ml
pH of the buffer (pH)	–	±0.2 units	2.80	3.20	3.0
Injection time (Inj_time)	Seconds	±0.5 s	2.5	3.5	3.0 s
The column temperature (Temp)	°C	±2°C	18	22	20°C
The column supplier (Column)	Different vendors	CMS[a] and Beckman	CMS	Beckman	CMS[a]
Rinse time solvent 1: water (Rinse_1)	Minutes	±0.2 min	1.8	2.2	2.0 min
Rinse time solvent 2: buffer electrolyte (Rinse_2)	Minutes	±0.2 min	3.8	4.2	4.0 min
Dummy factors	–	±1	−1	+1	0

Reprinted with permission from reference 18.
[a]Composite metal services.

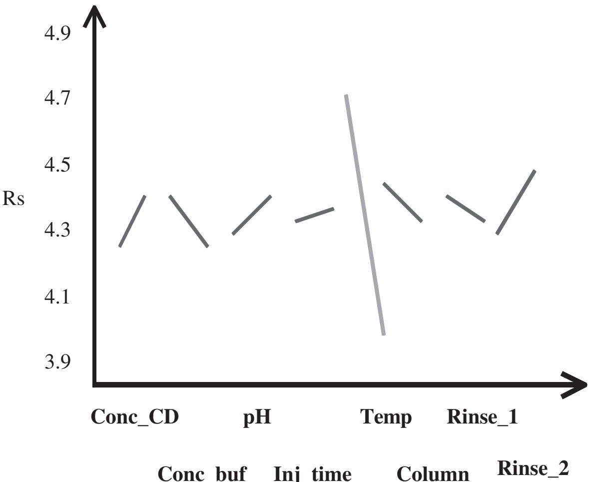


FIGURE 15 Example of a main effect plot (for the critical resolution) clearly showing the extent of the effects relative to each other. Factor temperature appears to be the most important factor on the resolution. Reprinted with permission from reference 18.

demands on controlling the injection volume. Injection is typically done indirectly by controlling the time of a flow created either by a hydrodynamic pressure difference or a flow due to electromigration. As a result the injection precision is typically less than observed in, e.g., HPLC methods. Another consequence of the nanotechnology is that the concentration

TABLE 2 System Suitability Test Parameters Typically Applied in Testing of Drug Substance and Drug Product Materials

Parameter	Limit assay DS main compound[a]	Limit assay DP main compound[a]	Limit purity and chiral methods	Comments
System repeatability	≤1.0% RSD	≤2.0% RSD	≤10.0% RSD	For at least five replicate injections of a reference solution(s)
Reporting threshold	0.10% (w/w)	0.10−0.20% (w/w)	0.10−0.20% (w/w)	Main compound peak should be readily integrated
Accuracy of reference weighing	98.0−102.0% recovery API	98.0−102.0% recovery API	75−125% recovery API	Tested by analysis of a second reference solution
System drift	98.5−101.5% recovery API	98.0−102.0% recovery API	75−125% recovery API	Tested by injecting a control reference solution (this is similar to the reference solution for calibration) after injection of the samples

The limits described are tentative and need to be defined on an ad hoc basis depending on the product-specific needs.
[a]According to internal or external standard calibration approaches.

sensitivity is typically very poor. Concentrations down to 0.1% levels for impurities can be challenging, but doable. As can be observed in Figure 16, at optimal conditions detection of impurity levels down to 0.01% is possible.

In addition, contamination and capillary plugging frequently lead to system failures due to current flow breakage. The capillary on its own can be of concern in many system failures. Due to contamination the capillary can be blocked (even by air bubbles) leading to current breaking and therefore reproducibility issues of separation. A frequently observed contamination is the settling of salt crystals (and other solids) on the outer surface of the capillary, as shown in Figure 17. The salt crystals may induce current leakage and eventually a breaking of the current flow completely. The capillary conditioning routine discussed before is designed to bypass this issue through the dip step, where the outer surface of the capillary and electrodes are rinsed prior to start of the separation.

Because of the risk for extra column variances in the sample zones, in-line detection is not possible which makes on-column UV detection obligatory. At the detection spot the polyimide coating of the capillary is burned out to create a detection window, leading to exposure of the fragile bare silica glass material of the capillary. Frequently the capillary breaks at this weak point (Figure 18), leading to current breakage and analysis failure.

2. High Speed and Efficiency Analysis

CE is known from literature to result in fast and high efficiency separations. As a result the ability to detect different constituents may lead to high demands on resolving many

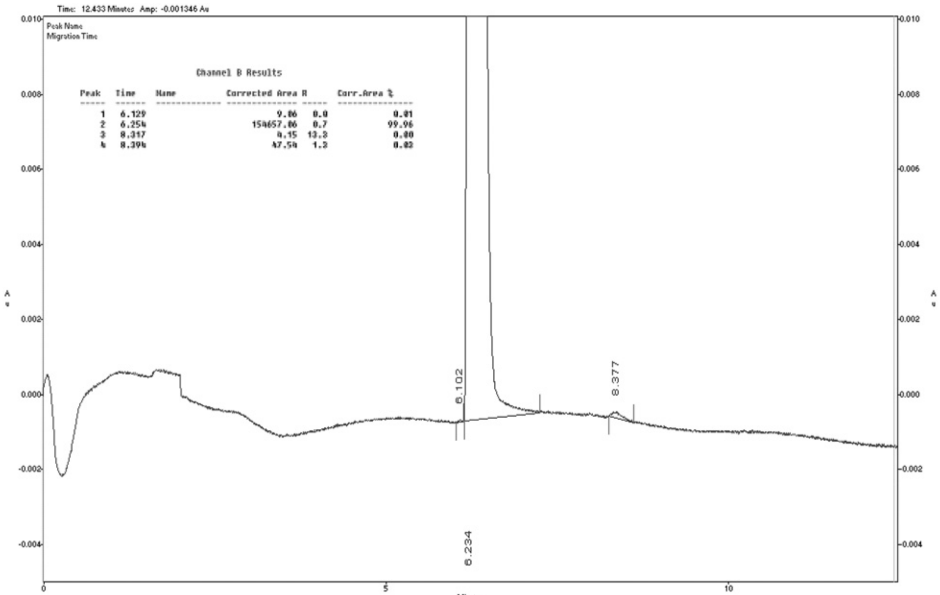


FIGURE 16 **Determination of the enantiomeric impurity (migration time = 6.102 min) of an experimental drug. At optimal conditions the detectability can be very good, even with the typical shortcomings of an UV detector in CE.**

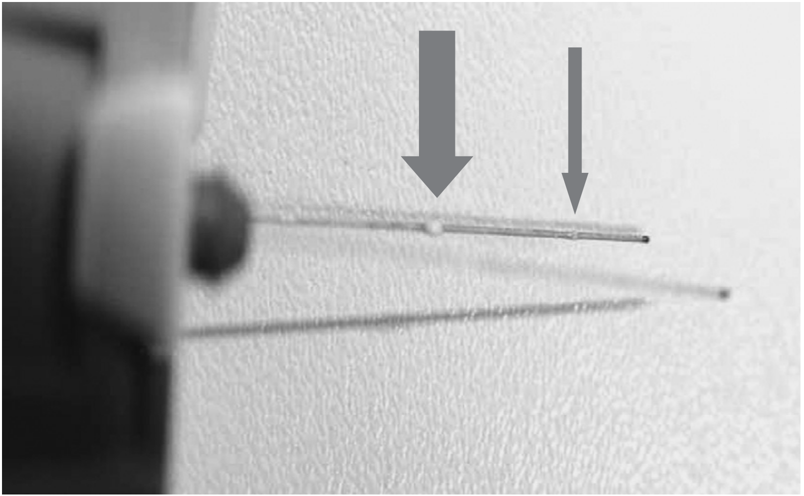


FIGURE 17 **During daily analysis in CE, the outer surface of the capillary is easily contaminated with residues of buffer electrolyte constituents, e.g., salts, cyclodextrins, etc. To ensure consistent performance in the QC labs it is important to rinse this systematically (capillary preconditioning routine).**

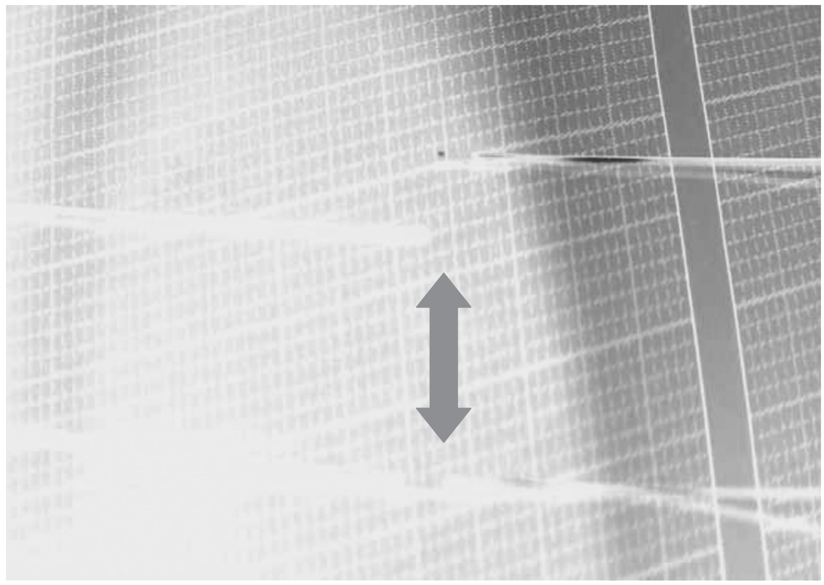


FIGURE 18 **At the detection window of the capillary the polyimide layer of the capillary outer coating is burned. At this spot the capillary is extremely fragile and may break easily.**

unknown peaks that can easily come from contamination. Within a QC environment such activities are not always welcomed with great enthusiasm. Faster separations may create controlling troubles, e.g., control of the EOF through the regeneration of the initial conditions at the capillary wall prior to each sample injection and thereby avoiding system drift. As mentioned before, the capillary rinsing routine proposed earlier may help to guarantee consistent performance.

There are also hardware considerations to mention as well. In high-speed analysis a proper autosampler is crucial for optimal performance. The Beckman MDQ CE system is one of the most suitable CE systems for a QC performance and is being applied extensively for this purpose in the industry. As a result more experience is obtained with these equipment compared to others. Therefore, the following discussion is focused on this type of systems only. Other machines may show more severe drawbacks, but are not described since they are not extensively applied in a QC environment. The Beckman MDQ capillary−electrode configuration is comparable to that of the Beckman PA/CE 5000 series instrumentation, however, the openings of the vials are very different. The diameter of the PA/CE 5000 series vials is much larger than that of the MDQ vials, making the calibration of the X/Y/Z robotic arms of the autosampler a critical part. As can be observed in Figure 19, a minor offset of the robot may lead to capillary breaking and electrode bending due to punching of the electrodes in the vial caps.

Another performance disturbance that has been encountered in the QC labs is the sticking of vials on the electrode block of the MDQ system. Due to the sticking the vial hangs on the electrode block when the autosampler rack is lifted down at switching vials. At the next alignment of a vial for, e.g., injection, the autosampler rack will hit the vial still hanging on the electrode block, resulting in breaking of the capillary and bending of the electrode. A simple way to overcome this issue is to frequently clean the electrode block to remove buffer electrolyte residues or to use the sandwich rack approach. As is observed in Figure 20, a cover

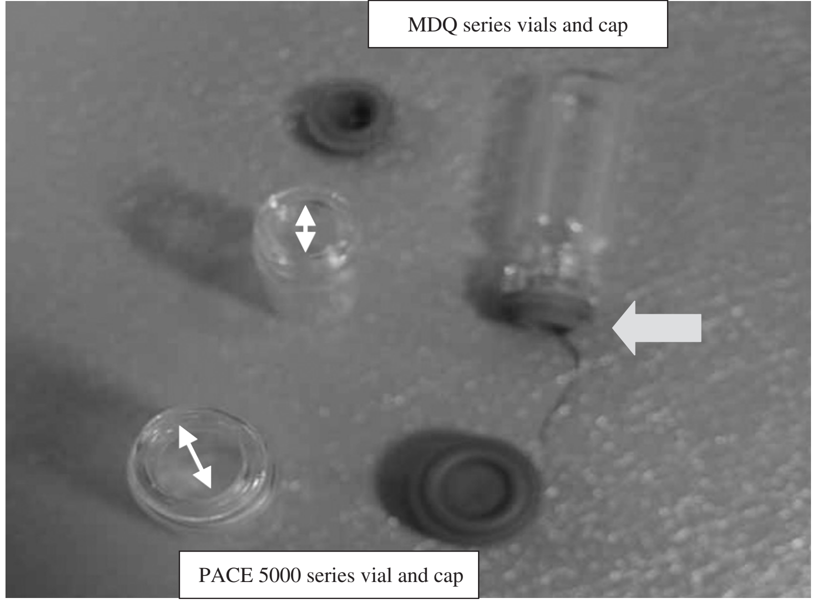


FIGURE 19 **A frequent system failure can be caused by punching of the electrode in the vial closure septum due to inaccurate outlining of the autosampler X/Y/Z robotic arms of the most frequently used QC performance Beckman MDQ CE system. Note that the opening of the vials with the PACE 5000 instruments was larger compared to the MDQ system, while the capillary/electrode interface of both systems is identical.**

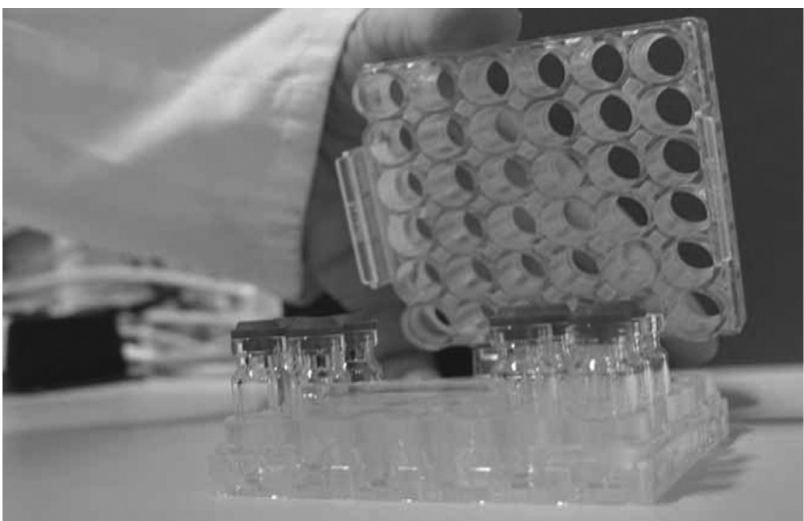


FIGURE 20 **A way to prevent frequent system failure caused by punching of the electrode in the vial closure septum due to sticking of vials at the capillary interface block of the most frequently used QC performance Beckman MDQ CE system is the use of sandwich racks. The cover of these racks avoids vials coming out of the racks.**

is used that nicely fits on the sample rack and avoids the vials to come out of the rack, while maintaining normal use of the vials.

3. New Technology

Although CE has been available now in the analytical environment for approximately two decades, it is still considered as new technology. As a result system handling is not fully understood by most of the analysts operating CE systems. Typically experience obtained during HPLC methodology is directly applied in CE, leading to obvious incompetence and frequent failures. CE requires dedicated attention and analyst training that is very different from typical chromatographic methods. It is poor practice to ask an HPLC analyst to start with CE methods without thorough training in the particularities of CE methodology.

Figure 21 shows some handy tools that can make life of a CE analyst in a QC environment easy. The first tool is a mould to facilitate cutting the capillary inlet and outlet at equal length. It has been demonstrated that this is very important in obtaining a stable baseline. The second is capillary cutting tool that allows a round cut of the capillary inlet and outlet. A smooth cut of the capillary end appears to be essential in avoiding baseline shifts at the start and end of a large peak (main compound) in the electropherogram. The third tool is a handy penlight that allows the analyst to do appropriate troubleshooting. Tool 4 is an ordinary GC injection micro-syringe mounted with a piece of Teflon tubing on the tip of the syringe. Sometimes it is possible to unplug a blocked capillary, by mounting it on the syringe through the Teflon tip and purge it with, e.g., methanol. The fifth tool is a remote mirror that can be helpful together with the penlight to visualize instrument parts that are difficult to see, e.g., when inspecting the back of the capillary tip or electrode for contamination. Tool 6 is a simple tooth brush that can do miracles when cleaning the electrode interface block to remove leftover material (e.g., cyclodextrins residues) sticking on the surface. Frequent cleaning of the electrodes, the

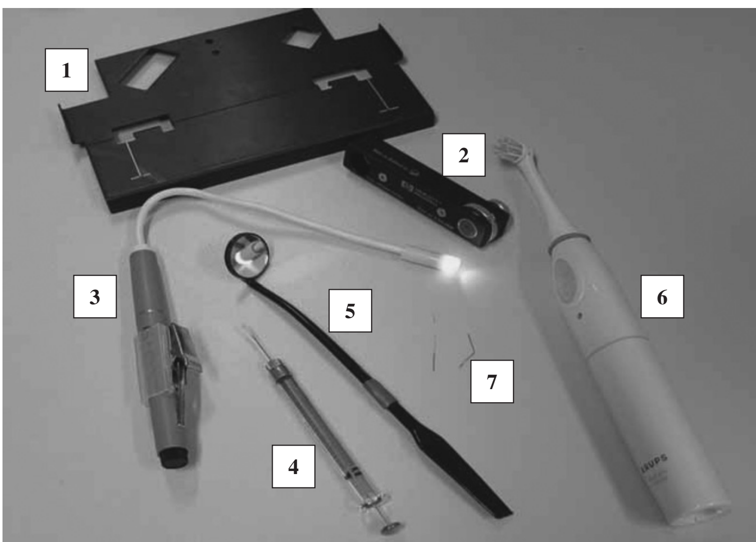


FIGURE 21 **Practical tools that can be very handy during handling and troubleshooting in CE. 1: capillary inlet and outlet length cutting mould, 2: capillary cutting knive, 3: pen light, 4: capillary unplugging tool, 5: remote mirror, 6: brush to clean electrode interface, and 7: examples of bended electrodes.**

capillary inlet and outlet, the electrode interface block, etc. is the key for a QC performance. In this way, horrible spikes in the electropherogram, current leaking, or even current breaking leading to loss of the electrophoretic run in a sequence can be avoided. Moreover, it will also lead to stable baselines making integration and data processing straightforward. Item 7 shows the electrodes of a Beckman CE system. As can be seen, one electrode is clearly bent. It is important to inspect the critical parts of a CE system prior to start of the analysis.

VII. DRAFT METHOD DESCRIPTION AND METHOD EVALUATION PHASE

The method development and optimization phase is completed with test method description drafted according to the method development results. The following method evaluation tests will continue with direct involvement of the customer (receiving laboratory). The goal is to check whether the developed late phase method performs adequately in different labs.

A. Analytical Method Evaluation Ring Test

The AMERT is an important part of the analytical method development process. The AMERT allows to verify whether the draft method performs adequately for its intended purpose and complies with the specific country requirements. The concept of performing an AMERT was introduced by Crowther et al.[2] and has been further discussed by Jimidar and De Smet.[1] Detailed description of the approach is presented in reference 1.

B. Method Capability Assessment

MSA[3] aims to assess the suitability of an analysis method in its application environment. It is important to refer to a system, since the outcome of an analysis is determined by many factors contributing to variance. The performance of a method not only depends on the quality of the method description but also the equipment, reagents, the lab environment, well trained analysts, good understanding of procedures, definitions, nomenclature, etc. MSA is performed via Gage R&R studies. The gage R&R study will tell whether the analytical method is capable of discriminating differences in the measured properties (e.g., assay value) of the batches (parts) and therefore can be used for process improvement/control and for acceptance testing. The repeatability and reproducibility of the measurement system is typically estimated from a designed trial across different batches and labs. Further reading on this topic is provided in reference 1.

VIII. METHOD VALIDATION PHASE

Method validation is only a minor, but important part in the overall method development process. The purpose is to demonstrate by experimentation that the method is suitable for the intended purpose. Method validation in CE is extensively discussed in various chapters of this book.

IX. METHOD TRANSFER PHASE

After validation the method is ready to be formally transferred to the application labs where it is intended to be applied during the entire product lifetime. Method transfer is

Laboratory: AO-STER
Method: XXXXX
Date:

Key Process Output Variable Feedback Sheet

General					SST								OOS	DEVIATIONS SST failure			
Date	System type	Initials Analyst	number of samples	Sample type	RSD	R	T	RT-check	Ref 2	Control references	Selectivity (separation pass/fail?)	Blank peaks (Y/N)	OOS reference number	NC reference number	Carry over Y/N	Other causes	Comments
22-Nov-01	MDQ	BHAE	6	R115777-DP	5. 5	5.0		116	101.1	102. 9	pass	N			N	N	N
27-Nov-01	MDQ	STHO	6	R115777-DP	2. 5	3.0		101	89.7	94. 6	PASS	N		112471	N	N	N
4-Dec-01	MDQ	STHO	6	R115777-DP	1. 1	5.0		90	103.0	101. 9	PASS	N			N	N	N
8-Nov-01	MDQ	BHAE	4	R115777-DS	1. 3	5.3		91	103.5	104. 1	PASS	N		112453	N	N	laatste select. opl.
23-Nov-01	MDQ	BHAE	4	R115777-DS	7. 9	4.5		91	98.5	106. 9	PASS	Y			N	N	N
11-Jan-02	MDQ	STHO	6	R115777-DP	1. 1	5.0		90	103.0	101. 9	PASS	N		212008	N	N	N
15-Jan-02	MDQ	STHO	6	R115777-DP	2. 1	3.6		112	104.6	110. 4	PASS	Y		212010	N	N	N
14-Feb-02	MDQ	STHO	2	R115777-DS	2. 4	3.5		87	98.4	99. 7	PASS	N			N	N	N
16-Jul-02	MDQ	DDEK	2	R115777-DP	1. 2	4.7		87	94.6	102. 1	PASS	N			N	N	KB: R115777-0012
29-Aug-02	MDQ	DDEK	3	R115777-DS	1. 9	5.3		93	97.9	100. 6	PASS	N			N	N	KB R115777-0016
24-Sep-02	MDQ	DDEK	2	R115777-DP	1. 9	4.0		84	95.7	100. 6	PASS	N		212314	N	N	KB: R115777-0024
30-ap-2002	MDQ	STHO	1	T002512		3.6		83			PASS	N			N	N	N
21-May-02	MDQ	DDEK	1	R096769		3.3		99			PASS	N			N	N	N
18-Jun-02	MDQ	DDEK	7	R096769							PASS	N			N	N	N
17-Jul-02	MDQ	DDEK	5	T002512							PASS	N			N	N	N
23-Aug-02	MDQ	STHO	2	T002512		3.5		99			PASS	N			N	N	N
16-Sep-02	MDQ	STHO	4	R096769		3.0		78			PASS	N		212305	N	N	N
27-Sep-02	MDQ	STHO	5	R096769		3.3		72			PASS	N		212307	N	N	N
10OKT2001	MDQ	STHO	4	R113675	1.4	3.4	1.1		98.1	96.3	PASS	N		112363	N	N	N
12-Nov-02	MDQ	STHO	6	R113675	3.2	3.9	1.0		105.4	99.6	PASS	N			N	N	N
7-Nov-02	MDQ	STHO	2	T002512		2.9		101			PASS	N			N	N	N
18-Dec-02	MDQ	STHO	9	R096769		2.7		58			PASS	N			N	N	N
												N				N	N
		Upper Limit			10.0	2.0	2.0	150.0	125.0	125.0							
		Lower limit						50	75	75							

FIGURE 22 Example of a feedback sheet that can be used to monitor performance of CE methods.

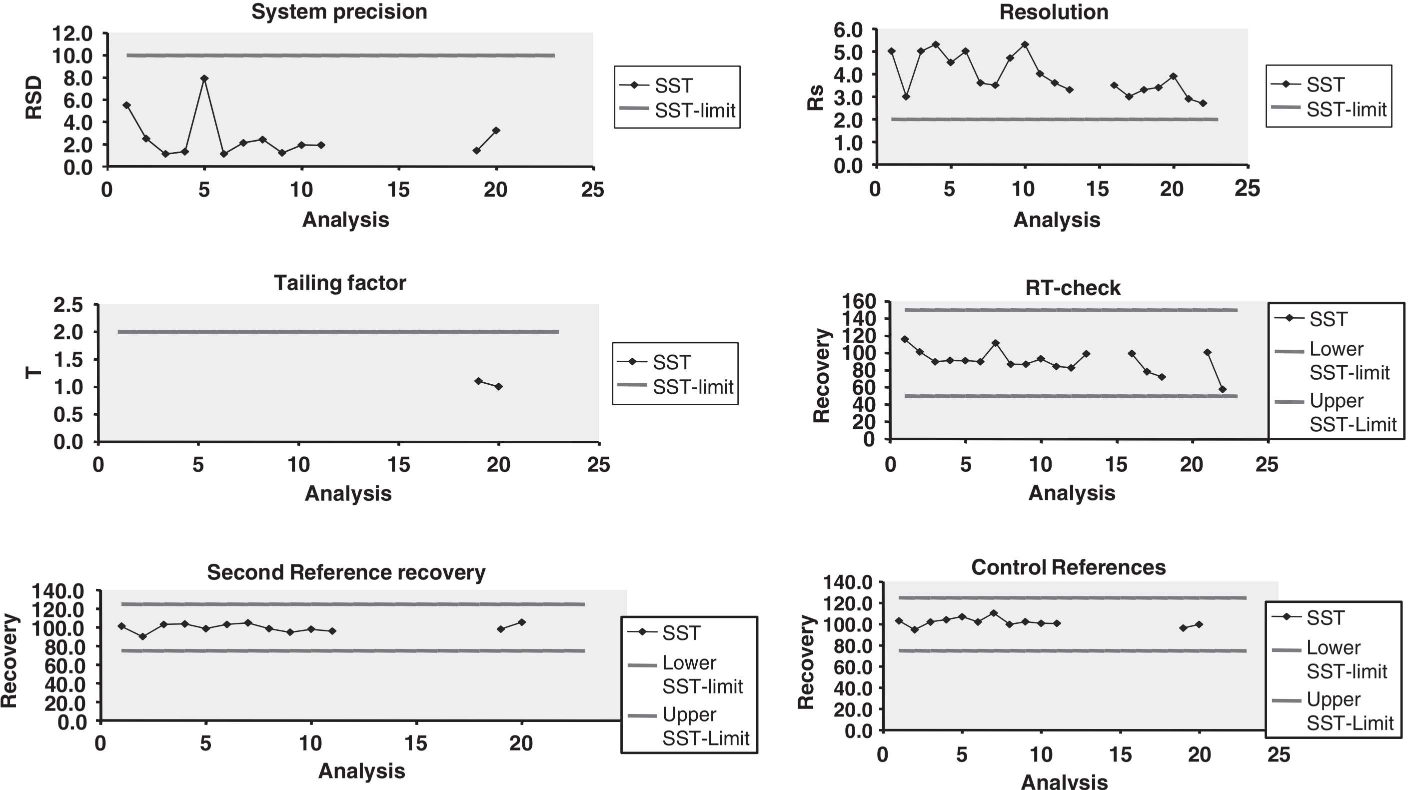


FIGURE 23 Quantitative method performance indicators can be plotted in control charts.

considered to be the real challenge of the method in the classical approach of method development. It is only at this stage that the receiving labs were involved in the process. Consequently, many issues were encountered at the transfer and required to be resolved. Within the advanced method development process of late phase methods there is a continuous involvement of the customers. The final test method is not "new" for the application labs as they have participated in the requirement setting and in the evaluation of the method. Moreover, the application labs have already tried the method out in their own environment. All issues on method applicability have been resolved prior to the transfer study. Method transfer is considered to be a formal process that is necessary to qualify the receiving lab to run the test method for release of products. By demonstrating equal method performance in the development and the application labs, the transfer is considered successful. Additional information on method transfer comparable to HPLC methods is provided in reference 16.

X. METHOD PERFORMANCE MONITORING AND FEEDBACK

It is recommended to monitor the performance of the methods during real time analysis in the application labs. Indeed, the best setting for method evaluation is really during actual application to sample batches. In this way, a historical source of objective data is made available that will be the basis for future discussions on method issues between the application and the development labs.

The development lab can benefit from valuable objective data to detect method shortcomings and to identify gaps in the method development process. The feedback on method performance should be discussed regularly. During the monitoring a number of key performance indicators are recorded and filled out in feedback sheets by the application labs (stability and operational labs) each time the method is applied. The method feedback sheet is sent together with the method description to the application labs at transfer. An example of such a feedback sheet is shown in Figure 22.

Several method performance indicators are tracked, monitored, and recorded, including the date of analysis, identification of equipment, identification of the analyst, number and type of samples analyzed, the system precision, the critical resolution or tailing factor, the recovery at the reporting threshold level, the recovery of a second reference weighing, the recovery for the control references (repeated reference injections for evaluation of system drift), the separation quality, blank issues, out of spec issues, carry over issues, and other non-conformances. The quantitative indicators are additionally visualized by plotting on control charts (Figure 23).

XI. SUMMARY AND CONCLUSIONS

It is important to include the receiving lab early in the development process of analytical methods. In this way, the receiving lab can provide critical input that may be primordial for a successful application in QC. In return, the receiving lab will be familiarized with the resulting method description and can receive proper training of analysts to perform the method, prior to final validation of the method. As a result method transfer activities are bound to be successful. This concept is essential for "new" technologies such as CE to be introduced in the QC environment.

When developing methods for QC, it has been demonstrated that a suitable pre-run rinse step is fundamental to guarantee a consistent performance. The proposed pre-run capillary rinse routine has shown to result in robust and reliable CE methods that can withstand the requirements of a QC lab. Because of many different variables affecting the outcome of a CE method, it is recommended to apply DOE approaches as much as possible during

development and optimization of CE methods. Moreover, CE-specific handling skills and expertise is required to allow both the method development lab as well as the receiving lab to operate CE methods adequately. Therefore, if you encounter a system failure do not start cursing the CE machine or the test method, but rather seek for more advice and training by CE practical experts.

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5

ROLE OF CE IN DRUG SUBSTANCE AND DRUG PRODUCT DEVELOPMENT

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ABSTRACT
I. INTRODUCTION
A. Benefits of CE
II. WHEN SHOULD CE BE APPLIED IN DRUG DEVELOPMENT?
III. CAPILLARY ELECTROPHORESIS IN THE DRUG DEVELOPMENT PROCESS, FROM CANDIDATE SELECTION TO THE MARKET
A. Preclinical Stage, from Research to the First Clinical Trial
B. Activities During Clinical Development
C. The Marketed Stage of a Mature Drug with Full Production Size for the Market
IV. PHARMACEUTICAL SUBSTANCES IN LITERATURE
V. SUMMARY AND CONCLUSIONS
REFERENCES

ABSTRACT

The use of capillary electrophoresis (CE) during the synthetic drug development is described from the preclinical development phase to the final marketed stage. The chapter comprises the determination of physicochemical properties, such as acid–base dissociation constants (pK_a), octanol–water distribution coefficients ($\log P$), and analysis of pharmaceutical counterions and functional excipients.

General requirements during clinical development and submission for CE methods, used for release, retest, and stability analysis, are illustrated by examples of CE applications. Guidelines for method development based on the nature of the analyte and the likelihood of a successful method development are given. Generic approaches and ready-to-use separation kits are described in order to minimize method development times. The specification setting process including examples for identity, purity, and assay tests is explained. Points to consider for a CE method transfer to another testing facility are discussed. Finally, further links to literature and review articles are referenced.

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I. INTRODUCTION

The development of a new drug for human use is a rather extensive process requiring time, workforce, and financial investments. The potential drug candidate must fulfill a series of rigorous requirements before it will receive its approval for marketing by the health authorities. This approval can only be obtained if the drug is effective, has a significant benefit for the patient, and, most important, is safe for the patient.

Focusing on physical and chemical properties, the requirements for a drug substance are as follows:

- The physicochemical properties of a drug substance must be known and well characterized (e.g., solubility, acidic constant (pKa), distribution coefficient in octanol water ($\log P$ value), pH-dependent distribution coefficient ($\log D$), and its physical state, e.g., morphology or salt stochiometry − if the drug forms a pharmaceutical salt).
- The drug substance must be pure and literally free of substances with high toxic or mutagenic potential. Impurities above a certain level must be quantified and identified. The drug substance must be stable during storage and transport for the stipulated storage period.
- The manufacturing route, raw materials, and chemicals must be well controlled and meet strict quality requirements (e.g., purity of raw materials, chemicals and intermediates, process steering control, cleaning verification).

The drug substance candidate that fulfills most of the properties described above will be selected for pharmaceutical formulation development. Focusing on bioavailability and stability, the requirements for a drug product are as follows:

- Sufficient bioavailability must be achieved by the chosen formulation with an appropriate selection of excipients. In general, dissolution rate tests and pharmacokinetic studies are used to assess the bioavailability of the drug product.
- It must be guaranteed that the product is stable through its assigned shelf life. Therefore, the potential drug product formulations are subjected to stability testing. A broad range of tests is performed to assess the chemical and physical stability of the drug product. The amount of possible degradation products needs to be monitored and quantified.
- It must be ensured that a drug product within a produced batch contains the same amount of the active ingredient, the same stability profile, and the same bioavailability. Furthermore all batches produced should be comparable in their properties as mentioned before.

Drug substance and drug product undergo enormous testing efforts before they can be administered to patients. The specifications to ensure the product quality are defined in the early development phase and will be monitored through the whole life cycle.

This chapter will focus on the potential fields of capillary electrophoresis (CE) used in the development process of drugs. Challenges, appropriate remediation to overcome limitations, as well as the benefits will be addressed and described.

Since its original development in 1981 by Jorgensson et al., CE has been undergoing ups and downs. The development of fully automated systems and further CE techniques, such as micellar electrokinetic chromatography (MEKC), capillary isotachophoresis (ITP), capillary isoelectric focusing (IEF), and capillary gel electrophoresis (CGE) in the early 1990s helped to spread the use of the CE technology. The human genome project and genetics in general boosted the development.

Because of its versatility and complexity CE should be regarded as a family of analytical techniques that are performed on one single instrument, rather than a single technique. Each

mode has its advantages and its limitations. To tap the full potential of CE, each single mode should be fully understood and implemented. This is a training challenge and a hurdle for the implementation of CE in the pharmaceutical development. CE is only successful if the resources necessary for its implementation are granted by the upper management. Successful implementation CE will make an invaluable contribution to the development of new drugs, by increasing separation speed and resolving new analytical challenges not addressable by other techniques.

Based on its nature (aqueous solutions, physiological conditions, well-investigated labeling, and staining reactions) and the historical transition from slab-gel electrophoresis to CE, the main targets are biological and bioequivalent samples such as proteins, peptides, polynucleotides, oligonucleotides, and carbohydrates.

In the first peak of the CE development in the mid-1990s, it was stipulated enthusiastically that CE would replace HPLC in the pharmaceutical industry. However taking a sober view after ten more years, this is not the case because of the following reasons:

- The majority of active pharmaceutical ingredients on the market are small molecules derived from a synthetic route; they are, partially badly, or not soluble in water.
- The separation ability of the most common CE technique CZE is based on the hydrodynamic volume:charge ratio, which makes it difficult to separate substances which are chemically very similar to the active ingredient such as by-products and degradation products.
- The small light path (50−100 μm) of the capillary and the non-linear behavior of the detection system and overloading effects are narrowing the concentration range that can be applied.
- CE requires more training and different knowledge and expertise than HPLC.
- Other chromatographic technologies, such as HPLC, gas chromatography (GC), and ion chromatography are in permanent competition with CE in many application fields and are well established and more widely used with less training requirements. Furthermore most of these techniques are supported by a large number of international companies that constantly produce improved instrumentation and applications. Despite its renaissance in the biotech industry, the number of companies producing CE instrumentation or supplies was on the decline and most of the innovations in the instrumentation were introduced a decade ago. Therefore it is a pleasant development that instrument manufacturers show new interest in CE and commit to its future support.
- The injection principle and the somewhat higher fragility of the system may lead to higher variations in results and higher failure rates of injections when working in a controlled quality environment.
- It is more challenging to obtain a good system-to-system transferability.
- Method transfer from development side to a contractor or a production side may be challenging. For HPLC, GC, or IC such transfers are performed permanently and most of the contractors and production sites have expertise in these technologies. In many cases, building up expertise at the production side lab is required for CE.

A. Benefits of CE

There are significant benefits of CE:

- Only very small amounts of sample are required for analysis (nanoliter range). This is a large advantage in early-phase drug discovery and a tremendous advantage for

biochemical applications where the amount of active ingredient is limited and extremely expensive.
- Based on the nature of separation, the peak capacity of the separation system is much higher than in liquid chromatographic techniques.
- The open tubular technique requires less sample preparation, shorter conditioning times, and, therefore, enables faster switching to other separation systems.
- Chemicals and consumables are considerably inexpensive and no or less organic solvents are used.
- The separation mechanism is quite different from other chromatographic techniques and a broader spectrum of possible impurities can be detected at the same system (e.g., inorganic small cations, anions by indirect detection, chiral separations by adding a chiral selector, proteins and peptides by adding a polymer to the separation buffer, etc.)
- Separations can be performed in physiologically equivalent environment (e.g., aqueous solutions, phosphate buffers, etc.)
- CE is a high-resolution technique and is the method of choice for the quality control of large biomolecules (e.g., DNA. RNA, monoclonal antibodies, etc.)
- Its versatility and its generic approach are the most important assets of CE. It can be challenging and time-consuming to find the optimized conditions for a separation. However, once optimized, the conditions can be used most often for a whole class of substances without further optimization. For drug development this means that in the majority of cases the drug substance method can be applied to the drug product with small adaptations like special rinsing steps to maintain the integrity of the capillary or sample cleanup steps in some cases. Sample preparation will be addressed later in this chapter.

A valuable introduction to the generic approach can be found in literature.[1] To cut down optimization times a good starting point for method development are kit solutions available from a broad range of suppliers. Table 1 is a summary of kit solutions for small molecules from several suppliers. For details on the content of the kit packages please refer to the corresponding supplier.

II. WHEN SHOULD CE BE APPLIED IN DRUG DEVELOPMENT?

This chapter will mainly focus on the majority of drug formulations based on synthetic drug substances and their quality control or both. For biotechnological, food,[2] medical,[3] and environmental applications[4] please refer to the relevant textbooks or other chapters of this book.

Table 2 summarizes in brief the CE techniques, possible drug substance chemo types (chargeable group, chromophore, water solubility), and competing quantification methods.

At present, most drugs on the market are based on synthetic drug substances that are chemically synthesized and contain either a chargeable group (amino or carboxyl function), a UV chromophore (typically heterocyclic aromatic rings), or both. However, solubility properties of a drug substance may vary strongly in coherence with the targeted effect in the organism. Therefore, not all techniques may be applied. The same is valid for molecules without charge. Due to its linear properties, quite broad range of application, and widespread distribution in industry, the UV detection is the most common technique for quantification in chromatographic techniques. For UV detection and alternative detection modes in CE please refer to Chapter 3. Table 3 may help evaluating the separation possibilities for a drug substance or the active ingredient in a pharmaceutical formulation, based on solubility,

TABLE 1 Commercially Available Kit Solutions for Small Molecules (Non-Exhaustive)

Supplier	Description	
Analis/Microsolv	Ceofix™ pH/CElexir-pH	A set of buffers in the pH range of 2.5–9.2 using an innovative double coating that grants high EOF reproducibility. Kits can be used for all kinds of positively charged absorbing molecules. A wide range of additives (e.g., organic modifier, cyclodextrins) enhance the versatility of these kits even more
	CEofix™ Anions/CElexir-OA	A set of buffers for absorbing negatively charged molecules (pH 2.5) and non-absorbing negatively charged molecules (pH 5.4–8.2)
	CEofix™ MEKC/CElexir-SDS	A kit solutions for non-charged molecules based on micellar electrokinetic capillary electrophoresis
	CEofix™ MS/CElexir-MS	A kit solution based on a volatile buffer for MS application and a coating solution granting high EOF reproducibility
Agilent	Plating Bath Analysis Kit	Kit solution for the indirect detection of inorganic and organic anions with metal cations (e.g., Ni^{2+}, Cu^{2+}, Co^{2+}) in plating bath solutions
	Forensic Anion Solutions Kit	Kit solution for the determination of toxic anions (e.g., arsenate, arsenite, azide, or cyanide) and other inorganic and organic anions with indirect UV detection
	Cation Solutions Kit	Kit for the separation of low-molecular mass organic cations like alkali metal ions, alkaline earth metal ions, and alkyl amines. Indirect UV detection is applied.
	Inorganic Anions Solutions kit	Kit for the separation of inorganic anions (e.g., chloride, bromide, sulfate, phosphate). Indirect UV detection is applied
	Organic Acids Solutions Kit	Kit for the separation of short alkyl chain carboxylic acids. Indirect UV detection is applied.
Beckman	P/ACE™ System MDQ chiral methods development	A complete package for chiral separation consisting of neutral capillaries, several cyclodextrins, and buffer solutions
	P/ACE™ System MDQ highly sulfated cyclodextrin trial kit	A complete package for chiral separation consisting of several highly sulfated cyclodextrins and buffer solution
	eCAP™ Amine Capillary Method Development Kit/Small Molecules	An extended package for the separation of basic molecules containing amine capillaries, buffers, and micellar agents
	eCAP™ Carbohydrate Labeling Kit	Kit solution for the labeling of *N*-linked (oligo) saccharides with APTS. Enables high sensitive detection by fluorescence.
Groton	Amino Acid Analysis	Several kit solutions for indirect detection of amino acids and carbohydrates and fluorescence detection of amino acids after derivatization.

TABLE 1 (Cont.)

Supplier	Description	
	Vitamin Analysis	Kit solution for the determination of vitamins and aromatic molecules
	Cell Culture Analysis	Kit for the determination of phosphate and organic anions in cell cultures
Prince Technologies	CE-Sure Anion kit	Analysis of Anions (CZE)
	CE-Sure Cation kit	Analysis of Cations (CZE)
	CE-Sure Drugs screening kit	Set of different buffers for drug screening
	CE-Sure MECC kit	Kit based on micellar electrokinetic capillary electrophoresis
	CE-Sure small organic acids kit	Kit for the analysis of small organic acids (CZE)
	CE-Sure Conductivity Cation kit	Kit for the analysis of small cations by conductivity detection
	CE-Sure Conductivity Anion kit	Kit for the analysis of small anions by conductivity detection

TABLE 2 Target Chemotypes, the Corresponding CE Technique and Competing Methods

CE technique	Target chemotype	Main competing methods
Capillary zone electrophoresis (direct detection)	Chargeable water soluble molecules with UV chromophore	HPLC, GC
Capillary zone electrophoresis (indirect detection)	Chargeable water soluble molecules without UV chromophore	Ion chromatography, HPLC MS
Chiral capillary electrophoresis	Neutral and chargeable molecules with chiral centers	Chiral HPLC, chiral GC
Micellar electrokinetic chromatography	Water soluble and insoluble molecules with UV chromophore	HPLC
Non-aqueous CE	In organic solvent chargeable, in solvent system soluble compounds, mainly with UV chromophore	HPLC
Capillary Isotachophoresis (cITP)	Chargeable water soluble molecules	Ion chromatography
Capillary electrophoresis with isoelectric focusing (cIEF)	Zwitterionic compounds with UV-chromophore, mainly oligopeptides and proteins	Slab-gel electrophoresis techniques
Size discriminating CE (Capillary gel electrophoresis, entangled polymer solutions, SDS-PAGE)	Oligo- and polynucleotides, oligo- and polypeptides, mono- and oligosaccharides	Slab-gel electrophoresis techniques

availability of a UV chromophore and charge. Based on the technique principles, the number of publications, and the degree of difficulty of the system, the success rate was given for the development of a purity method which is able to quantify 0.1 weight percent of a similar compound in a drug substance or the corresponding drug formulation.

TABLE 3 CE Purity Methods and Their Likelihood of Success

UV-chromophore	Solubility in aqueous systems (mg/ml)	Charge	Applicable CE-technique from Table 1	Success rate for a purity method (LOQ ≤0.1%)
Aromatic	>2	Yes	1,(2),3,4,5,6,(7,8)	Very high
Aromatic	>2	No	3,4	High
Aromatic	<2	Yes	3,4,5	Moderate
Aromatic	<2	No	Special system required	Very low
Others	>10	Yes	1,(2),3,4,5,6,(7,8)	High
Others	>10	No	3,4	Moderate
Others	<10	Yes	(5, indirect detection)	Low
Others	<10	No	Special system required	Very low
None	>10	Yes	2	High
None	>10	No	Special system required	Very low
None	<10	Yes	(5, indirect detection)	Low
None	<10	No	Special system required	Very low

For systems with moderate-to-low probability, CE might not be the chromatographic quantification method of choice, and other alternatives, such as HPLC and GC, should be considered. However, specific procedures (e.g., off-line concentration, stacking techniques, extended light path capillaries) and detectors may be applied to increase solubility and sensitivity of detection, such as derivatization (e.g., carbohydrates, amino acids, amines, etc.) or the use of a specific detector (e.g., contactless conductivity detection, coupling with mass spectrometry, etc.). However, increasing the complexity of the methodology may be counterproductive if it leads to a lower robustness and transferability of the system.

The pure drug can rarely be applied to the patients directly. A drug formulation is necessary. Nowadays a broad range of formulations (e.g., oral solutions, intravenous injections, tablets, inhalation devices, etc.) are available. The goal of the formulation is to transport the drug substance to its place of action in the patient's body and to grant high bioavailability. In addition the formulation needs to be stable through storage and shelf life. The formulations consist of a broad range of pharmaceutical excipients.

If the excipients do not interfere with the CE separation, the available drug substance method can also be applied to the drug product without further optimization. This is the most favorable option. Each additional sample preparation step decreases the sample throughput speed, increases the variability of the method, and requires more validation effort. Fortunately CE is a high-efficiency method and interference of excipient peaks with analyte peaks occurs rarely.

For high reproducibility of migration times and peak areas the sample matrix should be identical for all samples analyzed together. While this is challenging for forensic applications where analytes in whole blood or urine are determined, this requirement can be fulfilled easily in pharmaceutical analysis. After sample preparation of the drug product, the sample matrix is similar in most cases. The composition of blood or urine depends on its source. Thus, the changing sample matrix has more impact on the quality of the CE analysis.

For sample cleanup the typical methods like solid-phase extraction (SPE) and liquid–liquid extraction can be used in the same manner as they are used for HPLC. Please refer to the corresponding handbooks for a detailed background of these sample preparation methods.[5,6]

III. CAPILLARY ELECTROPHORESIS IN THE DRUG DEVELOPMENT PROCESS, FROM CANDIDATE SELECTION TO THE MARKET

Moving from drug candidate selection to the finally marketed drug, the requirements for each phase of research and development may vary strongly. Statistically seen, approximately 20% of new chemical entities entering a clinical trial phase will be finally approved and marketed. Each phase has therefore its own specific requirements for the physicochemical characterization of compounds. Although drug research and development is a continuous process, the following will try to classify some stages of the drug life cycle:

(A) The preclinical stage, from research to the first clinical trials.
(B) The clinical development stage up to the registration with health authorities.
(C) The marketed stage of a mature drug with full production size for the market.

A. Preclinical Stage, from Research to the First Clinical Trial

In the preclinical stage, the main activities are to find new promising chemical compounds or a group of chemically similar compounds (the so-called lead compounds) that will have the desired effect and efficiency in a specific therapeutic field. In general, only small amounts of a possibly active substance are available. Based on the high number of compounds, high-throughput screening approaches are required, delivering a broad range of data for each compound in a short time to assess the developability of a drug. Besides numerous other tests, physicochemical properties, such as solubility and permeability data, ionization constants, drug stability, and evaluation of the physical form and a first formulation concept will support the selection of a lead compound. CE techniques have found their application especially in areas where a versatile characterization and quantification using little amount of sample is required.[7]

Depending on the drug substance properties and the medical indication, the appropriate formulation is chosen. Drug substances which have a good solubility may be formulated in a simple tablet formulation, while drug substance candidates with low solubilities require special delivery systems like microemulsion formulations.

After the type of formulation (e.g., tablet, oral solution, intravenous injection, microemulsion formulation) is defined, the composition of the formulation is optimized. Most important at this stage is the excipient compatibility testing which is targeting to identify any possible interaction with the drug (e.g., induced degradation by excipients). Furthermore the bioavailability of the active ingredient is addressed. After the first in vivo studies for bioavailability, the determination of the dissolution rate of a solid dosage form is an important indicator to ensure batch-to-batch reproducibility and therefore verify a constant bioavailability. For most of these tests HPLC is the method of choice. Nevertheless some publications dealing with CE and dissolution rate exist.[8] To stabilize the formulation or to enhance bioavailability the so-called functional excipients are used. Most of these excipients are small ions that lack a chromophore. Thus they are an ideal analyte for CE with indirect UV detection.

1. Determination of Dissociation Constants

The fundamental principles of pK_a determination by CE rely upon measuring the ionic effective mobility of the solute as a function of pH. Consequently, the pK value is obtained by fitting an equilibrium equation to the effective mobility and pH data with a non-linear regression technique[9,10] (Figure 1). Depending on the chemical character of the compound

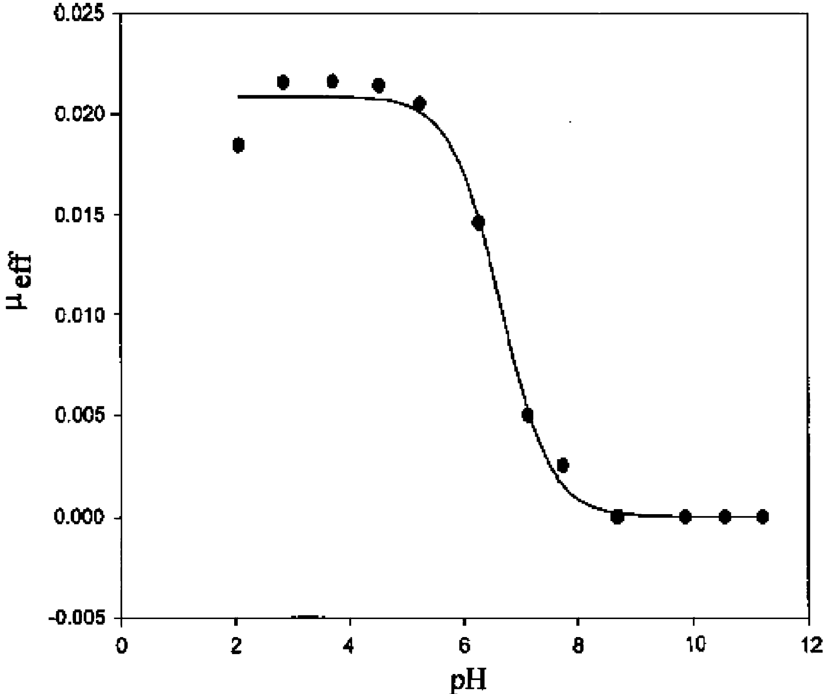

FIGURE 1 Plot of effective mobility versus pH for 2-aminopyridine (pK_a 6.7). The solid line corresponds to the fit of the data to the model equation for a mono base (taken from reference 10. With permission.).

and the type and ionic strength of the background electrolyte, the effective mobility (m_{eff}) of the ionized compound can be described in different equations, as given in literature[3] (Table 4).

The pK_a screening methodology was further standardized and optimized for high-throughput measurements.[11,12] Current developments are the use of a commercially available 96 capillary instrument including the corresponding evaluation software,[13] the use of commercially available separation kits and pressure-assisted CE to shorten the run times.[9,14]

2. Determination of Distribution Coefficients (log P Values)

Distribution coefficients were classically determined by the so-called "shake-flask" method where the distribution of a drug was measured by determining each concentration in the respective aqueous and organic phase. However, the classic procedure is rather slow and substance and solvent consuming. To increase the analysis speed and lower sample amounts, chromatography-based methods are a good alternative.[15] The most common CE method is based on the principles of MEKC and microemulsion electrokinetic capillary chromatography (MEEKC), where the electrophoretic mobility of a solute correlates with its hydrophobicity. The mobility is then correlated to a set of standards with known hydrophobicity[16–18] and good correlations are found (Table 5). To avoid charges of the molecule, the solute must be uncharged in the solution. Thus, two different systems for weak bases at high pH and weak acids at low pH are normally required. Current developments are the use of synthesized vesicles as carriers[19] for widening the hydrophobicity range of the system and high-throughput analysis with a 96 capillary instrument.[20]

TABLE 4 Model Equations from pK_a Determinations

Ionizable type	Model equation
Monoacid	$m_{eff} = \frac{m_a \times 10^{(-pK_a+pH+A)}}{1+10^{(-pK_a+pH+A)}}$
Monobase	$m_{eff} = \frac{m_b \times 10^{(pK_a-pH+A)}}{1+10^{(pK_a-pH+A)}}$
	$A = \frac{0.5085 \times z^2\sqrt{I}}{1+0.3281 \times a\sqrt{I}}$
Monoacid/monobase	$m_{eff} = \frac{m_{a1} \times [10^{-pH}]^2 + m_{a2} \times 10^{-pK_{a1}} \times 10^{-pK_{a2}}}{[10^{-pH}]^2 + 10^{-pK_{a1}} \times 10^{-pH} + 10^{-pK_{a1}} \times 10^{-pK_{a2}}}$
Diacid	$m_{eff} = \frac{m_{a1} \times 10^{-pK_{a1}} \times 10^{-pH} + m_{a2} \times 10^{-pK_{a1}} \times 10^{-pK_{a2}}}{[10^{-pH}]^2 + 10^{-pK_{a1}} \times 10^{-pH} \times 10^{-pK_{a1}} \times 10^{-pK_{a2}}}$
Dibase	$m_{eff} = \frac{m_{a1} \times [10^{-pH}]^2 + m_{a2} \times 10^{-pK_{a1}} \times 10^{-pH}}{[10^{-pH}]^2 + 10^{-pK_{a1}} \times 10^{-pH} \times 10^{-pK_{a1}} \times 10^{-pK_{a2}}}$

Note: A is the activity correction term, z is the charge of the ion, I is the ionic strength of the background electrolyte ($I = 0.5 \times \sum c_i \times z_i^2 = 0.05$), a is the hydrated analyte ion size parameter, set to 5Å, and m_a and m_b are the maximum mobility (constants for a given ion). For simplicity, the activity term A was not taken into account in some model equations.
Source: Adapted from reference 9. With permission.

TABLE 5 Log P Values Measured by MEEKC vs. Literature Values

Compound	Log k (MEEKC)	Log P_{ow} (MEEKC)	Log P_{ow} (literature)	ΔLog P_{ow}	pK_a (CE)
Atenolol	−0.67	0.49	0.15	0.34	9.58
Pilocarpine	−0.57	0.66	0.20	0.46	7.08
Aniline	−0.43	0.88	0.94	−0.07	4.61
N-Methylaniline	−0.07	1.46	1.65	−0.20	4.86
Acebutolol	0.14	1.79	1.75	0.04	9.41
Procaine	0.29	2.02	2.03	−0.01	9.04
Quinoline	0.22	1.91	2.15	−0.24	4.97
Quinidine	0.71	2.71	2.64	0.07	4.5; 8.57
Buspirone	0.71	2.70	2.78	−0.08	7.6
Papaverine	0.61	2.54	2.91	−0.37	6.38
3-Bromoquinoline	0.82	2.88	2.91	−0.03	2.74
Propranolol	1.06	3.25	3.35	−0.10	9.53
Chlorpromazine	1.88	4.56	5.34	−0.78	9.24

Source: Adapted from reference 17. With permission.

3. Analysis of Pharmaceutical Counterions and Functional Excipients

It is common practice in pharmaceutical industry to generate salt forms of a drug substance to improve solid-state properties and solubility. CE has proven its ability to analyze reliably organic acids (direct, indirect detection) and alkaline/earth alkaline metals and basic amino acids. For basic drugs, a non-toxic organic acid or inorganic acid is chosen as counterion. Acidic drug substances will usually be deprotonated by alkaline and earth alkaline

metal hydroxides or basic amino acids. As a salt formation has a high impact on permeability, solubility, and stability properties of a drug substance, a salt screening is performed in the preclinical stage, evaluating the possibilities to continue with an advantageous salt form. Upon discovery of a crystalline salt form, the identity of the salt form and the stoichiometry have to be evaluated. CE is competing with microtitration methods and ion chromatography. However, with sample amounts in the nanoliter range, inexpensive analysis, short conditioning times, and flexibility of the separation systems, CE is advantageous in this area. Especially the development of new commercially available kits using dynamic coatings improved the repeatability significantly. Figure 2 shows the separation of several salt-forming agents, using a commercially available kit for organic and inorganic anions.[21]

In principle, the same separation systems can be used to determine functional excipients like small organic acids or amines.

Besides CZE, recent advances were also made in ITP for the determination of salt-forming agents.[22]

B. Activities During Clinical Development

After the selection of a potential drug substance candidate and the corresponding pharmaceutical formulation, the preparations for a possible clinical trial will start. These include in vitro and in vivo toxicological studies and the modification of the drug substance synthesis. Upscaling, the manufacturing of pilot batches, and release and stability testing are performed on the drug substance and the drug product. In addition, potential impurities will be synthesized, analyzed, and toxicologically qualified.

To support the development activities, accurate and sensitive analytical methods are required. With progress in development, increasing method validation work is performed and the acceptance criteria for drug quality and test method precision are tightened.

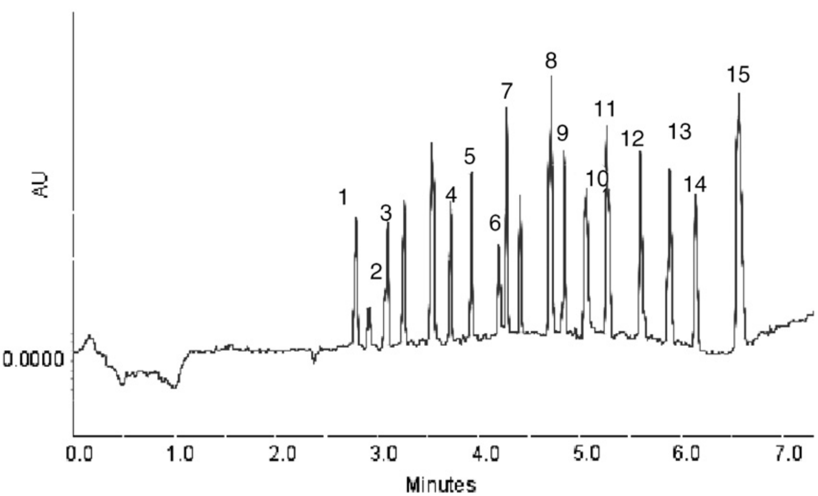

FIGURE 2 Separation of 15 acids used as pharmaceutical salt-forming agents, peak assignment: 1. Hydrochloric, 2. Nitric, 3. Sulfuric, 4. Tartaric, 5. Malic, 6. Citric, 7. Succinic, 8. Acetic, 9. Lactic, 10. Phosphate, 11. Propionic, 12. Butyric, 13. Pentanoic, 14. Hexanoic, and 15. Octanoic (taken from reference 21. With permission.).

The way to perform the analytical tests and the requirements for a drug for human use are strictly regulated in a harmonized way by North America, Europe, and Japan in the ICH guidelines, following the principles of good manufacturing practice (GMP). Table 6 summarizes the main guidelines that are directly related to drug substance and drug product development. Other guidance documents related to CE in general are provided in Chapter 7.

In addition, the country-specific pharmacopoeias describe in detail the test methods and procedures to be used for the characterization of chemicals. CE can be found as a technique in

- General Chapter 727 and 1053 in USP 31
- General Chapter 2.2.47 in the European Pharmacopoeia 5.8.

The Japanese pharmacopoeia has not yet included a general method description of CE. General chapter 8 in the Japanese Pharmacopoeia 15.

1. Specification Setting

As part of a quality system, specifications are set that define the quality chemical synthesis intermediate, raw material, the drug substance, and the drug product. Each batch of material produced will be compared with the test criteria set. If the results exceed the specified range or limit, the produced batch will not be released for human use or further production before a thorough quality investigation, corrective actions (e.g., reprocessing), and preventive actions (elimination of the causes for the specification mismatch in the future). The methods can be grouped into tests for description, identity, assay (content test of active compound), and impurities. Besides description tests, CE can be applied to the latter groups (see Chapter 4).

2. Identification Tests

The advantage of the use of CE for identification screening is based on the high peak capacity of the system and the separation ability to identify substance mixtures. Due to variations in the electroosmotic flow, the migration times in CE are less stable as in a HPLC system. Therefore, the identification tests in CE are based on the principle of calculating the

TABLE 6 ICH Guidelines Affecting the Analytical Testing of Drug Substances and Drug Products Valid as of November 2005

Guidelines	Title	Activity
Q1A (R2)	Stability testing of new drug substances and new drug products	Stability
Q1B	Stability Testing: Photostability Testing of New Drug Substances and Products	Stability
Q2 (R1)	Validation of Analytical Procedures: Text and Methodology	Method validation
Q3A (R2)	Impurities in New Drug Substances	Impurity testing
Q3B (R2)	Impurities in New Drug Products	Impurity testing
Q6A	Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (including Decision Trees)	Specification setting/ impurity testing

Source: Adapted from reference 51.

effective electrophoretic mobility μ_{eff} from its apparent mobility μ_{app} and the mobility of the electroosmotic flow μ_{EOF}:[23]

$$\mu_{eff} = \mu_{app} - \mu_{EOF}$$

The precision of the identification can be further improved by the use of standards for correction[24,25] and EOF stabilizing coatings.[26] The identification method can be widely applied with all CE technologies, starting from CZE for small ions up to proteins and small synthetic peptides. Application fields are therefore as wide as the range of CE technologies. Some examples are the identification and categorization of seized drugs,[27] counterfeit drugs,[28] and cleaning verification.[29,30] However, the most common use of CE identification methods remains in numerous applications for biosimilar synthetic drugs, such as small peptides[31] and oligonucleotides[32] and carbohydrates.[33]

As for similar relative identification methods, identification based on mobility may not be adequate alone and further spectral evidence by diode array detection and mass spectrometry may be needed. ICH Guideline Q6A claims that "identification solely by a single chromatographic retention time, for example, is not regarded as being specific." Thus for late-phase projects a second identification method is required for the active ingredient. Recently near-infra red (NIR) instruments replace the traditional thin layer technique used for identity testing. NIR is capable to perform high-throughput identity testing for tablets and liquids. NIR has its limitation in formulations that contain two, three, or even more active ingredients. For those combination products CE may be the choice for second identity determination.

3. Purity Tests

The ICH Guideline Q3A is very specific about the purity requirements of a drug substance for human use. Q3B is the corresponding guideline for drug products.

Based on the administered daily dose of a drug substance, thresholds for impurities were set as given in Tables 7 and 8.

Reporting Threshold: A limit above (>) which an impurity should be reported. (The limit of quantification should be less or equal to the reporting threshold.)

Identification Threshold: A limit above (>) which an impurity should be identified.

Qualification Threshold: A limit above (>) which an impurity should be qualified.

The percentages given are weight/weight percentages referred to the isolated drug substance. It should be mentioned that qualification means in this term the assessment of a possible biological effect of an impurity of the drug substance by toxicological investigation. In addition, these limits are only given for impurities that do not show any specific

TABLE 7 Thresholds for Impurities in a Drug Substance According to ICH Q3A

Maximum daily dose (g/day)	Reporting threshold (%)	Identification threshold	Qualification threshold
≤2	0.05	0.10% or 1.0 mg per TDI (whichever is lower)	0.15% or 1.0 mg per TDI (whichever is lower)
>2	0.03	0.05%	0.05%

TDI: Total daily intake.
Source: Adapted from reference 51.

TABLE 8 Thresholds for Impurities in a Drug Product According to ICH Q3B

Maximum daily dose	Reporting threshold
≤1 g/day	0.1%
>1 g/day	0.05%
Maximum daily dose	Identification threshold
<1 mg	1% or 5 μg TDI, whichever is lower
1–10 mg	0.5% or 20 μg TDI, whichever is lower
>10 mg–2 g	0.2% or 2 mg TDI, whichever is lower
>2 g	0.10%
Maximum daily dose	Qualification threshold
<10 mg	1% or 50 μg TDI, whichever is lower
10–100 mg	0.5% or 200 μg TDI, whichever is lower
>100 mg–2 g	0.2% or 3 mg TDI, whichever is lower
>2 g	0.15%

TDI: Total daily intake.
Source: Adapted from reference 51.

toxicological alert. If a toxicological assessment of an identified or potential impurity shows a high potential for toxicity (e.g., genotoxicity, mutagenicity), harsher limits in the ppm range (weight/weight) of a drug will be required. This is normally out of the range of a CE with UV detection, and other more sensitive detection techniques such as CE–MS should be considered.

Due to the small light path through the capillary, the response factors (absorption/concentration) for UV detectors are much smaller in comparison to HPLC. Therefore, the concentration of the sample injected is generally higher. The overloading effects, solubility limits of the drug, or non-linearity effects represent the upper limit of the concentration range. As a rule of thumb, the peak of the main drug substance should be in the range of 200 mAU. Calculating down to the reporting limit and assuming that the peak height will decrease linearly, a peak with height of 0.1 mAU (0.2 mAU for drug products) should be detectable and repeatedly quantifiable. Ways to improve the sensitivity of a CE method with UV detection are listed in Table 9.

4. General Purity Tests

General purity tests are performed to evaluate the overall purity of a drug substance or a drug product. Known and unknown impurities will be quantified and the sum of all impurities will be reported. In most cases, an additional specification for the sum of all impurities is set. Impurities may derive from the chemical synthesis (reagents, precursors, by-products, degradation during manufacturing) or from the degradation of a drug substance. An ideal purity method should separate and quantify all the above in a sensitive, precise, and robust way. The common standard in industry for this test is HPLC for small molecules. However, HPLC will separate compounds with similar structure and hydrophobicity. Some charged compounds or compounds very different in the chemical character will not be retarded on the HPLC column, adsorbed on the column head, or are very late in eluting from the column. Therefore it is crucial to evaluate the purity of a drug substance with a separate method giving

TABLE 9 Ways and Limitations to Increase Sensitivity of a UV-Based CE Purity Method

Action	Effect	Limitation
Increase concentration of drug substance in sample solution	Increased detector signal	Solubility of drug substance, decreasing linearity of signal, peak broadening or instable currents due to overloading effects
Increase injection volume of sample solution	Increased detector signal	Same as above
Increase diameter of capillary	Increased detector signal	Increase of current and Joule heating effects, decrease in linearity of signal
Change wavelength	Move to the optimum wavelength for lamp power/absorption of drug substance	Wavelength cut-off, spectral properties of drug substance, instrument wavelength precision robustness
Narrow the peak shape (stacking effects, shorter separation time, coated capillaries, etc.)	Higher peaks, easier to be distinguished from baseline noise	Influence on selectivity
Use capillaries with extended light path or high-sensitivity detection cells	Increased detector signal	Robustness of extended light path equipment (handling and fragility issues), peak broadening, permanent coatings not available

a different orthogonal separation mechanism. Orthogonal screening of the drug product may be advisable but is not a requirement today.

Furthermore CE and thin layer chromatography (TLC) have the advantage to detect retained substances in the original injection zone. In CE, either pressure can be applied or the EOF itself transports uncharged or slow molecules past the detector. In this case, a blank sample solvent injection should be compared with the sample to elucidate whether the peaks are caused by the injection plug itself or are compound-related.

When developing an orthogonal CE method, the emphasis should be on the detection of possible new unidentified substances, not on the separation of the already known substances. A screening with a set of generic methods with various separation and detection principles may be more advantageous than a single method developed to separate all identified impurities. Table 10 gives examples on simple generic methods published or found in literature. In addition, the use of ready-to-use separation kits will reduce the time for the test preparation. Recent developments in CE separation kits using a stabilizing dynamic coating, leading to stable EOF and higher reproducibility of the analysis, can be purchased from various vendors (e.g., Analis, Microsolv, TargetDiscovery).

In an early stage of development, the identity of some structurally similar by-products is not known, even though toxicologically qualified. In this case, the peak areas of unknown impurities are compared with that of the drug substance. In later development, the chemical structures were identified, a correction factor between the peak area of drug substance and the related impurities is often calculated after determining the UV response factors of both compounds in a separate experiment. This gives the advantage of only using one drug substance standard for routine analysis instead of injecting every possible impurity as separate standard. However, it should be mentioned that in both cases, the linearity over the whole concentration range of drug substance should be checked. If not linear, it is not recommended

TABLE 10 Examples of Simple Generic Methods

System	Type of system	Application for	Reference(s)
Phosphate pH 2.5	CZE with direct UV detection	Basic solutes	52
Borate	CZE with direct UV detection	Acidic solutes	52
Li-dodecyl sulfate, borate, beta-cyclodextrin	MEKC/Chiral system with direct UV detection	Neutral and charged solutes	52
SDS, octane, borate, butan-1-ol	MEEKC system with direct detection	Neutral and charged solutes	52
Chromate, TTAB, borate	CZE system with indirect detection	Inorganic anions	52
TTAB, phthalate, MES	CZE system with indirect detection	Organic acids	52
Formic acid, imidazole	CZE system with indirect detection	Metal ions	52
MeOH, ACN, Na acetate	Non-aqueous CE system with direct detection	Insoluble/soluble acids	52
MeOH, acetic acid, NH4 acetate	Non-aqueous CE system with direct detection	Insoluble/soluble acids	52
Phosphate buffer, HS-beta cyclodextrin (α,β,γ)	Chiral CE system with direct detection	Charged and uncharged chiral analytes	53
phosphate buffer, 5% sulfobutylether-cyclodextrin, β or γ-dimethyl cyclodextrin	Chiral CE system with direct detection	Mainly charged analytes	54

to use peak area% or calibrations in comparison to a high concentration standard solution. In those cases, a calibration standard in the range of the possible concentration of impurities should be injected.

5. Chiral Purity Tests

Based on the high peak capacity of CE, the separation speed, and the availability of numerous chiral selectors and the simplicity of the systems, chiral CE is superior to chiral HPLC separations. This is as well reflected by the high number of publications on chiral CE in recent years. Chiral HPLC is suffering from low peak capacity (broad peaks), system stability (often normal phase systems), pressure sensitivity of columns (often cellulose-based column materials), and as a consequence long separation times.

Based on the theory, the separation of enantiomers requires a chiral additive to the CE separation buffer, while diastereomers can also be separated without the chiral selector. The majority of chiral CE separations are based on simple or chemically modified cyclodextrins. However, also other additives such as chiral crown ethers, linear oligo- and polysaccharides, macrocyclic antibiotics, chiral calixarenes, chiral ion-pairing agents, and chiral surfactants can be used.[34] Few non-chiral separation examples for the separation of diastereomers can be found.

All chiral separation principles are based on a slightly different interaction between the chiral sample molecule and the chiral selector. The interaction is based on either a slight difference with complexation equilibrium of the compound or by the different hydrodynamic volumes of the drug–chiral selector complex. Therefore, two different concentration ranges, where chiral separation occurs can normally be found. The influence of complex formation is stronger at low concentration ranges of chiral selector. At a high concentration range of chiral selector, the predominant separation principle is the difference in hydrodynamic volume of the drug–selector complex. As the loadability of the system increases with the amount of chiral selector, it is preferable to operate at higher concentration ranges of chiral selector to improve the sensitivity of the method. A detailed overview of the theory and application on chiral separations by CE can be found in the book *Capillary Electrophoresis in Chiral Analysis* written by B. Chankvetadze.[35]

Chiral CE can be widely applied in release and stability testing, the chiral purity of intermediates, and raw materials. Various generic method development approaches have been developed and published recently.[36–38,54]

By complexation, compounds with low solubility in aqueous media can be solubilized. Chiral selectors can also enhance the separation of non-chiral impurities from the drug substance.

6. Specific Impurity Tests

Specific impurity tests are based on the same principle as general purity tests. However, the method is limited to the quantification of one or several compounds only and will not be used for the determination of unknown impurities.

Specific impurity tests are applicable for drug substance and drug product release analysis (specific impurities from the manufacturing process, related impurities, and degradation products not detected by the general purity method), process steering controls, and cleaning verification.[30] As the focus is only on one or a few compounds, fast separation methods, e.g., using short capillaries or short-end injection can be developed.[39] After separating the relevant peaks, the capillary can be flushed and quickly prepared for the next run.

Examples are residual organic amines, organic and inorganic acidic and basic impurities (acids, bases, salts[40]), and detergents.[29]

7. Assay Tests and Content Analysis

The assay of a drug substance determines the actual concentration of the active pharmaceutical ingredient versus a high purity standard. Differences in the content of a drug substance may be due to impurities, water take-up, residual solvents from the manufacturing process, and differences in the ratio with the salt-forming agent. The purity requirements for drug substances are normally in the range of 98–100%. However, for an accurate measurement of the content, the precision of the test method should be in the same range or better (compare with Table 11 for chromatographic assay methods). Assay determinations for drug products are used to assess the amount of API in the drug product formulation. Furthermore assay determination plays an important role in the assessment of the blend uniformity and the content uniformity.

Due to the lower injection precision of CE, it is not the method of first choice for the assay determination because the methods with higher precision are competing (HPLC, titration). However, a few examples in literature can be found[41] and as a rule of thumb a well-developed CE method including internal standards should be able to obtain a repeatability of injections around 1% while values below 0.5% are generally expected for HPLC.

TABLE 11 Repeatability Requirements of Injection for Chromatographic Assay Tests According to European Pharmacopoeia

Upper specification limit in 100+B%	Number of individual injections			
	3	4	5	6
	Maximal % permitted relative standard deviation			
$B = 2.0$	0.41	0.59	0.73	0.85
$B = 2.5$	0.52	0.74	0.92	1.06
$B = 3.0$	0.62	0.89	1.10	1.27

Source: Adapted from reference 55.

8. Stability Tests

The stability of a drug substance and drug products needs to be assessed in order to identify the maximum storage period, packaging type, and handling instructions. According to ICH Q1A the tests can be classified as follows.

Long-term testing: Stability studies under the recommended storage condition for the retest period or shelf life proposed (or approved) are performed with several drug substance batches in the stipulate packaging. In the general case, the storage condition is at a temperature of 25°C and 60% relative humidity.

Intermediate testing: Studies conducted at 30°C/65% RH are designed to moderately increase the rate of chemical degradation or physical changes for a drug substance or drug product intended to be stored long term at 25°C. In some cases the long-term data are generated at 30°C and 65% relative humidity only and no additional intermediate study will be necessary in this case.

Accelerated testing studies are designed to increase the rate of chemical degradation or physical change of a drug substance by using exaggerated storage conditions as part of the formal stability studies. Data from these studies, in addition to long-term stability studies, can be used to assess longer-term chemical effects at non-accelerated conditions and to evaluate the effect of short-term excursions outside the label storage conditions such as might occur during shipping. Results from accelerated testing studies are not always predictive of physical changes.

Similar to accelerated studies, stress tests give a general picture of the chemical stability and degradation pathways under exaggerated conditions, such as under extreme pH conditions (acids and bases), heat, oxidative or reductive conditions, hydrolysis, and light irradiation (light irradiation tests at not less than 1.2 million lux hours are formalized as described in ICH Q1B). These mostly non-formalized stress tests are only evaluated over a short term, e.g., 1 month.

For a new drug application, a dataset of at least 12-month-long stability and a 6-month accelerated (or intermediate) stability, light irradiation results are required. The minimum frequency of 3 months in the first year, 6 months in the second year, and yearly stability tests thereafter are required.

In general, the same principles as for purity and assay tests are applicable for tests used for stability assessment. It should be verified beforehand that the chosen test method is stability indicating and selective for the degradation products of the drug substance. This is usually done by evaluating the mass balance and selectivity of stressed bulk drug substance and degraded solutions in stress experiments, as described before. The mass balance is calculated by adding the results from the assay of the main ingredient and the sum of by-products and degradation products from the purity method. Theoretically, the sum should

be adding up to a value close to 100%. A strong deviation from 100% can indicate that some degradation products may not be detected. In addition, the peak purity of the main drug substance peak should be evaluated by means of spectral analysis (UV-DAD, MS) because co-eluting or co-migrating impurities may hide under the main peak.

Even if HPLC is chosen as the main stability indicating method, CE will be useful as orthogonal technique, especially in cases where the reason of mass balance deviations is unclear. In addition, due to its increased peak capacity and selectivity in comparison to HPLC, the detection of possible enantiomers, stereoisomers, and position isomers, having the same molecular weight and equal/similar spectra as the drug substance may be separated by a secondary CE method.

Figure 3 shows the comparison of the optimized HPLC and CE methods for a stressed sample of the basic drug substance LAS 35917.[42] While the main degradants (I1, I2, I3) co-elute in the HPLC method, they could be baseline separated in the optimized CE system.

Further examples for validated stability indicating methods can be found in literature,[43–45] please refer also to the literature section.

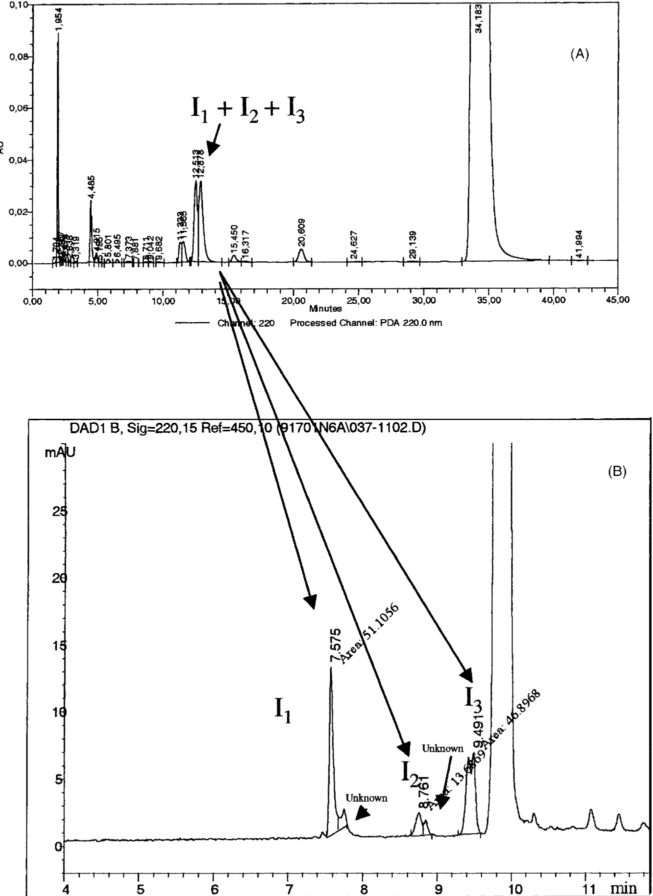

FIGURE 3 Comparison chromatogram (upper) and electropherogram (lower) of a batch of stressed sample of the drug substance LAS 35917 at 40°C for three months as (taken from reference 42. With permission.).

C. The Marketed Stage of a Mature Drug with Full Production Size for the Market

During the marketed stage, the same tests and investigations will be performed as during the development stage. However, the testing of numerous batches at multiple production and testing sites will be performed during the life cycle of a drug. Besides release testing, additional drug stability data have to be submitted to health authorities after the full upscale of the manufacturing process and to verify the same quality of drug substance and drug product produced at different manufacturing sites.

Before release and stability testing can be done in the production sites or service laboratories, a formal method transfer has to take place. Specific test criteria, based on intermediate precision comparison between the laboratories have to be fulfilled to ensure a correct test method transfer between the laboratories. Already during method development, an instrument-to-instrument and type-to-type precision test should be performed to ensure the transferability of the test method. Figure 4 shows the comparison of the voltage–current relationship on two different instrument models with the same buffer and capillary material. It can be clearly seen that, depending on the current chosen, a non-linear behavior is given at higher voltages. This is due to Joule heating, which has an impact on the separation quality of the system (e.g., peak broadening). In addition, a different behavior in two different instrument types is observed. An instrument-to-instrument transferability may only be feasible at a separation voltage below this effect. However, other factors should be considered and verified before method transfer:

- Instrument: e.g., cooling system, capillary types and lengths possible, type of detectors, instrument sensitivity, software and integration functions, maintenance status of instrument and qualification status, definition of instrument cleaning procedures

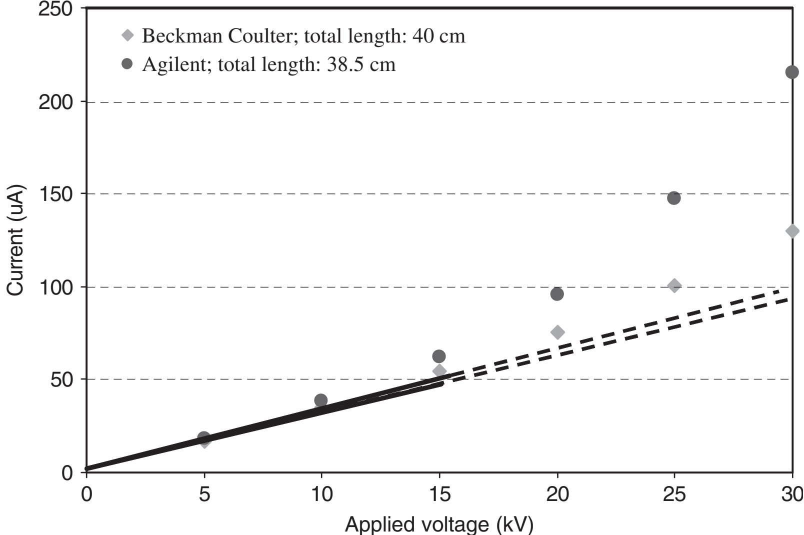

FIGURE 4 Comparison of voltage–current behavior between two different instrument models. Neutral capillary, 50 mm i.d.; *T*: 20°C, Electrolyte: HS a-CD: 2% (w/v), 18 crown 6 ether: 10 mM, methanol: 5% (v/v), H3PO$_4$: 15 mM, instruments: Beckman Coulter:P/ACE MDQ and Agilent: 3D CE (unpublished).

TABLE 12 Pharmaceutical Substances in Review Articles

Pharmaceutical substance	CE mode	Sample	Reference(s)
5-Aminosalicylic acid	MEKC	API	56
3,5-Dinitrobenzoyl leucine	Chiral CE	API or raw materials	57
3-(4-Methylbenzylidene)-camphor	Chiral CE	Cream	56
3,4-Methylenedioxy methamphetamine	NACE	Tablets	57
Acetaminophen	MEKC	API	57
Acetaminophenol	CZE	Tablets	56
Acetylcysteine	CZE	API	58
Acyclovir	MEKC	Cream	56
Adrenaline	Chiral CE	Local anesthetic solutions	56
Amitryptiline	NACE	API	57
Amoxicillin	MEKC	API	58
Anticholinergic drugs	Chiral CE	API	56
Apomorphine	Chiral CE	API	56
Atenolol	CZE	Tablets	57
Atenolol	NACE	API	57
Atropine	NACE	API	57
Baclofen	Chiral CE	API	57
Balaglitazone	Chiral CE	API, tablets	56
Benzodiazepines	CZE, dynamic. Coating	API	56
Benzodiazepines	MEKC	API	57
Benzylpenicillin salts	MEKC	API	56,57
Berberine	NACE	API	57
Betamethasone	MEEKC	API	56
Betaxolol	NACE	API	57
Bupivacaine	NACE	API	57
Butorphanol, cycloamine	Chiral CE	API, intermediate	56
Calcium levofolinate	Chiral CE	API	56
Candesartan	CZE, MEKC	API	56,57
Capozide	MEKC	API	58
Cefluroxime atexil	MEKC	Formulations	57
Cefoperazone	Chiral CE	Granulate	56
Cephalexin	MEKC	Capsules	58
Chlorepheniramine	CZE	Tablets	56
Chloroprocaine	NACE	API	57
Chlorothiazide	MEKC	API	58
Chlorpheniramine	MEKC	API	57
Chromanes	Chiral CE		59
Cinchocaine	NACE	API	57
Ciprofloxacin	CZE	API	56
Citalopram	Chiral CE	Tablets	56,57
Clothiapine	CZE	API	56
Clozapine	CZE	API	56
Cocaine	NACE	API	57
Codeine	CZE	Formulations	58
Cyclizine hydrochloride	CZE	Tablets, suppositories	56,57
Denopamine	Chiral CE		59
Desipramine	NACE	API	57

TABLE 12 (Cont.)

Pharmaceutical substance	CE mode	Sample	Reference(s)
Dexamethasone phosphate	MEKC	Tablets	57
Dihydrostreptomycin	CZE	Veterinary preparation	56
Diltiazem	MEKC	Tablets	58
Diltiazem HCl	Chiral CE		59
Diphenhydramine	CZE	Nasal drop	58
Domperidone	MEKC	API	58
Dothiepin	CZE	Tablets	58
Enalapril Maleate	MEKC	Tablets	58
Ephedrine	NACE	API	57
Ephedrine	MEEKC	API	56
Ephedrine	CZE-LIF	Tablets	57
Eprosartan	CZE, MEKC	API	56,57
Etodolac	Chiral CE	Tablets	56
Fenofibrate	MEKC	Capsules, tablets	57
Fenoprofen	Chiral CE	API	57
Fenoprofen	CZE-LIF	API	57
Flezelastine	Chiral CE		59
Fluparaxolan	MEKC	API crude	58
Flurbiprofen	Chiral CE	API	57
Flurbiprofen	CZE-LIF	API	57
Galantamine	NACE	API	56
Gentamicin	MEKC	API	57
Gentamycin	CZE	Injection solutions	58
Heroin	CZE, dyn. Coating	API	56
Hydrochlorothiazide	CZE, MEKC	API	56,57
Hydrochlorothiazide	MEKC	API	58
Ibuprofen	Chiral CE	API	57
Ibuprofen	CZE-LIF	API	57
Imipramine	NACE	API	57
Indinavir sulfate	CZE	Capsules	56, 57
Irbesartan	CZE, MEKC	API	56,57
Ketamine	NACE	API	57
Ketoconazole	CZE	Tablets, creams	56
Ketoconazole	Chiral CE	Substance, tablets, syrup, gel	56
Ketoprofen	Chiral CE	API	57
Ketoprofen	Chiral CE	API or raw materials	57
Ketoprofen	CZE-LIF	API	57
Ketorolac tromethamine	MEKC	API, tablets	56, 57
L-Carbidopa	Chiral CE		59
L-DOPA	Chiral CE	Tablet	56
Levetiracetam	MEEKC	API	56
Levetiracetam	Chiral CE	API	57
Lidocaine	NACE	API	57
Lisuride	Chiral CE	Substance	56
Loratadine	CZE	API	56
Losartan	CZE, MEKC	API	56,57
Matrine	NACE	API	57
Melagatran	Chiral CE	API	57
Meloxicam	CZE	Tablets	57
Mepivacaine	NACE	API	57

TABLE 12 (Cont.)

Pharmaceutical substance	CE mode	Sample	Reference(s)
Mepivacaine	Chiral CE		59
Metacycline	CZE	API	56
Methotrexate	Chiral CE	Tablets, injection solutions	56
Metoprolol	NACE	API	57
Minoxidil	CZE	Formulations	58
Moxiflaxin	Chiral CE	API, ophthalmic/otic solutions	56
N-Acetylcysteine	CZE	API	56
Naphazoline	MEKC	Nasal drops, aerosol	57
Naphazoline	CZE	Nasal drop	57
Naproxen	Chiral CE		59
Nicardipine	Chiral CE	API–CD complexes	56
Nortryptiline	NACE	API	57
Olanzapine	CZE	API	56
Omeprazole	Chiral CE	Capsules	56
Omeprazole	Chiral CE	Tablets	57
Ondansetron	Chiral CE		59
Organic disulfates	Chiral CE	API	57
Papaverine	NACE	API	57
Penicillin	CZE	API	56
Penicillin G	CZE	Veterinary preparation	56
Pheniramine	Chiral CE	Granulate	56
Phenobarbital	Chiral CE		59
Phenylephedrine	CZE	Tablets	56
Phenylephrine	MEKC	API	57
Phenylephrine	CZE	Nasal drop	57
Phenylephrine	MEKC	Nasal drops, aerosol	57
Pilocarpine	NACE	API	57
Pioglitazone	Chiral CE	API, tablets	56
Piribedil	MEKC	Tablets	56,57
Piroxicam	MEKC	Tablets	57
Pranoprofen	CZE-LIF	API	57
Pravastin	CZE	Tablets	56
Prednisolone	MEKC	Nasal drops, aerosol	57
Prilocaine	NACE	API	57
Prilocaine	Chiral CE		59
Procaine	NACE	API	57
Procaine	CZE	Veterinary preparation	56
Propranolol	Chiral CE	Tablets, injection solutions	56,57
Pseudoephedrine	MEEKC	API	56
Pseudoephedrine	CZE-LIF	Tablets	57
Pyridine-4-carboxylic acid	CZE	API	58
Quetiapine	CZE	API	56
Quinolizidine alkaloids	NACE	Chinese herbs	56
Quinolone	CZE	API	58
Racemorphan	Chiral CE		59
Ragaglitazar	Chiral CE	API, tablets	56
Raloxifene	CZE	Tablets	57
Ranitidine	CZE	API, solution	58

TABLE 12 (Cont.)

Pharmaceutical substance	CE mode	Sample	Reference(s)
Remoxipride	CZE	API	58
Rimantadine hydrochloride	CZE	Tablets	57
Rivastigmine	Chiral CE	API	56
Rofecoxib	CZE	API	56
Ropivacaine	Chiral CE		59
Salbutamol	Chiral CE	Tablets, syrup, oral solution	57
Salbutamol	CZE	API	58
Salicylamide	MEKC	API	58
Sertraline	Chiral CE	API	56
Simendan	Chiral CE	API	56
Sotalol	NACE	API	57
Sumatripan	CZE	Injection solutions	58
Tamoxifen	NACE	API	57
Telmisartan	CZE, MEKC	API	56,57
Terconazole			
Thiaglitazone	Chiral CE	API, tablets	56
Thiopental	Chiral CE		59
Tramadol	MEKC	API	57
Tramadol	Chiral CE		59
Trimetoquinol	Chiral CE		59
Tryptophan	Chiral CE		59
Urosdeoxycholic acid	CZE	Tablets	57
Valsartan	CZE, MEKC	API	56,57
Vancomycin	MEKC	API	56
Vasotec	MEKC	Formulations	58
Xanthine	MEKC	Tablets	58
Ximelagatran	CZE, cyclodextrin added	API	56
Ximelagatran	Chiral CE	API	57
Zopiclone	Chiral CE		59

- Operator: e.g., operator trained with CE, do's and don'ts, training with the test method, troubleshooting guide for test method available
- Location: room climate control, power supply, instrument position, humidity, air pressure supply
- Chemicals: quality of deionized water and chemicals for buffer preparation and rinsing, standards for quantification and selectivity verification, test samples, correct transport and storage conditions
- Consumables: glassware, buffer and test vials, filters, caps, capillaries, etc.

If feasible, on-site training at the receiving site should be performed by a person, who is experienced with the applied test method.

Recent results demonstrated the importance of an inter-laboratory comparison in order to ensure a correct method transfer.[46] Earlier inter-company collaborations were performed for small molecules[47,48] and biomolecules.[49]

IV. PHARMACEUTICAL SUBSTANCES IN LITERATURE

Table 12 gives an orientation help for CE separations sorted by pharmaceutical substances published in review articles. As this chapter focuses on the technical development of drug substances and products, only drug substances and drug formulations are covered. A useful compendium of CE applications in the pharmaceutical environment can be found in the book *Capillary Electrophoresis Methods for Pharmaceutical Analysis* written by G. Lunn.[50] The book covers more than 700 active pharmaceutical ingredients and contains short method descriptions, sample preparation steps, and references.

V. SUMMARY AND CONCLUSIONS

Though in competition with other analytical techniques, CE has proven its potential and necessity to be used for the characterization of small-molecule pharmaceuticals. Due to the versatility of the system, CE can be applied for the determination of physicochemical properties, identification, purity and stability analysis, and cleaning verification of the drug substance, its precursors, process chemicals, the drug product, and its excipients.

Further positive developments in this area will be linked to

- the major improvement of robustness, sensitivity, and precision of the instruments
- the acceptance from upper management to provide resources for implementation and training of the CE technology
- The further development of chemicals and capillaries, coatings and consumables, and ready-to-use generic methods
- The commitment of vendors and manufacturers to a worldwide accepted quality and testing system
- The further implementation of CE methods into the pharmacopoeias and the harmonization of the general chapter for CE.

For small molecules, the major driving force for the development of CE may be the emerging number of pharmaceutical compounds with multiple chiral centers and the development of complex molecules, such as synthetic oligopeptides and oligonucleotides and the large need for high-throughput technologies using minimal amounts of sample in research and early development.

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6

GENERAL CONSIDERATIONS TO IMPROVE PERFORMANCE OF CE METHODS

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ABSTRACT

This chapter discusses general considerations for improving capillary electrophoresis (CE) method performance from a robustness angle. Several method parameters are discussed and examples are offered of how CE procedures are handled to obtain optimal performance. The purpose of this chapter is to raise the awareness and help the development of more robust and sensitive methods.

I. INTRODUCTION

The performance demands for a method depend on its intended use. Assays demand a higher level of precision than low-level impurity determinations. A one-time-only special investigation by an experienced scientist requires less robustness and can make more use of special “tricks” than a quality control (QC) method that has to be transferred and run for years on a daily basis. It is important to consider the following questions: Do you need ultra-high efficiency or resolution, or do you go for good-enough-for-a-one-time-only investigation? Is speed/fast analysis important? Can you use the special feature of capillary electrophoresis (CE) (compared to liquid chromatography (LC)) that sample components not necessarily migrate in the same direction through the detector to separate your compounds of interest from disturbing matrix components? Is your method going to run by a specialized lab or by the basic training? Before starting any method development, you have to know the method purpose and from the method purpose you have to define the performance demands.

To develop robust methods, you have to keep in mind that simple methods are preferable. Also, if you have a chromatographic background, then the factors affecting CE precision (and to a lesser extent accuracy) are widely different to HPLC, which is currently the predominantly employed technique for drug assay.[1] There is no general preference for the choice between techniques: CE, LC, or other. The choice should be made on a scientific basis, supported by the relative merits of the techniques for the specific problem and factors such as the experience of the method-developing scientist. Robust CE methods have been successfully transferred to relatively inexperienced QC and Contract Research Organisation (CRO) labs.

This chapter discusses method parameters from the robustness point of view, not from the analyte or the specific analysis point of view. Certain aspects will be named under different parts, if they have impact on multiple aspects and overlap with other chapters is unavoidable. The chapter is not a review (Chapter 9), but will give illustrative examples. It is intended to help during method development and is based on the current status of equipment. The strategies provided are not considered to be “the only” way to address the issues discussed. They are offered as examples (for more discussion see other related chapters in this book) of

how CE procedures are handled to obtain optimal performance. Hopefully, this chapter will raise the awareness and help the development of more robust methods.

The chapter is a result of many years' experience within pharmaceutical industry, many discussions and sharing experiences with other CE scientists (e.g., at user meetings and dedicated sessions and short courses at symposia, sometimes referred to as "good CE practice"), and hints picked from the literature.

Some general advice to start with: It is important that a method is described explicitly and unequivocally. Be fair in the validation not to raise expectations that cannot be met in daily use.

II. CAPILLARY

A. What Capillary to Choose and Why

- *Length*: From an analysis time perspective, or to have the possibility to apply high field strengths, the capillary should be as short as possible. A short capillary is also advantageous for low diffusion. But complex separation problems need longer capillaries, as the plate number and resolution are linear with the effective length.
- *Internal diameter*: Generally, smaller diameters have better heat dissipation and therefore less band broadening.[2–6] For effective heat dissipation, forced convection is better than native convective cooling,[7,8] but most instruments still have parts that are outside the cooling system. Decreasing the electrolyte concentration in the BGE (background electrolyte) allows the use of wider capillaries,[5] although that also results in more electrodispersion. For better detection sensitivity and reduced integration errors, it is advantageous to use larger capillary diameters (e.g., reference 9). But when the currents are high and Joule heating is too much, problems like current breakdown for some injections in a long sequence or non-repeatable results can occur. Band broadening might even reduce sensitivity. In the case of the chiral determination of small amounts of adrenaline in local anesthetic solutions,[10] initially a 75 μm capillary was chosen in order to gain sensitivity. The result was however the reverse. When reducing to a 50 μm capillary, the band broadening was reduced and resolution increased to such an extent that the injection volume could be increased and sensitivity gained.
- *Capillary wall*: Generally, the most straightforward approach is to use an uncoated fused silica capillary. But sometimes this is not possible because of adsorption problems to the capillary wall, or other wall properties are needed to control the electroosmotic flow. In literature, there are multiple examples. Besides permanently coated capillaries, there are several descriptions of dynamic coatings available, e.g., triethanolamine, Triton X-100, Polybrene, and quaternary ammonium salts. The advantage of these dynamic coatings is that the coating can be renewed between injections, which could improve repeatability and reproducibility of the separation.
- *Detection*: If a small capillary diameter is desired for efficiency purposes, the detection part of the capillary can be adapted for better detection sensitivity. Examples are the bubble cell capillary and the Z-cell. In the bubble cell capillary, the capillary diameter is enlarged at the detection window so that better concentration sensitivity is obtained. If you implement a bubble cell capillary in your pharmaceutical analysis method, it is important to test different batches. Test also whether you need a bubble cell capillary or whether you can gain similar sensitivity increase with a proper injection procedure. Also, check the effect of the bubble cell on band broadening. An approximately three-times sensitivity enhancement is possible.

For the Z-cell, a special interface is constructed for the detection to take place in the length of the capillary instead of the diameter. Two different lengths of capillary with tapered ends on one side are connected with the interface. This takes some experience, so it is important to test putting the set together by different analysts, both experienced and inexperienced. The device is only useful if you have sufficient separation between the analytes of interest, since a longer plug is in the detection window. Consequently, if the analyte bands are simultaneously in the detection window, resolution is no longer observed. A sensitivity enhancement of a factor 10 is possible.

Since both the bubble cell and the Z-cell need high resolution in order to observe the sensitivity increase, test whether you can avoid the use by a clever injection procedure such as sample stacking or transient ITP (isotachophoresis) instead. Further information on detection approaches is provided in Chapters 3, 5, and 15.

B. Treatment of a New Capillary

Traditionally, new fused silica capillaries, both coated and uncoated, are treated with conditioning steps to activate the wall. Also traditionally, there exist multiple procedures, varying from lab to lab and method to method, while there is limited published research. It is common to treat bare fused silica with sodium hydroxide, concentrations varying from 0.1 to 1 M. The group of Wätzig investigated the surface chemistry of fused silica capillaries and found silica−carbon bondings on the inner capillary surface. For improved robustness, they recommend, especially in the pH range 4−7, to flush with 1 M NaOH for at least 1 h.[11−13] Most coated capillaries are not stable when washing with NaOH, so check the vendor's instructions. For non-aqueous CE, avoid aqueous washing steps.

Additional rinsing strategies of the capillary are provided in Chapter 4.

C. Storage of a Capillary

For short-term storage, e.g., between sequences or overnight, it is often best to clean the capillary and then fill it with running buffer, making sure that the capillary ends are immersed in the BGE. For long-term storage, the bare fused silica capillary needs to be cleaned properly with, e.g., NaOH. The final washing step is an extensive water wash before the capillary is blown dry. The latter can be achieved by flushing with air via empty vials.

D. Pre-sequence Conditioning

At the start of a new sequence, the (dedicated) capillary is installed in the instrument and a pre-sequence conditioning is performed. Usually, it suffices to perform a shortened version of the new capillary treatment, e.g., a 10 min wash with NaOH and conditioning with BGE. The application of the voltage might shorten the conditioning time needed.

If a capillary is used day to day, it is usually better to clean the capillary and store it overnight in BGE. In that case, only a short BGE conditioning often suffices.

E. Capillary Ends

The capillary ends have to be straight and undamaged to prevent carryover or peak tailing. Remove the polyimide coating from the capillary ends to prevent excessive carryover

and/or adsorption of sample components. Beware that solvents such as acetonitrile swell the polyimide coating, so more polyimide needs to be removed to prevent swelled coating from extending over the capillary inlet.

F. Capillary History

Some BGE or sample components can adsorb strongly to capillary wall, resulting in permanent changes or very long equilibrium times. Therefore, keep track of your capillary's history, or better, dedicate the capillary to a certain method or BGE. Always check the final method conditions after method optimization on a fresh capillary.

G. Capillary Batch-to-Batch Variability

Batch-to-batch variability is dependent on the quality of the capillary, the washing procedures, and the composition of the BGE. Comparison between capillaries sometimes needs to be done through effective mobilities or relative mobilities rather than migration times.[14]

III. ELECTRODES

Different instruments have solved the positioning of the electrodes in different ways. In the Beckman instruments, the electrodes are separate and some distance away from the capillary, while the vial caps have holes. In the Agilent instrument, the capillary goes through the hollow electrodes. There is a pre-punching system so that properly capped vials can be used. Both setups have different advantages and disadvantages. Vial caps with holes result in higher evaporation rates of the solvents, while the close proximity of a capillary going through electrodes can increase carryover. To reduce the latter effect, Agilent also supplies shorter electrodes for its instrument.

In any setup, it is paramount that the electrodes get cleaned regularly. The minimum frequency, e.g., once a week, should be described in the instrument standard operation procedure (SOP), but for some methods or samples more frequent cleaning is necessary. An example is the determination of the enantiomeric purity of adrenaline in local anesthetic solutions.[10] The samples are isotonic and contain high concentrations of local anesthetics (5−20 mg/ml). The determination concerns very low concentrations of adrenaline (typically 5 μg/ml of l-form and only a few percent of that of the d-form) and the samples are therefore injected undiluted. Furthermore, relatively high concentrations of cyclodextrin are present in the BGE. Long sequences therefore require electrode cleaning for every sequence and this is thus described in the method procedure.[10]

Other ways to reduce carryover or adsorption to the electrodes are dipping the capillary after injection in a clean buffer vial, or applying some reversed voltage during preconditioning (see Sections V and VI A).

IV. TEMPERATURE

CE instruments are thermostated to dissipate excessive Joule heat.[7,8] Generally that covers only the main part of the capillary, and not, e.g., the autosampler with the buffer and sample vials. In some instruments, it is difficult to control the autosampler temperature due to the near presence of extraneous heating sources such as the detector lamp. Also, some labs

have better possibilities for internal climate control than the others. Temperature differences between sample or buffer vials result in viscosity and current differences, and therefore in migration time and injection precision variability. To reduce these effects, let the vials stand for at least half an hour in the autosampler before starting the main part of the sequence.[15] During this time, you can, e.g., perform the necessary capillary pre-sequence conditioning or a blank conditioning injection.

From the method development and robustness point of view, the temperature is a parameter that controls equilibria such as pK_a and enantiomer−chiral selector complexation, or induces structural changes in, e.g., proteins.[16] For chiral separations, generally a lower temperature results in better enantioseparation,[17] but even the opposite has been observed.[18] Sometimes a raise in temperature does not so much affect the enantiomeric separation, but increases the resolution between an enantiomer and a matrix component.[10]

V. PRECONDITIONING

The term preconditioning here is reserved for between-runs steps. Treatment of new capillaries is described in the Section II b, and pre-sequence treatment is described in the Section II d.

Describe the preconditioning explicitly in your method.[19,20] Since preconditioning is dependent on the aim of the method, the BGE and the samples, it should be an integral part of method development. Often sub-optimal preconditioning is a significant aspect of method problems.

To keep constant liquid levels in the run vials, preconditioning should not be performed from and to the vials that are used during the voltage separation step of the method. Therefore, besides programming the washing steps from different vials, a preconditioning procedure should also control the vials at the outlet during the different preconditioning steps. The waste vial should not be empty but contain some liquid to prevent a drop hanging down from the capillary. If the sequence is very long, or if the preconditioning procedure is extensive, check that the waste vial does not overflow. When a voltage step is programmed, the inlet and outlet vial should contain BGE.

Before you start to compose your preconditioning procedure, consider what effect is required. For example:

- precision through reproducible and repeatable mobilities,
- constant EOF (electroosmotic flow) or suppression of EOF,
- prevention of buffer depletion,
- reproducible reduction of wall interactions,
- prevention of carryover from highly concentrated sample components, and
- flushing out late-migrating components that are of no interest for the analysis.

There are many different preconditioning steps (Chapter 4), some more common than others. The most common steps are described here.

- *BGE*: The simplest preconditioning step is flushing with BGE, which cleans out sample components and refreshes the BGE to avoid buffer depletion effects. In many cases, a BGE wash is sufficient enough to obtain a constant EOF to reduce carryover, to flush out late-migrating sample components that are not of interest to the analysis, etc. If a BGE wash suffices, do not spoil your wall equilibrium with flushing other liquids. Some buffer components are known to adsorb to the surface, such as phosphate, which can affect the equilibration time needed.

- *Applied voltage*: Applying the voltage after a BGE flush step can stabilize the EOF. If you apply a voltage opposite to the voltage during the run, sample compounds that stuck to the capillary or electrodes might be removed. In that way, carryover or other disturbing effects can be reduced.
- *Water*: Short water steps can be used to bracket solvents that are not compatible, e.g., when there is a risk of precipitation of components if they come into contact with each other. It can also be used to moderate pH jumps for the capillary wall.
- *Sodium hydroxide*: A NaOH flush can be applied if harsher treatment of the capillary wall is needed between samples. For example, when you have highly concentrated samples (analyte and/or matrix), or sample components with strong wall interactions. Also, when testing several different BGE compositions for method development in a sequence, a NaOH wash step is usually part of the preconditioning. Hydroxide washing is not compatible with all capillary coatings. When running low-pH buffers, an acid or organic solvent wash can be preferable.
- *Strong acids*: Acids such as H_3PO_4 or HCl also give harsher treatment than BGE or water, but without deprotonation of the silanol groups of the wall. An acid wash is not compatible with all capillary coatings.

  There are some reports on the effect of basic or acid preconditioning on the EOF.[17] At a lower pH, only a limited number of the silanol groups of the capillary wall are hydrolyzed and a gel that is held together by the remaining bonds is formed on the surface. The zeta-potential is strongly decreased and the EOF is slower using the same pH in the BGE if acidic preconditioning has taken place.[20,21] The effect is less pronounced when EOF-suppressing modifiers are used.
- *Organic solvents*: Solvents can be used to clean out what cannot be cleaned out with BGE or with another simple treatment, or when working non-aqueous and no water-based wash is possible. Beware that some solvents such as acetonitril (ACN) can swell the polyimide coating on the outside of the capillary.
- *Wait-step*: It is not always necessary to flush through an expensive solution. Sometimes it is sufficient to be in contact with the capillary wall through a wait-step in the preconditioning procedure.
- *Detergent*: For complex sample matrices, such as in biological samples, a preconditioning with a detergent such as sodium dodecyl sulphate (SDS) in BGE, might be more beneficial than extensive caustic or acid cleaning. An extra benefit is a shorter re-equilibrium time. A slight amount of SDS in the running buffer can improve precision even more.[20] Another report described the combination of acetonitrile and sodium dodecyl sulphate (SDS) for this preconditioning between biological samples.[22,71] Be aware that some of the detergent can remain on the capillary wall and cause permanent changes.[20]
- *Dynamic coating solution(s)*: Dynamic coating components do not always need to be added to the BGE, sometimes it suffices to flush in between runs.[23]
- *Dipping capillary end*: Cleaning the capillary injection end by dipping into water after preconditioning and before the sample injection has been found to improve injection precision.[24]

VI. INJECTION

A. Injection Precision

The injection precision in CE can be 1% or better on automated commercial equipment when carefully controlling the injection procedure (e.g., references 1 and 25 and references cited therein).

Several ways of increasing injection precision are as follows:

- *Internal standard*: The use of an internal standard (IS) is highly recommended and compensates for variations in the amount injected even when the injection was faulty.[25,26] An IS might not be needed when quantifying by internal normalization or when reporting with a reduced number of significant digits, e.g., in an impurity-level test.[27] The IS can also be used to improve identification precision of compounds through relative migration times.[14]
- *BGE plug injection*: A buffer plug injection after the sample injection prevents sample loss by thermal expansion on switching on the high voltage.[24,28]
- *Injection mode*: Hydrodynamic injection is generally more reproducible than electrokinetic injection. The electrokinetically injected amount has a non-linear relationship with the injection time.[20]
- *Injection time*: Most modern instruments have a control function of the injection pressure that automatically corrects for hydrodynamic injection variability through the injected time. An injection time of at least 3 s is needed for this to function properly. Too short injection times decrease precision and too long injection times induce band broadening.[29] Rather increase pressure if possible.
- *Viscosity differences*: Different sample vial temperatures create different viscosities, and thus different amounts injected. To reduce the effect, use the instrument temperature control (see Section IV). However, often the sample and buffer vials reside outside the temperature-controlled area. The effect of this might vary depending on the climate system in your lab and how the lab temperature varies over the year. Besides temperature control, it is important to match samples and standards in terms of viscosity and conductivity.
- *Vial levels*: Difference in liquid levels between vials will create a hydrodynamic flow and disturb injection precision. Fill the sample vials to the same level and make sure when programming the instrument that there is a vial with a constant level of BGE at the capillary outlet when injecting.
- *Sample concentration*: Higher sample concentrations or larger sample volumes reduce integration errors and thus increase precision.[25]
- *BGE dip*: After sample injection, dip in the capillary inlet into a BGE vial that is not the same vial as the run vial to reduce carryover and/or to increase peak symmetry.[30] Check how often this dip vial needs to be replenished.

B. Standards

The standards should match the samples in order to accurately determine the latter. Viscosity differences result in injected volume differences. If a compound is determined in the presence of high concentrations of other compounds in the sample, these can influence the migration time and peak shape by electromigration dispersion (EMD), and should therefore be present in the standards as well (Figure 1).[31]

C. Injection Procedure

A lot of sensitivity and robustness can be gained by carefully designing the injection procedure, so do put some time and effort in this during method development. Do not forget to test injection parameters during the robustness evaluation. There are plenty of examples for

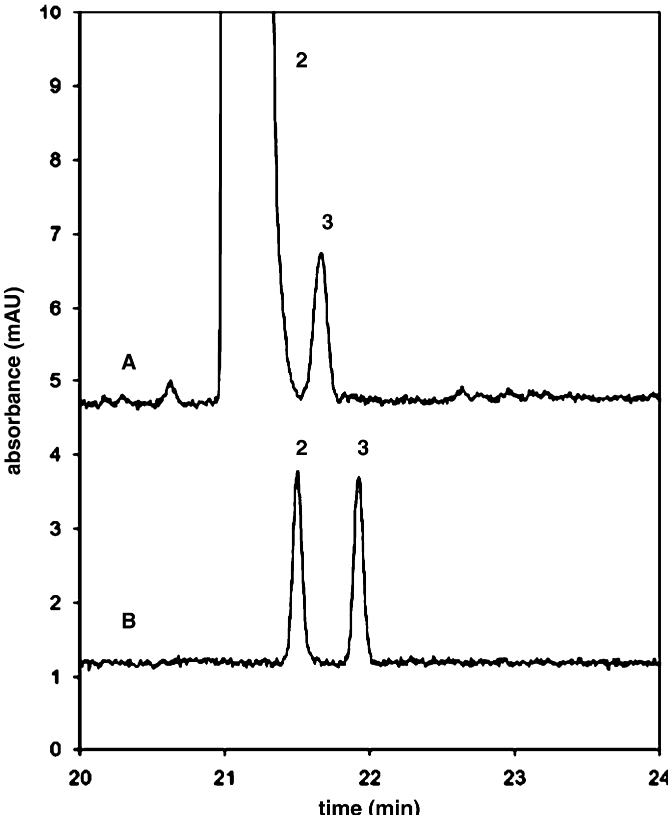

FIGURE 1 **Due to the relative high amount of sample injected, electrodispersion of compound 2 has a major influence on the migration and peak shape of compound 3. (A) 0.5 mg/ml of compound 2, 2.5 µg/ml of compound 3. (B) 2.5 µg/ml of compound 2, 2.5 µg/ml of compound 3 (with permission from reference 31).**

on-line sample concentration described in literature, but have robustness and reproducibility in mind even when using them. Some examples of simple procedures are given below.

- Sample dissolution in a low-conductivity solvent can induce sample stacking.[20,32,33] This might also be achieved by injecting a low-conductivity solvent plug before or after the sample plug.
- A buffering BGE co-ion gives better sample loadability.[34]
- pH Adjustment of the sample by dilution could improve the peak shape.[34]
- Injection of a stacking solution before or after the sample plug to induce transient ITP.[34] Generally, this should be a slower migrating compound with good solubility and preferably low UV absorption. An example is shown in Figure 2. A plug of 5 mg/ml tetrapentylammonium chloride (TPAC) was injected after the sample plug, thereby stacking the compound of interest that was broadened by EMD from the highly concentrated matrix component.
- Transient ITP by dilution of the sample in a stacking solution.[35]
- Dynamic pH junction. Use the difference between sample pH and BGE pH to selectively stack the compounds of interest.[36]

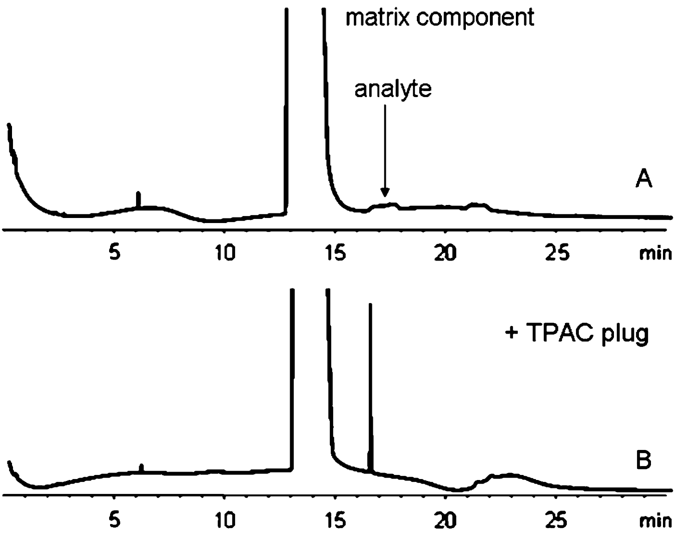

FIGURE 2 **Analyte stacking by injection of slower migrating compound, tetrapentylammonium chloride (TPAC), at 5 mg/ml. (A) Injection of BGE plug after sample injection. (B) Injection of TPAC plug after sample injection.**

VII. APPLIED VOLTAGE

A. Voltage Ramp

Whenever possible, ramp the applied voltage. As soon as the voltage is switched on, the sample components start migrating. But when the applied voltage is initially low, the thermal expansion of the capillary content is limited. While raising the voltage, the sample has already migrated from the inlet and further loss due to increasing thermal expansion is reduced.

For calculating effective mobilities for identification purpose, voltage ramping needs to be corrected.[37]

B. Current

Record the current during the runs, as this is a valuable tool for troubleshooting.[38,39] It is recommended to avoid current flows higher than 100 μA since the resulting amount of Joule heating may generate system failures.

VIII. BACKGROUND ELECTROLYTE

When developing a method, it is very important to have the intended use of the method clear in mind. Is the method to be transferred to a QC lab for use year after year, or is it only intended for a short-term study by experienced CE specialists? If a QC method is intended, often the more simple the BGE composition, the more robust the method. This means that a balance has to be found between a simple and robust BGE and sufficient separation, which can be quite difficult at times. But often it proves that the time spent on this process is worth it in the end.

TABLE 1 Some Generic BGE Compositions

BGE	Composition
BGE pH 2.15−3.0	0.10 M H_3PO_4 with 0.05−0.09 M tris(hydroxymethyl)-aminomethane (TRIS), with or without a cyclodextrin
BGE pH 2.15−3.0 (reversed EOF)	0.10 M H_3PO_4 with 0.05−0.09 M triethanolamine, with or without a cyclodextrin
BGE pH 9.3	20 mM Sodium tetraborate, with or without a cyclodextrin
SDS−MEKC pH 10	40 mM Borate pH 10.0, 40 mM SDS, 9% v/v acetonitrile,[40] with or without a cyclodextrin
SDS−MEKC pH 7.5	10 mM Phosphate buffer pH 7.5, 60 mM SDS, 10% v/v acetonitrile[41]
CTAB−MEKC pH 7.5	25 mM Phosphate buffer pH 7.5, 10 mM cetyltrimethyl-ammonium chloride (CTAC), 10% v/v acetonitrile[41]
micro-emulsion electrokinetic chromatography (MEEKC)	0.81% w/w Octane, 6.61% w/w butan-1-ol, 3.31% w/w SDS, 89.27% w/w 10 mM tetraborate pH 9.2[42]

In the final method, the BGE recipe has to be described unequivocally and it is preferred to have the precise composition over pH adjustments, e.g., "0.100 mol/l phosphoric acid, 0.090 mol/l triethanolamine, resulting in pH 3.0" instead of "0.100 mol/l phosphoric acid adjusted to pH 3.0 with triethanolamine."

When developing method, people often work from a series of simple buffers, sometimes called generic BGEs or methods, that have proven their usefulness over time. Some generic buffers are listed in Table 1, but even here you will find that different labs have different preferences. Also, for the development of chiral methods there exist general strategies that often give good starting points.[43−48]

Other BGE considerations are described in Section VIIIA−I.

A. Buffer Depletion

Choose a pH that is close to the buffer pK_a to reduce depletion effects. Determine how often the BGE has to be replenished in a specific method by measuring the pH change in the vials after running. Theoretical calculations are presented in references 25 and 49. The specific conductivity of several buffers as a function of the buffer capacity is presented in reference 50. A pragmatic way of increasing the use of the same set of vials is swapping the run vials between runs, which greatly increased migration-time stability using BGE of pH 4.[48]

The effect of buffer depletion gets even more pronounced if the BGE pH is close to the analyte pK_a. The choice of a pH close to the pK_as of the analytes can optimize the separation through maximizing the charge-to-mass ratio. But a careful choice of buffer is then paramount, since a very small change in buffer pH already affects the charge of the analytes and thus the mobilities. A BGE pH 1 unit from the buffer pK_a might then not be sufficient (Figure 3).[51] The buffer depletion rate increases using wide-bore capillaries or elevated temperatures.[52]

B. EOF Stability

The EOF is affected by several parameters such as temperature, pH, solvent, ionic strength, capillary wall, etc. All of these aspects need to be taken into account when very

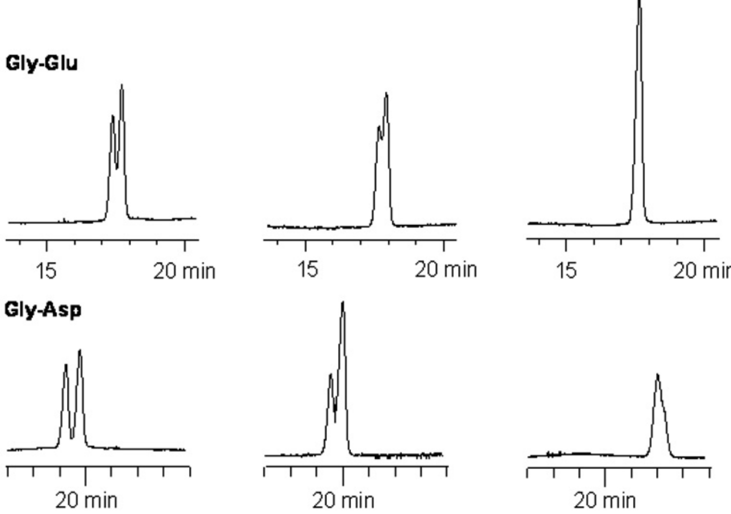

FIGURE 3 The effect of buffer depletion on repeated injections without replenishment. Conditions: 33.0 (24.5) cm × 75 µm ID fused silica capillary; BGE 10 mM DM-β-CD, 100 mM phosphoric acid, 88 mM triethanolamine (pH 3.0); $V = 10$ kV; $T = 30°$C. Calculated p*K*as for Gly-Glu are 3.0, 4.7, and 8.3. Calculated pK_as for Gly-Asp are 2.9, 4.5, and 8.3 (with permission from reference 51).

stable migration times are requested. Under certain conditions, even very small changes in the EOF can result in dramatically changed effective mobilities. Figure 4 shows an example of an micellar electrokinetic chromatography (MEKC) system where the effective mobility of micelles is almost zero. If the EOF varies slightly, the analytes migrate a lot faster or can be completely lost for detection (due to reversal of the micelle mobility), as the simulated electropherograms show. Such a system will never become robust, and although all compounds are very well resolved, a different BGE is preferred. In the present system, the EOF variability was probably caused by variations of the amount of acetonitrile in the BGE.[53] In the case of volatile buffer components, capped vials should be used and the effect of concentration changes of the volatile component on the EOF checked.

In the pH range 4−7, small pH changes have large effects on the charge of the fused silica capillary wall, and thus the EOF.[54] If a BGE pH in this range is needed, coated capillaries (permanently or dynamically) should be considered. Also, more robust systems are obtained if the BGE consists of both a buffering co-ion and a buffering counterion.[35]

To correct for small variations in the EOF, the relative migration time instead of the absolute migration time can be used for identification purposes.[14,55]

C. Ionic Strength

The ionic strength of the BGE affects the current generated in the system, and thus the maximum applicable voltage, as well as the amount of EMD or possible stacking. Figure 5 illustrates the effect of doubling the BGE concentration on the peak shape of the main component and thus the resolution.[56] Furthermore, a high ionic strength gives a decrease in zeta-potential and thus a slower EOF.[57] The ionic strength also affects the range of linearity of the method.[20]

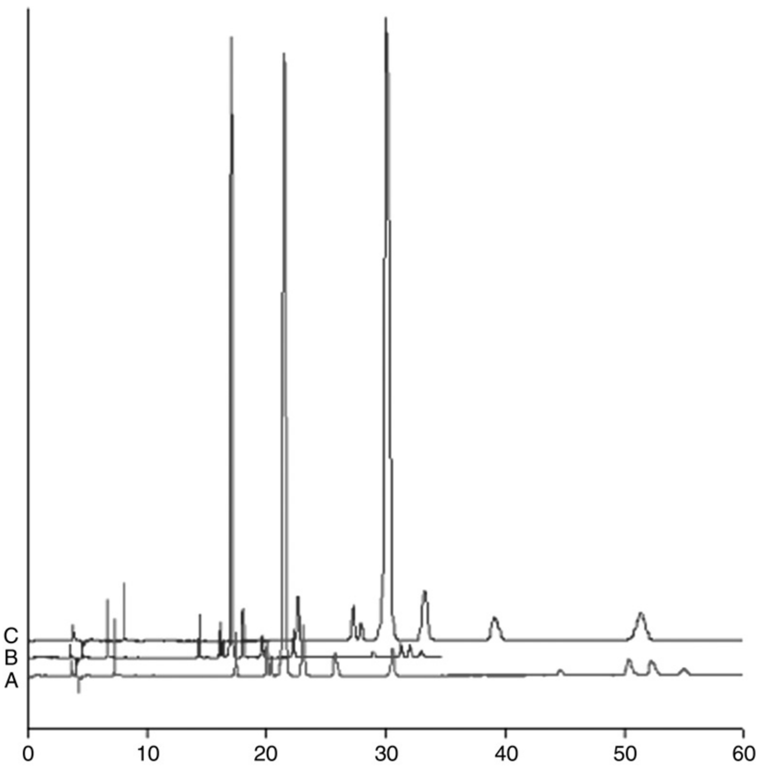

FIGURE 4 The effect of small variations in EOF when the effective mobility of the micelles is almost zero ($\mu_{mc} \approx -\mu_{EOF}$). A: EOF 4.25 min; B: EOF 4.00 min; C: EOF 4.50 min. A is the recorded electropherogram, and B and C are calculated from A by adjusting the EOF.

D. System Peaks

The number of system eigen zones is the same as the number of constituents of the BGE. Especially in BGEs using multiple constituents, there is a risk of co-migration of a system zone and an analyte zone.[58] There is a software available to calculate eigen zones.[59]

E. Sample Loadability

For better loadability of sample in a system, the BGE should be carefully selected. If the BGE co-ion is the buffering component, higher sample concentrations are possible.[34]

F. Degassing

Filter or degas the BGE shortly before use. Mostly, filtering through 0.45 μm pore size filter is sufficient even for degassing, but sometimes better degassing, e.g., by helium purging is needed. Figure 6 illustrates such an occasion when filtering alone did not suffice and small gas bubbles were formed during run, which resulted in small peaks and spikes throughout the electropherogram. Degassing the water with helium before preparing the BGE solved this problem.

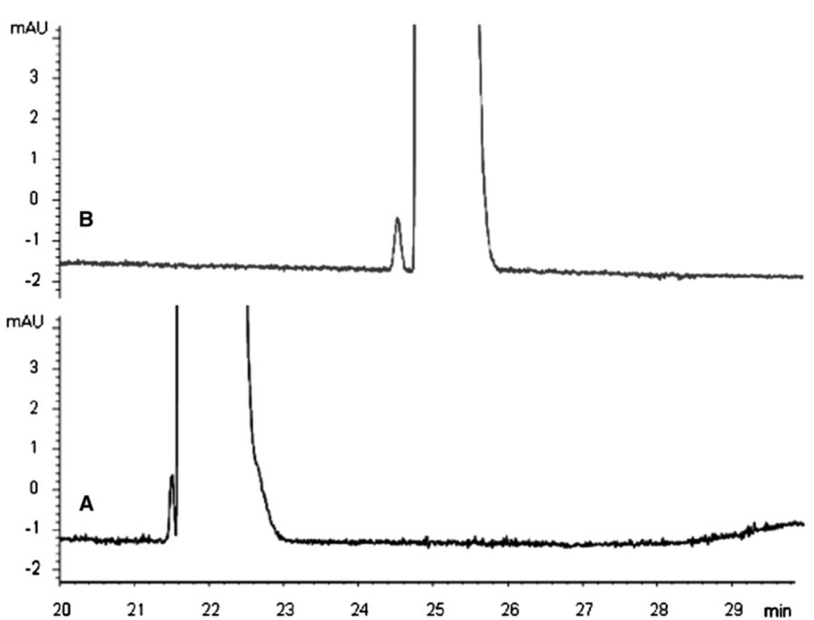

FIGURE 5 **The effect of the BGE concentration on the enantiomeric separation of ropivacaine. (A) BGE 50 mM phosphoric acid, 44 mM triethanolamine, 10 mM DM-β-CD. (B) BGE 100 mM phosphoric acid, 88 mM triethanolamine, 10 mM DM-β-CD (with permission from reference 56).**

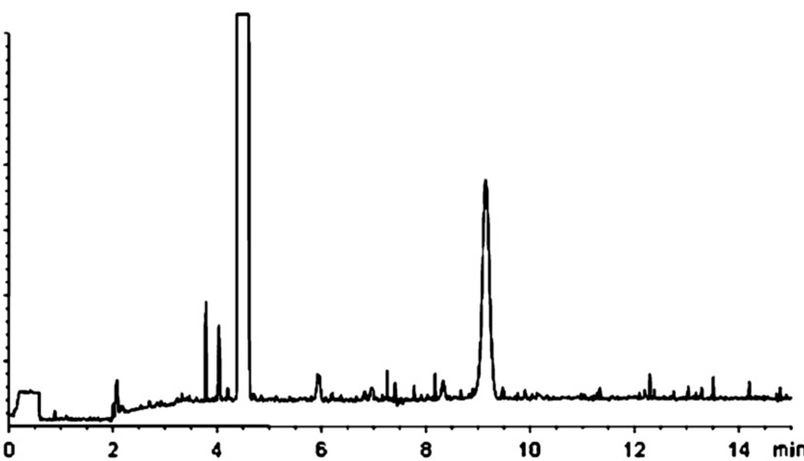

FIGURE 6 **Small spikes and peaks appearing in an electropherogram, caused by gas bubbles. Degassing the Milli-Q filtered water with helium solved the problem.**

G. Mobility Matching/Electromigration Dispersion

Peak shape can be regulated through the choice of the BGE co-ion. Generally, when the mobility of the BGE co-ion is similar to the analyte mobility, symmetric peak shapes are obtained.[60,61] However, other aspects also play a role.[58] Bohuslav Gas and co-workers have

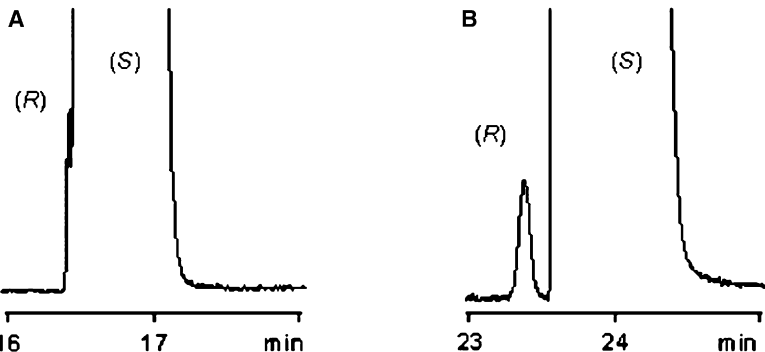

FIGURE 7 **Increased resolution by less electromigration dispersion and reversed EOF when changing from (A) NaOH to (B) triethanolamine as BGE co-ion. Sample: 0.1% (*R*)-ropivacaine in (*S*)-ropivacaine. Conditions: 80.5 (72.0) cm × 50 μm ID fused silica capillary; BGE 10 mM DM-*β*-CD, 100 mM phosphoric acid, 88 mM triethanolamine (pH 3.0); *V* = 30 kV; *T* = 30°C (with permission from reference 62).**

developed a simulator that can predict EMD and peak shape, which can be useful for method development.[59] Figure 7 shows the enantioseparation of ropivacaine.[62] Changing from sodium to triethanolamine as co-ion in the BGE inversed the EOF and reduced the EMD, and better resolutions were obtained. Furthermore, the dynamic coating of the capillary wall with triethanolamine resulted in a very robust method, which is now adopted in the United States pharmacopoeia (USP) and European pharmacopoeia (EP).

H. BGE Additives

Additives are often used to increase selectivity. They are paramount in chiral separations, but they are also frequently used in non-chiral separations, e.g., cyclodextrins (CDs).[63] In our lab, BGEs with and without a cyclodextrin are part of our generic protocol. Figure 8 demonstrates that although one can more or less predict interaction with the additive from the chemical structures, it is still difficult to predict separation.[64] Batch-to-batch variability and variability between suppliers can be a problem of (chiral) additives and a check of different batches has to be part of the robustness test (e.g., reference 56). If the additive is charged and has one or more pK_as around the pH of the BGE, extra care should be taken to control the pH. Alternatively, better robustness might be obtained with another uncharged additive, even if this results in lower resolution.

The concentration of additive that results in maximal electrophoretic mobility differences is not automatically the concentration that gives maximum resolution, since other aspects such as viscosity or ionic strength play a role as well.[64]

I. Volatile Buffers for CE–MS

For CE–MS, volatile buffers are common. To use MEKC systems combined with mass detection, volatile micelles have been tested.[65] When using atmosferic pressure photo-inonization (APPI), non-volatile BGE constituents do not deteriorate the mass signal to

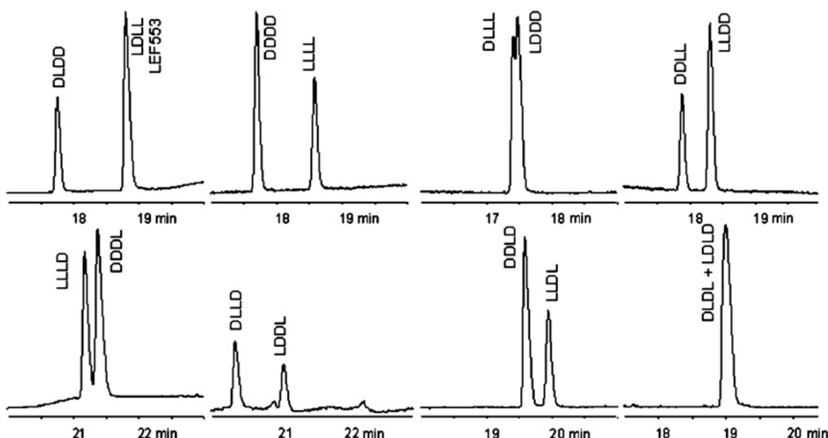

FIGURE 8 **Differences in enantiomeric separation of the eight enantiomer pairs of a tetrapeptide under the same conditions. Sample: Tyr-Arg-Phe-Phe-NH2. Conditions: 64.5 (56.0) cm × 50 µm ID fused silica capillary; BGE 10 mM DM-β-CD, 100 mM phosphoric acid, 88 mM triethanolamine (pH 3.0); $V = 25$ kV; $T = 30$°C (with permission from reference 64).**

the same amount as when using electrospray ionization (ESI).[66] Further details on CE-MS hyphenation are provided in Chapter 18.

IX. DETECTION AND SIGNAL INTEGRATION

A. Detection Wavelength and Bandwidth

Often lower detection wavelengths are possible in CE than in LC due to the aqueous character of the BGE. Wavelengths of 200 nm are routinely used and often result in higher sensitivity than longer wavelengths. However, it is important to check that the UV spectrum of the compounds of interest is not too steep to avoid precision problems. Also the detection wavelength bandwidth should match. It is worth the effort to check sensitivity and precision for different bandwidths at the detection wavelength of interest. Furthermore, thermally induced fluctuations of the baseline are more pronounced at the low wavelengths and if problems arise, a longer wavelength might be profitable.[67]

B. Response Time/Rise Time

The response time or rise time of the detector should be fast enough to be able to get sufficient data points in the narrow CE peak. The resulting absorbance at a certain time point is the average of the signal during the time interval determined by the detector response time. This means that the shorter the response time, the noisier the detector signal. So a proper balance between signal noise and peak width is needed. Do not choose a response time shorter than needed, since short response times give increased detection noise and thus decreased integration precision.

C. Integration

Unfortunately, integration can still be a problem for CE signals. Many integration software programs are not able to integrate small peaks properly, making automatic integration of impurity determinations difficult. The peak areas of the impurities are mostly overestimated. In the case of quantifying a minor impurity through external calibration, this could mean that a multilevel calibration curve will be "tilted" by the low-concentration standards and not go through the origin. In that case, a one-level calibration at the reporting threshold can give more reliable results. Integration errors of the internal standard can imply that the precision of a method decreases instead of increases, due to the propagation of errors if the integration error is dominant over the injection error.[67,68]

D. Resolution

Another typical problem for impurity determinations is the calculation of the resolution between a minor impurity peak and a big main component peak, since the latter is often triangular due to EMD. The usual resolution calculations are derived from Giddings equation for Gaussian peaks of similar height. Software-calculated values for resolution are therefore sometimes hard to interpret. Figure 9 shows that a calculated resolution value as little as 0.09 can still mean baseline resolution.[10] A pragmatic solution to get values that are comparable to Giddings resolution values would be to calculate on the facing halves of the peaks in question. Other solutions are peak-to-valley values or the Kaiser peak separation index,[69] but these have the disadvantage that increased resolution over baseline resolution does not result in higher values than 1. When optimizing or robustness testing with multifactorial/chemometric designs, one would like to give measure to these improvements for the better fitting of the models.

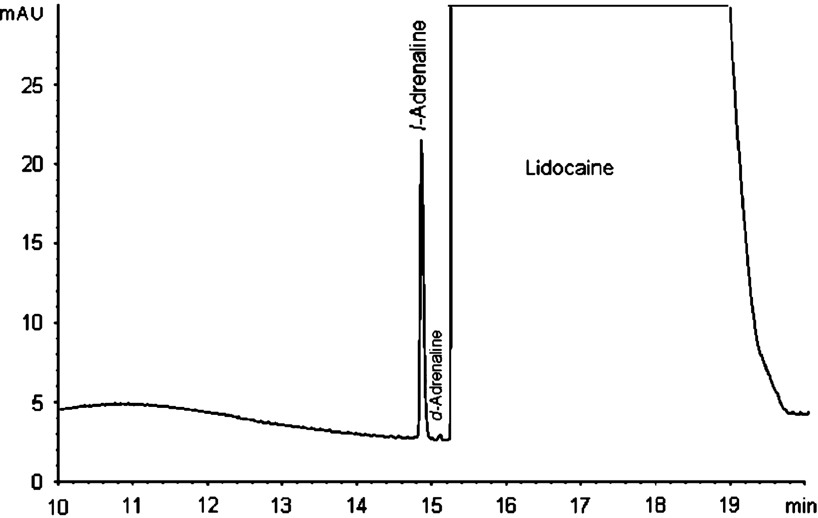

FIGURE 9 Resolution calculated by ChemStation software between l-adrenaline and d-adrenaline: 3.0; between d-adrenaline and lidocaine: 0.09 (with permission from reference 10).

E. Corrected Peak Areas

Since CE is an on-column detection technique, analytes migrate with different velocities through the detection window. Thus, slower migrating compounds will have the same peak height but a larger peak area than faster migrating compounds. Therefore, it is common to work with corrected peak areas, i.e., peak area divided by migration time.[55] The work on a racemic mixture of enantiomers demonstrates the importance of this correction.[70,72]

F. Linearity

The dynamic range of UV detectors for CE can be more limited than for LC. Linearity should therefore always be tested for the main component as well as the impurities. A general solution when the main peak is off-range is to inject a diluted solution to determine the response for the main component and a concentrated solution for the impurities. Alternatively, a small and a large injected volume of the same solution can be compared. In both cases, an internal standard is needed to be able to compare the two injections.

X. SUMMARY AND CONCLUSIONS

Before starting any method development, you have to know the method purpose, and from the method purpose you have to define the performance demands. To improve the performance of CE methods, parameters such as instrumental settings, the injection procedure, the composition and preparation of the BGE, sample, and standards all need to be considered carefully. It is important that the final method is described explicitly and unequivocally in all aspects.

It is important to remember that if CE were not be a robust technique, the human genome project HUGO would not have been completed today.

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7

OVERVIEW OF CURRENT REGULATORY GUIDANCE

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ABSTRACT

Capillary electrophoresis (CE) has become a valuable technique in the analytical toolbox for pharmaceutical analysts. CE methods have been successfully applied for identification, assay, purity determination, and chiral separation. ICH guidelines should be followed in meeting regulatory approval if CE methods are used in a registration dossier. Here, the validation parameters required for different analytical procedures are described and a comprehensive overview of CE validation studies presented in literature is given.

The harmonized general chapters on CE in the European, Japanese, and United States Pharmacopoeias are discussed. In addition, specific pharmacopoeial monographs prescribing CE methods in the current pharmacopoeias are described and the analytical instructions evaluated.

I. INTRODUCTION

During the last decade capillary electrophoresis (CE) has become a mature separation technique for pharmaceutical analysis. Numerous validated methods from pharmaceutical R&D laboratories and academia have been reported in literature, including identity confirmation, main component assay, purity determination, enantiomeric separation, and stoichiometry determination. In addition, CE is frequently applied as an orthogonal technique during the development of stability indicating liquid chromatography methods. As a result CE

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has been included in various regulatory submissions by different pharmaceutical companies. The use of CE as analytical tool within the pharmaceutical sciences has been elaborated in several review articles.[1−14]

The growing interest and application of CE as an advanced separation technique in the area of pharmaceutical analysis has been recognized by the regulatory authorities. CE has been included as a specific analytical technique in different guidance documents from the United States Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH). A general monograph on CE has been included in the European Pharmacopoeia (Ph.Eur.),[15] the United States Pharmacopeia (USP),[16,17] and the Japanese Pharmacopoeia (JP).[18] In addition, CE is included in a number of specific monographs for several products as, e.g., identity confirmation test or (enantiomeric) purity test. In order to prevent differences in nomenclature, recommendations on the terminology for analytical capillary electromigration techniques have been published by the International Union of Pure and Applied Chemistry (IUPAC).[19]

II. REGULATORY GUIDANCE DOCUMENTS

A. Method Validation

Analytical procedures used as part of a registration dossier in Europe, Japan, or the United States of America should be validated according ICH guideline Q2(R1) "Validation of Analytical Procedures: Text and Methodology."[20] The objective of analytical method validation is to demonstrate that the analytical procedure is suitable for its intended purpose. Depending on the type of analytical procedure, evaluation of different validation parameters is required. The four most common types of analytical procedures described in this ICH guideline are

- Identification tests
- Impurities: quantitative tests
- Impurities: limit tests
- Assay, i.e., quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

In Table 1, the typical validation parameters required for the different types of analytical procedures are listed. For all these analytical procedures CE might be an appropriate analytical technique. In fact numerous validated CE methods for pharmaceutical analysis have been described in literature during the last decade.[21,22] In Table 2, an overview is listed of the ICH validation parameters included in several reported CE validation studies. Since chiral purity determination is an important application area of CE methods, this test is listed separately as a specific analytical procedure. In addition, the determination of drug counterions has been included as a separate application. This overview illustrates that in general the required validation parameters are addressed in reported CE validation studies. It should be noted, however, that the validation parameters included in Table 2 are not necessarily evaluated exactly according ICH requirements in the reported references. Many pharmaceutical companies apply a phase-related validation approach in which the depth of validation depends on the clinical phase of development of the product involved.

B. Analytical Instructions

To be able to perform CE experiments correctly, the analytical instructions should contain sufficient information. In order to exclude any doubt on the experimental conditions and the performance of the CE experiments, the analytical instructions should be described to

TABLE 1 Validation Parameters that should be Considered for Different Types of Analytical Procedures[20]

	Type of analytical procedure			
		Testing for impurities		
Validation parameter	Identification	Quantitative	Limit test	Assay
Accuracy	−	+	−	+
Precision				
Repeatability	−	+	−	+
Intermediate precision	−	+[a]	−	+[a]
Specificity[b]	+	+	+	+
Detection limit	−	−[c]	+	−
Quantitation limit	−	+	−	−
Linearity	−	+	−	+
Range	−	+	−	+

(−) Signifies that this characteristic is not normally evaluated and (+) signifies that this characteristic is normally evaluated.
[a]In cases where reproducibility has been performed, intermediate precision is not needed.
[b]Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).
[c]May be needed in some cases.

an appropriate degree of detail. In the FDA draft guidance for industry "Analytical Procedures and Methods Validation, Chemistry, Manufacturing, and Controls Documentation,"[23] the following information on CE as a specific analytical methodology is provided regarding the analytical instructions:

> At a minimum, the parameters listed below should be specified for a capillary electrophoretic analytical procedure. Additional parameters may be included as required by the procedure. If method development has indicated that capillaries from only one commercial source are suitable, this information should be included as part of the analytical procedure. If more than one capillary is suitable, a listing of capillaries found to be equivalent should be included.

1. Capillary
   - Capillary dimensions: length, length to detector, internal diameter, external diameter
   - Capillary material
   - Capillary internal coating (if any).
2. Operating parameters
   - Capillary preparation procedure: procedure to be followed before the first use, before the first run of the day, before each run (e.g., flush with 100 mM sodium hydroxide, flush with running buffer)
   - Running buffer: composition, including a detailed preparation procedure with the order of addition of the components
   - Injection: mode (e.g., electrokinetic, hydrodynamic), parameters (e.g., voltage, pressure, time)

TABLE 2 Validation Parameters Included in Several Reported CE Validation Studies

Analytical procedure	Validation parameter								Compound(s)	Reference(s)
	Accuracy	Repeatability	Intermediate precision	Specificity	Detection limit	Quantitation limit	Linearity	Range		
Chiral purity	✓	✓	✓	✓	✓	✓	✓	✓	Etomidate	38
	✓	✓	✓	✓	✓	✓	✓	✓	*S*-Timolol	39
	✓	✓	✓	✓	–	✓	✓	✓	BIX (investigational new drug)	40
	✓	✓	✓	✓	✓	✓	✓	✓	Citalopram	41
	–	✓	✓	✓	✓	✓	✓	✓	Etodolac	42
	✓	✓	–	✓	✓	✓	✓	✓	SLV307	43
	✓	✓	–	✓	–	✓	✓	✓	R209130	44
	✓	✓	✓	✓	✓	✓	✓	✓	Ketoprofen	45
	✓	✓	✓	✓	✓	✓	✓	✓	Melagatran and ximelagatran	46
	✓	✓	–	✓	✓	✓	✓	✓	Galantamine hydrobromide	47
	✓	✓	✓	✓	✓	✓	✓	✓	Ropivacaine hydrochloride	48,49
	✓	✓	–	✓	–	✓	✓	✓	LY231514	50
	–	✓	–	✓	✓	✓	✓	✓	Fluparoxan	51
Purity	✓	✓	✓	✓	✓	–	✓	✓	Recombinant monoclonal antibody	52
	–	✓	–	✓	✓	✓	✓	✓	LAS35917	53
	✓	✓	✓	✓	✓	✓	✓	✓	Ragaglitazar and arginine	54
	✓	✓	✓	✓	✓	✓	✓	✓	Calcium levofolinate	55
	–	✓	✓	✓	✓	✓	✓	✓	Kanamycin sulfate	56
	–	✓	✓	✓	✓	✓	✓	✓	Aminopyridine and diaminopyridine	57
	✓	✓	✓	✓	✓	✓	✓	✓	Bisphosphonate and phosphonate impurities in clodronate	58

	–	✓	–	✓	✓	✓	✓	✓	5-Aminolevulinic acid	59
	✓	✓	✓	✓	✓	✓	✓	✓	Protegrin IB-367	60
	✓	✓	✓	✓	✓	✓	✓	✓	Ibuprofen and codeine phosphate	61
	–	✓	✓	✓	✓	✓	✓	✓	Amoxicillin	62
	–	✓	–	✓	✓	✓	✓	✓	Ranitidine hydrochloride	63
	–	✓	✓	–	✓	✓	✓	✓	Chlortetracycline	64
	✓	✓	✓	✓	✓	✓	✓	✓	Naphazoline hydrochloride	65
	✓	✓	–	✓	✓	✓	✓	✓	Mirtazapine	66
	✓	✓	✓	✓	✓	✓	✓	✓	Cephradine	67
	✓	✓	✓	✓	–	✓	✓	✓	Amino acids	68
	✓	✓	–	✓	✓	✓	✓	✓	Glutathione	69
	–	✓	–	✓	✓	✓	✓	✓	Ciprofloxacin	70
Assay	✓	✓	✓	✓	–	✓	✓	✓	Isoflavones	71
	✓	✓	✓	✓	✓	✓	✓	✓	Rosiglitazone and metformin	72
	✓	✓	✓	✓	✓	✓	✓	✓	Rabeprazole sodium	73
	✓	✓	✓	✓	✓	✓	✓	✓	Codeine, diphenhydramine, ephedrine, and noscapine	74
	–	✓	✓	✓	✓	✓	✓	✓	Adenosine	75
	✓	✓	✓	–	✓	✓	✓	✓	Denatonium benzoate	76
	✓	✓	–	–	–	–	✓	✓	Clothiapine, clozapine, olanzapine, and quetiapine	77
	✓	✓	✓	✓	✓	✓	✓	✓	Raloxifene	78
	✓	✓	✓	✓	✓	✓	✓	✓	Imipramine, amitriptyline, desipramine, and nortriptyline	79
	✓	✓	✓	✓	–	–	✓	✓	Rimantadine hydrochloride	80
	✓	✓	✓	✓	✓	–	✓	✓	Ragaglitazar	81
	✓	✓	✓	✓	✓	✓	✓	✓	1,4-Dideoxy-1,4-imino-d-arabinitol	82

TABLE 2 (Cont.)

Analytical procedure	Validation parameter								Compound(s)	Reference(s)
	Accuracy	Repeatability	Intermediate precision	Specificity	Detection limit	Quantitation limit	Linearity	Range		
	✓	✓	✓	✓	✓	✓	✓	✓	Metabisulfite and sulfate	83
	✓	✓	✓	✓	✓	✓	✓	✓	Meloxicam	84
	–	✓	–	–	–	–	✓	✓	Benzalkonium chlorides	85
	✓	✓	✓	✓	✓	✓	✓	✓	Amiodarone and desethylamiodarone	86
	✓	✓	–	✓	–	–	✓	✓	Rufloxacin hydrochloride	87
	✓	✓	–	✓	–	–	✓	✓	Diazepam and otilonium bromide	88
	✓	✓	✓	✓	✓	–	✓	✓	Synagis® (monoclonal antibody)	89
	✓	✓	✓	✓	✓	✓	✓	✓	Fluoxetine and fluvoxamine	90
	–	✓	✓	✓	✓	✓	✓	✓	Polymyxin B sulfate	91
	✓	✓	–	✓	–	–	✓	✓	Diclofenac sodium	92
	✓	✓	✓	–	✓	✓	✓	✓	Acarbose	93
	–	✓	✓	–	✓	✓	✓	✓	Atropine, homatropine, and scopolamine	94
	✓	✓	–	✓	–	–	✓	✓	Oxytetracycline	95
	✓	✓	✓	✓	✓	✓	✓	✓	Atenolol	96
	–	✓	✓	–	–	–	✓	–	Ribonuclease A	97
	✓	✓	✓	✓	–	–	✓	✓	Minoxidil	98
	✓	✓	✓	✓	✓	✓	✓	✓	Captopril	99
Counterions	✓	✓	✓	✓	✓	✓	✓	✓	Sulfate	100
	✓	✓	✓	✓	✓	✓	✓	✓	Sulfate	101
	✓	✓	✓	✓	✓	✓	✓	✓	Calcium	102
	✓	✓	–	–	✓	✓	✓	✓	Potassium	103

- Detector
- Typical migration time and total run time
- Model of CE equipment used
- Voltage (if constant voltage)
- Current (if constant current)
- Polarity (e.g., polarity of electrode by detector).

3. System suitability testing
   - Each analytical procedure should include the appropriate system suitability tests defining the critical characteristics of that system. Other parameters may be included at the discretion of the applicant.
   - If an internal standard is used, the minimum acceptable resolution between the internal standard and one or more active ingredient should be specified. If the analytical procedure is used to control the level of impurities, the minimum resolution between the active ingredient and the closest eluting impurity, or the two peaks eluting closest to each other, should be given.

III. CAPILLARY ELECTROPHORESIS IN PHARMACOPOEIAS

A. General Chapters

As a result of the pharmacopoeial harmonization process,[24,25] general chapter 2.2.47. of the Ph.Eur.[15] and general chapter 8 of the JP[18] (*Capillary Electrophoresis*) and general chapter 〈1047〉 of the USP[17] (*Biotechnology-Derived Articles − Tests, Capillary Electrophoresis**) have been harmonized to a major extent. At present some minor differences exist between the text and a few equations in the pharmacopoeia. In these chapters, the following definition of CE is given:

> Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

In the section *General Principles*, a comprehensive description is given of the basic principles of the capillary electrophoretic separation process. The concepts of electrophoretic mobility and electroosmotic mobility as well as band dispersion phenomena and resolution are described, using the equations listed in Table 3. A remarkable difference exists between the equations in both chapters in which the electroosmotic velocity and/or the electroosmotic mobility is used. In the Ph.Eur., the terms $+v_{eo}$ and $+\mu_{eo}$ are used, whereas in the USP the terms $\pm v_{eo}$ and $\pm \mu_{eo}$ are used in the corresponding equations, with the sentence added: "The sum or the difference between the two velocities (v_{ep} and v_{eo}) is used depending on whether the mobilities act in the same or opposite directions."

In the *Apparatus* section, the basic parts of a CE apparatus are described as

- a high-voltage, controllable direct current power supply
- two buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions

* The entire chapter 〈1047〉 is harmonized with the Japanese and the European Pharmaceopoeias. In order to have a parallel organization of the harmonized documents, the current chapter 〈1047〉 will be divided and replaced by six general information chapters, including 〈1053〉 *Biotechnology Derived Articles − Capillary Electrophoresis*.

TABLE 3 Equations used in European Pharmacopoeia Chapter 2.2.47,[15] Japanese Pharmacopoeia Chapter 8,[18] and United States Pharmacopeia Chapters ⟨727⟩[16] and ⟨1047⟩[17]

Section	Equation: Ph.Eur. chapter 2.2.47 and JP chapter 8	Equation: USP chapter ⟨1047⟩	Equation: USP chapter ⟨727⟩
General Principles[a]	$v_{ep} = \mu_{ep} \times E = \left(\frac{q}{6\pi\eta r}\right) \times \left(\frac{V}{L}\right)$ $v_{eo} = \mu_{eo} \times E = \left(\frac{\varepsilon\zeta}{\eta}\right) \times \left(\frac{V}{L}\right)$		$v_{EP} = \mu_{EP} \times \left(\frac{V}{L}\right) \quad \mu_{EP} = \frac{q}{6\pi\eta r}$ $v_{EO} = \mu_{EO} \times \left(\frac{V}{L}\right)$
	$v = v_{ep} + v_{eo}$ $t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo})V}$ $N = \frac{(\mu_{ep} + \mu_{eo})Vl}{2DL}$ $R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\overline{\mu_{ep}}\mu_{eo})}$	$v = v_{ep} \pm v_{eo}$ $t = \frac{l}{v_{ep} \pm v_{eo}} = \frac{l \times L}{(\mu_{ep} \pm \mu_{eo})V}$ $N = \frac{(\mu_{ep} \pm \mu_{eo})Vl}{2DL}$ $R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\overline{\mu_{ep}} \pm \mu_{eo})}$	$t = \frac{l}{E(\mu_{EP} + \mu_{EO})} = \frac{l \times L}{V(\mu_{EP} + \mu_{EO})}$ $N = \frac{(\mu_{EP} + \mu_{EO})V}{2D}$ $R_s = 0.18(\mu_{EP1} - \mu_{EP2})\sqrt{\frac{V}{D(\overline{\mu_{EP}} + \mu_{EO})}}$
Capillary Isoelectric Focusing	$\Delta pI = 3 \times \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$		
Micellar Electrokinetic Chromatography	$k = \frac{t_R - t_0}{t_0[1 - (t_R/t_{mc})]} = K\frac{V_S}{V_M}$ $R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b}{k_b + 1} \times \frac{1 - (t_0/t_{mc})}{1 + k_a(t_0/t_{mc})}$		$t_R = \frac{(1 + k')t_0}{1 + (t_0/t_{MC})}$ $k' = \frac{(t_R/t_0) - 1}{1 - (t_R/t_{MC})}$ $k' = \frac{t_R}{t_0} - 1$ [b] $R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_2}{k'_2 + 1} \times \frac{1 - (t_0/t_{MC})}{1 + (t_0/t_{MC})k'_1}$

System Suitability	$N = 5.54\left(\frac{t_R}{w_h}\right)^2$	$N = 5.54\left(\frac{t}{b_{0.5}}\right)^2$	$k' = t_R - t_0[1 - (t_R/t_{MC})]$ $N = 16\left(\frac{t_R}{W}\right)^2 \quad N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$ $\text{TPM} = 1600\frac{(t_R/W)^2}{L}$
	$R_s = \frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}, \quad t_{R2} > t_{R1}$ $\frac{p}{v} = \frac{H_p}{H_v}$ $A_s = \frac{w_{0.05}}{2d}$ $\frac{S}{N} = \frac{2H}{h}$	$R_s = \frac{1.18(t_b - t_a)}{b_{0.5b} + b_{0.5a}}, \quad t_b > t_a$ $\frac{c}{d} \leq x$ $A_s = \frac{b_{0.05}}{2A}$ $\frac{S}{N} = \frac{2H}{h_n}$	$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2}, \quad t_2 > t_1$ $T = \frac{W_{0.05}}{2f}$

Parameter	Description
A	Distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20th of the peak height
A_s	Symmetry factor
$b_{0.5}$, $b_{0.5a}$, $b_{0.5b}$	Width of the peak at half height
$b_{0.05}$	Width of the peak at 1/20th of the peak height
D	Distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20th of the peak height
D	Molecular diffusion coefficient of the solute in the buffer
E	Intensity of electric field
f	Distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20th of the peak height
h	Range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to 20 times the width at the half height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found
H	Height of the peak corresponding to the component concerned, in the electropherogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half height
H_p	Height of the smaller peak
H_v	Height of the valley
k, k_a, k_b, k', k'_1, k'_2	Retention factor of the solute
K	Partition coefficient of the solute
l	Capillary effective length

TABLE 3 (Cont.)

Parameter	Description
L	Total length of the capillary
N	Number of theoretical plates
p/v	Peak-to-valley ratio
q	Effective charge of the solute
r	Stoke's radius of the solute
R_s	Resolution
S/N	Signal-to-noise ratio
t, t_R, t_{R1}, t_{R2}, t_1, t_2, t_a, t_b	Migration time of the solute
t_0	Analysis time of an unretained solute
t_{mc}, t_{MC}	Micelle migration time
T	Tailing factor
TPM	Number of theoretical plates per meter
V	Velocity of the solute
v_{eo}, v_{EO}	Electroosmotic velocity
v_{ep}, v_{EP}	Electrophoretic velocity
V	Applied voltage
V_M	Volume of the mobile phase
V_S	Volume of the micellar phase
w_h, w_{h1}, w_{h2}, $w_{1/2}$	Width of the peak at half height
$w_{0.05}$, $W_{0.05}$	Width of the peak at 1/20th of the peak height
W, W_1, W_2	Analyte peak width at baseline
α	Selectivity
ΔpI	Separation achieved
ε	Dielectric constant of the buffer
ζ	Zeta potential of the capillary surface
η	Viscosity of the electrolyte solution
μ_{ep}, μ_{EP}	Electrophoretic mobility
μ_{eo}, μ_{EO}	Electroosmotic mobility
$\overline{\mu_{ep}}$, $\overline{\mu_{EP}}$	Mean electrophoretic mobility of the two analytes=$\frac{1}{2}(\mu_{epb}+\mu_{epa})$ or $\frac{1}{2}(\mu_{EP1}+\mu_{EP2})$
μ_{epa} and μ_{epb}, μ_{EP1} and μ_{EP2}	Electrophoretic mobilities of the two analytes separated
dpH/dx	pH gradient
$-d\mu$/dpH	Variation of electrophoretic mobility of the analyte with pH

[a]The corresponding equations of USP ⟨727⟩ are included in section *Principles of Capillary Zone Electrophoresis*.
[b]In the monograph it is stated that for practical purposes, k' is calculated by this equation.

- two electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply
- a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph
- a suitable injection system
- a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time; it is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric, or mass spectrometric detection can be useful for specific applications; indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds
- a thermostatic system capable of maintaining a constant temperature inside the capillary is recommended to obtain a good separation reproducibility
- a recorder and a suitable integrator or a computer.

The different modes of injection, i.e., gravity injection, pressure or vacuum injection, and electrokinetic injection, are addressed. Also, several practical experimental issues are described, such as correct treatment of electrolyte solutions and the importance of a rigorous capillary rinsing procedure.

Subsequently four different CE modes are described in the sections *Capillary Zone Electrophoresis*,[†] *Capillary Gel Electrophoresis*, *Capillary Isoelectric Focussing*, and *Micellar Electrokinetic Chromatography (MEKC)*, respectively. The fundamental principles of the specific separation modes are briefly explained, using appropriate equations where required. In Table 3 all equations are listed. In addition, the influence of both instrumental parameters and electrolytic solution parameters on the optimization of separations is described.

In the *Quantification* section, the necessity for using corrected peak areas, i.e., peak areas divided by the corresponding migration time, is emphasized. Corrected peak areas are used in order to

- compensate for the shift in migration time from run to run, thus reducing the variation of the response
- compensate for the different responses of sample constituents with different migration times.

In the *System Suitability* section, different parameters are described which can be applied in order to check the behavior of the CE system. The choice of the appropriate parameters depends on the mode of CE used. The system suitability parameters include retention factor (k) (only for MEKC), apparent number of theoretical plates (N), symmetry factor (A_s), resolution (R_s), area repeatability, migration time repeatability, and signal-to-noise ratio. Practical equations to calculate different system suitability parameters from the electropherograms are presented, which are also included in Table 3.

In addition to the general information chapter ⟨1047⟩ *Biotechnology-Derived Articles – Tests, Capillary Electrophoresis*, the USP also provides a general monograph ⟨727⟩ *Capillary Electrophoresis* in the *General Chapters* part under *General Tests*

[†] Denoted as *Free Solution Capillary Electrophoresis* in the USP.

and Assays, Physical Tests and Determinations.[16] In this general monograph CE is described as follows:

> Electrophoresis refers to the migration of charged electrical species when dissolved or suspended in an electrolyte through which an electric current is passed. Cations migrate toward the negatively charged electrode (cathode), while anions are attracted toward the positively charged electrode (anode). Neutral particles are not attracted toward either electrode. The use of capillaries as a migration channel in electrophoresis has enabled analysts to perform electrophoretic separations on an instrumental level comparable to that of high-performance liquid chromatography (HPLC), albeit with some distinct operational differences, advantages, and disadvantages relative to HPLC. This method of analysis is commonly known as capillary electrophoresis (CE).

After a brief description of the electrophoretic separation process in capillaries, five major modes of operation are distinguished in this chapter: capillary zone electrophoresis (CZE), also referred to as free solution or free flow CE; micellar electrokinetic chromatography (MEKC); capillary gel electrophoresis (CGE); capillary isoelectric focusing (CIEF); and capillary isotachophoresis (CITP). It is stated that CZE and MEKC are the most commonly utilized CE techniques. The separation mechanism of both modes are briefly discussed in the sections *Principles of Capillary Zone Electrophoresis* and *Principles of Micellar Electrokinetic Chromatography*, respectively. For the sake of completeness, the equations used in these sections are also included in Table 3. Notice that two different equations for the calculation of k for neutral species in MEKC are given. The equation for k, generally known for chromatographic separations (i.e., without t_{MC}), is stated to be included "for practical purposes." From a theoretical point of view this equation is however incorrect, and it has not been included in the new harmonized general chapter on CE. Subsequently, instrumental considerations, analytical considerations, and operational parameters are included in the general chapter as well. In the *Instrumental Considerations* section, the primary instrumentation issues *Capillary Type and Configuration*, *Sample Introduction and Injector Technology*, *Power Supply*, and *Detector Modes* are addressed, respectively. In order to provide some insight in the optimization of experimental parameters, the influence of *Capillary Dimensions*, *Voltage Effects*, *Ionic Strength Effects*, and *pH Effects* on the separation process is described in the *Analytical Considerations* section. Finally, the major steps in operating a CE system are described in the *Operational Parameters* section, including *System Setup*, *Capillary Rinsing Procedure*, *Running a Sample*, *System Suitability*, *Sample Analysis*, *Data Handling*, and *System Shutdown*. In addition to the number of theoretical plates, N, the parameter number of theoretical plates per meter, TPM, is presented in the system suitability section of the monograph.

B. Specific Monographs in Pharmacopoeias

During the last few years, a number of specific monographs for different pharmaceutical products have appeared in pharmacopoeias in which CE is prescribed as one of the analytical procedures. Several comparative studies have been reported in which established analytical procedures described in pharmacopoeial monographs were compared with capillary electrophoretic methods[26–30] or in which CE was evaluated as a valuable additional technique.[31–33] Based on the results, some analytical procedures have been replaced by CE-based alternative methods in a number of monographs. For other products, CE has been included in the monograph from the initial version onwards. In addition, CE has been applied

for the establishment of replacement batches of the Ph.Eur. somatropin chemical reference substance[34] and for the comparison of impurity profiles of gentamicin samples[32] or amino acids[35] of different origin. In Table 4, an overview is presented of the different monographs in the Ph.Eur. and USP, in which CE is included. In Figure 1, the structural formulae of the compounds concerned are shown. At present CE is applied as analytical procedure for identification or for the determination of related substances for small organic compounds as well as for biotechnology-derived substances such as polypeptides or (glyco)proteins.

Aprotinin is a polypeptide consisting of a chain of 58 amino acid residues, which inhibits stoichiometrically the activity of several proteolytic enzymes such as chymotrypsin, kallikrein, plasmin, and trypsin. Aprotinin is obtained from bovine tissues and purified by a suitable process. It is stored as a bulk solution or lyophilized powder. The amount of two related substances des-Ala-des-Gly-aprotinin and des-Ala-aprotinin is determined by CZE with a 100% analysis. The relative migration times are 0.98 for des-Ala-des-Gly-aprotinin and 0.99 for des-Ala-aprotinin, and the specified limits are 8.0 and 7.5%, respectively.

Erythropoietin concentrated solution is a solution containing a family of closely related glycoproteins which are indistinguishable from the naturally occurring human erythropoietin in terms of amino acid sequence (165 amino acids) and average glycosylation pattern, at a concentration of 0.5−10 mg/ml. Erythropoietin is produced in rodent cells in vitro by a method based on recombinant DNA technology. CZE is one of the prescribed analytical procedures for identification. On the basis of a collaborative study among 12 laboratories,[28] the former isoelectric focusing procedure for the determination of isoform distribution was replaced by the present CZE procedure. A separation is obtained between eight different isoforms and the percentage content of each isoform, calculated from the corresponding peak area, should comply with the ranges listed in the monograph. In Figure 2, a reference electropherogram of erythropoietin is shown. Recently the overall isoform number, calculated from CZE data, was suggested to provide a new quality control measure to ensure batch-to-batch consistency of the active pharmaceutical ingredient of erythropoietin market products.[36]

Glutathione is a tripeptide composed of glutamate, cysteine, and glycine that plays an important role in antioxidant defense, nutrient metabolism, and regulation of various cellular events. Related substances are determined by CZE, using internal standardization. In Figure 3, a typical electropherogram of a glutathione sample is shown. Five specified impurities are included in the monograph, for two of them a correction factor for the UV response is given. For unspecified impurities a limit of 0.10% is set, and a disregard limit is defined at 0.05%.

Levocabastine hydrochloride is a antihistamine that is used for the treatment of nose and eye allergies by preventing the effect of histamine. Related substances are determined by MEKC, using sodium dodecyl sulfate as pseudo-stationary phase. A current gradient is defined in the analytical instructions, containing four steps with either a linear increasing or a constant current profile. The disregard limit is defined as 0.05%. In Figure 4, an electropherogram is shown of levocabastine hydrochloride including several related substances.

Ropivacaine hydrochloride is a long-acting local anesthetic, which is manufactured as the pure S-enantiomer. The enantiomeric purity is determined by CZE, using heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin as chiral selector. A resolution of 3.7 between the two enantiomers is required for the system suitability solution. The percentage R-enantiomer is calculated relative to the S-enantiomer in the same electropherogram, and should not exceed 0.5%. In Figure 5, a representative electropherogram is presented.

Somatropin is a protein having the structure (191 amino acid residues) of the major component of growth hormone produced by the human pituitary. Somatropin is produced by a method based on recombinant DNA technology. Recently the results of a collaborative study between 14 laboratories have been reported, in which a new CE method was compared with the existing test for "isoform distribution" by isoelectric focusing.[27] The CE method was found to be superior to the method of isoelectric focusing. As a result CE is applied for identification

TABLE 4 Overview of Specific Monographs in Pharmacopoeia in which CE is Included

				Analytical instructions			
Product	Pharma-copoeia	Analytical procedure	Compound type	Capillary dimensions	Capillary material	Capillary internal coating	Capillary preparation procedure
Aprotinin Aprotinin injection	USP	Related substances	Polypeptide	✓	✓	na	✓
Erythropoietin concentrated solution	Ph.Eur.	Identification[b]	Series of closely related glycoproteins	✓	✓	na	✓
Glutathione	Ph.Eur.	Related substances	Small organic compound	✓	✓	na	✓
Levocabastine hydrochloride	Ph.Eur.	Related substances	Small organic compound	✓	✓	na	✓
Ropivacaine hydrochloride	USP and Ph.Eur.[c]	Enantiomeric purity	Small organic compound	✓	✓	na	✓
Somatropin Somatropin bulk solution Somatropin for injection	Ph.Eur.	Identification and related substances	Protein	✓	✓	na	✓

na: not applicable.
[a]Referred to as standard solution.
[b]Confirmation of content of different isoforms.
[c]A draft monograph for the Ph.Eur. has been published in *Pharmeuropa*.[104]
[d]System suitability solution.

Analytical instructions

Running buffer	Injection mode	Injection parameters	Detector	Typical migration time and run time	Model CE equipment used	Voltage or current	Polarity	System suitability tests	Preparation test solution	Preparation reference solution	Preparation internal standard solution	Temperature	System shutdown
✓	✓	✓	✓	✓	–	✓	–	✓	✓	✓[a]	na	✓	–
✓	✓	–	✓	✓	–	✓	–	✓	✓	✓	na	✓	–
✓	✓	✓	✓	✓	–	✓	–	✓	✓	✓	✓	✓	–
✓	✓	✓	✓	✓	–	✓	–	✓	✓	✓	na	✓	–
✓	✓	✓	✓	✓	–	✓	–	✓	✓	✓[d]	na	✓	✓
✓	✓	✓	✓	✓	–	✓	–	✓	✓	✓	na	✓	–

Aprotinin

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RPDFCLEPPY TGPCKARIIR YFYNAKAGLC QTFVYGGCRA KRNNFKSAED
CMRTCGGA

Erythropoietin

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APPRLICDSR VLERYLLEAK EAENITTGCA
EHCSLNENIT VPDTKVNFYA WKRMEVGQQA
VEVWQGLALL SEAVLRGQAL LVNSSQPWEP
LQLHVDKAVS GLRSLTTLLR ALGAQKEAIS
PPDAASAAPL RTITADTFRK LFRVYSNFLR
GKLKLYTGEA CRTGD

Glutathione

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Levocabastine hydrochloride

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Ropivacaine hydrochloride

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Somatropin

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FPTIPLSRLF DNAMLRAHRL HQLAFDTYQE FEEAYIPKEQ
KYSFLQNPQT SLCFSESIPT PSNREETQQK SNLELLRISL
LLIQSWLEPV QFLRSVFANS LVYGASDSNV YDLLKDLEEG
IQTLMGRLED GSPRTGQIFK QTYSKFDTNS HNDDALLKNY
GLLYCFRKDM DKVETFLRIV QCRSVEGSCG F

FIGURE 1 Structural formulae of different compounds for which CE is prescribed in the pharmacopoeial monograph.

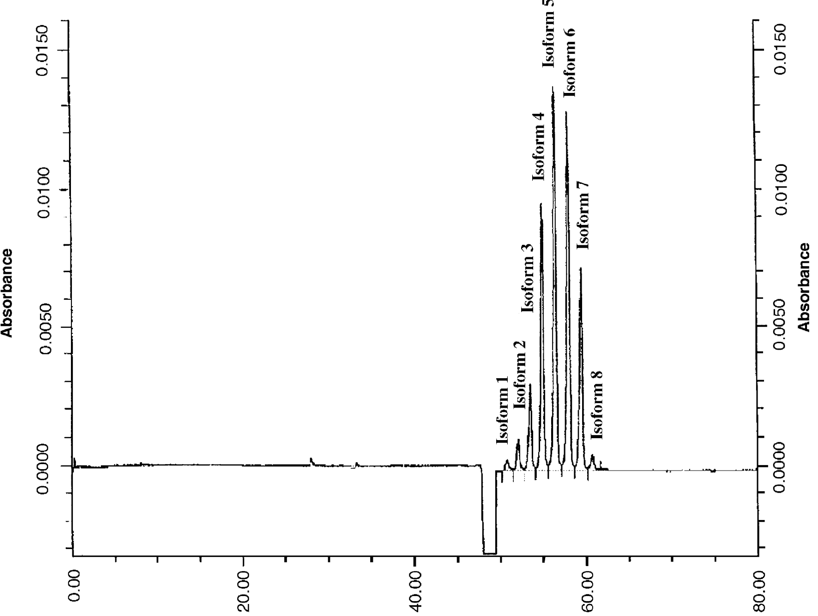

FIGURE 2 Reference electropherogram of erythropoietin. (Reprinted with permission from *Pharmeuropa* 11(2) (1999) 415.)

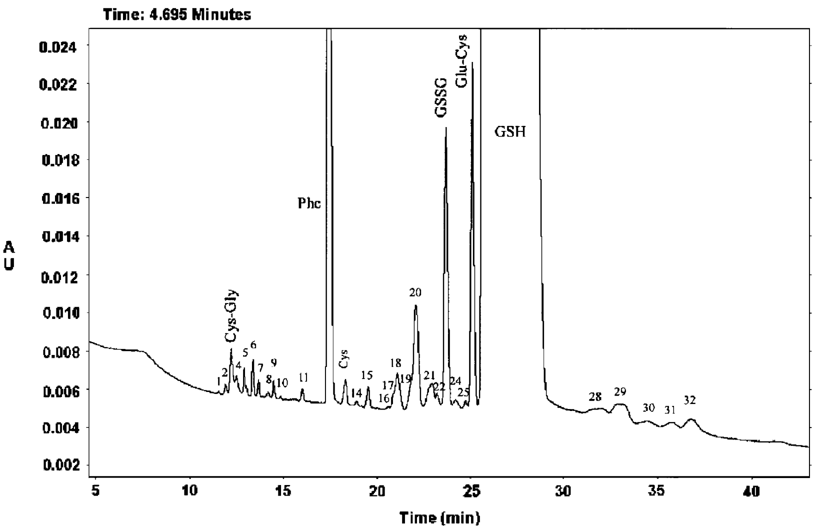

FIGURE 3 Electropherogram of a glutathione (GSH) sample (20 mg/ml) containing 1% (w/w) phenylalanine (Phe) as internal standard. (Reprinted with permission from reference 69.)

and for the determination of related substances of somatropin, somatropin bulk solution, and somatropin for injection in the current Ph. Eur. Identification is performed by a single experiment of a sample mixture of equal amounts of the test solution and the reference solution, which should result in one principal peak. Three related substances are defined in the

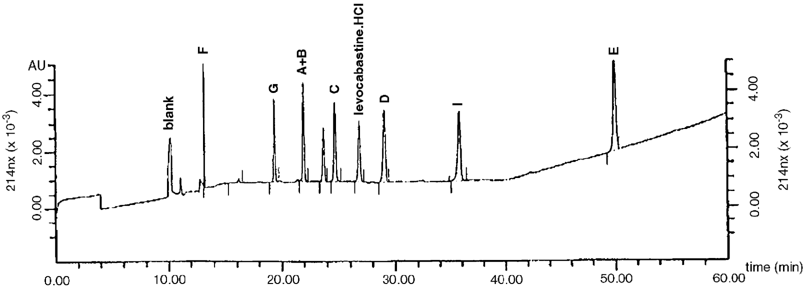

FIGURE 4 Electropherogram of levocabastine hydrochloride including several related substances. (Reprinted with permission from *Pharmeuropa* 10(2) (1998) 245.)

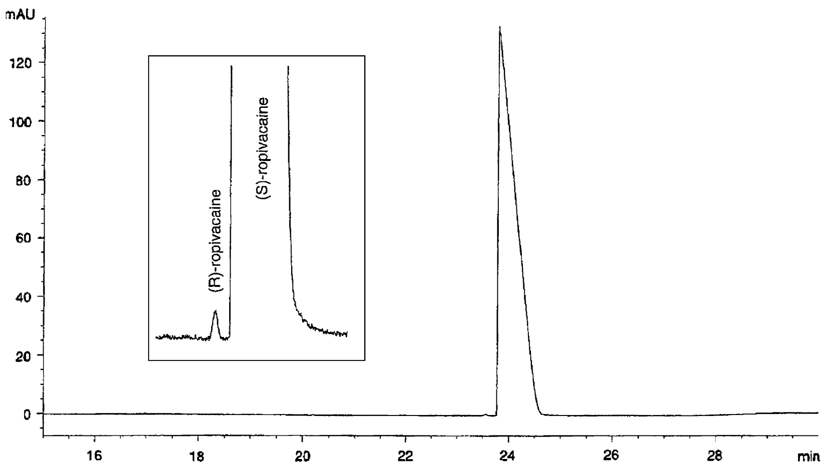

FIGURE 5 Electropherogram of (*S*)-ropivacaine containing (*R*)-ropivacaine at the LOQ level. (Reprinted from *J. Pharm. Biomed. Anal.*, Vol. 15, C.E. Sänger-van de Griend, H. Wahlström, K. Gröningson, M. Widahl-Näsman, A chiral capillary electrophoresis method for ropivacaine hydrochloride in pharmaceutical formulations: validation and comparison with chiral liquid chromatography, pp. 1051–1061, Copyright (1997), with permission from Elsevier.)

paragraph "charged variants," i.e., cleaved form, Gln-18 somatropin and deamidated forms (migrating as a doublet), having relative migration times with reference to somatropin of 0.94–0.98, 1.02–1.06, and 1.06–1.11, respectively. In Figure 6, a typical electropherogram of a somatropin sample is shown. The limits set are 5.0% for the deamidated forms (6.5% for the deamidated forms in somatropin for injection) and 2.0% for any other impurity.

In Table 4, an overview is presented of the different experimental parameters of the CE analyses that are included in the analytical instructions part of the specific monographs mentioned above. Notice that the first 13 parameters (from "capillary dimensions" up to "system suitability tests") are those described in the FDA draft guidance for industry described in paragraph II B.[23] From this overview it can be concluded that most experimental parameters required by the draft guidance are included in the specific monographs, currently published in the Ph.Eur. and USP. In the monograph for erythropoietin concentrated solution the injection parameters are not included. Only the injection mode (pressure or vacuum) is defined. Instead,

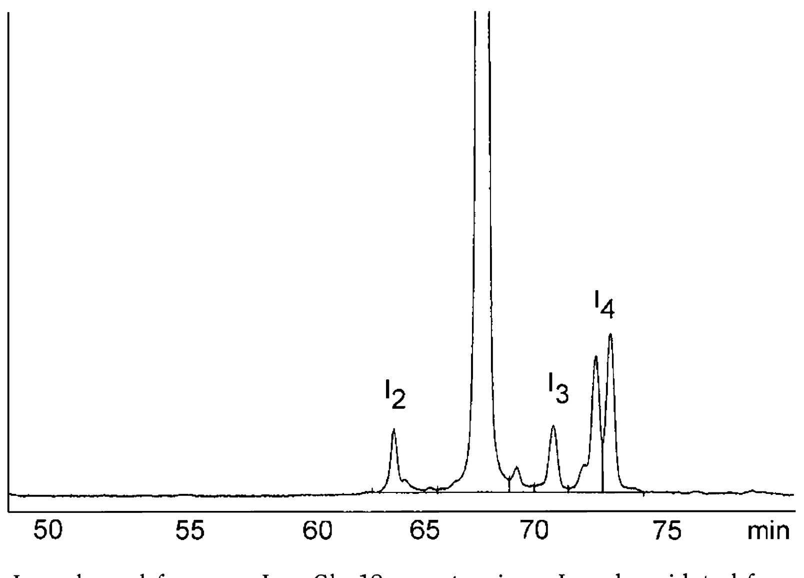

FIGURE 6 **Typical electropherogram of a somatropin sample. (Reprinted with permission from reference 27.)**

it is stated that "the largest peak is at least 50 times greater than the baseline noise. If necessary, adjust the sample load to give peaks of sufficient heights." At present coated capillaries are not applied. In none of the monographs the model of CE equipment used is prescribed. Obviously this parameter is considered not to be essential by the authors of the monographs. Also, the polarity is not mentioned specifically as such in the monographs. In all applications a positive polarity mode (normal mode) is applied, i.e., the anode is placed at the inlet side and the cathode at the outlet side of the capillary, respectively. In fact the polarity is included in the description of the field strength to be applied, which is defined as positive for this mode. In all monographs a description for the preparation of test solutions and reference solutions (also referred to as standard solution) or system suitability solution is included. The monograph of glutathione is the only analytical procedure to use internal standardization for the quantitative determination of related substances. All other quantitative determinations for related substances (including enantiomeric purity) are based on 100% analyses, which are according to the calculation procedure described in the harmonized pharmacopoeial general monograph on CE. In addition to the experimental parameters mentioned in the FDA draft guidance, the temperature is defined in all specific monographs. Actually this is not surprising since temperature plays a major role in the performance of CE analyses. It is worthwhile to mention that in the monograph of ropivacaine hydrochloride a specific description is included for system shutdown.

IV. CONCLUSIONS

CE is an important separation technique within the field of pharmaceutical analysis. CE may be an attractive choice as analytical procedure for identification, assay, or (chiral) purity determination. In addition, CE may provide distinct advantages over existing pharmacopoeial

methods and as a consequence several analytical procedures have been replaced by CE-based alternatives. Due to the growing interest in CE within the pharmaceutical sciences, CE methods have appeared in registration submissions for various new drugs during the last decade. These methods need to be validated according to the current ICH harmonized tripartite guideline on validation of analytical procedures. The extent of the validation depends on the intended purpose of the method. An extended overview of reported CE validation studies has been presented in Table 2, illustrating that the appropriate validation parameters according to the regulatory guidance are generally addressed.

CE has been included as a distinct analytical technique in a general monograph in the Ph.Eur., JP, and USP. These monographs have been harmonized and at present only some minor differences exist between the different pharmacopoeias. They give an overview of the general principles, instrumental considerations, and the different separation modes. Also, attention is paid to quantification and system suitability aspects.

At present a number of specific pharmacopoeial monographs exist in which CE is prescribed as analytical procedure for identification or (chiral) purity determination.

The requirements for the experimental conditions to be included in the analytical instructions for CE methods have been described in an FDA draft guidance for industry. Most parameters have been included in the current specific monographs prescribing CE, except for the model of CE equipment. The temperature has been included additionally in all these monographs.

Despite some distinct advantages of CE for pharmaceutical analysis, the current number of pharmacopoeial monographs which prescribe CE is rather limited.[37] Although it is difficult to point out the exact reason for this, an appropriate training for pharmaceutical analysts in CE-specific analytical characteristics certainly plays an important role. We do hope that this book may provide a contribution in this respect.

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8 QUALIFICATION OF CE INSTRUMENTATION

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ABSTRACT
I. INTRODUCTION
II. PARAMETERS FOR QUALIFICATION
A. Sample Compartment Parameters
B. Injection Parameters
C. Separation Parameters
D. Detection Parameters
III. COMPUTER SYSTEM
IV. KEEPING THE INSTRUMENT QUALIFIED
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ABSTRACT

Qualification of a capillary electrophoresis instrument is performed using failure mode, effects, and criticality analysis as the risk analysis tool. The instrument is broken down into its component modules and the potential failures of those components identified. The potential effect of those failures is defined and the risk characterized. Any current evaluation of those failures is identified and any recommended actions to mitigate the risk defined.

I. INTRODUCTION

Apart from the qualification dossiers provided by vendors there seems, at present, to be very little information published on the performance of an operational qualification for capillary electrophoresis (CE) instruments other than a chapter in *Analytical Method Validation and Instrument Performance*. The chapter, written by Nichole E. Baryla of Eli Lilly Canada, Inc,[1] discusses the various functions (injection, separation, and detection) within the instrument and provides guidance on the type of tests, including suggested acceptance criteria, that may be performed to ensure the correct working of the instrument. These include injection reproducibility and linearity, temperature and voltage stability, detector accuracy, linearity, and noise.

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However the latest thinking provided by the United States Food and Drug Administration (FDA) in the Guidance for Industry for Quality Risk Management[2] suggests that all qualification activities for analytical instruments should be performed using a risk-based approach.

To this end the following guideline describes a general approach toward risk management for a laboratory instrument and then applies it to the performance of the operational qualification of a CE instrument. Tests then need to be devised in order to determine the suitability of the instrument for its intended use and to fulfill those user requirements that have been defined by the operator.

It is widely understood within the industry that risk is defined as the combination of the probability of harm and the severity of that harm. Within the pharmaceutical industry whenever risk is considered the equipment or product being assessed must be viewed in the context of the "protection of the patient." From our perspective, analytical instruments may impact on the validity of data that determines the safety and efficacy of drug products, or on the quality of the drug product. They may also impact on the identity or potency of the drug product and therefore it is important to ensure that risk management is performed throughout the complete life cycle of the instrument.

The following passage from International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q9[3] provides a basic principle that enables the appropriate degree of risk assessment for the instrument to be determined.

> It is neither always appropriate nor always necessary to use a formal risk management process (using recognized tools and/or internal procedures, e.g., standard operating procedures). The use of informal risk management processes (using empirical tools and/or internal procedures) can also be acceptable.

However for an equipment such as a CE instrument the utilization of a systematic technique as opposed to an empirical approach is advisable in order to ensure that all of the functionality of the instrument are correctly assessed.

Figure 1 describes how risk management can be incorporated into the qualification process and the life cycle of the instrument.

There are a number of techniques available to perform this task and a selection of these can be found in Table 1.

From those techniques given in Table 1 my personal preference is for failure mode, effects, and criticality analysis (FMECA).[4] This technique can be applied to both equipment and facilities and can be used to methodically break down the analysis of a complex process into a series of manageable steps. It is a powerful tool for summarizing the important modes of failure, the factors that may cause these failures, and their likely effects. It also incorporates the degree of severity of the consequences, their respective probabilities of occurrence, and their detectability. It must be stressed, however, that the outcome of the risk assessment process should be independent of the tool used and must be able to address all of the risks associated with the instrument that is being assessed.

The list below shows the overall process flow of an FMECA:

1. Describe the system
2. Define functions or components
3. Identify potential failure modes
4. Describe effects of failure
5. Determine the causes

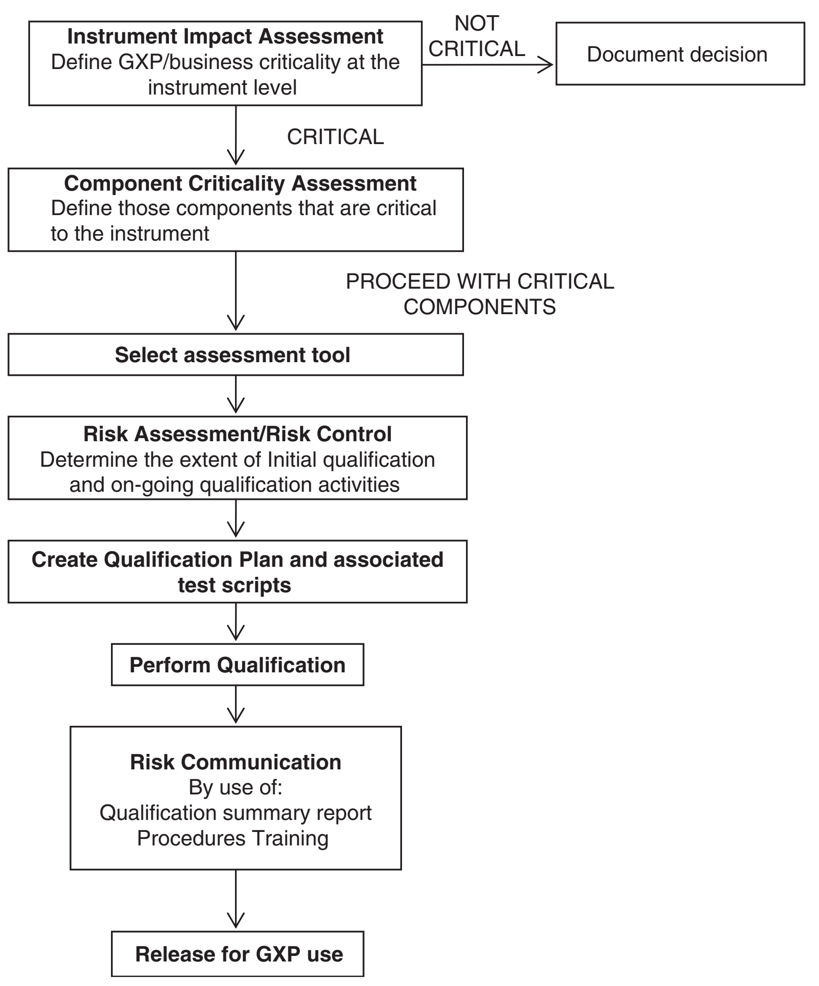

FIGURE 1 **Risk management during the qualification process and life cycle of an instrument.**

TABLE 1 **Typical Risk Management Tools**

Failure mode and effects analysis (FMEA)
Failure mode effects and criticality analysis (FMECA)
Empirical risk management (ERA)
Hazard operability analysis (HAZOP)
Fault tree analysis (FTA)
Hazard analysis and critical control points (HACCP)
Primary hazard analysis (PHA)

6. Detection methods/current controls
7. Calculate risk
8. Take action
9. Assess results.

II. PARAMETERS FOR QUALIFICATION

Performing an operational qualification ensures that the individual components of the instrument and the instrument as a whole are functioning correctly to certain defined specifications. The testing of individual instrument parameters and comparing the results to those specifications require the isolation of the parameters. Each identified parameter is related to a specific function within the instrument. Typical functions that should be subject to the qualification process and their associated parameters are described below.

A. Sample Compartment Parameters

As modern analytical instruments are designed to perform analytical run sequences unattended, it is essential that all samples and standards are injected in the correct sequence. Also, the temperature at which those standards and samples are stored within the instrument prior to analysis can affect the accuracy of the subsequent analysis.

B. Injection Parameters

To ensure quantitative results it is essential that the instrument is capable of performing reproducible injections. Also, an evaluation of the injection linearity would be necessary to ensure that injections of different sample volumes can be related in a quantitative manner.

C. Separation Parameters

There are two parameters that need to be controlled during the separation step of any CE analysis, the temperature of the capillary and the voltage that is applied across the capillary.

1. Temperature

The application of the voltage across the capillary will inevitably generate heat that, in turn, creates a radial temperature gradient across the capillary where the temperature decreases toward the capillary wall. This can cause a viscosity difference in the buffer that may give rise to band broadening within the capillary resulting in poor peak shape. This viscosity difference can also affect the sample injection and migration time of the analyte that, in turn, can affect the reproducibility of the analysis. Therefore a well-regulated temperature control for the capillary is essential to ensure reproducible analysis.

2. Voltage

During the separation a direct current power supply is used to provide either a constant voltage or current across the capillary. The accuracy and stability of this applied voltage or current is essential to ensure reproducible migration times.

D. Detection Parameters

There are a number of techniques that can be employed as detection mechanisms within a CE instrument. These include absorbance of ultraviolet (UV) light, laser-induced fluorescence, electrochemical and mass spectrometry. UV absorbance is, at present, the most commonly used technique.

1. Ultraviolet Absorbance

The parameters that require qualification for a UV absorbance detector are wavelength accuracy, linearity of response, detector noise, and drift. These determine the accuracy of the results over a range of sample concentrations and the detection limits of the analysis.

2. Laser-Induced Fluorescence

The wavelength accuracy and detector linearity and detector noise have the same effect on laser-induced fluorescence, as those of a UV absorbance detector.

3. Conductivity Detector

For a conductivity detector the parameters that need to be considered are cell response, detector linearity, detector sensitivity, and the effect of the detector's internal thermistor.

III. COMPUTER SYSTEM

The computer system attached to the instrument provides instrument control, data acquisition, data processing, and reporting. According to the draft Analytical Instrument Qualification chapter of the United States Pharmacopoeia (USP) <1058>[5] "The manufacturer should perform design qualification, validate the software and provide users with a summary of the validation. At the user site, holistic qualification that involves the entire instrument and software system is more efficient than modular validation of the software alone. Therefore the user qualifies the instrument control, data acquisition and processing software by qualifying the instrument."

This statement suggests that provided the manufacturer has performed and adequately documented the functions mentioned in the Analytical Instrument Qualification chapter no further qualification activities that are specific to the software is required by the user of the instrument.

For instruments being used in a GXP environment they must also comply with the requirements of 21 CFR Part 11;[6] however, this requirement is generic for all computerized systems and is beyond the scope of this chapter.

The outcome of the criticality analysis for determining the instrument parameters that need to be assessed for qualification.

Instrument module	Potential failure mode for the specified parameter
Sampler	Sample picking
	Inaccurate temperature
	Unstable temperature
Hydrodynamic injector	Inaccurate pressure
	Unstable pressure
	Inaccurate vacuum
	Unstable vacuum
	Inaccurate time
Electrokinetic injector	Inaccurate voltage
	Unstable voltage
	Inaccurate time
	Injection volume not linear

(Cont.)

Instrument module	Potential failure mode for the specified parameter
Capillary	Inaccurate temperature
	Unstable temperature
	Incorrect slit
	Broken cartridge
	Inaccurate voltage
	Unstable voltage
	Inaccurate current
	Unstable current
Photo diode array detector	Inaccurate wavelength
	Sensitivity problem
	Noise
	Linearity problem
	Broken optical fiber
Laser-induced fluorescence detector	Wavelength accuracy
	Detector linearity
	Noise
Conductivity detector	Cell response
	Sensitivity problems
	Linearity problems
	Thermistor inaccurate
Mass selective detector	Outside of the scope of this chapter

Having determined those parameters that need to be assessed, the potential failure effect for each can be described.

Instrument module	Potential failure mode for the specified parameter	Potential failure effect
Sampler	Sample picking	Incorrect analysis
	Inaccurate temperature	Precipitation or degradation. Imprecise/inaccurate injection volume
	Unstable temperature	
Hydrodynamic injector	Inaccurate pressure	Incorrect injection volume
	Unstable pressure	Incorrect analysis
	Inaccurate vacuum	Incorrect injection volume
	Unstable vacuum	Incorrect analysis
	Inaccurate time	Incorrect analysis
Electrokinetic injector	Inaccurate voltage	Incorrect analysis
	Unstable voltage	Incorrect analysis
	Inaccurate time	Incorrect analysis
	Injection volume not linear	Incorrect analysis
Capillary	Inaccurate temperature	Different migration times, poor peak shape, incorrect selectivity
	Unstable temperature	Peak shifting
	Incorrect slit	Poor sensitivity
	Broken cartridge	Leak error, no data acquired
	Inaccurate voltage	Inaccurate migration times
	Unstable voltage	
	Inaccurate current	
	Unstable current	

(Cont.)

Instrument module	Potential failure mode for the specified parameter	Potential failure effect
Photo diode array detector	Inaccurate wavelength	Sensitivity and linearity problems. Incorrect quantification of impurities. Inaccurate use of relative response factor(s)
	Sensitivity problem	Inaccurate determination of the impurity profile at the lower concentration level
	Noise	Inaccurate determination of the impurity profile at the lower concentration level
	Linearity problem	Incorrect analysis
	Broken optical fiber	Sensitivity problem
Laser-induced fluorescence	Wavelength accuracy problems	Incorrect analysis
	Detector linearity	Incorrect analysis
	Noise	Inaccurate determination of the impurity profile at the lower concentration levels
Conductivity detector	Cell response problems	Incorrect analysis
	Linearity problems	Incorrect analysis
	Sensitivity problems	Inaccurate determination of the impurity profile at the lower concentration levels
	Thermistor inaccurate	Incorrect analysis
Mass selective detector	Outside of the scope of this chapter	

Now that the potential failure effect has been identified the impact, level of occurrence, and the ease of detection of these failures need to be assessed by using the rating scales given in Table 2.

From this assessment Table 3 can then be used to determine the level and frequency of testing. From the example of FMECA in Table 4, the following actions should be performed:

- All of the parameters that have been identified as having a risk in the risk assessment need to be evaluated as part of the initial qualification.
- Those parameters that are not directly evaluated by system suitability testing during routine analysis must be checked periodically or the risk mitigated in some other way to ensure that the instrument is maintained in a qualified state. However for some critical parameters evaluation as part of the system suitability testing may not be sufficient and specific periodic testing may also be required.

TABLE 2 Rating Scales

Severity	Low (1)	Medium (2)	High (3)	Impact of the failure
Occurrence	Low (1)	Medium (2)	High (3)	Frequency of occurrence
Detection	Low (3)	Medium (2)	High (1)	Possibility that the failure will be detected during the analysis

TABLE 3 Qualification Effort

Risk ≤ 3 and detection = 1	At least initial qualification (IQ)
Risk > 3 and Detection = 1	At least initial qualification and system suitability Testing (SST) or periodic retest (PR)
Risk ≤ 3 and detection ≥ 2	
Risk > 3 and detection ≥ 2	At least initial qualification, system suitability testing and periodic retest

Note: Risk is the product of the severity and occurrence scores from the FMECA table. See Table 4.

Test scripts will then need to be devised to ensure that all of the identified parameters are tested and that those tests cover the complete range of the intended use of the instrument and also meet the required acceptance criteria suitable for that intended use.

Some examples of the tests that should be considered are shown below:

The temperature of the sample compartment and capillary column should be tested for both accuracy and stability at the lowest and highest settings that are expected to be used. Typically these would be between 10 and 60°C with an accuracy of ±1°C for the sample compartment and between 15 and 50°C with an accuracy of ±2°C for the capillary column.

The pressure module needs to be tested for accuracy at both low and high pressure. Typical values would be 0.5 and 7.5 psi with an acceptance criterion of ±0.2 psi for the low pressure and 20 and 80 psi with an acceptance criterion of ±1.0 psi for the high pressure.

The voltage and/or current should be tested for accuracy, stability, and repeatability at least at both ends of the range of use and preferably at a mid-point in the range. Typically these would be between 3 and 30 kV with an accuracy of ±4% and RSD of 0.5% over six replicate tests for the voltage and 25–300 μA with a similar accuracy and %RSD also over six replicate tests for the current.

Due to the nature of the specialized equipment that is required to perform these tests it is usual for the supplier to be asked to carry out the tests on behalf of the user. This would normally be performed as part of the initial installation or after a routine maintenance of the instrument. Following this the user would perform the rest of the initial qualification or periodic testing covering the other aspects of the instrument.

Repeatability of migration time can be used as an indication of the stability of the voltage or current. Therefore running a number of injections using constant voltage and examining the %RSD of the peak migration times give an assurance that the applied voltage is sufficiently stable for analytical purposes. Performing the same type of test in constant current mode gives similar assurance for current stability. Typical values that would be acceptable for migration time %RSD would be 1% over six replicate analyses.

Similarly the UV detector, whether it is variable wavelength or a photodiode array spectrophotometer, should also be qualified over the anticipated wavelength range; however, at present there is no material commonly available that covers the normal range of use. The current industry standard for this test is the use of holmium oxide in perchloric acid as the UV-absorbing species but this only covers the range from 241 to 651 nm and it is not unusual for wavelengths below 241 nm to be routinely used for detection in CE. It is, however, currently considered by both industry and the regulatory authorities to be a sufficient test of wavelength accuracy to enable the entire working range of the detector to be used. The normal acceptance criterion for this test would be in the order of ±3 nm.

Detector linearity would normally be tested as part of an overall holistic test that examines the linearity of the complete instrument, the injector, as well as the detector. The test would normally be designed to cover the range up to 2 absorbance units (AU).

TABLE 4 An Example of Capillary Electrophoresis Failure Mode, Effect and Criticality Analysis

Instrument module	Potential failure mode for the specified parameter	Potential failure effect	Severity	Occurrence	Current Evaluation	Detection	Risk priority no.	Recommended actions		
								IQ	PR	SST
Sample compartment	Sample picking	Incorrect analysis	3	1	May be detected during evaluation by the analyst or review after the analysis	1	3	X	–	X
	Inaccurate temperature	Precipitation or degradation	1	1	None	3	1	X	X	–
	Unstable temperature	Imprecise/ inaccurate injection volume	3	1	None, may be detected during SST	3	3	X	X	–
Hydro-dynamic Injector	Inaccurate pressure	Incorrect injection volume	2	2	SST: reporting threshold not met	3	4	X	X	X
	Unstable pressure	Incorrect analysis	3	1	SST: repeatability of injection	3	3	X	–	X
	Inaccurate time	Incorrect analysis	2	1	Electronically controlled and no wearing parts	1	2	X	–	–
Hydro-dynamic Injector	Inaccurate vacuum	Incorrect injection volume	2	2	SST: reporting threshold not met	3	4	X	X	X
	Unstable vacuum	Incorrect analysis	3	1	SST: repeatability of injection	3	3	X	–	X
Electro kinetic Injector	Inaccurate voltage	Incorrect analysis	2	2	SST: reporting threshold not met	3	4	X	X	X
	Unstable voltage, inaccurate time	Incorrect analysis	3	1	SST: repeatability of injection	3	3	X	X	–
	Injection volume not linear	Incorrect analysis	2	1	Electronically controlled and no wearing parts	1	2	X	–	–

TABLE 4 (Cont.)

Instrument module	Potential failure mode for the specified parameter	Potential failure effect	Severity	Occurrence	Current Evaluation	Detection	Risk priority no.	Recommended actions		
								IQ	PR	SST
Capillary compartment	Inaccurate temperature	Different migration times, poor peak shape, incorrect selectivity	3	1	If applicable evaluated using a selectivity batch	1	3	X	X	–
	Unstable temperature	Peak shifting	2	1	If applicable evaluated using a selectivity batch, repetition of migration time	2	2	X	X	–
	Incorrect slit	Poor sensitivity	3	2	SST: reporting threshold	1	6	X	–	X
	Broken cartridge	Leak error, no data acquired	1	1	Considered to be a consumable item	1	1	–	–	–
	Inaccurate voltage	Inaccurate migration times	3	1	SST: Migration time selectivity batch	1	3	X	–	X
	Unstable voltage		2	1	SST: migration time selectivity batch	2	2	X	–	X
	Inaccurate current		3	1	SST: Migration time selectivity batch	1	3	X	–	X
	Unstable current		2	1	SST: migration time selectivity batch	2	2	X	–	X
Variable wavelength or PDA Detector	Inaccurate wavelength	Sensitivity and linearity problems. Incorrect quantification of impurities. Inaccurate use of relative response factor(s)	3	1	SST: reporting threshold	3	3	X	X	–

	Sensitivity problem	Inaccurate determination of the impurity profile at the lower concentration level	3	2	SST: reporting threshold	1	6	X	–	X
	Noise	Inaccurate determination of the impurity profile at the lower concentration level	3	1	SST: reporting threshold	1	3	X	–	X
	Linearity problem	Incorrect analysis	3	1	SST: reporting threshold, linearity check	1	3	X	–	–
	Broken optical fiber	Sensitivity problem	3	1	Considered to be a consumable item	3	3	X	X	–
Laser Induced Fluorescence	Wavelength accuracy problems	Incorrect analysis	3	1	SST	3	3	X	–	X
	Linearity problems	Incorrect analysis	3	1	SST	2	3	X	–	X
	Sensitivity problem	Inaccurate determination of the impurity profile at the lower concentration levels	3	2	LOD and SST	1	6	X	–	X
Conductivity Detector	Cell response problems	Incorrect analysis	3	1	SST	3	3	X	–	X
	Linearity problems	Incorrect analysis	3	1	SST	2	3	X	–	X
			3	2	LOD and SST	1	6	X	–	X

TABLE 4 (Cont.)

Instrument module	Potential failure mode for the specified parameter	Potential failure effect	Severity	Occurrence	Current Evaluation	Detection	Risk priority no.	Recommended actions		
								IQ	PR	SST
	Sensitivity problem	Inaccurate determination of the impurity profile at the lower concentration levels								
	Thermistor inaccurate	Incorrect analysis	3	1	No current evaluation	3	3	X	–	X

Other types of detectors such as mass selective detectors would need to have their own assessment performed. It should be clearly understood that the outcome of the risk assessment example is a personal assessment and is not meant to be definitive in any way.

A test for noise and drift would normally follow the recommendations of the instrument vendor.

It cannot be stressed enough that whatever tests are devised must meet the intended use of the instrument and that the acceptance criteria are set at appropriate levels. There is no need to test an instrument to a level more stringent than that required by the process for which it is being used.

IV. KEEPING THE INSTRUMENT QUALIFIED

In order to ensure that the instrument is kept in a qualified state it is important to assess what impact any changes made to the instrument may have, whether they are due to a breakdown of the instrument, any preventative maintenance performed, or simply a change of use. This would also apply to any changes to the software used to control the instrument or acquire and process any data that are generated. These changes must be documented along with the corresponding impact assessment or criticality analysis. Sufficient tests must then be performed to ensure that those changes have not adversely affected the instrument and that it is still performing according to the defined or, if necessary, revised specifications.

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9

ROBUSTNESS TESTING OF CE METHODS

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ABSTRACT

In biomedical and pharmaceutical analysis, and particularly in the pharmaceutical industry, much attention is paid to the quality of the obtained analytical results because of the strict regulations set by regulatory bodies. Proper method validation demonstrates the fit of an analytical method for a given purpose. In this context, robustness testing has become increasingly important.

The setup and treatment of results of such a robustness test are discussed in this chapter. All steps of the test are considered. Finally, a literature review and critique of applications of robustness testing of CE methods has been provided.

I. INTRODUCTION

In biomedical and pharmaceutical analysis, especially within the pharmaceutical industries, much attention is paid to the quality of the analytical results. After developing an analytical method, it needs to be fully validated. In pharmaceutical analysis, compared to other domains of the analytical chemistry, often a more extensive method validation is required in order to meet the strict regulations set by regulatory organizations. One characteristic that needs verification is the robustness or ruggedness of the analytical method. In the past, robustness or ruggedness tests often were not performed because of their complexity, and the many possible experimental approaches and data analysis methods, but nowadays their importance has grown considerably. Several definitions of robustness and/or ruggedness are available. Some only use the term ruggedness,[1] some distinguish between robustness and ruggedness,[2] while others consider them synonyms.[3–5]

To our knowledge, in the first approach described, Youden and Steiner[1] introduced the term *ruggedness* test for a setup in which by means of an experimental design the influences of minor but deliberate and controlled changes in the method parameters or factors are evaluated in order to detect non-robust factors, i.e., with a large influence on the response. Controlling the latter factors avoids problems in the following interlaboratory study.

In the approach presented by the United States Pharmacopeia (USP),[2] *ruggedness* is defined as "The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc." In this approach, no deliberate changes in method parameters are introduced and the method is simply performed under different test conditions. The influences of these conditions (factors) are evaluated using a nested design or a nested ANOVA.[6] However, the above definition will not be applied in this chapter, since it is equivalent to that of intermediate precision (within-laboratory variations) or reproducibility (between-laboratory variations), depending whether or not the test is performed in one laboratory. For these types of precision, detailed ISO (International Organization for Standardization) guidelines exist.[7,8]

The USP definition of robustness is equal to that of the ICH (International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use),[3] which is given below.

The ICH guidelines define *robustness* as "The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage."[3] Thus, the robustness of a method is a measure of its capacity to be transferred to another laboratory without affecting the analytical result. A robustness test is the experimental setup applied to evaluate the robustness of the method. The ICH guidelines also state that "One consequence

of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution tests) is established to ensure that the validity of the analytical procedure is maintained whenever used."[4] The latter definition for robustness will be considered in this chapter since it is the most widely applied. Although robustness tests are not obligatory yet in the ICH guidelines, they are demanded by the Food and Drug Administration (FDA) for the registration of drugs in the United States of America.[5]

Initially, robustness testing was performed to identify potentially important factors, which could affect the results of an interlaboratory study.[1] Therefore, the robustness test was executed at the end of the method validation procedure, just before the interlaboratory study. However, a method found to be non-robust should be redeveloped and revalidated, leading to a waste of time and money. For these economical reasons, nowadays, method robustness is verified at an earlier stage in the lifetime of the method, i.e., at the end of method development or at the beginning of the validation procedure.[9]

II. AIMS/OBJECTIVES AND STEPS IN A ROBUSTNESS TEST

A robustness test examines potential sources (factors) of variability in one or more responses of the considered method. In pharmaceutical analysis, chromatographic and electrophoretic separation methods are most commonly evaluated.

To identify potential sources of variability, a number of factors, usually described in the operating procedure of the method, are selected. These factors are then varied in an interval, representative for the fluctuations in the nominal factor levels, which can be expected when transferring a method between different instruments or laboratories.[9,10] The nominal level is the one described in the operating procedure of the method or that set during normal application of the method. The selected factors are not individually evaluated, but simultaneously by means of an experimental design approach.[6,9] The considered responses may describe quantitative aspects of the method, such as the estimated concentrations or percentage recoveries of the main compound(s) and/or related compound(s). On the other hand, during the development of separation methods, the robustness of the separation itself also can be evaluated. Responses for which system suitability test (SST) limits can be defined, such as for chromatographic methods, resolution, tailing or asymmetry factors, retention factors, selectivity factors, or column efficiency, and for electrophoretic methods, migration time and resolution, also can be studied.[9,11–13] After determining the response(s) for the designed experiments, the effects of the selected factors on the responses are estimated.[6,9,11,14] This allows determination the factors with an important influence on the results and enables establishing boundaries/limits to control the levels of these factors, if necessary.

Another aim of performing a robustness test can be to define SST limits.[9,12,13,15–17] The SST limits are then determined based on the experimental data from a robustness test, while frequently they are chosen arbitrarily based on the experience of the analyst.

In a robustness test, the following steps can be distinguished:[6,9,17,18]

(1) selection of the factors to be evaluated and their levels,
(2) selection of the experimental design,
(3) selection of the responses,
(4) planning and execution of the experimental setup, and determination of the responses,
(5) calculation of the factor effects on the responses,
(6) graphical and/or statistical interpretation of the estimated effects,

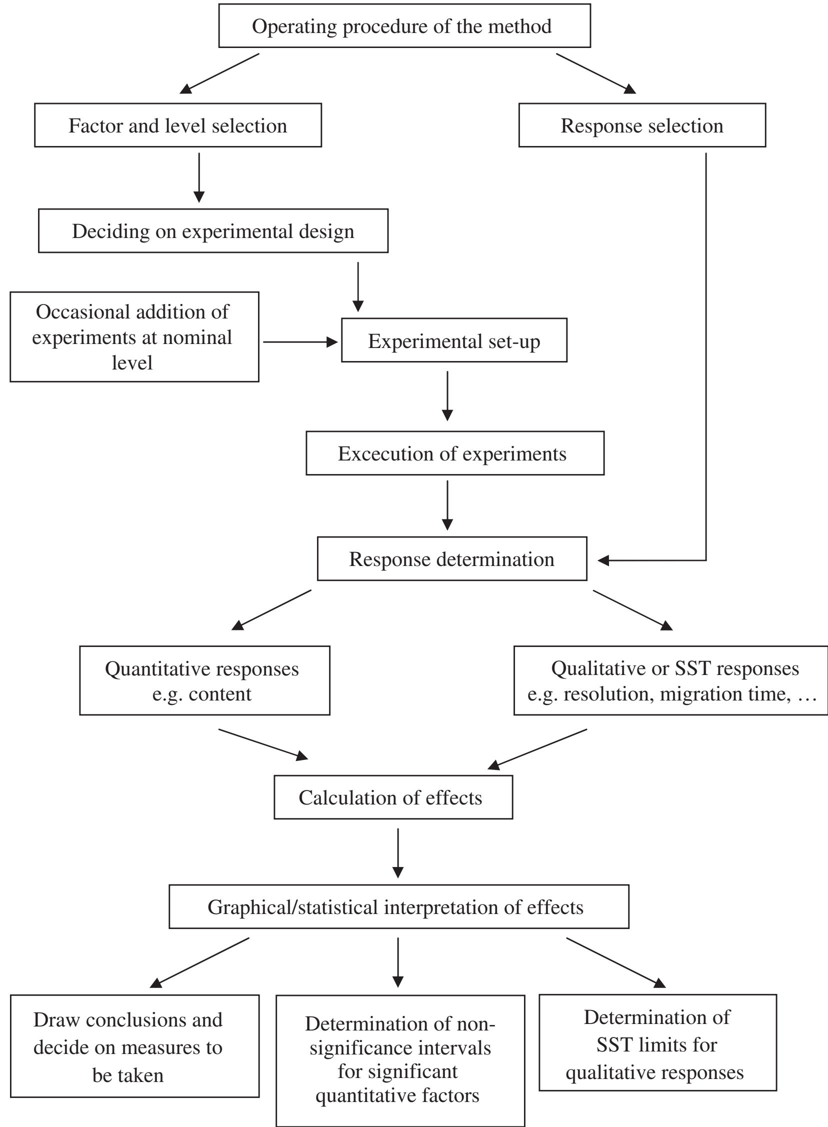

FIGURE 1 Schematic representation of the different steps in a robustness test.

(7) drawing chemically relevant conclusions and if necessary, taking precautions to improve the method performance,
(8) determining non-significance intervals for significant quantitative factors, and finally,
(9) defining SST limits for certain qualitative responses.

These different steps are represented in Figure 1, discussed in more detail below (Sections III–VIII), and illustrated with two examples, taken from the literature. In Section IX, a critical review of some published case studies, describing robustness tests of CE methods, is given.

III. SELECTION OF FACTORS AND LEVELS

A. Operational and Environmental Factors

Defining and selecting the factors to be examined should be carefully thought through before starting a robustness test. Usually, the factors in a robustness test are *operational or environmental factors*.[6,9–11,17,19] The former are selected from the operating procedure of the method, while the latter are not necessarily specified in this procedure. Those factors, which are most likely to vary when a method is transferred between different laboratories, analysts or instruments, are selected.

Table 1 gives an overview of potential factors to be considered when performing a robustness test on a capillary electrophoretic (CE) method. In references 6, 9, 17, and 19, lists of factors for high-performance liquid chromatographic (HPLC), gas chromatographic (GC), and/or thin-layer chromatographic (TLC) methods, can be found.

The selected factors are either mixture-related, quantitative (continuous), or qualitative (discrete).[6,9–11,18] A mixture-related factor is, for instance, the fraction organic solvent in the buffer system. Examples of quantitative factors are the electrolyte concentration, the buffer pH, the capillary temperature, and the voltage, and of qualitative factors the manufacturer or the batch number of a reagent, solvent, or capillary. Sample concentration (see Table 1) is a factor sometimes included. However, the aim of the method tested is to determine this concentration through the measured signal, from a calibration procedure. Thus, one evaluates the influence of the sample concentration on the sample concentration, which we do not consider a good idea.

Table 1 also contains some *peak measurement/analysis parameters* that might be investigated in a robustness test (see Section III.C).[17,20] However, except from the detection wavelength, these parameters are hardly ever evaluated in a robustness test, even though they can have a large influence on the electropherogram.

TABLE 1 **Potential Factors in the Robustness Testing of Capillary Electrophoretic Methods**

Operational and environmental factors	Peak measurement/analysis parameters
Additives concentrations:	Detection:
Organic solvents	Detection wavelength (VIS, UV, or fluorimetric detection)
Chiral selectors	Reference wavelength
Surfactants	Detection wavelength bandwidth
Capillary:	Reference wavelength bandwidth
Batch	Integration: sensitivity
Manufacturer	Peak detection:
Capillary temperature	Peak width
Concentrations of rinsing liquids	Threshold
Electrolyte concentration	Signal processing:
pH of the buffer	Data acquisition rate
Rinse times	Type of filtering
Sample concentration[a]	Amount of filtering
Sample injection time	
Voltage	

[a] See text Section III.A.

1. Mixture-Related Factors

Mobile phases in chromatography and buffer systems in electrophoresis are examples of frequently used solvent mixtures.[9,10] In a mixture of p components, only $p-1$ can be varied independently, which means that maximally $p-1$ mixture-related variables can be examined in the type of experimental designs typically used in robustness testing. The value of the pth variable is determined by those of the other and used as adjusting component to complete the mixture. If one of the mixture components has an important effect on a response, then the composition of the whole mixture is important and should be strictly controlled.[9,10]

Suppose a buffer system in CE consists of methanol/buffer 10:90 (v/v). When the methanol fraction is selected as factor in a robustness test, the buffer fraction serves as adjusting component to sum the fractions to 1.

2. Quantitative Factors

Quantitative factors are those most often evaluated in robustness tests.[6,9–11,18] These factors are preferably defined in such a way that the effects can be linked to a physical aspect of the method. The following example illustrates this. A buffer can be defined either by the concentrations of its acid (C_a) and basic (C_b) compounds or by a given pH and ionic strength μ.[9] Considering the first possibility, the individual effects of C_a and C_b do not directly represent physically interpretable information and the significance of one factor (C_a or C_b) should lead to a strict control of both, as for mixture-related variables. To relate C_a or C_b to pH and μ, they are combined in such a way, C_a/C_b, that their effect corresponds to a change in pH and/or ionic strength μ.[9] The latter approach for C_a or C_b might be preferred since it gives the analyst a better physical understanding of the calculated effects.

3. Qualitative Factors

Qualitative factors are also frequently considered in a robustness test.[6,9–11,18] For CE methods, factors such as the batch or manufacturer of the capillary, reagent or solvent can be selected. When evaluating the influence of such qualitative factor, the analyst should be aware that the estimated effect is only valid or representative for the examined discrete levels and not for any other level of that factor, and certainly not for the whole population.[6,9] For example, when examining two capillaries X and Y, the estimated effect only allows drawing conclusions about these two capillaries and not about other capillaries available on the market. Such approach allows evaluating whether capillary Y is an alternative for capillary X, used, for instance, to develop the method.

B. Selection of Levels

In robustness tests, usually the factors are examined at two extreme levels.[6,11,17] For mixture-related and quantitative factors, these levels usually are chosen symmetrically around the nominal. The range between the extreme levels is selected so that it represents the variability that can occur when transferring the method.[6,9–11,19] However, specifications to estimate such variability are not given in the ICH guidelines.[3,4] Often the levels are chosen based on personal experience, knowledge, or intuition. Some define the extreme levels as "nominal level $\pm x\%$."[19] However, this relative variation in factor levels is not an appropriate approach, since the absolute variation then depends on the value of the nominal level.[10] Another possibility is to define the levels based on the precision or the uncertainty, with which they can be set and reset.[9,10] The uncertainty can be estimated for each factor level.[10,21] For example, if the uncertainty or absolute error on a measured pH value is 0.02, this means that

the true pH value with 95% certainty is situated in the interval “measured pH ± 0.02.” To define the extreme levels, the latter interval is extended to simulate potential variability caused by the transfer between laboratories or instruments and to compensate for potential sources of variability that were neglected during the estimation of the uncertainty. The uncertainty is multiplied with a constant k, chosen arbitrarily and usually $2 \leq k \leq 10$. Thus, the extreme factor levels are given by “nominal level $\pm k^*$ uncertainty.”[9,10] The minimal k value should be 2 to enable a clear distinction between the factor levels. Often $k = 5$ is used as default value.[9,10] Nevertheless, the analyst is free to choose a different value. The lower the k value for a factor, the smaller the examined interval, and the stricter that factor is to be controlled during later use, since only robustness in a narrow interval is verified. On the other hand, higher k values increase the probability that a significant effect occurs in the examined interval, but allow a less strict control of those factors if no important effects were observed. Examples of the above approach to define factor levels can be found in references 9 and 10.

As already indicated, the extreme levels usually are chosen symmetrically around the nominal. However, for some factors, an asymmetric interval might give either a better representation of the reality or better reflect the effect occurring. One example is the capillary temperature. Suppose a temperature of 15°C is prescribed then the symmetric levels, selected based on uncertainty could, for instance, be 10 and 20°C. These levels can be applied when the instrument disposes of a cooling system that can cool until 10°C. However, when such system is not present, the lowest extreme level will be taken equal to the nominal value (15°C). Another example is the detection wavelength. Suppose a signal is measured at the maximum absorbance wavelength, λ_{max} or λ_{nom1} (see Figure 2), then a small decrease in detection wavelength often has a similar effect on the response as a small increase. This leads to an estimated effect, E_{nom1}, close to zero, when evaluating the change between the extreme levels. Therefore, examining an asymmetric interval seems more representative and often only one extreme level is chosen for the robustness test in combination with the nominal. On the other hand, when the nominal wavelength is in a slope of the spectrum, λ_{nom2} (see Figure 2), then a symmetric interval seems best, since the response is continuously increasing or decreasing as a function of the factor levels, resulting in a proper effect estimation, E_{nom2}.

For qualitative factors, only discrete values are possible, e.g., capillaries X, Y, or Z. As already indicated, this means that only conclusions can be drawn about the examined capillaries and no extrapolation to other capillaries can be made. The most logical in a robustness test is to compare the nominal capillary with an alternative one.

C. Peak Measurement/Analysis Parameters

In robustness tests, peak measurement/analysis parameters can also be considered.[17,20] Such parameters are related to the measurement of the detector signal and they affect responses, such as peak areas, peak heights, retention time, and resolution. They allow improving the quality of these responses. These factors can be found in the data treatment software of an instrument, where often only the default settings are used by the analyst.

They can be divided into photodiode array or ultraviolet (PDA/UV) detection parameters, signal processing parameters, and peak detection parameters.[20] Examples of PDA/UV detection parameters are the detection and reference wavelengths and their bandwidths; of signal processing parameters, the data acquisition rate and the type and amount of filtering; and of peak detection parameters, the peak width and the threshold (see Table 1). Also, the integration sensitivity can be chosen as factor. However, except for the detection wavelength, none of these factors usually is adapted or selected for a robustness test. The values of these factors are often kept at their default values and many analysts are not even aware of their potential importance. However, their influence can be seen from Figure 3,

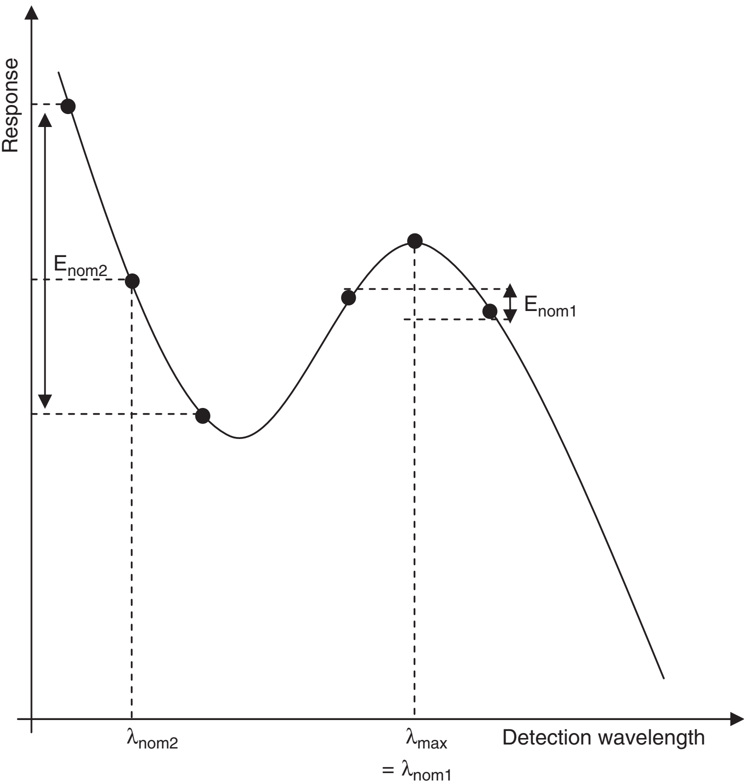

FIGURE 2 Response (e.g., absorbance, peak area, or height) as a function of detection wavelength.

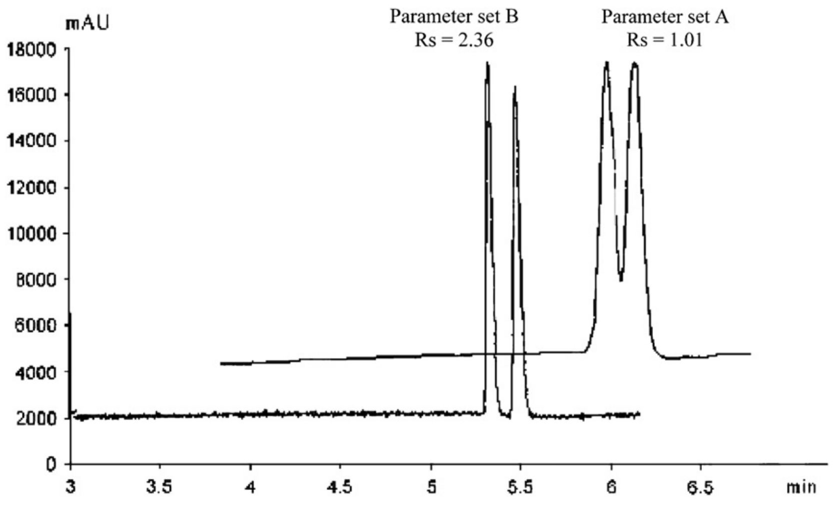

FIGURE 3 Two electropherograms from one run, recorded with different settings for peak measurement/analysis parameters.

where two electropherograms are shown, extracted from reference 20. They were recorded only differing in the above-mentioned parameters, while the chemical experimental conditions remained constant. It is clear that only changes in peak measurement/analysis parameters can lead to very different values of some responses. For instance, the peak resolution in Figure 3 was 1.01 and 2.36 for parameter sets A and B, respectively.

D. Examples of Some Factors and Their Levels

The factors and their levels examined in the robustness testing of some chiral separations[22,23] are given in Tables 2 and 3.

The factors in Table 2 were selected from the chiral separation methods for propranolol, praziquantel, and warfarin.[22] All factors were quantitative and their extreme levels situated symmetrically around the nominal.

The factors in Table 3 were selected from a non-aqueous chiral separation method for timolol.[23] One qualitative factor (1), i.e., the type of CE equipment, was examined. Two HPCE systems, A and B, with different software versions for equipment control, data acquisition, and handling were compared. Six quantitative factors ((2) till (7)), for which the extreme levels usually were situated symmetrically around the nominal, also were

TABLE 2 Factors and Their Levels Investigated in the Robustness Testing of the Chiral Separations of Propranolol, Praziquantel, and Warfarin[22]

Factor	(−1) Level	Nominal (0) level	(+1) Level
(1) Concentration of chiral selector (% m/V)	4.5	5.0	5.5
(2) pH of BGE	2.2	2.5	2.8
(3) Concentration of BGE (mM)	45	50	55
(4) Capillary temperature (°C)	17	20	23
(5) Separation voltage (kV)	8.5	9.4	10.3
(6) Rinse volume with NaOH (μl)	4.27	5.12	5.98
(7) Rinse volume with BGE (μl)	4.27	5.12	5.98
(8) Injection time (s)	2.7	3.0	3.3

BGE, background electrolyte; (−1) and (+1), extreme levels.

TABLE 3 Factors and Their Levels Investigated in the Robustness Testing of a Non-Aqueous Chiral Separation of Timolol[23]

Factor	(−1) Level	Nominal (0) level	(+1) Level
(1) Type of CE equipment	A	B	B
(2) Capillary temperature (°C)	15	15	17
(3) Detection wavelength (nm)	290	295	300
(4) Separation voltage (kV)	23	25	27
(5) Injection time (s)	7	8	9
(6) Concentration chiral selector (mM)	27.5	30.0	32.5
(7) Concentration camphorsulfonate (mM)	27.5	30.0	32.5

(−1) and (+1): extreme levels.

investigated. Only for the capillary temperature, asymmetric levels were chosen, i.e., the lowest level was equal to the nominal, since the equipment did not allow cooling below the nominal value of 15°C.

IV. SELECTION OF EXPERIMENTAL DESIGNS

The influence of the selected factors is evaluated by means of an experimental design, which is an experimental setup that allows studying simultaneously a number of factors in a predefined number of experiments. Several types of experimental designs are described in the literature.[6,9,14–17,24–26]

In robustness testing, two-level screening designs, such as fractional factorial (FF) or Plackett–Burman (PB) designs, are most often applied.[6,9,18,19] Each factor is examined at two levels in these designs. They allow evaluating the effects of a relatively high number of factors f on a response, in a relatively small number of experiments ($N \geq f+1$). The number of factors determines the number of experiments required. The designs used in robustness tests can be constructed by the analyst, as discussed thoroughly in references 6 and 9. However, they can also be selected using (commercial) software packages.[19,27–37] To evaluate a given number of factors both FF and PB designs can be used. The most frequently applied designs in robustness testing have 8, 12, or 16 experiments. In reference 9, several FF and PB designs are proposed for varying numbers of factors. For a given number of factors, usually two FF and two PB designs are presented. They differ in design properties and number of experiments.

The analyst should avoid creating impossible factor combinations.[6,9] This occurs, for instance, when choosing both the batch number and the manufacturer of the capillary as factors in a robustness test by means of a two-level design. It is impossible to define two unique batch numbers that exist for both manufacturers. The way to examine both factors is by using nested designs.[6]

Three- or more-level response surface designs, such as three-level full factorial, central composite (CCD), and Box–Behnken designs, are applied in some case studies.[16,17,38,39] However, the need for these designs to evaluate the method robustness seems unnecessary for several reasons. First, these designs require more experiments than the two-level screening designs to examine a given number of factors. For example, to examine three factors, a three-level full factorial design requires $3^3 = 27$ experiments and a CCD at least 15 experiments, while two-level screening designs with 8 (FF or PB) or, theoretically, even with 4 experiments (FF design)[14] can be used. Secondly, these designs become unpractical when more than three factors are examined, since the number of experiments increases dramatically.[14,16,17] In robustness testing, usually (much) more than three factors are evaluated. Thirdly, response surface designs are used to model the response, usually in the context of method optimization.[14] For optimization, the chosen interval of the factors is much larger than in robustness testing. The responses also often have a curved behavior in the optimization interval examined. In robustness testing, on the other hand, only a small interval is chosen. Regardless whether the response is linear or not in the broad interval evaluated during optimization, the response usually can be considered linear in the small interval examined in the robustness test. This is represented in Figure 4. Suppose that during method optimization the response was examined as a function of a factor (levels 0–100) and a curved relation was obtained. The most optimal response is, for instance, the largest, which is obtained at a factor level near 100. Often in optimization the best value is found near one of the extreme levels; not so many responses show an intermediate optimum. During robustness testing, a small interval around this nominal value 100 would be examined, e.g., 95–105 or even smaller. Although the global relation between the response and the factor is curved, it can be considered linear in the small range, as can be seen in Figure 4.

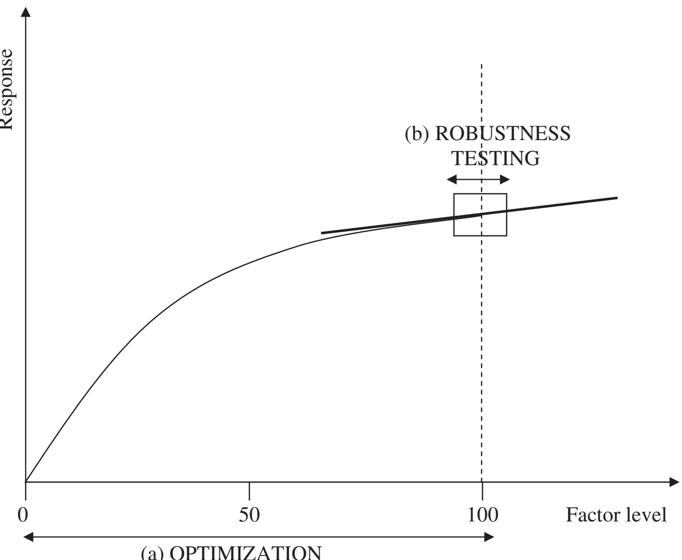

FIGURE 4 **Factor level intervals examined during (a) method optimization and (b) robustness testing.**

However, if for some reason in a robustness test it is expected that the effects between [−1,0] differ from those between [0,+1], it is worthwhile investigating the factors at three levels. For example, when measuring at the maximum absorbance wavelength and when the analyst still prefers studying a symmetric interval around the nominal level, it is wiser to examine at least this factor at three levels.

Another possibility to examine the factors at three levels in a robustness test, and perhaps a better alternative than the three- or more-level response surface designs, is the reflected designs.[6,15,16,27,40] Reflected designs are two-level full factorial, FF, or PB designs (f factors in N experiments) that are duplicated, i.e., executed once with the factor levels between [−1,0] and once between [0,+1]. One experiment is common for both designs, resulting in a reflected design requiring $2N-1$ experiments to examine f factors. An advantage of reflected designs over response surface designs is that more factors can be examined in a feasible number of experiments. For example, a CCD requires at least 15 experiments to examine three factors, while a reflected FF or PB design with 15 experiments can examine up to seven factors. A second advantage is the easier data analysis of reflected designs. To analyze reflected designs, the methods described further in Section VII are used twice, i.e., once to estimate the effects between [−1,0] and once between [0,+1], while for the analysis of response surface designs, mathematical models need to be build, which in general are less easy to construct and to understand for the average analytical chemist.

A. Fractional Factorial Designs

A full factorial design contains all possible combinations (L^f) between the different factors f and their levels L, with $L = 2$ for two-level designs. It allows estimating all main and interaction effects between the factors. A FF design will only perform a fraction of the full factorial. A two-level FF design $2^{(f-v)}$ examines f factors, each at two levels, in $2^{(f-v)}$ experiments, with $1/2^v$

TABLE 4 2^{6-2} **Fractional Factorial Design to Examine Six Factors in 16 Experiments (Design Generators E = ABC and F = BCD, Design Resolution = IV; for More Information, see references 6, 9, 14, and 41)**

	Factors					
Experiment	A	B	C	D	E	F
1	−1	−1	−1	−1	−1	−1
2	1	−1	−1	−1	1	−1
3	−1	1	−1	−1	1	1
4	1	1	−1	−1	−1	1
5	−1	−1	1	−1	1	1
6	1	−1	1	−1	−1	1
7	−1	1	1	−1	−1	−1
8	1	1	1	−1	1	−1
9	−1	−1	−1	1	−1	1
10	1	−1	−1	1	1	1
11	−1	1	−1	1	1	−1
12	1	1	−1	1	−1	−1
13	−1	−1	1	1	1	−1
14	1	−1	1	1	−1	−1
15	−1	1	1	1	−1	1
16	1	1	1	1	1	1

TABLE 5 2^{6-3} **Fractional Factorial Design to Examine Six Factors in Eight Experiments (Design Generators D = AB, E = AC, and F = BC, Design Resolution = III; for More Information, see references 6, 9, 14, and 41)**

	Factors					
Experiment	A	B	C	D	E	F
1	−1	−1	−1	1	1	1
2	1	−1	−1	−1	−1	1
3	−1	1	−1	−1	1	−1
4	1	1	−1	1	−1	−1
5	−1	−1	1	1	−1	−1
6	1	−1	1	−1	1	−1
7	−1	1	1	−1	−1	1
8	1	1	1	1	1	1

representing the fraction of the full factorial ($v = 1, 2, 3, \ldots$).[6,9,14] For example, a full factorial design for five factors at two levels requires $2^5 = 32$ experiments, while a quarter-fraction factorial design evaluating these factors only demands $2^{5-2} = 8$ experiments. The construction of FF designs has been thoroughly described in references 6, 9, 14, and 41. To examine a given number of factors, different FF designs can be selected. For example, for six factors, both a 2^{6-2} FF design requiring 16 experiments (Table 4) and a 2^{6-3} FF design with only 8 experiments (Table 5) can be used. The difference between both designs is their so-called confounding pattern, i.e., the (number of) interaction effects which are estimated together.

B. Plackett–Burman Designs

PB designs are saturated factorial designs that examine up to $N-1$ factors in N (a multiple of four) experiments.[6,9,14,19,42] In these designs, two-factor and higher-order interaction effects are confounded with the main effects.[6,9] This phenomenon also occurs in the design given in Table 5. PB designs are constructed by means of a cyclic permutation of the first line of the design, which was given by Plackett and Burman (Tables 6 and 7).[42] This permutation is repeated $N-2$ times and finally a row of -1 signs is added. The construction has been thoroughly described in references 6 and 9. To examine a given number of factors, different PB designs can also be applied. For example, to examine six or seven factors, a PB design with 12 or one with only 8 experiments can be selected. These designs are shown in Tables 6 and 7, respectively. When the number of method parameters, selected to be evaluated in an experimental design, is lower than the number of factors that potentially can be examined in the PB design ($N-1$), the remaining columns are defined as dummy factor

TABLE 6 Plackett–Burman Design to Examine up to 11 Factors in 12 Experiments

	Factors										
Experiment	A	B	C	D	E	F	G	H	I	J	K
1	1	1	−1	1	1	1	−1	−1	−1	1	−1
2	−1	1	1	−1	1	1	1	−1	−1	−1	1
3	1	−1	1	1	−1	1	1	1	−1	−1	−1
4	−1	1	−1	1	1	−1	1	1	1	−1	−1
5	−1	−1	1	−1	1	1	−1	1	1	1	−1
6	−1	−1	−1	1	−1	1	1	−1	1	1	1
7	1	−1	−1	−1	1	−1	1	1	−1	1	1
8	1	1	−1	−1	−1	1	−1	1	1	−1	1
9	1	1	1	−1	−1	−1	1	−1	1	1	−1
10	−1	1	1	1	−1	−1	−1	1	−1	1	1
11	1	−1	1	1	1	−1	−1	−1	1	−1	1
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1

TABLE 7 Plackett–Burman Design to Examine up to Seven Factors in Eight Experiments

	Factors						
Experiment	A	B	C	D	E	F	G
1	1	1	1	−1	1	−1	−1
2	−1	1	1	1	−1	1	−1
3	−1	−1	1	1	1	−1	1
4	1	−1	−1	1	1	1	−1
5	−1	1	−1	−1	1	1	1
6	1	−1	1	−1	−1	1	1
7	1	1	−1	1	−1	−1	1
8	−1	−1	−1	−1	−1	−1	−1

columns. A dummy factor is an imaginary variable for which a change in its levels does not correspond to any physical or chemical change.

The two-factor interaction effects and the dummy factor effects in FF and PB designs, respectively, are often considered negligible in robustness testing.[9] Since the estimates for those effects are then caused by method variability and thus by experimental error, they can be used in the statistical analysis of the effects.[6,9,11] Requirement is that enough two-factor interaction or dummy factor effects (≥ 3) can be estimated to allow a proper error estimate (see Section VII.B.2.(b)).

C. Examples of Applied Designs

The applied designs in the robustness testing of the chiral separation methods of propranolol, praziquantel, and warfarin,[22] and of timolol[23] concern PB designs, i.e., one with 12 (Table 6)[22] and one with 8 (Table 7)[23] experiments.

In reference 22, the effects of eight factors are examined in 12 experiments, which means that three dummy factors were included in the design, e.g., in columns I, J, and K. On the other hand, in reference 23, seven factors are evaluated in eight experiments, leaving no room for dummies.

V. SELECTION OF RESPONSES

A. Quantitative Responses

In the first instance, the considered responses usually represent quantitative aspects of the method, such as the concentrations or the percentage recoveries of the main and/or related compound(s).[9,11] An analytical method is considered robust, if no significant effects are found on the response(s) describing the quantitative aspect of the method.[12]

B. Qualitative or SST Responses

However, additionally, qualitative responses, i.e., providing information on the qualitative aspects of the method and for which occasionally SST limits can be defined, may also be examined in a robustness test.[9,11–13] Regardless whether or not the method is considered robust concerning its quantitative aspect, these SST responses often are “non-robust,” i.e., they contain significant effects.[12] For electrophoretic methods, the migration time, the peak height or area, the selectivity factor, the resolution between peaks, the signal-to-noise ratio, etc. can potentially be considered as qualitative or SST responses. When evaluating the robustness of a separation, responses describing the quality of the separation are studied, such as the selectivity factor or the resolution.

C. Examples of Responses Studied

In reference 22, only qualitative responses were examined in the 12-experiment PB design, i.e., the selectivity factor and the resolution of the propranolol, praziquantel, and warfarin enantiomers. In Table 8, the resolution results of the three chiral substances are given.

In reference 23, both quantitative and qualitative responses were considered in the eight-experiment PB design. The content of the *R*-timolol impurity in test samples of *S*-timolol, expressed as percentage, is a quantitative response, while the migration times of pyridoxine,

TABLE 8 Results of the 12-Experiment Plackett–Burman Design[22]

	Responses		
Experiment	Rs_{pro}	Rs_{pra}	Rs_{w}
1	2.30	9.07	4.15
2	2.63	10.12	4.45
3	2.42	8.27	3.38
4	2.37	8.86	4.24
5	2.41	9.17	3.42
6	2.55	8.75	3.65
7	2.42	9.43	3.58
8	2.56	9.05	4.43
9	2.71	11.08	4.07
10	2.63	8.04	4.30
11	2.19	9.99	3.28
12	2.60	9.62	3.99

Rs_{pro}, the resolution of Propranolol; Rs_{pra}, the resolution of Praziquantel; and Rs_{w}, the resolution of Enantiomers.

TABLE 9 Results of the Eight-Experiment Plackett–Burman Design[23]

	Responses			
Experiment	$MT_{S\text{-}t}$ (min)	$Rs_{S/R}$	$C_{R\text{-}t,R1}$ (%)	$C_{R\text{-}t,R2}$ (%)
1	13.8	4.9	0.62	0.59
2	9.3	3.5	0.63	0.65
3	10.9	4.2	0.72	0.60
4	11.4	5.1	0.63	0.61
5	14.4	4.7	0.72	0.70
6	13.7	5.8	0.66	0.55
7	10.7	3.8	0.64	0.63
8	13.0	5.2	0.65	0.66

$MT_{S\text{-}t}$, the migration time of *S*-timolol; $Rs_{S/R}$, the resolution between the *S*- and *R*-timolol peaks; and $C_{R\text{-}t,R1}$ and $C_{R\text{-}t,R2}$, two replicated measurements of the % content of *R*-timolol in a test sample.

S-timolol, and *R*-timolol, and the resolutions between the pyridoxine/*S*-timolol and *S*-timolol/*R*-timolol peaks, were qualitative or SST responses. In Table 9, the results for three of these responses are shown.

VI. PLANNING AND EXECUTION OF EXPERIMENTAL WORK

After selection of the experimental design, the experiments can be defined. For this purpose, the level symbols, −1 and +1, as given in Tables 6 and 7, are replaced by the real factor values, as for instance shown in Tables 2 and 3, respectively, yielding the factor level combinations to be performed. Dummy factors in PB designs can be neglected during the execution of the experimental work.

It is often advised to perform the experiments in *a random sequence*, in order to minimize uncontrolled influences on the calculated effects.[6,9] A *time effect* represents time-dependent changes in a response, which are larger than the experimental error. An example in electrophoresis is the change in migration times, due to fluctuations in the voltage and/or the temperature. A *drift* of a response is a special case of a time effect and occurs when the response changes continuously in one direction (increase or decrease). However, when a response is indeed subject to a time effect, then randomization of the experiments does not always solve the problem, since the time effect still will affect the estimated effects of some factors, depending on the sequence of the experiments.[43] The time effect will, to a larger or a smaller extent, be confounded with some factor effects. One way to overcome this problem, is by using *anti-drift designs*.[9,44] In these designs, the sequence of the experiments is such that the factor effects are not or only minimally confounded with the drift. However, the drift effect is then (mainly) confounded with the interaction terms or dummy factors in FF or PB designs, respectively. This results in an overestimation of the experimental error when the effects of the latter are used in the statistical interpretation (see Section VII.B.2.(b)).

A possibility to trace and correct drift or time effects is by repeating experiments at regular time intervals.[6,9,43] These *replicated (nominal) experiments* are performed before, at regular times between (e.g., every four or six design experiments) and after the design experiments. They allow verifying the method performance before and at the end of the robustness test, and checking and correcting for time effects.[9,11,43] A drift plot (Figure 5) can be drawn by plotting the replicated response, usually measured at nominal levels, as a function of time. In this plot, a drift or a time effect is visualized. An estimation quantifying the drift is given by the following equation:[11]

$$\%\mathrm{Drift} = \frac{y_{\mathrm{nom,end}} - y_{\mathrm{nom,begin}}}{y_{\mathrm{nom,begin}}} \times 100 \qquad (1)$$

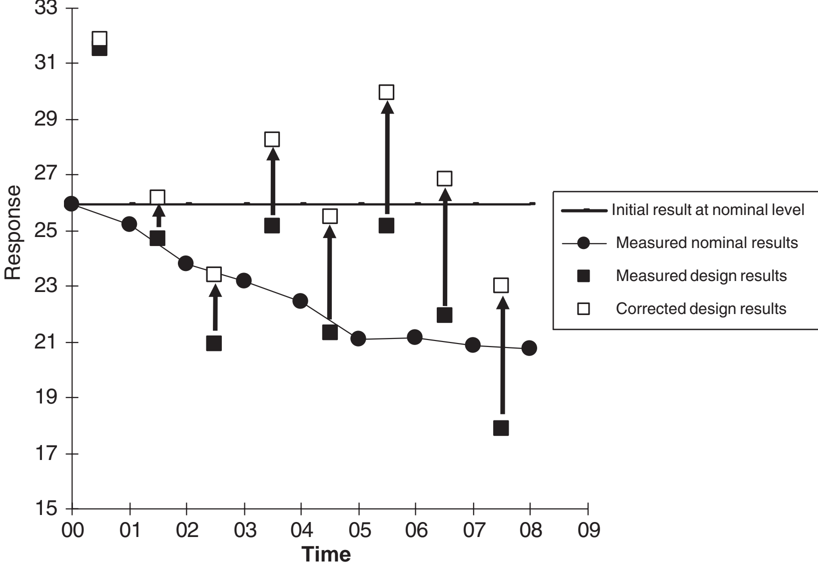

FIGURE 5 Drift plot: nominal responses, measured as a function of time. Design responses and corresponding corrected design responses are also shown.

where $y_{\mathrm{nom,begin}}$ and $y_{\mathrm{nom,end}}$ are the nominal responses measured before and after the design experiments, respectively.

When a time effect or drift is present, the responses are corrected relative to the nominal result obtained before the design experiments. Otherwise possibly wrong decisions on the significance of the factor effects are drawn. These corrected responses, calculated with Equation (2),[9,11,43] are then used to estimate the factor effects, which thus are estimated free from the drift effect. The correction of design results is also illustrated in Figure 5.

$$y_{i,\mathrm{corrected}} = y_{i,\mathrm{measured}} + y_{\mathrm{nom,begin}} - \left(\frac{(p+1-i)y_{\mathrm{nom,before}} + iy_{\mathrm{nom,after}}}{p+1}\right) \quad (2)$$

In Equation (2), it is assumed that the replicated experiments are at nominal levels, $i = 1, 2, \ldots, p$ and p is the number of design experiments between two consecutive nominal experiments. $y_{i,\mathrm{corrected}}$ is a corrected design response, $y_{i,\mathrm{measured}}$ the corresponding measured design response, $y_{\mathrm{nom,begin}}$ the nominal response at the beginning of the design experiments, and $y_{\mathrm{nom,before}}$ and $y_{\mathrm{nom,after}}$ the nominal responses measured before and after the $y_{i,\mathrm{measured}}$ that is being corrected, respectively.

For example, in reference 22, nominal experiments were added to the experimental setup at the beginning, halfway, and at the end of the experimental design. For the response resolution of warfarin enantiomers, the results of these nominal experiments were 4.11, 4.08, and 4.05, respectively. The estimated %drift is −1.46% (Equation (1)). In this example, the drift is considered non-existing and corrected responses (Equation (2)) are not calculated. Thus, for each particular response, it is evaluated whether a time effect occurs.

Although it is often advised not to do, frequently, for practical reasons, experiments are *blocked* or *sorted* by one or more factors.[6,9,11,43] For example, when the manufacturer or the batch of the capillary is a factor, it is easier and less time-consuming, when first all experiments are performed with this factor at one level, and afterwards at the other. Usually, the experiments are randomized within one block, while a check for drift is recommended, at least before and after each block. These latter experiments also allow observing and correcting occasional block effects.

The solutions, measured at each of the required experiments, are one or more samples and one or more standards, representative for the future use of the analytical method, i.e., the typical concentration range and matrices should be evaluated.[14] When the future samples are situated in a broad concentration interval, it is advised to evaluate the robustness in a large range, i.e., by several samples covering the range. When all samples have a similar concentration, the robustness is evaluated in a small interval (80–120%) around the expected (100%). When only the robustness of a separation is examined, it suffices to measure a sample or standard with representative composition.

VII. ANALYSIS OF RESULTS

A. Calculation of Effects

Effects can be estimated from the measured or corrected design responses, depending on the absence or presence of drift in the considered response. When drift is absent, both effect estimates are similar, while in the presence of drift, they are different for the factors mostly affected by the drift. The effect of factor X, E_X, on a response Y is calculated with the following equation:[6,9,11–14,17,43,45,46]

$$E_X = \frac{\sum Y(+1) - \sum Y(-1)}{N/2} \quad (3)$$

Here $\sum Y(+1)$ and $\sum Y(-1)$ represent the sums of the responses where factor X is at (+1) and (−1) levels, respectively, and N the number of design experiments. Often the normalized effect of factor X, $E_X(\%)$, is also calculated.[6,9,11,43,45–47]

$$E_X(\%) = \frac{E_X}{\overline{Y}} \times 100\% \tag{4}$$

In the absence of drift, $\overline{Y}$ is the average nominal result or the average design result.[6,9,11,43,45–47] When drift is present, the corrected responses are preferably used to estimate the factor effects, and then $\overline{Y}$ is replaced by the nominal result measured before the design experiments.[9,11]

B. Interpretation of Effects

After estimation of the factor effects, they usually graphically and/or statistically are interpreted, to determine their significance.

1. Graphical Interpretation of Effects

The graphical identification of the most important effects is usually performed by drawing normal probability[6,9,11,14,24–26] or half-normal probability plots.[6,9,18,48–51] The normal probability plot is constructed by plotting expected values from a normal distribution as a function of the estimated effects, while the half-normal probability plot, also called Birnbaun plot, is created by plotting the absolute values of the estimated effects as a function of so-called rankits, derived from a normal distribution. More information about the construction of these plots can be found in references 9, 18, 25, and 26 or they can be constructed using the appropriate statistical software.[29–37]

Examples of a normal and a half-normal probability plot are given in Figure 6 for the estimated effects on the resolution of praziquantel enantiomers (see Table 10).[22] Non-significant effects, i.e., only due to experimental error, are normally distributed around zero. In both plots, non-significant effects tend to fall on a straight line through the origin (0,0), while significant effects deviate from this line. In Figure 6, the effects of factors H and D clearly deviate from the straight line and can thus be considered important. However, the important effects cannot always be identified easily by means of the graphical methods, e.g., it is not always evident how to draw the straight line through the non-significant effects. This is, for instance, the case when the number of plotted effects is low. Further, some software automatically draws a least-squares line through all points (see Figure 6a). This line is largely influenced by the significant effects and obscures interpretation rather than being a help. Therefore, it is advised to combine the graphical with a statistical evaluation of the effects.

2. Statistical Interpretation of Effects

The statistical evaluation leads to a limit value, i.e., a critical effect, and all effects, E_X, that are in absolute value larger than or equal to the limit value are considered significant. The limit value is usually based on the t-test statistic given in the following equation:[6,9,11,43,45–47]

$$t = \frac{|E_X|}{(\mathrm{SE})_e} \Leftrightarrow t_{\mathrm{critical}} \tag{5}$$

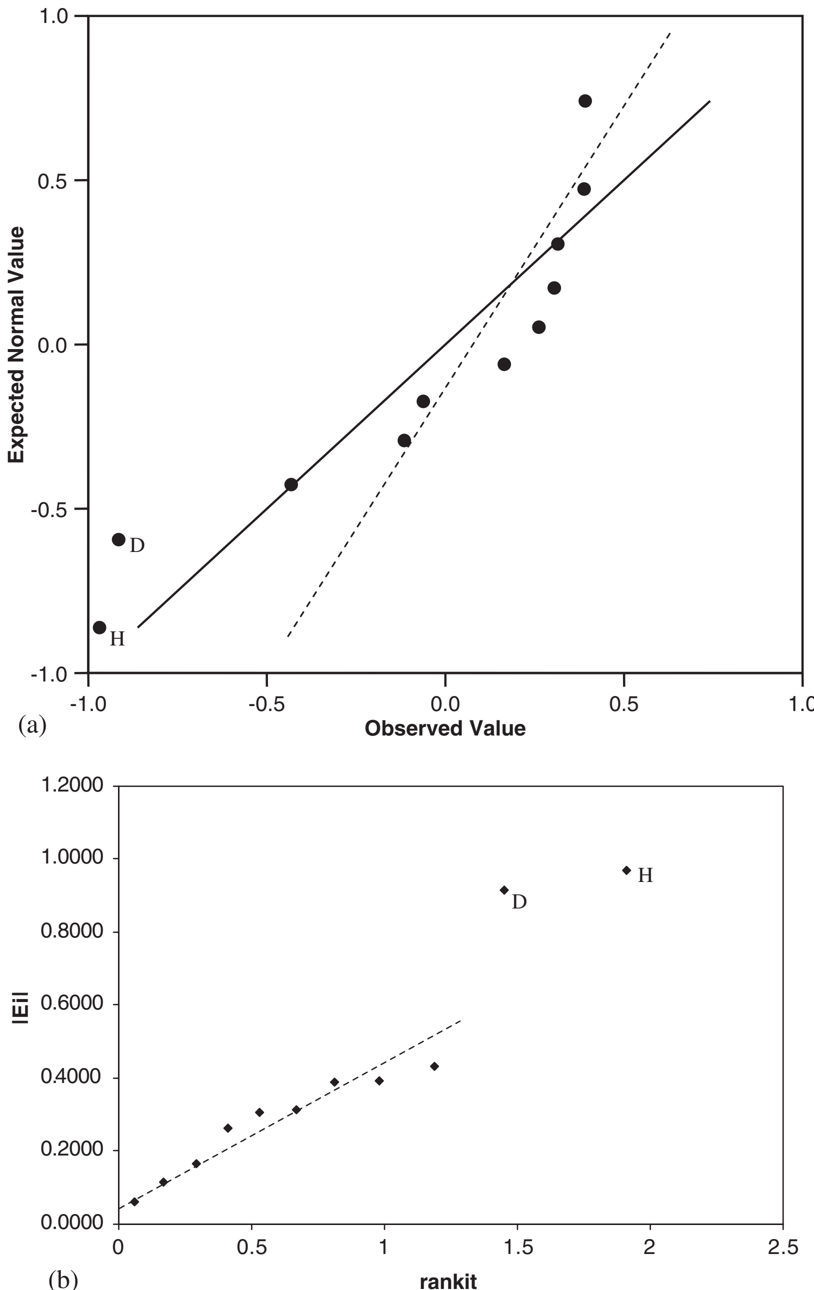

FIGURE 6 **(a) Normal probability plot and (b) half-normal probability or Birnbaun plot, for 11 effects on the response resolution of praziquantel enantiomers, estimated from a 12-experiments Plackett–Burman design. (——) least-squares line through all effects; (- - - -) assumed line through non-significant effects.**

TABLE 10 Effects on the Responses of Table 8 and Critical Effects According to Different Statistical Interpretation Methods

	Responses		
Factor	**Rs_{pro}**	**Rs_{pra}**	**Rs_{w}**
A	−0.098	0.388	−0.193
B	0.102	0.165	0.723
C	0.032	0.315	−0.190
D	−0.145	−0.915	−0.157
E	−0.192	0.305	−0.117
F	−0.008	−0.432	0.003
G	0.068	0.262	−0.033
H	−0.028	−0.968	−0.040
$I = d_1$	−0.035	0.392	−0.127
$J = d_2$	0.042	−0.062	−0.100
$K = d_3$	0.028	−0.115	0.073
Method to estimate $(SE)_e$	**Critical effects**		
R replicates at nominal level ($R = 3$)			
$\alpha = 0.05$	0.066	0.176	0.075
$\alpha = 0.01$	0.152	0.407	0.172
Duplicated design experiments			
$\alpha = 0.05$	–	–	–
$\alpha = 0.01$	–	–	–
Variance of design experiments			
$\alpha = 0.05$	0.197	1.064	0.543
$\alpha = 0.01$	0.278	1.501	0.766
Dummies			
$\alpha = 0.05$	0.113	0.758	0.326
$\alpha = 0.01$	0.207	1.392	0.598
Algorithm of Lenth			
$\alpha = 0.05$	0.167	1.376	0.473
$\alpha = 0.01$	0.288	2.370	0.815
Algorithm of Dong			
$\alpha = 0.05$	0.160	1.064	0.269
$\alpha = 0.01$	0.227	1.501	0.383

(–) Not possible to calculate from reported setup.

where $(SE)_e$ is the standard error of an effect, which is an estimation for the experimental variability of the method. Equation (5) can be rewritten as follows:[6,9,11,43,45–47]

$$|E_X| \Leftrightarrow E_{critical} = t_{critical}(SE)_e \tag{6}$$

The critical effect, $E_{critical}$, depends on the (tabulated) critical t-value, $t_{critical}$, and on $(SE)_e$. The $t_{critical}$ depends on the number of degrees of freedom associated with $(SE)_e$ and is usually considered at a significance level $\alpha = 0.05$ (occasionally also $\alpha = 0.01$). An effect is considered significant if $|E_X| \geq E_{critical}$. In a robustness test, $(SE)_e$ can be estimated in different ways:[6,9,11,12,14,17,18,43,45–47,52,53] from the variance of (replicated) experiments (Section VII.B.2.(a)), from a priori declared negligible effects (Section VII.B.2.(b)), or from a posteriori defined negligible effects (Section VII.B.2.(c)).

(a) The Variance of (Replicated) Experiments (Intermediate Precision Conditions): $(SE)_e$ can be derived from intermediate precision estimates (Equation (7)),[6,9] using the propagation of error rules on the equation to calculate effects.

$$(SE)_e = \sqrt{\frac{s_a^2}{n_a} + \frac{s_b^2}{n_b}} \Rightarrow (SE)_e = \sqrt{\frac{2s^2}{n}} \quad \text{when } s_a^2 = s_b^2 = s^2;\ n_a = n_b = n \tag{7}$$

where s_a^2 and s_b^2 represent variances of two sets of measurements, and n_a and n_b their corresponding numbers of measurements. When s_a^2 and s_b^2 are estimated by the same variance, s^2, and $n_a = n_b = n$ is the number of experiments performed at each factor level, the simplified equation for the standard error of an effect is obtained (Equation (7)). The variance, s^2, can be estimated using replicates at nominal level, duplicated design experiments, or the design results themselves.

- When using the variance from R replicates at nominal level, $n = N/2$ in Equation (7), with N the number of design experiments. The number of degrees of freedom for $t_{critical}$ in Equations (5) and (6) is equal to $R-1$.[6,9] These replicates should be measured at intermediate precision conditions, since when using repeatability conditions, the experimental error is underestimated and most effects will be considered significant. One should not forget that a robustness test simulates reproducibility conditions, i.e., those occurring in different laboratories. Another requirement is that enough replicates ($R \geq 4$) should be measured to obtain at least three degrees of freedom, as is also recommended for the estimation of error based on a priori declared negligible effects (see Section VII.B.2.(b)). Otherwise ($R < 4$), the value of $t_{critical}$ becomes too large, resulting in unrealistically high critical effects.
- When using duplicated design experiments, then $s^2 = \sum d_i^2/2n$, with d_i the difference between the duplicated experiments, and both n (Equation (7)) and the number of degrees of freedom associated with $t_{critical}$ (Equations (5) and (6)) are equal to the number of design experiments N.[6,9,14,17,25] For the duplicated design experiments, the same remark concerning the measurement conditions is valid as for the replicated nominal experiments.
- A third option, sometimes applied in the literature, is to use the variance of the design experiments ($y_1, y_2, \ldots, y_N$), $s^2 = \sum(y_i - \bar{y})^2/(N-1)$, with $\bar{y}$ the average design response.[45,54] Then $n = N/2$ (Equation (7)), and the number of degrees of freedom for $t_{critical}$ (Equations (5) and (6)) is equal to $N-1$. However, it is not advised to use this variance to estimate $(SE)_e$[6,54] because s^2 is largely affected by the significant factors. This results in an overestimated error estimate. The consequence is that either no or at the most one significant effect can be detected. Other significant effects may remain hidden due to the overestimated error value.

(b) A Priori Declared Negligible Effects:

Another way to estimate $(SE)_e$ is using effects that are a priori considered negligible, such as two-factor interaction effects[6,9,11,14,43,45,46] and dummy factor effects[6,9,11,12,45,47] in FF and PB designs, respectively (Equation (8)). Such effects are considered solely due to the experimental error of the method.[6,9,11,14]

$$(SE)_e = \sqrt{\frac{\sum E_N^2}{n_N}} \tag{8}$$

E_N represents a negligible effect, and n_N the number of such effects. The number of degrees of freedom associated with $t_{critical}$ (Equations (5) and (6)) is equal to n_N.[11] To obtain a good estimation of $(SE)_e$, at least three negligible effects are recommended.[9] For designs that do not allow estimating three such effects, it is better to use alternative methods, such as, the estimation of $(SE)_e$ according to the algorithm of Dong, i.e., based on a posteriori defined negligible effects.

(c) A Posteriori Defined Negligible Effects:

$(SE)_e$ can also be estimated from a posteriori defined negligible effects, by using the algorithms of Lenth[18,52] or Dong.[9,18,53] These negligible effects can originate both from examined factors and interaction or dummy factors. Both algorithms calculate an initial estimate of the standard error, s_0, from the median of the absolute effects, using the following equation:

$$s_0 = 1.5 \times \text{median}|E_i| \tag{9}$$

where E_i is the effect of factor i.

The algorithm of Lenth then calculates a pseudo standard error, PSE, based on the median of all effects E_j that are in absolute value smaller than $2.5s_0$ (Equation (10)).[18,52] The algorithm of Dong calculates a final error estimate, s_1, based on effects considered non-important, i.e., the m effects E_k that are in absolute value smaller than or equal to $2.5s_0$ (Equation (12)).[9,18,53] Consecutively, both algorithms estimate a critical effect, called the margin of error, ME, equal to the product of the error estimate and a critical t-value. Usually $\alpha = 0.05$ or $\alpha = 0.01$ is chosen as significance level. The number of degrees of freedom for the t-value, df, equals $f/3$ for the Lenth algorithm, with f being the number of factor effects (Equation (11)),[18,52] while for Dong df is m (Equation (13)).[9,18,53]

$$\text{PSE} = 1.5 \times \text{median}|E_j| \quad \text{for all } |E_j| < 2.5s_0 \tag{10}$$

$$E_{\text{critical}} = \text{ME}_{\text{Lenth}} = t_{(1-(\alpha/2),\text{df})}\text{PSE} \tag{11}$$

$$s_1 = \sqrt{m^{-1}\sum E_k^2} \quad \text{for all } |E_k| \le 2.5s_0 \tag{12}$$

$$E_{\text{critical}} = \text{ME}_{\text{Dong}} = t_{(1-(\alpha/2),\text{df})}s_1 \tag{13}$$

Factor effects $|E_X|$ that are larger than or equal to $E_{critical}$ are considered significant. The algorithm of Dong performs better than that of Lenth and is preferred, i.e., the obtained $E_{critical}$ is practically more relevant than that obtained with Lenth.[18,53,55,56]

(d) Examples:

For the two examples, described in references 22 and 23, the estimated effects on the responses (Tables 8 and 9) are given in Tables 10 and 11, respectively. Their significance according to the different statistical interpretation methods was determined when possible.

The most appropriate approaches estimate the error based on a priori considered negligible effects, such as dummies, or on the algorithm of Dong. They usually result in similar critical effects.[9,18,45,56,57]

TABLE 11 Effects on the Responses of Table 9 and Critical Effects According to Different Statistical Interpretation Methods

Factor	Responses		
	$MT_{S\text{-}t}$ (min)	$Rs_{S/R}$	$C_{R\text{-}t}$ (%)
A	0.500	0.500	−0.050
B	−0.200	−0.850	0.013
C	−0.450	−0.100	−0.028
D	−3.150	−1.000	−0.005
E	0.950	0.150	0.015
F	0.100	0.250	0.005
G	0.550	−0.050	0.023
Method to estimate $(SE)_e$	**Critical effects**		
R replicates at nominal level			
$\alpha = 0.05$	–	–	–
$\alpha = 0.01$	–	–	–
Duplicated design experiments			
$\alpha = 0.05$	–	–	0.049
$\alpha = 0.01$	–	–	0.071
Variance of design experiments			
$\alpha = 0.05$	3.048	1.286	0.058
$\alpha = 0.01$	4.510	1.903	0.086
Dummies			
$\alpha = 0.05$	–	–	–
$\alpha = 0.01$	–	–	–
Algorithm of Lenth			
$\alpha = 0.05$	2.800	1.179	0.088
$\alpha = 0.01$	6.102	2.569	0.193
Algorithm of Dong			
$\alpha = 0.05$	1.305	1.033	0.058
$\alpha = 0.01$	1.978	1.565	0.086

(–) Not possible to calculate from reported setup.

The error estimate based on replicates at nominal level results in underestimated critical effects, and consequently a high number of effects is considered significant, which practically are not relevant, e.g., the effects of A, C, D, I, E, and J on response Rs_W at significance level $\alpha = 0.05$ (Table 10). A possible reason is that the replicates are measured under repeatability conditions. For duplicated design experiments, a similar problem might occur. However, in Table 11 it is not the case or the critical effect is only slightly underestimated. In case underestimation occurs for a response related to the quantitative aspect, the method would incorrectly be considered non-robust, since effects considered significant occur. This is fundamentally not a problem, because one will react when it is not necessary. It just leads to a waste of time and money. The opposite situation is worse.

The error estimate based on the variance of the design experiments themselves leads to similar critical effects as the algorithm of Dong when no or small significant effects occur. However, when a large effect is present, e.g., that of factor B on Rs_W in Table 10 or that of factor D on response $MT_{S\text{-}t}$ in Table 11, the error is overestimated, compared to the algorithm of Dong.

When using the algorithm of Lenth, the critical effects are (slightly) overestimated compared to those from dummies or the algorithm of Dong. This confirms the statements of references 18 and 56. Usually, overestimation becomes worse when the number of design experiments decreases. This can be explained by the fact that the number of degrees of freedom in ME_{Lenth} equals the number of factor effects divided by three ($df = f/3$), resulting in higher critical t-values and thus larger critical effects.

C. Non-significance Intervals for Significant Quantitative Factors

When significant effects are found on the response(s) describing the quantitative aspect of the method, the results from the robustness test can be used to set restrictions on the levels of significant continuous factors. When factor X has a significant effect, the initially examined interval is reduced and the levels can be estimated, where the effect is eliminated. The non-significance interval limits are estimated as follows:[9]

$$\left[X_{(0)} - \frac{|X_{(+1)} - X_{(-1)}|E_{\text{critical}}}{2|E_X|}, X_{(0)} + \frac{|X_{(+1)} - X_{(-1)}|E_{\text{critical}}}{2|E_X|}\right] \tag{14}$$

For example, the effect of factor A on response $C_{R\text{-}t}$ at $\alpha = 0.05$ was found significant when using the variance from duplicated design experiments to estimate the critical effect (see Table 11).[23] However, since this factor represents different CE equipments, i.e., is discrete, calculating a non-significance interval is irrelevant.

Suppose a factor X has 45, 50, and 55 as extreme low, nominal, and extreme high levels, respectively, and an effect of 100 on response Y, with the critical effect equal to 80. Then the non-significance interval limits for this factor are [46.0,54.0], which means that when restricting the levels of X to this interval, the quantitative aspect of the method is considered robust. It can be noticed that the interval is symmetrically around the nominal level and meant for factors thus examined, i.e., with extreme levels symmetrically around the nominal.

VIII. DETERMINATION OF SST LIMITS

A SST is an integral part of many analytical methods.[4] It verifies the suitability and the efficacy of the instrument or the setup for the intended purpose of the method. SST limits for some responses are occasionally derived from the method optimization and validation results, but quite often based on the experience of the analyst.

As an alternative, SST limits can be determined from the results of a robustness test,[9,12,13,15–17] as recommended by the ICH.[3,4] It can be done using the worst-case results for the response, derived from the experimental design results. This allows defining SST limits for responses such as resolution or peak asymmetry. The main idea behind the approach is that the most extreme results are considered, obtained under experimental conditions resulting in acceptable quantitative determinations. SST limits can thus only be meaningfully derived when the tested method is considered robust concerning its quantitative aspect. Then, nowhere in the domain, described by the experimental design, a problematic quantitation occurs, even not at the conditions where the SST responses are worst.

The conditions with the worst response value can be derived from the estimated effects.[9] The worst-case situation is that combination of factor levels resulting in the worst result, e.g., the lowest resolution. The worst-case conditions are set using only the effects significant at

significance level $\alpha = 0.10$.[9,12,13] The other effects are considered to solely represent experimental error,[9,12] and are put at nominal level in the worst-case conditions setting.

For example, when using the algorithm of Dong (Table 11), the quantitative response, $C_{R\text{-}t}$ (%), is considered robust since no significant effects were found at $\alpha = 0.05$ or $\alpha = 0.01$. To derive the SST limit for response, $MT_{S\text{-}t}$ (min), the significant factors are considered. Only factor D is considered significant at $\alpha = 0.10$ ($ME_{Dong} = 1.037$). For the worst-case conditions, the non-significant factors are set at their nominal level ($F_i = 0$). Since the effect of factor D on $MT_{S\text{-}t}$ is -3.150 and since the worst-case situation has the highest migration time, the level of D resulting in the worst result is $F_D = -1$.

SST limits can then be derived either mathematically or experimentally. In the first situation, the limits are calculated as follows:[9,13]

$$Y = b_0 + \left(\frac{E_1}{2} \times F_1\right) + \left(\frac{E_2}{2} \times F_2\right) + \cdots + \left(\frac{E_k}{2} \times F_k\right) \tag{15}$$

where Y is the calculated SST limit, b_0 the average design result for the considered response, E_i the effect of factor i, and F_i the level of factor i. Significant factors have $F_i = -1$ or $+1$, while non-significant are at their nominal level ($F_i = 0$).

For example, for response $MT_{S\text{-}t}$ (min) of Table 11, the mathematically obtained SST limit is $Y = b_0 + (E_D/2)F_D = 12.15 + (-3.150/2)(-1) = 13.73$ min, i.e., the migration time of future routine experiments should be below 13.73 min.

A second possibility consists of experimentally determining the SST limits from measurements at the worst-case conditions (n measurements with standard deviation s).[9,12,13] The SST limit is defined as the lower or upper limit of the one-sided 95% confidence interval around the worst-case average result. For example, for resolution, the lower limit will be considered, while for migration time it would be the upper. The confidence intervals are defined as in Equations (16) and (17),[14] when considering the lower or the upper limit, respectively,

$$\left[\overline{Y}_{\text{worst-case}} - t_{\alpha,n-1}\left(\frac{s}{\sqrt{n}}\right), +\infty\right] \tag{16}$$

$$\left[-\infty, \overline{Y}_{\text{worst-case}} + t_{\alpha,n-1}\left(\frac{s}{\sqrt{n}}\right)\right] \tag{17}$$

where $\overline{Y}_{\text{worst-case}}$ is the average of n replicated measurements with standard deviation s executed at the worst-case conditions. The t-value is determined by the significance level α and the number of degrees of freedom for s, here $n-1$. For example, for response $MT_{S\text{-}t}$ (min), the experimentally obtained SST limit is given in Table 12. The upper limit was calculated, since the highest value represents the worst-case result. It can be noticed that the number of replicates is quite low, resulting in a low number of degrees of freedom to estimate the SST limit.

When no significant effects are found on a response, one can still determine some SST limits, according to the above procedure, but now the measurements are performed at nominal level.[9] For such situation, it anyway is often still necessary to use some arbitrarily defined minimum or maximum limits, since the experimentally obtained limits are not sufficiently extreme to be of practical use. As an alternative to replicated nominal experiments, all experiments in the domain defined by the design could be used and the average and the standard deviation of these results applied to define SST limits.

TABLE 12 Results at Worst-Case Conditions to Estimate the SST Limit for the Response MT_{S-t} (min) of Tables 9 and 11

	MT_{S-t} (min)
1	14.90
2	14.80
3	14.60
Average	14.77
Standard deviation	0.1528
n	3
$t_{(\text{one-sided},\alpha = 0.05,\text{df} = 2)}$	2.920
Upper limit	15.02

IX. REVIEW OF CASE STUDIES

In this part of the chapter, a critical review is given of case studies studying the robustness of CE methods. The case studies are divided into robustness tests using the one-variable-at-a-time (OVAT) procedure (see Section IX.A) and those applying experimental design procedures (see Section IX.B). Both approaches are critically discussed. Depending on the type of design used, the latter section is subdivided into a full factorial (see Section IX.B.1), fractional factorial (see Section IX.B.2), Plackett–Burman (see Section IX.B.3), and response surface design (see Section IX.B.4) section. For each case study, the chosen factors, the selected design, the considered responses, and the analysis of the results are reviewed and discussed.

HPLC methods can usually be transferred without many modifications, since most commercially available HPLC instruments behave similarly. This is certainly true when the columns applied have a similar selectivity. One adaptation, sometimes needed, concerns the gradient profiles, because of different instrumental or pump dead-volumes. However, larger differences exist between CE instruments, e.g., in hydrodynamic injection procedures, in minimum capillary lengths, in capillary distances to the detector, in cooling mechanisms, and in the injected sample volumes.[58] This makes CE method transfers more difficult. Since robustness tests are performed to avoid transfer problems, these tests seem even more important for CE method validation, than for HPLC method validation. However, in the literature, a robustness test only rarely is included in the validation process of a CE method, and usually only linearity, precision, accuracy, specificity, range, and/or limits of detection and quantification are evaluated. Robustness tests are described in references 20 and 59–92. Given the instrumental transfer problems for CE methods,[93,94] a robustness test guaranteeing to some extent a successful transfer should include besides the instrument on which the method was developed at least one alternative instrument.

The terminology applied in the different papers might vary. For example, both the terms buffer concentration and electrolyte concentration are frequently used and usually refer to the same. The same occurs for the terms buffer pH and electrolyte pH, and buffer ionic strength and electrolyte ionic strength. However, for the exact meaning or practical implications, we refer to the corresponding literature.

Another example is the confusion between the terms efficiency, efficacy, and selectivity of a separation. It is not always mentioned how these responses are obtained. Usually, the efficiency is either expressed as the number of theoretical plates or plate number, N, or as the height equivalent to a theoretical plate or plate height, HETP or H.[95] With efficacy, usually efficiency, thus plate count, is meant, although it is not always clearly specified. The selectivity, in general, is found by calculating the selectivity or separation factor, α, which is a measure for the

difference in migration of two compounds.[95] In the following discussion, the terms as used in the respective publications were applied, and for their exact meaning, we refer to the corresponding literature.

A. One-Variable-at-a-Time Procedure

In references 59–67, CE robustness tests were performed with a OVAT procedure. Analytical CE methods included were the chiral separations of drug substances[59,60,64,66,67] and drug products,[64] and the separations of drug substances which are structural isomers,[62,63] of angiotensin-converting enzyme (ACE) inhibitors in drug substances and products (tablets),[61] or of raloxifene in drug products or human plasma.[65]

The influence of several factors, such as buffer or electrolyte pH,[59–63,65–67] chiral selector concentration[59,60,64,66,67] and batch,[60] buffer or electrolyte concentration,[60–62,65–67] buffer or electrolyte ionic strength,[64] capillary temperature,[60,66,67] ion-paring reagents concentrations,[61] voltage,[62,63,65–67] detection wavelength,[62] organic solvent concentration,[64] capillaries,[59,64] and instruments[64] was determined. The OVAT approach varies the level of a given factor while keeping the other at nominal level, to verify the effect of the varied factor on one or several responses. Responses such as migration time of given compounds,[59–63,66] resolution,[59–62,64,66,67] peak area,[62,63,66] selectivity,[63] and peak efficiency[65] have been considered. The result obtained after varying one factor is then compared to that with all factors at nominal level. This univariate approach is, although often performed and described in the literature, not recommended to draw conclusions about the method robustness for several reasons.

First of all, such approach would require too many experiments when the number of factors becomes too large. To vary f factors independently between -1 and $+1$ with the OVAT approach, at least $2f+1$ experiments are required. For example, to vary 10 factors with the OVAT approach, at least 21 experiments are needed, i.e., 20 experiments with one factor varying (once at -1 and once at $+1$) and 1 experiment with all factors at nominal level (center point). With an experimental design approach, on the other hand, these 10 factors can be examined in 12 experiments.

Secondly, with the OVAT approach the importance of interactions is not taken into account. An interaction between two factors is present when the effect of one factor depends on the level of another factor. Since only one factor at a time is varied, the presence or absence of interactions cannot be verified. However, this is not dramatic, since in robustness testing the interaction effects are considered negligible. The evaluation of such interactions is more important in method optimization.

Finally, the most important reason why the OVAT approach is not recommended is that the factor effects are estimated for a small domain, much smaller than with the experimental design approach. By applying an experimental design, the effects of a given factor are calculated at different level combinations of the other factors, while with the OVAT approach this is only at one, i.e., the nominal conditions. Thus, in an experimental design, a reported factor effect is an average value for the whole domain. This is illustrated in Figure 7, where the experimental domains are shown when examining two and three factors with either the OVAT or an experimental design approach.

In reference 68, a different approach was used to verify the robustness of a CE separation of ibuprofen, codeine phosphate, degradation products, and impurities in a drug product (tablet). Small variations around the optimal conditions obtained during method optimization were introduced and the results were predicted from the response model.[68] The variations in the factor levels during the robustness evaluation were smaller than those evaluated during method optimization. Since both migration times and resolutions were acceptably predicted, the method was considered robust with respect to the small changes. The examined factors

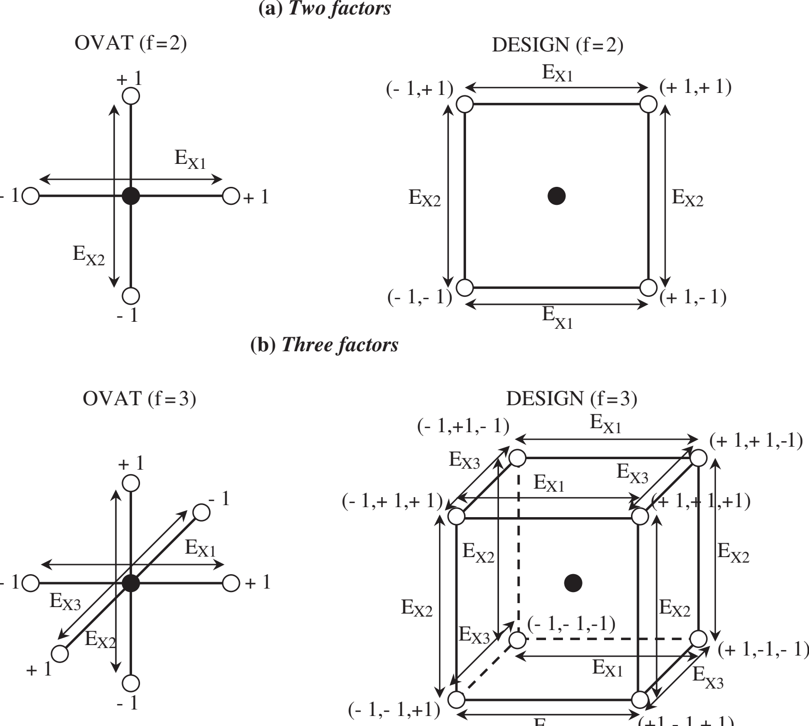

FIGURE 7 **Experimental domains when examining (a) two and (b) three factors with either the OVAT or an experimental design approach. (●) Nominal level; E_X, effect of factor *X*.**

were surfactant concentration, buffer or electrolyte pH, organic solvent concentration, buffer or electrolyte concentration, field strength, and capillary temperature. Information regarding the method robustness was gathered from the experimental design used in the method optimization,[96] where a 2^{6-1} FF design was used to examine six factors at two levels in 32 experiments, a rather high number of experiments. To check for curvature in the response surface, and to obtain an error estimate, the center point was replicated ($R = 3$).[96]

These six factors could also have been examined in a 2^{6-2} FF design, requiring 16 experiments, or even in a 2^{6-3} FF design with only 8 experiments. The applied 2^{6-1} design allows two-factor interactions to be investigated, because each two-factor interaction is only aliased with a four-factor interaction (design resolution = VI). In the 2^{6-2} design, the two-factor interactions are aliased with each other (design resolution = IV), while for the 2^{6-3} design, main effects are aliased with two two-factor interaction effects (design resolution = III).[26] From the 2^{6-1} FF design used during optimization, all factors were found significant on both responses,[96] possibly due to the error estimate that was obtained from three replicates of the center point under repeatability conditions, leading to an underestimation of the critical effect.

B. Experimental Design Procedures

As already mentioned, an experimental design approach is preferred to evaluate method robustness. It is a multivariate approach, evaluating the factor effects on the responses by varying the factors simultaneously, according to the experimental conditions defined by the design.

The robustness tests described in references 20 and 69–92 used an experimental design approach, but often not all information is provided to repeat. Several analysts rely on software packages to set up and interpret a robustness test. Applied software packages are Modde (Umetrics, Umea, Sweden),[69,91,92] Statgraphics (Manugistics, Rockville, USA),[70] Design Ease and Design Expert (Stat-Ease, Minneapolis, USA),[71,72] Nemrod (LPRAI, Marseille, France),[20,75,76,78–80,89] and Minitab (Minitab, Pennsylvania, USA).[88,90] However, just referring to the software does not explain the setup or the data analysis, since in each software several approaches to analyze the data exist. A proper description should include the selected factors and their levels, the experimental design used, the responses considered, the planning and execution of the experimental work, and finally the analysis of the results (graphically and/or statistically). All choices made should be clear. Certain responses, such as resolution, can be calculated in different ways. For such responses, an equation or a proper reference should be provided, so that no doubt exists about the data reported. As will be seen further, the above is not always fulfilled. Especially the data analysis part is often poorly described.

In the following, a division, according to the type of design, into full factorial (see Section IX.B.1), fractional factorial (see Section IX.B.2), Plackett–Burman (see Section IX.B.3), and response surface designs (see Section IX.B.4) is made. For each type of design, the factors, the specific design, the responses, and the analysis of the results from some case studies are described and discussed. An overview of the applied designs is given in Table 13.

1. Full Factorial Designs

Two-level full factorial designs were used to determine the CE robustness of a chiral separation of the local anesthetic ropivacaine in injection solutions[69] and of a separation of the macrolide antibiotic tylosin and its main related substances.[70] Table 13a shows the applied designs.

(a) Factors:

Examined factors were the chiral selector concentration,[69] the buffer or electrolyte concentration,[69] the buffer or electrolyte pH,[69,70] the capillary temperature,[69] and the mixture of surfactants.[70]

(b) Experimental Designs:

Full factorial designs allow the estimation of all main and interaction effects, which is not really necessary to evaluate robustness. They can perfectly be applied when the number of examined factors is maximally four, considering the required number of experiments. In references 69 and 70, four and three factors were examined at two levels in 16 and 8 experiments, respectively. When the number of factors exceeds four, the number of experiments increases dramatically, and then the full factorial designs are not feasible anymore.

(c) Responses:

Studied responses were resolution[69] and selectivity.[70]

(d) Analysis of Results:

In reference 69, results were analyzed by drawing response surfaces. However, the data set only allows obtaining flat or twisted surfaces because the factors were only examined at two levels. Curvature cannot be modeled. An alternative is to calculate main and interaction effects with Equation (3), and to interpret the estimated effects statistically, for instance, with error estimates from negligible effects (Equation (8)) or from the algorithm of Dong (Equations (9), (12), and (13)). For the error estimation from negligible effects, not only two-factor interactions but also three- and four-factor interactions could be used to calculate $(SE)_e$.

TABLE 13 Designs Applied in CE Robustness Case Studies

Design	*N*	*f*	Reference
(a) Full factorial designs			
2^4	16	4	69
2^3	8	3	70
(b) Fractional factorial designs			
–	20	7	71
–	–	6	72
2^{7-4}	8	7	73
Reflected 2^{4-1} (three levels)	15	4	74

Design	*N*	*f*	*d*	Reference
(c) Plackett–Burman designs				
Two-level	8	5	2	75
	12	8	3	76
	12	9	2	77
	8	4	3	78
	8	5	2	79
	12	9	2	80
	12	11	0	81
Reflected two-level (three levels)	15	7	0	82
	15	7	0	83
	(15)	7	0	84
	(15)	7	0	85
	15	7	0	86

Design	*N*	*f*	*n*	Reference
(d) Response surface designs				
Face-centered central composite design	15	3	1	20[b]
Central composite design[a]	17	3	3	87
Box–Behnken design	15	3	3	88
Circumscribed central composite design	19	3	5	89[b]
Box–Behnken design	27	4	3	90[b]
Face-centered central composite design	27	4	3	91[b]
Full factorial design	27	3	0	92[b]

N, number of experiments; *f*, number of examined factors; *d*, number of dummies; and *n*, number of center points; (–) not specified; ( ) assumed.
[a]Type of CCD not specified.
[b]Robustness information derived from optimization.

In reference 70, a graphical and statistical interpretation using standardized Pareto charts was applied. Such chart contains a bar for each effect, sorted according to importance (Figure 8). The bar lengths equal the standardized effects, which are in fact equal to the calculated *t*-values (Equation (5)). Often a vertical line is drawn, corresponding to the critical *t*-value at a given significance level, usually $\alpha = 0.05$. The bars exceeding this line are statistically significant at the considered significance level. The $(SE)_e$ from Equation (5) was calculated using the three two-factor interaction effects that could be calculated besides the main effects.

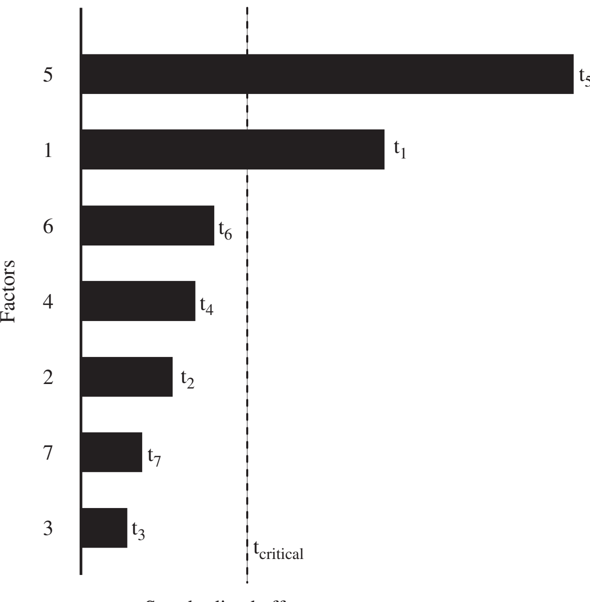

FIGURE 8 **Standardized Pareto chart. t_X, standardized effect of factor *X*; $t_{critical}$, critical *t*-value.**

The standardized Pareto charts are usually provided by commercial software packages. However, from publications reporting these charts, it often is not clear how the experimental error, resulting in the critical *t*-value, is obtained. Moreover, such software packages provide different possibilities to estimate the error, which affects the critical *t*-value. It can, for instance, result from what is called "pure error" or "total error," or an external error estimate can be entered by the user. Those different error estimates can be very different, resulting in very different critical *t*-values and very different conclusions concerning the significance of effects. Moreover, it is not always clearly defined from the software manuals how the different error estimates are calculated. It is not always evident for the user how to adjust the error estimation, and often one prefers to use the default estimation. Therefore, one should be careful in interpreting the results, unless it is clear how the critical *t*-values were obtained.

2. Fractional Factorial Designs

Other two-level screening designs are more commonly applied to evaluate robustness. The first type is the FF design. They execute a fraction of the full factorial. When the number of factors exceeds four, they require a still feasible number of experiments. Such designs were applied in the robustness tests of CE methods, described in references 71–74. Table 13b shows the applied designs. The methods tested were separations of a wide range of basic[71] or acidic[72] drug substances, of carnitine and acyl-carnitines drug substances,[73] and of three non-steroidal anti-inflammatory drug (NSAID) substances, i.e., indomethacin, ketoprofen, and sodium diclofenac.[74]

(a) Factors:

Examined factors were buffer or electrolyte concentration,[71–73] buffer or electrolyte pH,[71,73,74] buffer or electrolyte ionic strength,[74] rinse times,[71–73] capillary temperature,[71–73] injection time,[71,72,74] voltage,[71–74] and detection wavelength.[73]

(b) Experimental Designs:

In references 71 and 72, the use of FF designs is reported, but the type is not specified. In reference 71, the effects of seven factors were evaluated in 20 duplicated experiments. However, for FF designs, the number of experiments is equal to a power of two, which 20 is not. For PB designs, the number of experiments equals a multiple of four. Possibly such design was applied, although it is not evident to use a PB design with 20 experiments to examine only seven factors. PB designs with 12 experiments, or even with only 8, seem more evident alternatives for the given situation. Another possibility is that a 2^{7-3} FF design was executed with four center points. However, given the fact that the experiments were duplicated, this possibility is not evident either. In reference 72, the number of design experiments was not reported.

In reference 73, a 2^{7-4} FF design was applied to examine seven factors at two levels in eight experiments, which is the minimal number of experiments possible to evaluate such number of factors. In reference 74, a two-level 2^{4-1} FF design with eight experiments was reflected, to evaluate the effect of four factors at three levels. A reflected design is a two-level screening design that is duplicated.[6,15,16,27,40] Once the factors are examined between levels [−1,0] and once between [0,+1]. Since one experiment is common for both designs, the effects of the four factors at three levels are investigated in 8+8−1 = 15 experiments.

(c) Responses:

Studied responses were resolution,[71,72,74] migration time,[72–74] peak area or height,[73,74] and drug content.[74]

(d) Analysis of Results:

In references 71 and 72, SST limits are defined based on experience, and the examined responses should fall within these limits. The two papers do not provide much information concerning the robustness test performed. Therefore, it is not evident to comment on the analysis applied, or to suggest alternatives. In reference 73, a graphical analysis of the estimated effects by means of bar plots was performed. In reference 74, a statistical analysis was made in which an estimation of error based on negligible two-factor interaction effects was used to obtain the critical effects between levels [−1,0] and [0,+1].

3. Plackett–Burman Designs

The second type of commonly applied screening designs in robustness testing is the PB design. PB designs are used in the CE robustness tests, described in references 75–86. Table 13c shows the applied designs. The methods tested were chiral separations of citalopram[83] and omeprazole[85] in pharmaceutical formulations, of catechins in *Theobroma cacao* beans,[80] and of salbutamol,[75] and citalopram and metabolites[81] in urine. Other methods were determinations of calcium acamprosate,[76] ximelagatran and related substances,[77] rufloxacin hydrochloride,[78] ketorolac tromethamine and its impurities[79] in drug substance and/or pharmaceutical formulations, of gleevec and its main metabolites,[82] of tamoxifen, imipramine, and their metabolites,[84] and of propranolol and metabolites[86] in urine.

(a) Factors:

Examined factors were organic solvent concentration,[75,77,82,84] buffer or electrolyte concentration,[75–80,82–86] buffer or electrolyte pH,[75,78–81,83,85,86] voltage,[75–86] chiral selector concentration[75,77,80,81,83,85] and supplier,[77] active pharmaceutical ingredient (API) concentration,[76] internal standard concentration,[76] injection time[76,77,81–84,86] and pressure,[81,83] detection wavelength,[76,82,84–86] rinse times,[76,83,85,86] capillary temperature[76–80,82,84–86] and conditioning time,[77] filtration,[77] surfactant concentration,[79,80] % solvent used in

extraction,[80] extraction temperature and time,[80] time (days),[81] capillaries,[81] reagent lots,[81] solid phase extraction (SPE) elution volume,[81] SPE washing buffer volume,[81] and selectivity additive concentration.[81]

(b) Experimental Designs:

In references 75–81, PB designs were used with the minimal number of experiments needed to examine the considered number of factors (see Table 13c). In reference 76, initially a PB design was used to screen for significant factors on both quantitative and qualitative responses. The quantitative response was found to be robust. In a next step, a response surface design (CCD) was applied to examine the most important factors on the qualitative response resolution more closely, in order to predict the best resolution in the same domain as for the PB design, i.e., where the method is robust. In references 84 and 85, the number of experiments of the applied design is not specified. However, it was assumed to be the same as in references 82, 83, and 86, i.e., 15 from a reflected two-level PB design, since the papers are from the same research group, also three levels were investigated for each factor, and the same data analysis was applied.

(c) Responses:

Studied responses were resolution,[75–77,79,81–86] analysis time,[75] migration time,[76,77,80,86] plate count,[76,81–83,85,86] tailing factor,[76] tablet content,[76] peak area,[77,80–86] peak height,[85] peak width,[86] and peak area/migration time ratio.[78]

(d) Analysis of Results:

In reference 75, ANOVA was used. In the literature, such ANOVA approach with *F*-tests is regularly used to evaluate the results of screening designs.[6,24,97] In fact, this approach is equivalent to the *t*-test approach described earlier.[6,97] In reference 76, a graphical analysis by means of standardized Pareto plots and a statistical analysis, based on three dummies to estimate the critical effects, were used. In reference 77, results were analyzed with multiple linear regression (MLR) and partial least squares (PLS) models. The reason to consider the latter method is not very clear, since it uses latent variables and not the examined factors. Alternatively, the applied 12-experiments PB design with two dummies allows calculating the factor effects (Section VII.A and Equation (3)), and critical effects based on the algorithm of Dong (Section VII.B.2.(c) and Equations (9), (12), and (13)). In reference 78, graphically, a bar graph, indicating the absolute effect value of each factor, was drawn, and statistically the error was calculated based on three dummy effects. In reference 79, the estimated effects were analyzed graphically, but the type of plot was not specified or shown. Critical effects based on the algorithm of Dong could alternatively have been calculated. Defining critical effects based on the two available dummies[77,79] seems less appropriate since the t_{critical}-values increase dramatically when the number of df is smaller than three, resulting in overestimated critical effects and important factors incorrectly considered non-significant. In reference 80, results were treated graphically and statistically with the NEMROD software,[30] but no further details were provided. In reference 81, a graphical analysis by means of normal probability plots was performed.

In references 82–86, the results were treated statistically. Main effects and standard errors were calculated. In references 83, 85, and 86 also a graphical interpretation by means of bar plots was performed. Both positive and negative effects were seen on these plots, but all effects between levels [−1,0] are negative, while all those between [0,+1] are positive. Possibly, the length of the bars represents the absolute value of the factor effects, and all effects for the interval [−1,0] seem to be given a negative sign, while all those for [0,+1] a positive. However, the above are assumptions since no details were provided. In references 83 and 86, critical effects are drawn on the bar plots.

An alternative approach to analyze the data from the reflected PB designs[82–86] would be to calculate the effects between [−1,0] and [0,+1], and then calculating critical effects between [−1,0] and [0,+1] with the algorithm of Dong (Section VII.B.2.(c)). Since normally there is no reason why the error estimates for the intervals [−1,0] and [0,+1] would be different, they could be pooled,[14] resulting in one error estimate and one critical effect.

4. Response Surface Designs

In references 20 and 87–92, response surface designs were applied to evaluate the robustness of CE methods. In references 20 and 89–92, information regarding the robustness was gathered from the response surface design used in optimization, while in references 87 and 88 such design was specifically chosen for the robustness test. Table 13d shows the applied designs. The methods tested concern determinations of ranitidine and related substances,[87] ethambutol and impurity,[88] and didanosine and impurities[91] in drug substance and/or pharmaceutical formulations. Also, chiral separations of a new NSAID,[89] dimethindene,[20] a new chiral compound[90] in drug substances, and of adrenaline in local anesthetic solutions[92] were examined.

(a) Factors:

Examined factors were voltage,[87,88,90,91] buffer or electrolyte concentration,[87,88,90–92] buffer or electrolyte pH,[88,89,91] chiral selector concentration,[89,90,92] capillary temperature,[89] detection wavelength[20] and its bandwidth,[20] reference wavelength[20] and its bandwidth,[20] peak width,[20] threshold,[20] data acquisition rate,[20] filter[20] and its peak width,[20] and surfactant concentration.[91]

(b) Experimental Designs:

Table 13d shows the applied response surface designs. Although in references 89–91 the designs were performed during method optimization, the factor level intervals are not that large and can in fact also be considered as robustness test intervals, though they are somewhat larger than intervals based on uncertainty (Section III.B). In reference 92, on the other hand, the intervals are larger. This will increase the probability that a given factor has a significant effect in the interval examined.

(c) Responses:

Studied responses were resolution,[20,87–92] migration time,[87–90] concentration,[88] plate count,[88] peak area,[20] signal-to-noise ratio,[20] and run time.[91]

(d) Analysis of Results:

To analyze response surface designs, a model is fitted to the data for each response. Usually the results are visualized in response surface plots, showing the change in response as a function of two factors.[20,87–92] These plots allow deciding on the optimal conditions. However, as already mentioned in Section IV, these response surfaces seem not so useful when only small variations around the nominal conditions are examined.

In reference 88, response surfaces from optimization were used to obtain an initial idea about the method robustness and about the interval of the factors to be examined in a later robustness test. In the latter, regression analysis was applied and a full quadratic model was fitted to the data for each response. The method was considered robust concerning its quantitative aspect, since no statistically significant coefficients occurred. However, for qualitative responses, e.g., resolution, significant factors were found and the results were further used to calculate system suitability values. In reference 89, first a second-order polynomial model was fitted to the data and validated. Then response surfaces were drawn for

each response and Derringer's desirability functions[14,98,99] were used to optimize the responses simultaneously. The method was considered robust since a flat surface was observed around the optimal conditions, which means that the responses were not influenced by variations in method conditions. In reference 90, a second-order polynomial model was fitted to the data. Derringer's desirability functions and a so-called "separation dashboard" (effect plots), describing the influences of the factors on each of the responses, were used to optimize the responses simultaneously. In reference 91, a PLS2 model was used to simultaneously model several examined responses. Bar plots of the regression coefficients and response surface plots of the responses as a function of the significant factors were drawn. Conclusions about the method robustness in references 87–92 were mainly drawn from evaluating the response surface plots around the nominal conditions.

In reference 20, a typical robustness test is not performed, but a study on the influence of peak measurement parameters is reported on the outcome. The study is special in the sense that no physicochemical parameter in the experimental runs is changed, but only data measurement and treatment-related parameters. These parameters can largely affect the reported results, as shown earlier, and in that sense they do influence the robustness of the method. The different parameters (see above) were first screened in a two-level D-optimal design (9 factors in 10 experiments).[14] The most important were then examined in a face-centered CCD, and conclusions were drawn from the response surfaces plots.

X. SUMMARY AND CONCLUSIONS

In this chapter, the possibilities to set up and treat the results of a robustness test were reviewed (Sections I–VIII). Robustness usually is verified using two-level screening designs, such as FF and PB designs. These designs allow examining the effects of several mixture-related, quantitative, and qualitative factors, on one or several responses, describing either quantitative and/or qualitative aspects of the analytical method.

Most frequently, the design results, or more specifically the factor effects, are analyzed graphically and/or statistically, to decide on method robustness. A method is considered robust when no significant effects are found on responses describing the quantitative aspects. When significant effects are found on quantitative responses, non-significance intervals for the significant quantitative factors can be defined, to obtain a robust response. However, no case studies were found in CE where such intervals actually were determined.

The method can be robust concerning its quantitative aspect, but non-robust regarding one or more qualitative aspects, i.e., significant effects are found on responses, such as, resolution. Then SST limits can be mathematically or experimentally derived, based on the results of the robustness test. These SST limits correspond to the interval in which a qualitative response is allowed to vary, to still obtain a quantitatively robust method.

Finally, a review of robustness testing of CE methods was made and the tests were critically discussed (Section IX). Some researchers use the OVAT procedure, which seems less appropriate for a number of reasons. Some use response surface designs, which also seems less preferable in this context. Another remarkable observation from the case studies is that only in a minority the quantitative aspect of the method is considered in the responses studied, even though that was the initial idea of proposing the robustness tests.

Given the instrumental transfer problems for CE methods, a robustness test should include besides the instrument on which the method was developed at least one alternative instrument, when the method is to be applied on different instruments or in different laboratories. However, obtaining an analytical CE method that is considered robust from a test that included different instruments does not guarantee a successful transfer to a third instrument or the absence of problems during an interlaboratory study.

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10

VALIDATION OF ANALYTICAL METHODS USING CAPILLARY ELECTROPHORESIS

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ABSTRACT

This chapter deals with the validation of capillary electrophoresis (CE) methods. It describes the various validation characteristics, namely accuracy, precision, specificity, detection limit, quantitation limit, linearity, and range in accordance with the official guidelines. Practical aspects related to the calculation of these parameters and factors affecting them in CE analysis have also been described. Validation requirements have been described according to the goal of the method. The chapter contains numerous tables and diagrams to illustrate these ideas. It also covers other related aspects such as instrument qualification, revalidation, and method transfer.

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I. METHOD VALIDATION

A. Introduction

The validation of analytical methods is a task which is generally accepted to be an inherent part of enfolding quality assurance systems. There are various approved references (recommendations and guidelines) for realization and interpretation of assay performance and proficiency testing for the quality control of analytical methods. For pharmaceutical methods, guidelines set by the United States Pharmacopoeia (USP), the International Conference on Harmonization (ICH), and the Food and Drug Administration (FDA) provide a framework for performing such validations (cf. Section I.B). Thus, when evaluating electrophoretic procedures, the validation must be in stringent compliance with these current guidelines to be accepted in all parts of the world. Where there is reliable experience of the use of an analytical technique (such as high performance liquid chromatography (HPLC) or gas chromatography (GC)), the validation can scale down to several significant parameters. For capillary electrophoresis (CE), as a mature but still younger technique, validation studies need to provide stronger evidence and more parameters should be considered in detail.

Validation is the process of proving that a method is acceptable for its intended purpose. It is important to note that it is the method not the results that is validated. The most important aspect of any analytical method is the quality of the data it ultimately produces. The development and validation of a new analytical method may therefore be an iterative process. Results of validation studies may indicate that a change in the procedure is necessary, which may then require revalidation. Before a method is routinely used, it must be validated. There are a number of criteria for validating an analytical method, as different performance characteristics will require different validation criteria. Therefore, it is necessary to understand what the general definitions and schemes mean in the case of the validation of CE methods (Table 1). Validation in CE has been reviewed in references 1 and 2. The validation of calibrations for analytical separation techniques in general has been outlined in reference 3. The approach to the validation of CE method is similar to that employed for HPLC methods. Individual differences will be discussed under each validation characteristic.

B. Requirements and Guidelines

ISO defines validation as: "Conformation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled." This is decided by using a number of performance characteristics. These are specificity, linearity, range, accuracy, precision, detection limit (DL), quantitation limit (QL), and robustness. System suitability testing (SST) is an integral part of many analytical procedures. Definitions of these terms based on the recommendations of the ICH Guideline Q2 (R1) are given in Table 2.[4–6]

Considering the variety of analytical methods, it becomes obvious that different test methods require different validation schemes. ICH distinguishes mainly four different cases shown in Table 3. It is the responsibility of the applicants to choose the validation procedure and protocol most suitable for their method because different performance characteristics will require different validation criteria.

The FDA acknowledges the USP Validation of Compendial Methods as the legally recognized specifications to determine compliance in the USA[7,8].

The USP requirements for assay validation are very close to the ICH proposal.[9] Here, three categories are distinguished. Category I corresponds to ICH assay, category II corresponds to ICH determinations of impurities. The additional category III includes analytical methods for the determination of performance characteristics (e.g., dissolution, drug release). For this category, the ICH assay characteristics are always sufficient. The objective of the analytical procedure

TABLE 1 Important Aspects of Method Validation[1,2,20]

Aspects which are generally relevant to validate analytical methods

Precision
- Wavelength accuracy and reproducibility; reproducibility of sensitivity/response factors
- Heteroscedasticity
- Short- and long-term precision of migration time and peak area (= repeatability, intermediate/day-to-day precision, etc.)
- Rules to exclude outliers

Specificity
- Peak purity (peak homogeneity) of all relevant peaks, freedom from matrix interferences
- Peak shapes and efficiencies

Linearity
- Rectilinearity

Range

Detection limit (DL)

Quantitation limit (QL)

Robustness

Accuracy
- Cross-validation
- Recovery

Additional aspects which are relevant to validate CE methods

EOF stability
Rinsing procedures
Stability and shelf life of buffers and sample solutions
Reproducibility between capillaries

should be clearly understood since this will govern the validation characteristics which need to be evaluated. In some cases, tests for linearity, accuracy, and specificity are considered to be not necessary. According to the USP, tests for ruggedness are generally recommended (Table 3).

Although there is a general agreement about what type of studies should be done, there is a great diversity in how they are performed. The guidelines and the Pharmacopoeia intentionally give only very general definitions in order to allow reasonable procedures for the validation of many different techniques. This chapter discusses an approach for performing CE validation studies. Validation of analytical procedures for biological and biotechnological products could in some cases be differently approached due to their complex nature. Commonly, there are certain requirements and guidelines analyzing these products.

C. Basic Principles of Method Validation

During the method development, key method parameters are determined and used for subsequent validation steps to ensure that the validation data are generated under conditions equivalent to the final procedure (risk analysis).[10] Aims of the method development are summarized in the list that follows.

- detection of all compounds of interest (purity control)
- separation of all compounds of interest
- quick method development

- short analysis time
- reduced need for sample pretreatment
- high reproducibility of migration time
- high reproducibility of peak area, relative peak area (main component assay)
- accuracy (main component assay)
- ruggedness
- low costs
  - during method development and
  - during routine analysis

A validation protocol adapted from the experiences during the method development defines the scope of the validation study (goal of the study, regulating guidelines, key method parameters, etc.). To investigate the adequate method performance, these features (e.g., range of analyte concentration), together with a statement of any fitness-for-purpose criteria, have to be specified in the validation protocol. A basic check has to provide that the reasonable assumptions about the principles of the method are not seriously flawed. In this process, sources of error in analysis have to be listed (Table 4) and their effects have to be checked. The validation should, as far as possible, be conducted to provide a realistic survey of the number

TABLE 2 Glossary for Method Validation According to ICH[5] and Others

Name	Definitions
Accuracy	Closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value[5]
Intermediate precision (day-to-day precision)	Precision including within-laboratory variations, e.g., different days, different analysts, etc.[5]
Detection limit (DL)	Lowest amount of analyte that can be detected but not necessarily quantitated as an exact value[5]
Quantitation limit (QL)	Lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy[5,6]
Precision	Closeness of agreement between a series of measurements[5] (general term includes repeatability, intermediate precision, and reproducibility)
Range	Interval between the upper and lower concentrations[5]
Recovery	Ratio of measured and spiked amount[4,5]
Repeatability (intra-assay precision)	Precision under the same operating conditions over a short interval of time (e.g., 10 subsequent injections)[1,5,6,34]
Reproducibility (ruggedness)	Precision between laboratories, usually determined by collaborative studies[5]
Robustness	Capacity to remain unaffected by small, but deliberate, variations in method parameters[5,6]
Ruggedness (reproducibility)	Degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions[9]
Sensitivity	Slope of the calibration function[58]
Specificity	Ability to assess unequivocally the analyte in the presence of components which may be expected to be present[5,6]
Validation	Process of proving that a method is acceptable for its intended purpose[4]

TABLE 3 Validation Requirements[5]

	Type of analytical procedure			
		Testing for impurities		
Performance characteristic (cf. Table 2)	**Identification**	**Quantitation**	**Limit**	**Assay**
Accuracy	−	+	−	+
Precision	−	+	−	+
Repeatability	−	+	−	+
Intermediate precision	−	+[a]	−	+
Specificity[b]	+	+	+	+
Detection limit (DL)	−	−[c]	+	−
Quantitation limit (QL)	−	+	−	−
Linearity	−	+	−	+
Range	−	+	−	+

"−" Signifies that this characteristic is not normally evaluated.
"+" Signifies that this characteristic is normally evaluated.
[a]In cases where reproducibility has been performed, intermediate precision is not needed.
[b]Lack of specificity of one analytical procedure could be compensated by the other supporting analytical procedure(s).
[c]Essential to some extent.

TABLE 4 Source of Error in Analysis[10]

Random error of measurement (repeatability)
Run effect (seen as a bias in one run, and random variation over several runs)
Laboratory effect (seen as a bias by a single laboratory)
Method bias
Matrix variation effects

and range of effects operating during normal use of the method. The performance of the required validation parameters (Sections I.D−I.I) set by the guidelines (cf. Section I.B) should occur against the background of the basic principles of method validation and the results should be documented in a validation report.

Generally, the weak points of methods should be quickly identified and eliminated, thus every validation parameters should be tested as early as possible. If the method fails with respect to one of the parameters, the entire method has to be changed. Consequently, the validation must be started again. The development and validation may be an iterative process (Diagram 1). However, it is beneficial to keep the iterative steps to a minimum.

D. Range

Before a calibration is started, the lowest and highest concentration, i.e., the concentration range of interest, must be defined. Then the method must be validated over the entire range. The range of an analytical method is the interval between the upper and

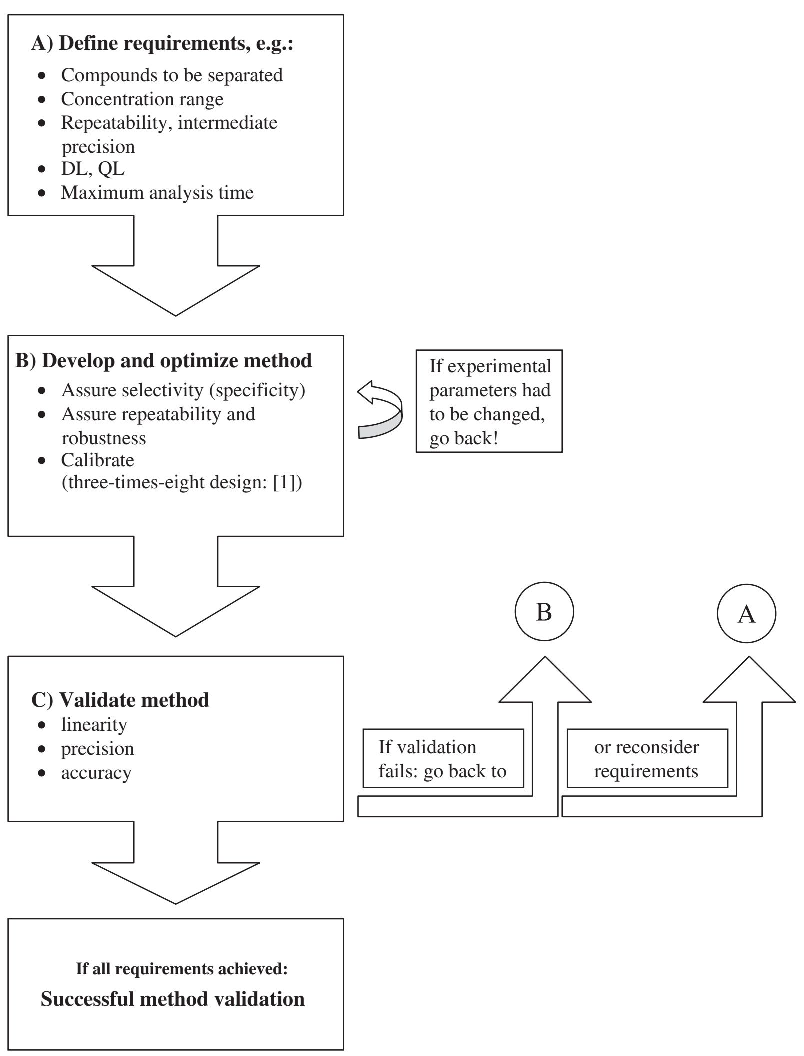

DIAGRAM 1 **Validation concept.**

lower levels of expected analyte concentrations or amounts (including these levels) over which acceptable accuracy, linearity, and precision are required.

Sample variabilities and the measurement error must be considered (risk analysis, cf. Section I.C) to avoid that an analyte signal y_0 will be measured outside the calibrated range. Thus, the range shall be chosen a little wider than the expected range of analyte concentrations.

A rather wide range must be calibrated for pharmacokinetic measurements. The value x_{max} must exceed the highest expected body fluid concentration; x_{min} is the QL. The range is smaller for a stability test, where x_{max} corresponds to the highest concentration that may be expected from the drug formulation, and x_{min} corresponds to the lowest concentration after a time of decay; usually x_{min} will be still about 90% of x_{max}. A very small range is often sufficient in an assay. In this case, a one-point design (only one standard concentration) is appropriate for routine analysis. A one-point design practically means the comparison of one standard with the

analyte. If the signals are the same, then so are the concentrations. If the one-point design is planned for routine use, for method development the later-described calibration design will be preferred because it allows to test for linearity.[3]

E. Specificity (Selectivity)

Specificity is an important validation parameter that should be established as early as possible. Specificity means to measure accurately in the presence of all potential sample components. Depending on the analytical problem, these components can be excipients, dissolving solvents, synthesis intermediates, and process impurities including extraction solvents, the internal standard, or degradation products. The response of the analyte in test mixtures containing the analyte and all potential sample matrices is compared with the response of a solution containing only the analyte. These should be spiked into a test solution at least at their expected level. Any contribution of the matrix in response leads to constant or proportional systematic error as such methods are referred as non-specific.[11] It is also possible to compare the results with those of other independent methods, preferably based on different separation principles.[12] Because the peak width depends strongly on the injected amount, the effects of a possible sample overloading must be investigated.[8,13] Appropriate selectivity of the method requires the complete separation of all analytes of interest and all other detectable sample components.

If there is no reference material for degradation products, the analyte should be exposed to stress conditions such as light (600-foot candle),[11] heat (50°C), acid and base (0.1 M HCl and 0.1 M NaOH), and oxidant (3% H_2O_2).[1,4,5]

The validation of the peak purity is important for selectivity. The efficiency should be optimized to avoid co-elution of different analytes. Two-dimensional detection is a quick and convenient method to check peak purity. CE/UV coupling is most common,[14–19] and instruments are commercially available.[20–22] The possibilities of CE/MS have already been discussed. The use of several separation systems with different selectivity is a more time-consuming method to test peak purity.

In some cases, the signal is still influenced by the sample matrix despite peak purity, e.g., because of sample pre-treatment operations. If matrix effects are considered to be very unlikely, the following check can be skipped. They would still be discovered testing for accuracy. However, matrix effects lately discovered imply a lot of extra work, since the method must then be changed and validated once again.

To check influences caused by the matrix, analytes in different concentrations (at least at the upper and lower ends of the range) are spiked into the expected matrix. This should be blank, which means contain everything of the real sample (e.g., excipients, body fluids, etc.) but the analyte. The obtained signals are compared to signals from samples without matrix (recovery). If these signals do not significantly differ, this also gives strong evidence for accuracy (cf. Section I.J).

If the matrix just causes an additional signal which is the same for all analyte concentrations, the calibration results are not negatively affected. However, a change in sensitivity caused by the matrix may occur if the formation of complexes is hindered or favored by the matrix. In these cases, sensitivity changes in UV/Vis spectroscopy, and may change for derived techniques like CE/UV.[3]

Working with blank matrix, the calibration standards are spiked into the matrix to avoid false estimations. It should be checked if the same sensitivity is obtained using different batches of blank matrix.

However, sometimes blank matrix is not available. For example, this can occur with minerals, plant ashes, and lyophilized materials.[3,4] Here, it is necessary to use the design of standard addition. It should be emphasized that standard addition is restricted to this

situation only. If and only if the sensitivity of an analytical method is influenced by the sample matrix, there is no blank matrix, and there is no appropriate alternative technique, the method of standard addition must be used.[3] It is also necessary when using substituted cyclodextrins (CDs) for enantiomeric separations to confirm that CDs from different suppliers give similar selectivity, as different degrees of substitution, polydispersity, or purity of the CDs, even using identical nominal reagents, may alter the selectivity.[23]

F. Robustness

CE is not yet as widespread as analytical techniques such as HPLC and GC. Especially as a more recent technique, it needs to demonstrate sufficient robustness to be considered as a conventional routine analytical technique. Robustness (see Chapter 9) of a method should always be tested before starting to calibrate. If the method is later found to be not robust, parameters have to be changed. Then the calibration and consequently its validation have to be repeated.

For each technique all those parameters should be varied during robustness testing which are expected to slightly vary during routine use in one laboratory. All parameters that are relevant for the analytical result should be observed, worst case changes should be considered (Table 5). In CE, robustness testing can be easily performed in comparison with HPLC because of the short equilibrium time when changing the composition of electrolyte. In addition, the separation is generally rapid, which means that robustness testing may be performed within a short time.[12]

In general, analytical solutions of different age should be included. In contrast to HPLC in which the mobile phase is usually prepared daily because of the considerable volume that is required, in CE there is a low consumption volume (typically 10−20 ml per day) of electrolyte solution, which can be prepared for several days, weeks, or even months. Hence, a longer shelf

TABLE 5 Relevant Parameters for Robustness

Parameters to vary in order to test robustness
Analysts
Buffer pH
Instruments, or at least detector lamps
Age of analytical solutions
Temperature
Ionic strength, buffer concentrations
Rinse times
Equilibration conditions
Additive concentrations
Batches of reagents
Detector wavelength
Sample loading conditions (t_{inj}, Δp)
Capillaries (lots and/or suppliers)
Parameters to measure in order to test robustness
Resolution of critical peak pairs
Efficiencies (mean and standard deviations for all relevant peaks should be given for)
Migration times (especially t_{EOF})
Peak areas
Relative peak areas (to the migration time)

TABLE 6 Experimental Parameters to Define Method

Buffer: pH, molarity, recipe: weight or volume of all chemicals used
Sample solvent
Separation: pole outlet, *U*, *I*
Capillary: material, i.d., *l*, *L*
Injection: *t*, $U/\Delta p$
Detection: wavelength, instrumentation
Temperature
Rinsing procedures (*t*, reagents, Δp); equilibration times
Shelf time of solutions, if relevant

life has been assigned for several buffer solutions (e.g., for a phosphate-borate, 3 months has been assigned when stored at room temperature, unprotected from light).[24] However, some buffers need to be prepared freshly every day.[25] Similarly, storage times and conditions should be established for sample solutions. If buffers or standard solutions are stable, they should be used for longer times to save time. However, if solutions are not prepared freshly prior to use, this must be mentioned in the description of the method (Table 6).

More than one analyst should conduct the analysis exactly as detailed in the analytical method. Each analyst should independently prepare his or her own sample solutions, capillaries, and reagents. The results generated by one analyst should not differ significantly from those generated by another.

If there are CE instruments of various manufacturers in one laboratory, and all these shall be used to run the method under investigation, it is advisable to test the method on different instruments as well. However, if there is only one CE instrument in the laboratory, it is not necessary to prove that a method will run on another, although it may become sometimes necessary to substitute an instrument. Difficulties in transferring a method from instrument to instrument are only found when the thermostating system is differently constructed. Therefore, it is important to test the method for robustness against temperature changes. If this is obtained, the method will be readily transferable to another instrument. If there is only one CE instrument available for the test of robustness, it is a good idea to keep older detector lamps. Older lamps give more noisy baselines. If the lifetime of a lamp was specified as 1000 h, e.g., a new lamp and another one used for approximately 900 h may be applied for robustness tests.

It is important to use pure reagents, or at least reagents of constant quality. It is necessary to demonstrate that it is possible to repeat the separation using different batches of reagents and materials. Long-term availability of all reagents used must be guaranteed. This is not a problem if a reagent is frequently used. However, less common reagents are rather seldom sold; companies may only temporarily produce them. Thus, these reagents are usually not only expensive but may cause a problem of availability in constant quality (Table 7). Differences in selectivity have been reported using the nominal identical reagents, especially for chiral selectors.[26–29] The use of defined mixtures of single isomers of CDs gives small variation in resolution. For the development of a robust method of enantioseparation, it is possibly more advisable to apply mixtures of defined single isomer CDs for resolution enhancement rather than randomly substituted CD derivates.[30] It is crucial to use well-characterized buffer additives for CE.

It is still not common to validate integration parameters. Several software packages for peak integration do not provide the option to steplessly change the integration parameters, as would be required for a robustness test. In the method descriptions, a range for suitable integration parameters and even video integration is tolerated. A possibility to achieve validated peak integration software has been described in reference 31. However, this software is not yet

TABLE 7 Desirable Reagent Properties

Well soluble in water
Stable in aqueous solution
UV transparent (consider UV absorbing impurities); exception: BGEs for indirect detection
Highly pure, or at least available in constant quality
Well characterized
Long-term commercially available
Not too expensive
Non-toxic

• Separation:	pole at outlet: cathode, U=25 kV (but P<5 W/m)
• Capillary:	fused-silica, 50 µm i.d., as short as possible
• Capillary conditioning:	rinsing with 0.1 M sodium hydroxide for 30 minutes. Then it is filled with the buffer and equilibrated for at least 1 hour. During this time a voltage of 25 kV is applied.
• Injection:	t* Δ p = 2 psi ∗ s (e.g. 4 sec with 0.5 psi), or t* Δ h = 172.5 cm ∗ s (e.g. 17.25 cm during 10 s)
• Detection:	λ_{max} of the analyte of interest, for which weakest signal is expected because of low concentration or low UV absorbance; if unknown: ≈ 230 nm, rise time is 1 s
• Temperature:	25°C
• Rinsing procedures:	running buffer for 2 minutes
• Sample:	dissolved in pure water

DIAGRAM 2 Sensible Setting for Initial CE Experiments.

commercially available. To get the best possible conditions, it is necessary to describe a detailed record of the integration parameters and the integration software in the validation plan.

For robustness testing, it is important to observe all relevant parameters, which include resolution of critical peak pairs and efficiencies. The means and additionally the standard deviations for all relevant peaks should be given for migration times (especially t_{EOF}), peak areas (PAs), and relative PAs (Table 5).[1,20,32]

Before robustness testing is started, precise specification of all method parameters is certainly needed (Table 6); else it may not be clear later which method has been validated. Possible settings for initial CE experiments are shown in Diagram 2. In particular, it is important to specify the buffer composition, e.g., by buffer recipes (Table 8). Otherwise, inadvertent variations in pH or ionic strength can lead to variability in selectivity.

Moreover, rinsing procedures and equilibration conditions should be carefully described. Note that each type of instrument uses different pressure settings, so rinsing times cannot be directly transferred. Therefore, it is preferable to specify a rinsing volume[32] or the product of pressure difference and rinsing time[20] in the method which can be considered instrument independent.

A fractional factorial design is often suggested to observe several parameters at the same time.[1,2,4] Advantages are, among others, the formal sampling plan, which is easy to evaluate by supervisors and auditors. Moreover, a fractional design only needs a fraction (usually about 50%) of experiments compared to a design that tests the relevant parameters one by one.

However, fractional factorial designs are less flexible. The range in which the parameters should be varied must be known beforehand. Thus, it is much better to overestimate rather than to underestimate this range; else the whole experiment might become meaningless. It is possible to include additional data within this experimental design, if the range for one parameter was largely overestimated.

TABLE 8 Standard Buffer Recipes for Initial Experiments

pH	Buffer System	Acid	Base
	Phosphate	85% H_3PO_4[a]	KH_2PO_4[a]
2.0		395.3	349.9
2.5		205.3	574.3
3.0		81.4	720.5
	Acetate	1 M CH_3COOH[b]	CH_3COONa[a]
3.5		5.67	26.6
4.0		5.08	75.8
4.5		3.81	174.6
5.0		2.13	317.6
5.5		0.89	419.1
	Phosphate	$NaH_2PO_4 \cdot H_2O$[a]	$Na_2HPO_4 \cdot 2H_2O$[a]
6.0		779.2	61.9
6.5		692.8	174.3
7.0		512.2	407.2
7.5		280.7	705.9
8.0		115.5	919.0
	Borate	H_3BO_3[a]	$Na_2B_4O_7 \cdot 10\ H_2O$[a]
8.0		320.9	77.3
8.5		232.7	213.2
9.0		59.3	480.6
		$Na_2B_4O_7 \cdot 10\ H_2O$[a]	0.1 M NaOH[b]
9.5		371.0	41.77
10.0		371.0	52.72

Amount[a] of substance (in mg) or solution[b] (in mL), calculated for 100 ml of 60 mmol/L buffer.

It is often claimed that a fractional factorial design saves plenty of working time. However, all experiments of one design have to be evaluated together. This can become a major drawback. Consider a one-by-one test of robustness parameters that might take 2 or 3 weeks. The fractional factorial design may need only 1½ weeks; however, during this time parameters may change, which are beyond the control of the analysts, such as air pressure and moist. The lamp performance will certainly change during the time of the test. What is more, a capillary may break during 10 days and needs to be exchanged. All these parameters would influence the results of a factorial design in an unpredictable way. During a one-by-one design, every parameter is tested within one day. Uncontrollable influences are much less likely. When the capillary breaks, it is just replaced, and only a small part of the series has to be repeated.

A check of robustness includes preliminary experiments on precision. During robustness testing, a single standard is repetitively analyzed before starting the actual calibration. Without sufficient precision at a single concentration, it is fruitless to calibrate. However, the RSD obtained from repeated injections underestimates the overall error by a factor of up to 3.[33]

G. Detection Limit

The DL can be determined without calibration. It is usually defined using the signal-to-noise (S/N) ratio[5]:

$$\frac{S}{N} = \frac{2H}{h_n}, \qquad (1)$$

where H is the height of the measured peak related to the average baseline. The peak-to-peak noise h_n is defined as the difference between the maximal and minimal (baseline) signal measured in a section of about 20 peak widths at half the height, at both sides of the peak. The DL can now be arbitrarily defined as concentration that guarantees an S/N ratio of, e.g., 2 or 3.[5]

DLs for impurities should correspond to less than 0.1% of the main compound;[34] compounds that take effect in lower amounts may require lower DLs. For example, imipramine *N*-oxide hydrochloride impurities and salicylamide impurities[35] have been determined at the 0.01% level. DLs and QLs in CE are generally to some extent higher (in concentration) than in HPLC because of the small optical path (50−100 μm) used for ultraviolet (UV) detection and small injected volume (2−20 nl) compared with HPLC 10 mm optical length and 10−200 μl injected volume.[12]

Several factors could be optimized at the stage of method development to improve DLs and QLs. One possibility is to use a sample solvent of low conductivity to increase stacking effects and increase the analyte signal. The stacking phenomenon in capillary electrophoresis is described in details in reference 36. Using a bubble cell at the detection window will increase the optical path and so improve the detection. Using a larger capillary diameter to have an increased optical path and injected volume, the limit is the increased current and subsequent Joule effect which causes band broadening. Detailed information about effective path length in CE is discussed in reference 37. The DL can also be improved by increasing the injection volume to increase the signal, but the sample should be well solvable in the solvent, and this may have a detrimental effect on the resolution. Detection at a low wavelength (200 nm or lower), which is very common in CE because of the transparency of the separation electrolyte, may often be used for a better DL.[12]

H. Calibration Design

The remaining parameters can only be checked after a test calibration. Here a “three-times-eight” design is suggested. Eight measurements each are performed at the lowest and highest concentration as well as in the middle of the range. This design is beneficial to obtain a low standard deviation of the regression parameters and of the estimated analyte concentrations. At the same time, this design allows to test linearity and homoscedasticity.[3]

I. Linearity

Linearity tests have been comprehensively reviewed and explained in [39]. PAs are generally used as response in CE; however, they can be corrected by dividing them by their respective migration time (t_M) to take into account any possible drift of this parameter.[12] For the establishment of linearity, a minimum of five concentrations has been recommended.[5]

Assessing linearity is an important aspect in calibration work since lack-of-fit will usually lead to biased results. When a simple linear regression model is chosen, the more general test of goodness-of-fit becomes a test of linearity.

Significant lack-of-fit can be detected by various sensible methods. However, the sample correlation coefficient r does not belong to the pool of these methods to assess linearity. The sample correlation coefficient may be misleading and is, despite its widespread use, to be discouraged for two reasons: First, r depends on the slope. That is, for lines with the same scatter of the points about the line, r increases with the slope.[38] Second, the numerical value of the correlation coefficient cannot be interpreted in terms of degree of deviation from linearity. Put differently, a correlation coefficient of 0.99 may be due to random error of a strictly linear relationship or due to systematic deviations from the regression line.

Systematic deviations from the assumed model yield systematic patterns in the residuals and can, therefore, be detected by checking independence of the residuals. Residuals plot, particularly the residuals e_i versus expected values $\hat{y}_i$ plot, is also well suited to detect non-linearities since the plot will show curved patterns instead of randomness.[39]

Formal tests are also available.[39] The ANOVA lack-of-fit test[40] capitalizes on the decomposition of the residual sum of squares (RSS) into the sum of squares due to pure error $SS\varepsilon$ and the sum of squares due to lack of fit SS_{lof}. Replicate measurements at the design points must be available to calculate the statistic. First, the means of the replicates $\bar{y}_k$ ($k = 1, \ldots,$ m $\equiv$ number of different design points) at all design points are calculated. Next, the squared deviations of all replicates $(y_{j,k}\bar{y}_k)^2$ [$j = 1, \ldots,$ number of replicates] from their respective mean are calculated and summed. This sum of squares is the estimator of pure error and is called sum of squares due to pure error $SS\varepsilon$. Suppose all means of the replicates ($\bar{y}_k = \hat{y}_k$) were lying on the fitted regression line, then, the RSS would be identical with $SS\varepsilon$ and there would be no lack of fit. If not all means are lying on the regression line (the usual case), RSS consists of two portions: the sum of squares due to pure error $SS\varepsilon$ and the sum of squares due to lack of fit SS_{lof}. Subtracting $SS\varepsilon$ from RSS yields SS_{lof}. If the deviation of the individual means from the regression line gets severe, then SS_{lof} will make up the main portion of the RSS, i.e., the portion of $SS\varepsilon$ that is subtracted from RSS will be small. If so, lack-of-fit is indicated. To test for lack-of-fit, the mean squares (MS) are calculated, i.e., $SS\varepsilon$ and SS_{lof} are divided by their respective degrees of freedom [$SS\varepsilon/(n-l)$, $SS_{lof}/(l-p)$, $p =$ number of parameters, equals 2 for a straight-line model], and the variance ratio of the resulting MS_{lof} and $MS\varepsilon$ can be formed (F-test), which is, in the case of goodness-of-fit, a random variable that follows an $F_{(lp),(nl),\alpha}$ distribution.

The ANOVA test, which is also recommended by the Analytical Methods Committee of The Royal Society of Chemistry (UK), can be generalized to other regression models, and it can be extended to handle heteroscedasticity. For a more detailed prescription and the extension of the test see further reading.[41]

It should be emphasized that if the concentration range is greater than one order of magnitude (long-range calibration), violations of the constant variance assumption of OLS are frequent.[39,42] Especially, homoscedasticity is rarely found; hence, a more general model needs to be applied.

The detector linearity for a trace enantiomer in the presence of the main peak has been successfully demonstrated in several examples (references 1 and 43 and references cited therein). Usually the linearity of PAs can readily be shown in CE. However, the use of peak heights is restricted because they are often non-linear. Linearity and a significantly positive intercept β_0 can be due to an undesirable siphoning effect during injection. For an active ingredient assay in a formulation, the linearity is assessed in the range 50−150%, 80−120%, or 60−140% of the target concentration.[44]

For an impurity, the linearity should be tested in the presence of the main component around the maximum tolerated level of impurity (e.g., from the QL to 200% of the maximum tolerable level).[12]

For dissolution testing, the range should cover $\pm 20\%$ over the range specified for batch release.[45] One should keep in mind that the linear range of the UV detection in CE is more restricted than in HPLC because of the circular geometry of the capillary used for detection, which increases light scattering. However, the linear range is sufficiently extended for most applications, even if assay and purity determinations are performed in a single injection.[12]

J. Precision and the QL

A method must be acceptable for its intended purpose over the entire range. Thus, precision and accuracy must be acceptable over the whole range and should be determined at least at the upper and lower ends. There are four types of precisions that can be determined for

an analytical method, namely instrument precision or injection repeatability, repeatability or intra-assay precision, intermediate precision, and reproducibility. Repeatability of both PA and migration time is used to test precision. Instrument precision is determined by repeated measurement of one sample solution so as to test instrument performance. The repeatability or intra-assay precision is obtained by repeatedly analyzing independently prepared homogenous samples in one laboratory, by one operator, using one piece of equipment and set of reagents on one day. At least five determinations of three concentrations at low, medium, and high range of calibrations are performed and the percent relative standard deviation (RSD) is calculated.[11]

Repeatability is automatically tested during robustness testing. It should be better than 2% RSD for the area of a main peak without IS, less than 1% using relative PAs or analyte/IS, and better than 10% RSD for a trace impurity. Repeatability in CE is generally lower than in HPLC because of the small injection volume (between 2 and 20 nl), but can be significantly improved by the use of internal standard.[12]

Intermediate precision has been tested by repetitive analysis on five separate days.[46] For each day the electrolyte, sample, and standard solutions should be freshly prepared to include errors from, e.g., weighing and diluting. Variations to be studied also include multiple analysts, multiple equipment, and multiple sets of reagents in the same laboratory.[5]

From a statistical point of view, precision can best be described using Equation (2), the confidence interval of the analytical result:

$$\mathrm{cnf}\{x_0(y)\} = x_0(y) \pm t_{n2}^{1\alpha/2} \frac{\hat{\sigma}}{\hat{\beta}_1} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(x_0(y)\bar{x})^2}{\sum(x_i\bar{x})^2}} \tag{2}$$

The simplified term $\hat{\sigma}/\hat{\beta}_1$ (standard deviation of the residuals divided by the estimated slope of the calibration line), the procedural standard deviation, will often be accepted as a measure of precision.

The dependence of precision on different parameters has already been discussed. Precision is strongly dependent on the constancy of migration data. Thus, the stability of the EOF is most important. Buffer recipes describe clearly the preparation and avoid errors caused by, e.g., a poorly calibrated pH electrode.

The alteration of the capillary walls by adsorption or other effects can be diminished by suitable equilibration and rinsing procedures. Routine analysis should be done on dedicated capillaries.

Drifts of migration times can partly be compensated by calculating the mobility for analyte identification and using corrected PAs or internal standards for quantitation.

The control of temperature is most important because the reproducibility of sampling, electroosmosis, and electrophoretic mobility depends on it. The power should be limited to avoid excess heat.

Besides constant migration times, a constant current is a good indicator for stable conditions.[47] Thus, the current should always be documented. Sometimes it is favorable to use constant current instead of constant voltage.[48]

To determine the QL, it is necessary to agree on acceptable limits for precision and accuracy. Commonly, precision is given as a maximal acceptable RSD of the analytical result, $\mathrm{RSD}_{\mathrm{max}}$. Then the QL can be calculated using

$$\mathrm{QL} = \frac{100\%}{\mathrm{RSD}_{\mathrm{max}}} \cdot \frac{\hat{\sigma}}{\hat{\beta}_1} \tag{3}$$

This also is the ICH proposal. For $\mathrm{RSD}_{\mathrm{max}}$, a limit of 10% RSD seems sensible. Steps for the development of quantitative CE methods and precision requirements are summarized in Diagram 3.

- **avoid adsorption**
- **use a short capillary**
- **increase the sample concentration**
  - limited by solubility and
  - separation efficiency
- **choose detection wavelength at maximum absorbance of the analytes**
  - using short wavelengths (< 250 nm): degas buffers,
  - use buffers with high UV transparency
- **use Internal Standards or relative peak areas to calculate results**
  - if RSD% < 2% is required
- **if repeatability (n=10, cf. I.B., Table 2) still much worse than 1% RSD% for relative peak areas**
  - consider[54]
  - check instrument (**cf. II**)
  - consider troubleshooting[57]
- **if intermediate precision (cf. I.B., Table 2) still much worse than 1.5% RSD% for peak areas**
  - reconsider migration time precision

DIAGRAM 3 Quantitation.

K. Accuracy

Accuracy is determined after a test calibration using one out of the four following ways. The difference between precision and accuracy is clearly shown in Figure 1. In the first case, if available, e.g., from the National Institute of Standards and Technology (NIST), reference samples (e.g., pharmaceutical in matrix) with defined true reference values are analyzed. Then true and measured values can directly be compared, but this case is rare.

The second possibility is called cross-validation. The test samples are measured by a reference method. However, the reference method cannot provide true values because measurement error occurs here as well. Nevertheless, well-characterized methods can provide generally accepted values, which are then compared to the ones obtained using the test calibration. Note that this comparison must be done using particularly suited regression methods,[39] because the error for both methods will be in the same order of magnitude. Especially, cross-validation of CE and HPLC has been frequently reported.[1,43]

For comparison of impurity levels quoted as % area/area, the normalized PA [area divided by the respective MTs, often stated as corrected PA (A_C)] must be used in CE to compensate for the residence time difference of the species in the detector. In HPLC, the separation takes place on the column. After the column, all analytes travel through the detector at the same speed (that of the mobile phase) and hence have the same residence time in the detector cell. However, in CE, the electrical field also takes effect in the detection cell. Therefore, the residence time of the species that have a higher apparent mobility (as shorter t_M) will give a lower response than species with a lower mobility, for species with the same absorptivity and concentration.[12]

In a third case, known amounts of analytes are spiked into a blank matrix. Then the recovery is determined, which is the ratio of measured and spiked amount[4,5] (see examples[1]).

The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a

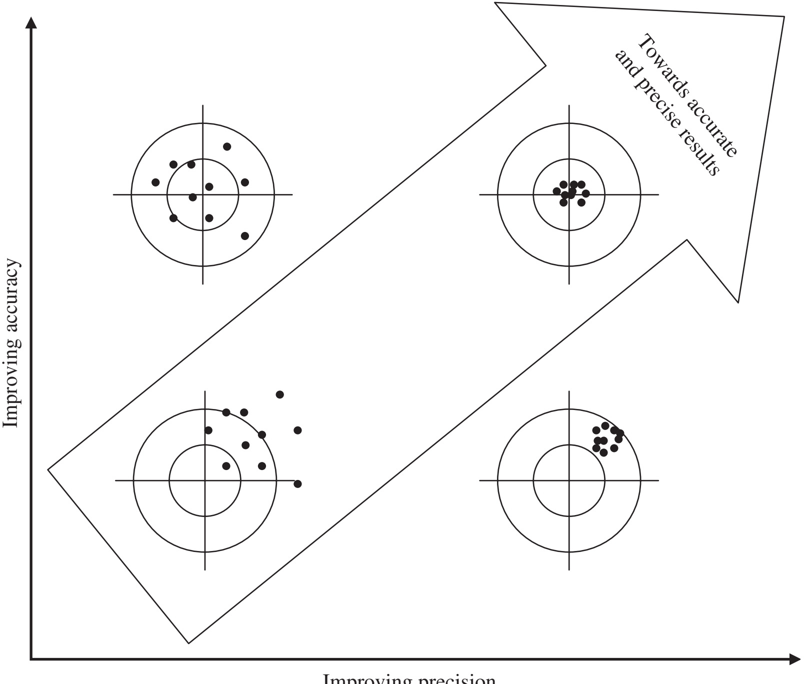

FIGURE 1 Accuracy and Precision.

blank sample matrix without the presence of the analyte, as is the case for lyophilized material in which the speciation in the lyophilized material is significantly different when the analyte is absent.[11]

It is beneficial to develop "generic methods,"[2] this means methods that are already validated for some aspects, e.g., EOF stability and freedom of matrix interferences. Generic methods that are suitable to separate a number of analytes can be found in CE, especially due to the high separation efficiency. Some aspects, like specificity and repeatability, are still analyte specific, but the entire method validation is substantially speeded up when a generic method already exists.

L. Revalidation

According to the ICH guideline, revalidation of a CE method is necessary in case of a major change, e.g., in the synthesis of the drug substances, in the composition of the finished product, or in the analytical procedure [ICH Q2 (R1)].[5]

II. INSTRUMENT QUALIFICATION

Similar to method validation, it is important to be able to demonstrate that analytical instrumentation is fit for its intended purpose and that it is calibrated and maintained in an appropriate state of readiness.[49] The verification of instrument qualification (see Chapter 8)

TABLE 9 Important Aspects of Instrument Qualification/Validation and System Suitability (Cf. References 1, 2, and 20)

Relevant parameters for full instrument validation
Reproducible slope (= sensitivity, response factor); linearity
Wavelength accuracy
Efficiencies, peak shapes
Short- and long-term precision of migration time, peak area, etc. (= repeatability, intermediate/day-to-day precision, etc.)
Detection limit (DL), Quantitation limit (QL)
Relevant parameters for system suitability
Repeatability of migration time and peak area
Resolution
Parameters that should be re-validated after method transfer
Repeatability of migration time and peak area
If trace impurities are investigated: DL

means the systematic inspection of all relevant instrumental parameters. Most parameters can only be tested by a reference method. This method must be validated, and parameters obtained with a validated instrument using this method must be known. When this reference method is used with another instrument, the obtained parameters can be compared to the ones obtained with a validated instrument. Aspects of importance are listed in Table 9.

The CE instrument should be validated by a standard method with known migration data and S/N ratio.[50,51] The test method described in reference 20 has proven to be very useful for instrument validation as well. Note that flushing times and conditions (e.g., pressure) must be adjusted for method transfer (Section III) between different instruments. Typical values for performance parameters of several CE instruments are listed in reference 20. Wavelength accuracy can be checked by filling the capillary with a standard solution with well-known spectral properties (e.g., *p*-hydroxyacetophenone).[52] No voltage is applied. When the wavelength is subsequently changed, a spectrum of the standard solution is obtained. If the measured values are out of specification, troubleshooting may be required.

Full instrument validation should be regularly repeated: at least half a year routinely, even if routine measurement was successfully performed every day. Unscheduled validation is recommended if valuable samples will be analyzed next, if the lamp exceeds its specified lifetime, or if unusual performance data are obtained although validated methods were used.

In addition to that a quick system-suitability check should be done every day and when a system is restarted. The system-suitability test particularly emphasizes on resolution and sensitivity (single/concentration) or the DL. To save time, the system suitability test should restrict to repeatability of migration time and PA, and to resolution of relevant peak pairs.

SST must be performed before and throughout all regulated assays. It is no longer sufficient to apply SST at the beginning of the electrophoretic run and to assume that the system will function properly during the experiment. Moreover, a single-component calibration solution to check system suitability is not adequate because the separation capability of the system is not demonstrated. The use of statistical analysis [e.g., Plackett and Burman or other fractional factorial designs (cf. Section I.F) on data gathered during method optimization or validation] is in line with guidance from ICH, which regards SST as one of the method validation steps.

A validation test certificate, at least according to the system-suitability protocol, should be supplied when an instrument is sold.

III. METHOD TRANSFER

Methods have successfully been transferred to various laboratories in inter-company cross-validation exercises for a chiral separation,[32] for an assay of the main component in a formulation[53] and for drug stoichiometry.[1,54] Revalidation is an alternative to method transfer in case of changes in product composition or analytical procedure (cf. Section I.L). Although a method transfer in CE is not a major difficulty, some aspects have to be considered, especially if a method is transferred to an instrument of another manufacturer.

When a method is transferred to another instrument of the same type, a check for repeatability is sufficient. If repeatability is not obtained, usually the instrument is the reason.

Different types of CE instruments have different thermostating systems, have different detectors, use capillary of different lengths, have detection windows at different distances from the injection point, and have different injectors. Thus, additional tests may be required after a method transfer.

The temperature is the most important parameter for CE method transfer. A reproducible method is defined by a temperature set for one instrument. However, if the same temperature is set for another instrument, this does not mean at all that the same temperature is obtained within the capillary. It is not possible to have the whole capillary thermostated. A part must remain unthermostated, e.g., the part that dips into the buffer and the part in the detector cell. The proportion of the non-thermostated part differs for different instruments.

Moreover, the indicated temperature does not even correspond to the temperature of a certain section of the capillary. The temperature is never measured inside the capillary, but at best right at the outside. In many cases, there is even a considerable distance between temperature sensor and capillary. Furthermore, even the sensor does not give a true temperature but it has to be calibrated, which may be done in different ways.

Therefore, it is very hard to compare the set temperature from one instrument to another. If it is intended to transfer a method to an instrument of another type, the only solution is to develop a method that is robust against temperature changes (cf. Section I.F).

Thermal effects may also cause baseline disturbances and system peaks.[55] These can be different from instrument to instrument as well.

Each type of instrument uses different pressure settings, so injection times cannot be directly transferred. Therefore, it is preferable to specify an injection volume[32] or the product of pressure difference and injection time[20] in the method which can be considered instrument independent.

Fortunately, the detector performance is in the same order for commercially available instruments.[20] Still a re-validation of the DL is recommended for methods that investigate trace impurities. A significant change of precision data by a different S/N ratio would be found during the repeatability test.

To successfully transfer CE methods, the use of buffer recipes and defined rinsing procedures is strongly recommended.[1,56,57]

If a method from literature cannot be transferred, the reason is often a difference in the EOF velocity. Means to influence this parameter may be tried to adjust the method.

Note that difficulties in method transfer are frequently observed because one of the instruments does not work properly. Therefore, a qualification of all relevant instruments is recommended before the transfer.

A revalidation of linearity should not be required. Deviations from linearity are rare when PAs are used for calibration. If they occur, they are certainly dependent on the method (e.g., overload effects).

IV. SUMMARY

This chapter sheds light on the different validation requirements and methods to investigate them. Evaluation of the typical validation characteristics, namely accuracy, precision, specificity, DL, QL, linearity, and range in CE, has been discussed in details. Validation in CE is similar to validation in other separation techniques such as HPLC, but in CE, the capillary surface properties and namely the EOF have to be especially addressed. Further, the instrument performance has to be carefully considered during validation and method transfer. Here, the condition of the lamp and the thermostating system is of particular importance.

ABBREVIATIONS

BGE	Background electrolyte
CD	Cyclodextrin
FDA	Food and Drug Administration
ICH	International Conference on Harmonization
ISO	International Organization for Standardization
DL	Detection limit
QL	Quantitation limit
RSS	Residual sum of squares
SST	System-suitability testing
USP	The United States Pharmacopoeia

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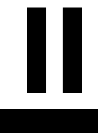

THE NEED FOR CE METHODS IN PHARMACOPOEIAL MONOGRAPHS

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ABSTRACT

The international pharmacopoeias such as USP, EP, and JP, being responsible for the quality of drugs are in a continuous process of revision of their monographs. Despite the fact that a drug's production might have changed and a different impurity profile has to be expected, the development of new analytical methods is mirrored in the pharmacopoeias. In the beginning color reactions were performed for identification and purity evaluation purposes. Nowadays the pharmacopoeias make use of chromatographic methods and try to replace the less sensitive thin layer chromatography (TLC) methods with high performance liquid chromatography (HPLC) tests. However, capillary electrophoresis (CE) methods are rarely used even when they are often more appropriate for the impurity evaluation of a drug than HPLC. Especially in the case of peptides and proteins CE is currently applied in the European and the United States pharmacopoeias. These methods and perspectives for new applications are given in this chapter.

I. INTRODUCTION

"The purpose of the European Pharmacopoeia is to promote public health by the provision of recognised common standards for use by health-care professionals and others

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concerned with the quality of medicines. Such standards are to be of appropriate quality as a basis for the safe use of medicines by patients and consumers." For 500 years the assurance of the quality of drugs as described in the introduction of the 5th European Pharmacopoeia (EP)[1] and similarly in the United States Pharmacopoeia (USP) XXX[2] has been the aim of regional (for towns in medieval age), national, international, and transcontinental pharmacopoeias, as well as the International Conference on Harmonization (ICH).[3] Thus, a monograph of every pharmacopoeia consists of the name of the described drug, the chemical structure, the definition, the characters, the identification, the tests, and the assay – the latter being mostly a titration since ages, because it is the most accurate method. In former times chemical reactions resulting in a color or a precipitate of a defined melting point were usually employed to identify a drug and look for and limit impurities, whereas chromatography has now replaced many of these reactions. At first thin layer chromatography (TLC) was introduced to identify a drug and limit its impurities. Since the materials of stationary phases, e.g., particle size of silica gel decreased, were continuously improved, and detection became more reliable, e.g., by introduction of TLC scanners for quantitative purposes, the TLC was normally able to limit an impurity to less than 0.5% and sometimes to less than this limit. However, the high performance liquid chromatography (HPLC) was the next step forward due to a much higher selectivity and along with that higher resolution than TLC. Typical detectors, such as UV and fluorescence ones, show a very high sensitivity, which make a limit of quantification in the femto to nanomolar range possible. Electrochemical and mass detectors may have an even higher sensitivity and selectivity. Consequently, HPLC started to replace TLC in the major international pharmacopoeias, although in the beginning some analysts doubted the reproducibility of HPLC. However, these concerns are not an issue any more. In most cases it is easy to limit an impurity to 0.1%. Thus, recently the EP commission started to replace the TLC tests with HPLC methods wherever HPLC is superior. In addition, a transparency list of all possible impurities of each "substance for pharmaceutical use" will be added to the monographs. In the USP XXX the tests are mostly performed with HPLC as in the EP, whereas the Japanese Pharmacopoeia (JP XIV) and its supplements make extensive use of TLC rather than HPLC.[4]

Comparing the monographs of the national and international pharmacopoeias of some 200 years reveals the development of analysis to be mirrored in the monographs. However, there is always a lack of time before new techniques expend into the quality assessment of the drugs. This was especially true for the TLC, for the isocratic and, later on, for the gradient HPLC.

For 25 years capillary electrophoresis (CE) and related techniques have been well-known methods that are characterized by an even higher efficiency and peak capacity than HPLC and by a very high speed of analysis. Early inter-company cross-validation exercises initiated by Altria from Glaxo[5–7] demonstrated an acceptable precision and repeatability for the determination of sodium levels in the sodium salt of an acidic drug, the chiral analysis of clenbuterol, and the quantitative determination of paracetamol levels in capsula, respectively. Thus, a successful transfer of CE methods between independent laboratories of seven pharmaceutical companies was possible. The advantages and the initial excellent experiences induced the optimism that CE would be able to completely replace HPLC. However, there are some disadvantages that have to be faced. On the one hand CE methods may suffer from a lack of sensitivity due to the short detection pathway of the light that is identical to the diameter of the capillary and to the low amount of sample (nL instead of μL in HPLC) injected to the capillary. The latter can be an advantage at the same time because a small sample volume (nl instead of μl in HPLC) is needed only. On the other hand the CE relies on more and more complicated physical effects resulting in high number of parameters that have to be considered when optimizing and validating a method.

For evaluation of impurities, the various methods of CE can be employed,[8,9] i.e., capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC),

capillary gel electrophoresis (CGE), electrochromatography (EC),[10,11] cyclodextrin-modified CE,[12–15] and isotachophoresis (ITP).[16,17] This diversity of methods makes not only the analysis of ionic and chargeable drugs possible, e.g., with CZE, but also the analysis of neural molecules by using MEKC and more recently by microemulsion electrokinetic chromatography (MEEKC).[18,19] Besides the classical procedure to develop a method[20] by optimization of all parameters step by step, generic methods were recently proposed to speed up the method development. This is especially true for chiral analysis.[21–23] Taken together, meanwhile a comfortable number of different CE techniques are available to separate and quantify drugs and their components as well as the corresponding impurities and to determine the enantiomeric excess (EE) by applying the cyclodextrin-modified CE.

As can be read in the reviews cited above CE has been established as a reliable method in pharmaceutical analysis for more than 10 years. On the one hand the "traditional" methods were more and more improved and refined, and new methods established such as the EC and the MEEKC and on the other hand validation and robustness testing are in the focus[24] of special issues now (e.g., *Electrophoresis* issue 12, 2005, edited by Wätzig and issue 12, 2006, edited by Wätzig and Scriba). Thus, CE methods comply with all the requirements for application in pharmaceutical quality control and stability evaluation of drugs. However, the pharmaceutical industry,[25,26] licensing authorities, and the international pharmacopoeias, e.g., the EP[1] and the USP,[2] do not make use of CE with exceptions of amino acids and peptide and protein compounds, i.e., erythropoietin, levocarbastine, and glutathione as well as products of recombinant DNA technology and products such as human coagulation factor VIII in the EP. These compounds are highly suitable to be analyzed by CE techniques because they are easily chargeable by applying a corresponding pH due to the amino and carboxylic groups. Charges are a prerequisite for performing CZE. However, this requirement of being chargeable can be fulfilled by about 90% of all drugs. Neutral compounds can be analyzed by MEKC whose charged micelles make uncharged compounds move into the electric field. One of the great stories of success is the chiral analysis by means of cyclodextrin-modified CE that is in almost all cases superior to chiral HPLC. Surprisingly, only one example can be found, the EE determination of ropivacaine in the USP which will also be introduced in the EP soon. However, the JP XIV does not even have a general description of the CE method.

Considering the tremendous progress CE and related techniques have made in the last years the behavior of the authorities is difficult to understand because there is still a need of highly selective and sensitive methods for quality assessment of drugs and CE can contribute to the issue on a very high level. As already mentioned above every new technique needed time to find their way into the pharmacopoeias; therefore, there is still a chance for CE.

This chapter seeks to promote CE techniques by demonstrating the power of the method by means of CE tests that are already in the EP and USP, and by comparing HPLC and CE methods for quality assessment of some representative drugs. It will be demonstrated that CE can be equivalent or superior to HPLC. Since CE methods are orthogonal and complementary to chromatography it can give additional information about the quality of a drug or examine the validity of chromatographic method.

II. KEY FACTOR FOR THE DEVELOPMENT OF A ROBUST CE METHOD AND VALIDATION

The prerequisites for the development of a robust method are simple; robust equipment, robust staff, and a robust development are needed. Even though the number of CE equipment manufacturers is low, the available instruments can be considered to be robust. In contrast, qualified staff for CE analysis is not often available. Since CE is based on absolutely different physics, i.e., electrophoretic and electroosmotic mobility,[16,27] than HPLC,

i.e., adsorption and distribution, it is not possible to switch from HPLC to CE without having understood the rules.

Robust method development has to consider both the separation of the components itself, which is described in Chapter 4 by Jimidar, and the development of appropriate rinsing procedures. As the latter is rarely discussed in scientific papers but may determine the success and repeatability of a separation, some critical factors to be considered are given here. A new capillary has to be washed before being used in order to remove possible contaminants from the manufacturing procedure. A sequence of rinsing procedures with 1 M NaOH for 30 min, pure water for 15 min, and air for 5 min is suitable for conditioning. Prior to each analysis, the capillary should be rinsed 15 min with 0.1 M NaOH and/or 10% H_3PO_4, 10 min with water, and 5 min with methanol (and 5 min with air). It can be useful to allow a waiting step of the capillary in the buffer solution to get rid of crystals outside the capillary that might produce ghost peaks. Similar rinsing procedures before daily use and between each run are critical to robustness.

Capillary coating can also stabilize the migration times and resolutions. This is in particular necessary in the case of peptide and protein analysis, because proteins tend to stick to capillary walls. Often low-concentration polyethylene oxide solutions are recommended[21] as well as dynamic bilayer coating formed by a non-covalent adsorption of polybrene and polyvinylsulfonate (PVS).[28,29] Due to the stability of the EOF, the variation of intra- and intercapillary migration time was less than 1% relative standard deviation (RSD) with basic analytes and peptides.

Since buffer additives may remain at the capillary walls, each capillary has its own history and should be dedicated to one application only.

Buffer depletion often results in a change of the migration times. Thus, the stability of the buffer has to be checked and as a consequence the buffer electrolyte vials have to be replaced after a certain number of runs.

For validation the following robustness factors should be considered: different lots of the capillary, temperature (~$\pm$2°C), applied voltage/current (~$\pm$2% relative), buffer electrolyte concentration (~$\pm$10% relative), pH ($\pm$0.1), concentration of additives, e.g., organic modifiers or chiral additives (~$\pm$10% relative), injection time (~$\pm$0.5 s), detection wavelength (~$\pm$2 nm), batch-to-batch variation of chiral selectors (~2−3 different lots), CE instruments (two instruments of two different manufacturers preferentially).

In turn, the international pharmacopoeias define suitability criteria that have to be fulfilled, otherwise the method is not valid for the determination of related substances. The adjustment of the chromatographic conditions being necessary "to satisfy the system suitability criteria without fundamentally modifying the methods," listed for each kind of chromatography, is described in HPLC chapter 2.2.46 of the EP 5.[1] Till now, corresponding information is almost completely missing in the CE Chapter 2.2.47 and, thus, has to be introduced as soon as possible because they are a prerequisite for a successful transfer of a method from laboratory to laboratory. However, CE is more critical against small variations of a method than HPLC: small variations of the pH or the buffer concentration may result in a principle change of the method. Even the preparation of the buffer may be critical. Thus, the technical guide of the EP should give more precise information on the development and description of a method that is intended to be introduced in the EP.

III. OVERCOMING THE PROBLEM OF LOW SENSITIVITY

In comparison to UV-HPLC the sensitivity in UV-CZE is lower, resulting in poor concentration limits of detection (LOD). Increasing the detection pathway by means of wide-bore capillaries, bubble cells, and Z-shaped cells enhances the sensitivity only 2−40-fold.

Simple sample stacking given an electrically focused sample zone can increase the LOD by a factor of 30–100. Field amplification, (transient) ITP, isoelectric focusing, sweeping concentration and other online-concentration techniques (e.g., field-amplified sample stacking (FASS), large-volume sample stacking (LVSS), or pH-mediated stacking) enhance the sensitivity up to 1000–100,000-fold.[30] Offline pre-concentration techniques such as solid-phase extraction, liquid–liquid extractions, liquid-phase microextraction and microdialysis are further possibilities to increase the LOD.[31] Employing fluorescence, sometimes in combination with derivatizations, laser-induced fluorescence, amperometry, conductometry, and mass spectrometry can also enhance the sensitivity and, often additionally, the selectivity. Further details can be found in the corresponding chapters.

IV. METHODS IN THE EUROPEAN PHARMACOPOEIA

As already mentioned the EP wants to replace old TLC tests with separation methods of higher efficiency; for example, the purity of amino acids is currently evaluated by a TLC test for "ninhydrin-positive substances" that is only able to find and limit amino acids to 0.5%. However, this test is only valid in the case the amino acids are produced by the cleavage of peptides/proteins and purification. The ninhydrin method is also used in the "amino acid analysis" of peptides, utilizing a cation-exchange chromatography with a post-column derivatization and a subsequent UV/Vis detection. This method is often used in industries for purity evaluation of amino acids.

In contrast, many amino acids are often chemically synthesized or produced by fermentation. In both cases related substances other than amino acids have to be expected and thus limited.[32]

The monograph of levocarbastine has already been revised. The determination of the related substances is performed by means of MEKC using an electrolyte solution composed of sodium dodecyl sulfate as a micelle-forming agent in addition to hydroxypropyl-β-cyclodextrin in a boric acid buffer of pH 9.0. Due to the very good specificity and robustness the method is able to baseline separate the nine specified and detectable impurities and the drug substance. It is easy to meet the system suitability ($Rs \geq 4$); the resolution between levocarbastine and impurity D was found to be 6.4 and the content of related substances less than 0.5% (see Figure 1A and B).

An initial study of a representative series of amino acids utilizing the MEKC method in combination with derivatization with 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) demonstrated the problem of insufficiency of the "ninhydrin-positive substance" TLC test currently in the EP: for example 15 unknown impurities were found in histidine batches.[33,34] Furthermore, the analysis of isoleucine batches revealed the presence of a number of impurities including the amino acids glycine, leucine, and valine (see Figure 2). None of these impurities was found by the "ninhydrin-positive substance" TLC test. However, all impurities were found to be below the qualification limit of 0.1%, but an impurity in the ppm range of concentration in tryptophan produced the eosinophilia–myalgia syndrome in hundreds of patients and some deaths 15 years ago. Thus, the revision of the amino acid monographs is urgently needed.

However, the revision of many naturally occurring amino acids is under progress. For limitation of other amino acids various methods such as ion chromatography, amino acid analysis, HPLC, and CZE are available and have to be tested case by case. In addition, tests for other non-amino acid related substances have to be developed. Possibly substances of different origin (production by synthesis, by fermentation, or by protein hydrolysis) may need different methods. In addition, a test for enantiomeric purity has to be included.

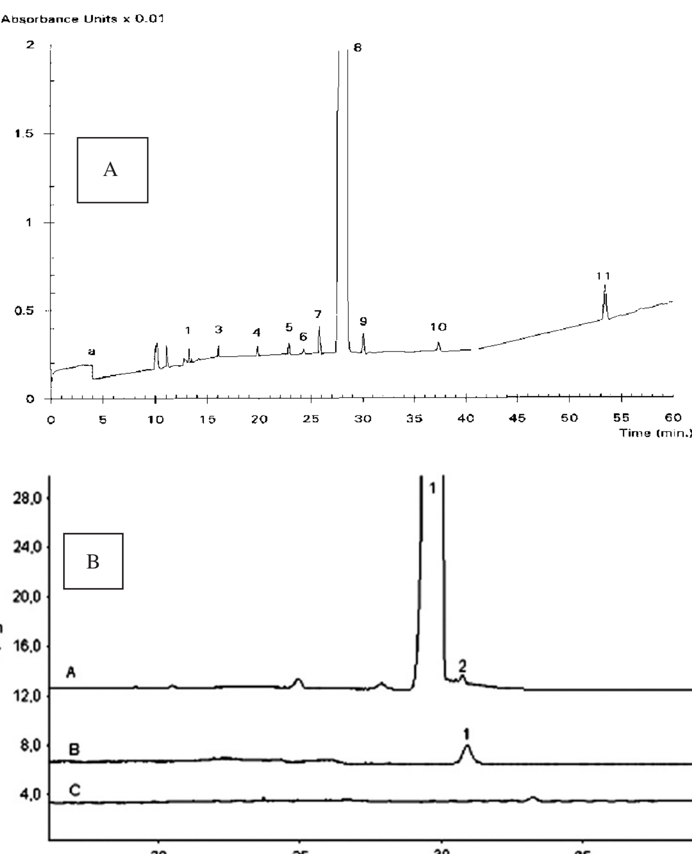

FIGURE 1 (A) Electropherogram of levocarbastine with all impurities (provided by Jimidar). (B) Electropherogram of real batch levocarbastine: (a) electropherogram of the EP test solution – levocabastine hydrochloride 2.5 mg/ml; (b) electropherogram of the reference solution b – 0.5% solution of the test solution; (c) blank peak identity: (1) levocabastine impurity D; (2) levocabastine hydrochloride. Conditions: fused silica capillary, effective length of 50 cm, 75 μm ID; injection 5 s with 0.5 psi; capillary temperature of 50°C; running buffer: 225 mM borate buffer, pH 9.0, 75 mM SDS, 9 mM hydroxypropyl-β-cyclodextrine, 2-propanol; current gradient as described in the monograph; detection at 214 nm.

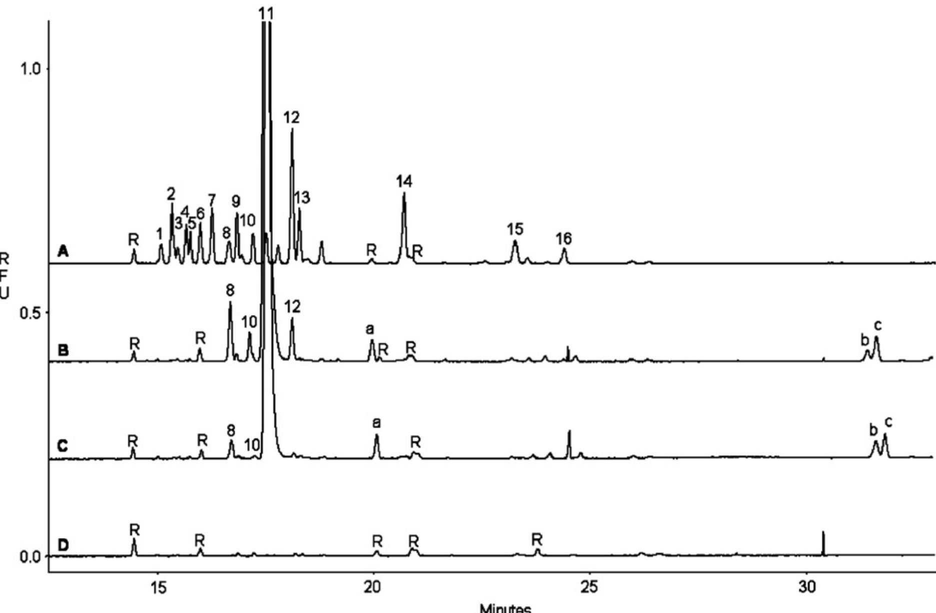

FIGURE 2 **Electropherograms of real batches of isoleucine after derivatization with CBQCA.[35] Conditions: fused silica capillary, total length 70 cm, 75 μm ID; running buffer: 20 mM tetraborate buffer pH 9.3, containing 100 mM SDS; 20 kV; temperature: 25°C; injection: 3448 Pa, 5 s; LIF detection: excitation wavelength 488 nm, emission wavelength 520 nm. (A) Model mixture of amino acids 0.00045 mg/ml each. (B) Isoleucine 0.45 mg/ml, manufacturer A. (C) Isoleucine 0.45 mg/ml, manufacturer B. (D) Blank. Peak identity: (1) Met; (2) Gln, His; (3) Thr; (4) Asn; (5) Tyr; (6) Ser; (7) Cys; (8) Val; (9) Ala; (10) Gly; (11) Ile; (12) Leu; (13) Phe; (14) Arg; (15) Glu; (16) Asp; (a) to (c) peaks of unknown impurities; (R) reagent peaks.**

The tripeptide glutathione is composed of l-γ-glutamyl-cysteinylglycine that easily oxidizes to give GSSG. The single amino acids of the tripeptide as well the corresponding dipeptides have to be regarded as impurities, i.e., cysteine, l-cysteinylglycine and l-γ-glutamyl-l-cysteine. The JP and the Japanese manufacturers make use of HPLC methods[26] which fail to evaluate the impurities without degradation of the sample during the HPLC run. In contrast, the CE method introduced in the EP is able to separate and limit the impurities down to the level of 0.1% without any degradation[36] (Figure 3). Furthermore, the degradation can also be observed by means of the CE method.[37]

Taking into account that quite a lot of fundamental CE work with peptides, such as with aspartame and many related compounds, has already been done,[38,39] CE has good chances to be considered in the EP. However, proteins have found their way into the EP. The "erythropoietin concentrated solution" is identified by means of CZE. Using a running buffer containing tricine, NaCl, Na-acetate, urea, and putrescine all isoforms of erythropoietin are baseline separated and give a typical pattern caused by their content.[40] Thus, the number of isoforms can be easily determined.[41] Recently Neusüss et al. published a CE–mass spectrometry (electrospray-time-of-flight) method that performs even better.[42] This method is able to distinguish 44 glycoforms and in total about 135 isoforms of recombinant human erythropoietin, including acetylations of the protein. Due to the CE–MS hyphenation a volatile running buffer composed of aqueous acetic acid and 20% methanol at pH 2.6 was applied.

Recently, a CE method was introduced for impurity profiling of the human growth hormone somatropin produced by the recombinant DNA (rDNA) technology.[1]

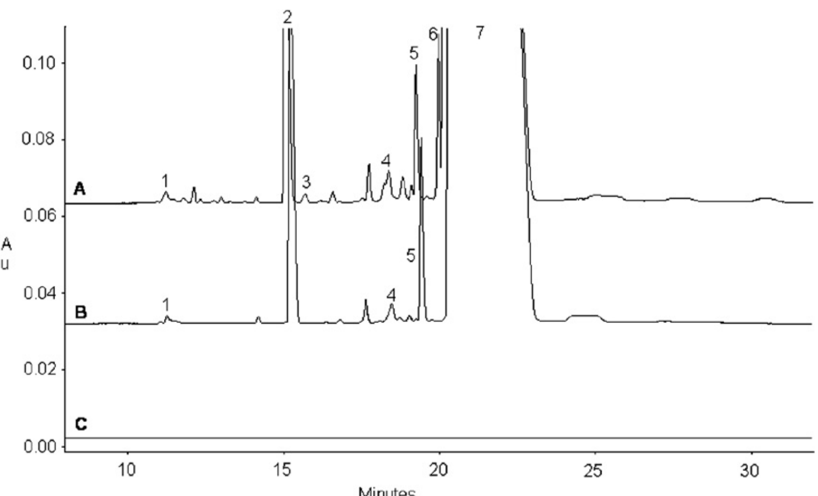

FIGURE 3 Electropherograms of glutathione. Conditions: fused silica capillary, total length 60 cm, 75 μm ID; running electrolyte: 50 mM phosphate buffer pH 1.8; 20 kV; temperature: 25°C; injection: 3448 Pa, 5 s; UV detection: 200 nm. (A) Glutathione reduced, manufacturer A, 20 mg/ml in running electrolyte, spiked with phenylalanine as internal standard 1% w/w. (B) Glutathione reduced, manufacturer B, 20 mg/ml in running electrolyte, spiked with phenylalanine as internal standard 1% w/w. (C) Blank. Peak identity: (1) impurity A: l-cysteinylglycine; (2) internal standard; (3) impurity B: l-cysteine; (4) impurity E; (5) impurity C: glutathione oxidized; (6) impurity D: l-γ-glutamyl-l-cysteine; (7) glutathione.

The EP limits charged variants of somatropin, e.g., deamidated forms, by a CZE method that is characterized by an ammonium phosphate buffer at pH 6.0 as an electrolyte, an uncoated fused silica capillary, and a UV detection at 200 nm (see Figure 4; for robustness see reference 43).

Correspondingly, the test for des-Ala-aprotinin and des-Ala-des-Glyaprotinin in aprotinin will be performed by means of CE in both the EP and USP XXX.[44,45] Using a phosphate buffer of pH 3.0 as running buffer, an uncoated fused silica capillary and a UV detection at 214 nm, both impurities can be limited to 8.0 and 7.5% (normalization procedure).

This is the consequence of the traditional application of CE in the process and product monitoring of rDNA-derived biopharmaceuticals in biotechnological industries.[46] However, related proteins, and dimer and related substances of higher molecular mass of somatropin, and aprotinin are evaluated by means of HPLC and size exclusion chromatography, respectively, by the EP.

Taken together, impurity profiling of peptides and proteins is one of the emerging fields of CE methods, and further applications are likely to be expected in the EP. However, the EP has

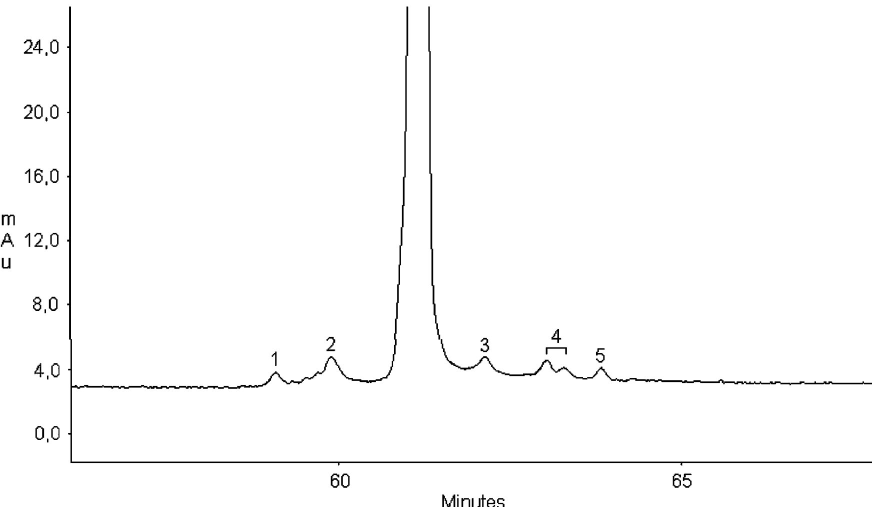

FIGURE 4 **Electropherogram of somatropin CRS: (A) Electropherogram of the EP reference solution somatropin CRS 1.0 mg/ml; (B) blank. The major peak corresponds to somatropin. The peaks 1–5 correspond to the impurities described by the Ph.Eur.: (2) cleaved form; (3) Gln-18 somatropin; (4) deamidated forms. Temperature: 30°C; voltage program 0–17.4 kV. For more conditions see text.**

not taken the chance to introduce superior MEKC methods in the case of the antibiotics gentamicin[47] and bacitracin[48,49] (for comparison see Figure 5). In both cases an HPLC method of low selectivity and efficiency is currently described.[26,37]

V. CHIRAL ANALYSIS

The number of pure enantiomers of the drugs on the market and in international pharmacopoeias is increasing. Currently the enantiomeric purity is often determined by means of optical rotation, which is a quite insensitive method, especially in the case of small angles of optical rotation. Since chiral HPLC utilizing chiral stationary phases has a long-standing tradition a couple of applications can be found in both the USP and the EP. However, the methods always suffer from the drawback that chiral columns are not very robust. In addition, chiral columns are extremely expensive. In comparison, chiral CE, especially cyclodextrin-modified CE being the major method, is cheaper, often much more efficient, and the method development is much faster. Utilizing especially negatively charged CDs such as sulfated CD, SBE-CD, and HDAS-CDs of varying cavity size (i.e., α-, β-, and γ-CD) as chiral additives to the BGE, almost all racemates can be separated which is proved by thousands of applications reported. Thus, the USP has utilized CD-modified CE recently. The enantiomeric purity of ropivacain is tested by means of *heptakis*(2,6-di-O-methyl)-β-CD in a phosphate buffer of pH 2.9–3.1. The method is able to limit 0.5% of the R-enantiomer in presence of 99.5% of the S isomer. The EP is going to copy this method. A method for levothyroxin is in discussion.

Further applications have to be expected because in several inter-laboratory trials the CE has performed to be as good as and often better than chiral HPLC. The recently reported separation of timolol enantiomers using a *heptakis*(2,3-di-O-methyl-6-O-sulfo)-β-cyclodextrin under non-aqueous buffer conditions is indicative of the good performance of the method.

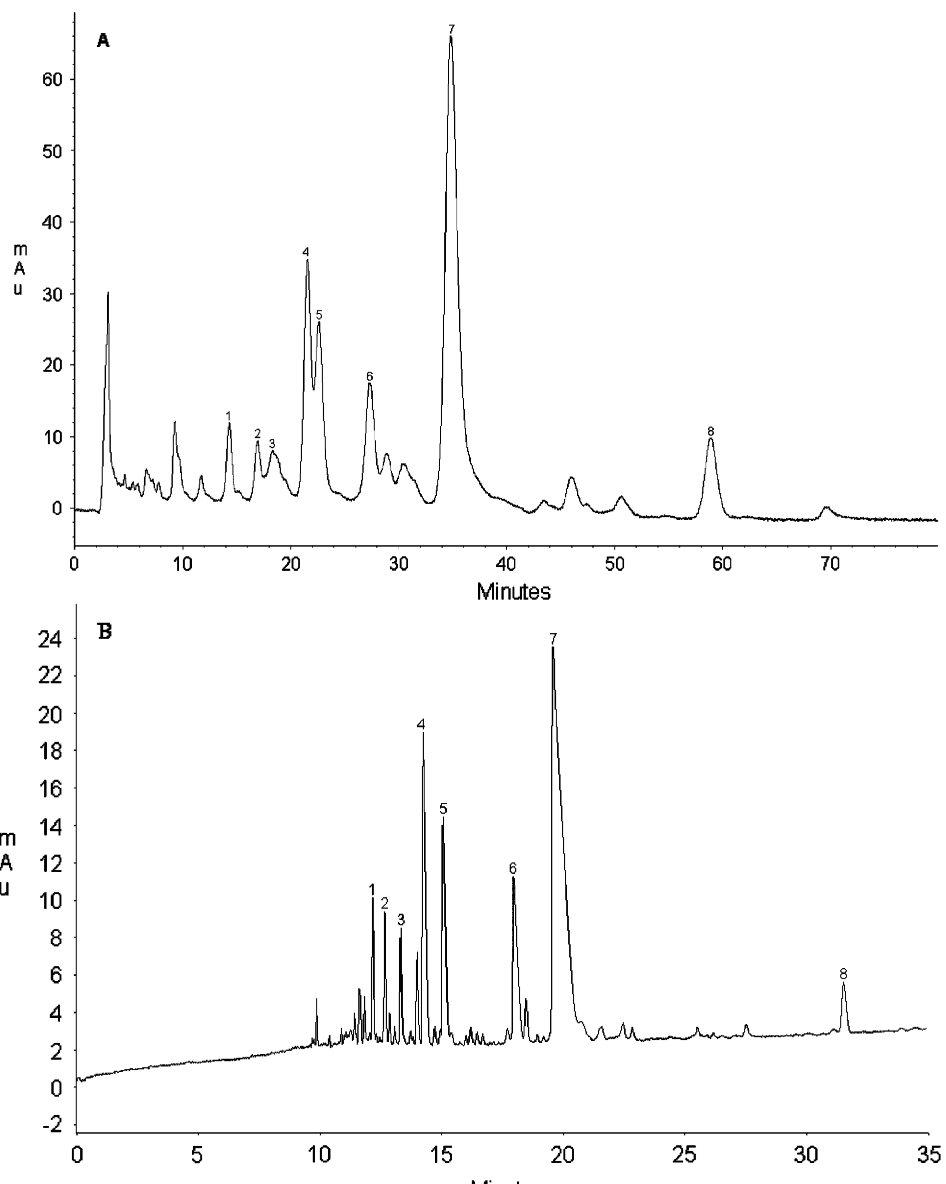

FIGURE 5 **(A) HPLC chromatogram of bacitracin; Hypersil ODS 18 column (250 × 4.6 mm, 5 μm), mobile phase composition: 51% of a mixture of 50 volumes of CH_3CN and 520 volume of MeOH and 49% of a mixture of 300 volumes of H_2O and 100 volumes of phosphate buffer, pH 6; detection at 254 nm; further details see EP.[4] Peak identity: 1 Bacitracin C1; 2 Bacitracin C2; 3 Bacitracin C3; 4 Bacitracin B1; 5 Bacitracin B2; 6 Bacitracin B3; 7 Bacitracin A; 8 Bacitracin F. (B) Electropherogram obtained by means of MEKC: uncoated fused-silica capillary (375 μm OD, 50 μm ID), running buffer 90 mM Tris, 17 mM PAPS, 03% m/V Brij 35 in water, pH 2.5 adjusted with 1.5 M phosphoric acid. For further details see reference 49.**

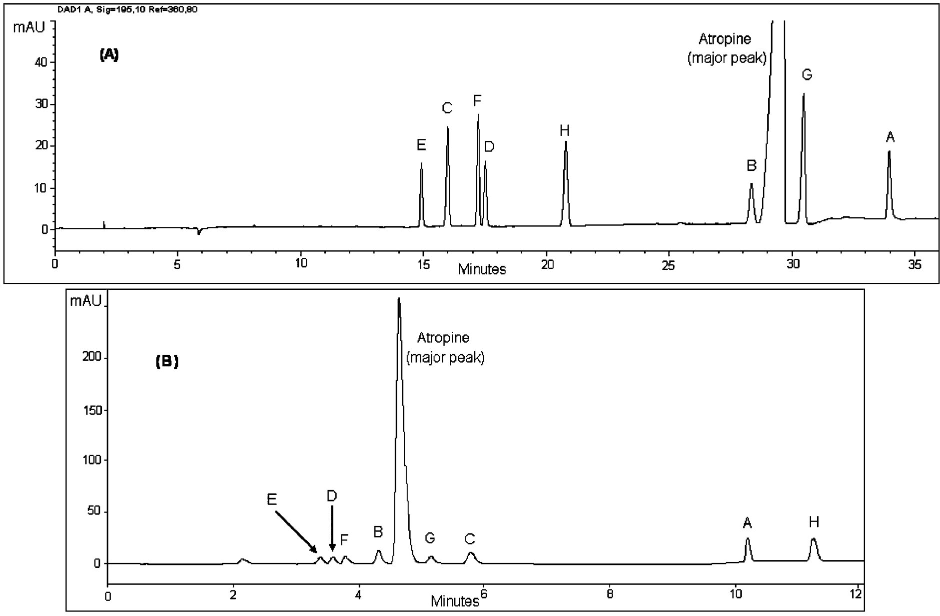

FIGURE 6 (A) Electropherogram of atropine and its related substances being other alkaloids and decomposition products obtained by MEEKC. Conditions BGE: 0.8% octane, 6.62% butanol, 2.0% isopropanol, 4.45% SDS, 86.15% tetraborate buffer (10 mM, pH 9.2); fused silica capillary; voltage 15 kV; temperature 30°C, UV Detection at 195 nm. (B) Chromatogram of atropine and its related substances. Conditions: Thermo Hypersil Aquasil C18 analytical column (5 μm particle size, 125 $\times$ 4 mm ID), gradient elution with 20 mM phosphate buffer, pH 2.5 (adjusted with conc. phosphoric acid) and acetonitrile; 25% acetonitrile from 0 to 4 min operating at a flow rate of 0.6 ml/min, 23% acetonitrile from 4 to 6 min at a flow rate of 1.0 ml/min and 45% acetonitrile up to 12 min at a flow rate of 1.0 ml/min, post-run with 25% acetonitrile for 2 min. For peak assignment see EP,[1] impurity (H) atropic acid.

0.1% of the R-enantiomer could be precisely determined in the presence of 99.9% of S-timolol in 12 different laboratories, indicating a good robustness of the method.[50]

VI. CONCLUSIONS

The fact that the EP wants to replace old TLC methods with more selective, efficient, and sensitive separation methods provides the chance for the introduction of more CE methods. The continuous development of analytical methods is reflected in the national and international pharmacopoeias. This might be demonstrated for atropine sulfate. Whereas the Deutsches Arzneibuch, 7th Edition (DAB 7) only limits the tropic acid by extraction and titration with NaOH and phenolphthalein indication, the 4th edition of the EP looked for foreign alkaloids and decomposition products by means of TLC with a potassium iodobismuthate for detection. By intensity comparison of the obtained spots, it was possible to limit these impurities to 0.5%. The EP 5 utilizes an ion-pair HPLC method that is able to limit most of the impurities to less than 0.2%. To make the method more robust, an HPLC method using a polar embedded was applied, which might be the next step for the EP.[51] However, recently the same authors have reported on a MEEKC method being as robust and precise as the latter HPLC method (see Figure 6) but far more sensitive[52] and, therefore, a future perspective for the EP.

Time will tell which new methods will be introduced to the major international pharmacopoeias – USP, EP, and JP. The CE is one of the main candidates to be used more often. One indication is the fact that besides the traditional instrument new manufacturers start to build CE instruments. The resulting competition will animate the market and stimulate the development of new and more effective techniques.

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12 CE IN IMPURITY PROFILING OF DRUGS

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ABSTRACT

This chapter illustrates possible applications of capillary electrophoresis in impurity profiling. Due to the large peak capacity of the technique, it is extremely well suited to separate the main drug compound from its possible impurities that often have a very related chemical structure. Moreover, the high efficiencies obtained, as well as the low reagent consumption make it a viable alternative to liquid chromatography in many cases of drug analysis.

After a short introduction into the relevance of impurity profiling for regulatory authorities, public health, and the pharmaceutical industry, an overview is presented based on the various modes of capillary electrophoresis that have been used in drug impurity analysis. The applications of capillary zone electrophoresis, non-aqueous capillary electrophoresis, micellar electrokinetic capillary chromatography, microemulsion electrokinetic capillary chromatography, capillary gel electrophoresis, and capillary electrochromatography are presented consecutively.

I. INTRODUCTION

This chapter has the aim of emphasizing that capillary electrophoresis (CE) can be a very valuable analytical technique in impurity profiling of drugs. It is common knowledge that

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bulk drug materials most often if not always contain impurities of various natures. This chapter will focus mainly on the determination of organic impurities in bulk drugs.

In order to constantly safeguard the patients' health and well-being, it is essential to check the quality of the drugs on the market. At the moment the regulatory guidelines issued by the International Conference on Harmonisation (ICH) demand that impurities in newly synthesized compounds are investigated from a certain limit upwards.[1,2] For a daily dose lower than or equal to 2 g/day, impurities need to be reported from 0.05% (m/m) on. They need to be chemically identified from 0.10% (m/m) and they need to be qualified toxicologically from 0.15% (m/m). When the daily dose exceeds 2 g/day, these limits decrease to 0.03, 0.05, and 0.05% (m/m), respectively. Good analytical separation techniques will be needed to reach down to these relative percentages.

As stated above, these regulations mostly apply to new drug substances, but even for the older drugs and for non-synthetic compounds such as fermentation products like antibiotics, it is common practice to characterize the final drug as thoroughly as possible. The ICH rules are widely accepted and followed while editing official monographs. More information on current regulatory issues can be found in Chapter 7.

The determination of impurities in drugs is a substantial challenge because the impurity profiles can be quite complex. Substances of structure highly similar to the main drug are usually involved, which necessitates a highly selective separation process. The impurity profile can change in function of the site of production, i.e., the origin of the compound. In this respect, CE is exactly the proper technique because the high peak efficiency of this analytical tool is an advantage and strength. Within impurity profiling it is important to reach as many separated peaks as possible, which is facilitated by high efficiencies. The variety of CE modes and selectivities that can be applied also improve separation capability.

Some difficulties can be encountered when using CE in impurity profiling. An inherent disadvantage is the rather low sensitivity when using a UV detector due to low optical path length. In the determination of impurities this needs particular attention because of the low concentrations of impurity that need to be detected. Moreover, at these low levels, the lower area and migration time repeatability that are often obtained with CE versus, for example, liquid chromatography (LC) can play a negative role. It might also be difficult to prove the peak purity by, for example, the use of a diode array detector (DAD), since it is difficult to generate nice UV-spectra from very small peaks. However, proving peak purity is important in impurity profiling as well as in support of stability studies.

The hyphenation of CE with techniques allowing structural determination of impurities is the object of Chapter 18 "Coupling CE and Microchip-based Devices and Mass Spectrometry" of this book, but the potential of CE coupled to mass spectrometry (CE/MS) in impurity profiling has also been discussed elsewhere.[3]

It has been discussed before that CE has several particular advantages making it useful in drug analysis[4] and "the need for CE methods in Pharmacopoeial monographs" is discussed by the same author in Chapter 11.

Several strategies have been applied in the development of CE methods for impurity profiling. These are discussed in more detail in the following overview of applications, subdivided according to the technique, with capillary zone electrophoresis (CZE) applications being discussed first. Many applications of micellar electrokinetic capillary chromatography (MEKC), microemulsion electrokinetic capillary chromatography (MEEKC), capillary gel electrophoresis (CGE), capillary electrochromatography (CEC), and non-aqueous capillary electrophoresis (NACE) in impurity profiling have appeared and these are described in subsequent subdivisions of this chapter. This contribution is not meant to give an exhaustive overview of the vast literature existing on this topic. Separation of chiral compounds from their related substances is discussed throughout the chapter.

A recent review on CE of small-molecule pharmaceuticals dedicates one part to the determination of drug-related impurities.[5]

Ample additional background information on the relevance of impurity profiling of drugs can also be found in reference 6.

II. OVERVIEW OF CE APPLICATIONS IN IMPURITY PROFILING

A. Capillary Zone Electrophoresis

Upon starting method development for the separation of a drug and its main impurities, CZE is preferred owing to its simplicity and compatibility with various detection modes. Details about this variant are described in Chapter 2. To introduce supplementary selectivity, a diverse set of additives to the background electrolyte (BGE) can be used. Organic solvents such as methanol and acetonitrile (ACN) are commonly utilized as buffer modifier to tune the selectivity by altering viscosity and polarity of the buffer. As a consequence, both the electroosmotic flow (EOF) and the electrophoretic mobility of the analytes will be affected. Complexation, e.g., with borate or cyclodextrins (CDs), is a different means of improving resolution between closely related compounds. Several detection modes can be coupled to CZE. Direct UV detection is still most abundantly present in literature in spite of its relatively low sensitivity caused by the restricted internal diameter of the capillary and the concomitant short optical path length. Indirect UV detection is employed as well but it imposes stringent requirements on the separation conditions. Electrochemical detection (ECD) is a powerful and sensitive technique for monitoring easily oxidized species. Yet, the selection of material used to fabricate the working electrode is decisively significant for determination of the analytes. CZE equipped with an MS can be helpful for identification and structural elucidation purposes. More detailed information about these different CZE strategies in impurity profiling is given in the following sections. It can be seen that a broad range of compounds is amenable to separations by CZE.

1. Antibiotics and Other Antibacterials

The concept of impurity profiling is very important for antibiotics, since most of them are still produced by fermentation or by semisynthesis starting from fermentation products. Antibiotics are typically complex mixtures of several components and their composition depends on the fermentation conditions. Impurities due to degradation occur frequently. Commercial samples usually contain significant amounts of impurities with only minor structural differences among them, but differing widely in their pharmacological activities. These impurities can exhibit antibiotic activity, but in many cases they are inactive and sometimes even toxic. The applicability of CE in the analysis of antibiotics has been reviewed elsewhere.[7–9] The use of CZE in impurity analysis of antibiotics is discussed in detail below.

An overview of CE analysis of aminoglycosides shows that CZE is by far the most commonly used technique for analyzing this type of antibiotic.[10] One of the major drawbacks of aminoglycosides, however, is their lack of UV chromophore and/or fluorophore, which limits straightforward detection. A number of techniques can be utilized to circumvent this problem. The first major work on the use of CZE in the study of aminoglycosides employed indirect UV detection at 214 nm by incorporating a strong UV absorbing substance, imidazole, in the BGE.[11] In alkaline pH, polyols, such as aminoglycosides, form a complex with borate not only in its tetrahydroxyborate form, but also as a more highly condensed polyanion such as triborate and tetraborate. A conventional UV detector operating at 195 nm provided detection for these negatively charged aminoglycoside–borate complexes.[12] Twelve aminoglycosides were studied and separated to demonstrate identification capabilities. The

borate buffer system permitted the detection of minor impurities such as precursors and closely related fermentation products. One drawback concerning the use of borate complexation for UV detection is that absorbance is dependent upon the strength of the complex. Low sensitivity and, to a lesser extent, selectivity were the major limitations of this method. Aminoglycosides contain a number of hydroxy and amino groups that can be oxidized easily when brought into contact with metal electrodes, especially in alkaline medium. Hence, ECD is another way of overcoming the aminoglycoside detection hurdle. Using an alkaline BGE and amperometric detection at a nickel disk electrode, a CZE method was developed for the determination of seven aminoglycosides.[13] The method has been successfully employed to determine the purity of kanamycin samples. Another mixture of seven aminoglycosides was analyzed by CZE with ECD at a copper-based electrode.[14] A copper microparticle-modified carbon fiber electrode was fabricated and employed in CZE analysis for the simultaneous determination of netilmicin, tobramycin, lincomycin, kanamycin, and amikacin.[15] Baseline separation was achieved in relatively short analysis time. Detection of aminoglycosides can also be accomplished by derivatization, mostly off-line, followed by direct UV detection, and different derivatizing agents have been described. To obtain repeatable results, the reaction step needs to be carefully controlled as both the yield and the stability of the resulting derivative depend on time, temperature, and presence/absence of light. CZE, following derivatization with *o*-phthaldialdehyde (OPA) and mercaptoacetic acid (MAA), was applied to separate eight related substances of kanamycin and several minor unknowns from the main component.[16] Separation took place in a borax buffer pH 10.0 containing 16% (v/v) methanol and the thioisoindole derivatives were detected at 335 nm. The same derivatizing agents, OPA and MAA, were used for the determination of gentamicin.[17,18] Baseline separation of gentamicin C_1, C_{1a}, C_2, C_{2a}, C_{2b}, sisomicin, and several minor components was achieved employing pre-capillary derivatization and a BGE containing 30 mM sodium tetraborate pH 10.0, 7.5 mM β-CD, and 12.5% (v/v) methanol.[17] The method was converted into a fully automated and validated procedure, utilizing in-capillary derivatization with OPA and MAA based on electrophoretically mediated microanalysis (EMMA).[18] Comparable selectivity to the pre-capillary method was shown (see Figure 1). In addition, three impurities, garamine, paromamine, and 2-deoxystreptamine, were separated and identified for the first time. The same group investigated the potential of a pre-capillary derivatization with OPA and mercaptopropionic acid for the CZE analysis of tobramycin.[19] Successful results were obtained with a simple BGE containing 30 mM sodium tetraborate pH 10.2 and ACN (75:25, v/v). Under these conditions, baseline separation of tobramycin from kanamycin B and an unknown peak was accomplished. The method showed good validation data in terms of precision, limit of quantitation (LOQ), limit of detection (LOD), specificity, and linearity, and was found to be suitable for analysis of bulk tobramycin pharmaceutical samples. A CZE method has been developed for determination of the components of paromomycin after pre-capillary derivatization with OPA and MAA.[20] The BGE consisted of sodium tetraborate buffer, β-CD, and methanol. Finally, it should be mentioned that direct UV detection at lower wavelengths has been described for streptomycin analysis. One study reports the simultaneous determination of streptomycin, dihydrostreptomycin, and their related substances by CZE with direct UV detection at 205 nm.[21] Good selectivity was obtained within short analysis time. During validation, quite high LODs and LOQs were observed, as expected, due to the presence of only a weak chromophore. It can be highlighted that most of the studies, described above, compare the developed CZE method to an established LC method. In general, a similar selectivity is obtained and CZE provides a faster, but less precise, separation with respect to LC.

Tetracyclines commonly comprise a plethora of related substances and degradation products, resulting from dehydration and epimerization reactions, and separating all of these constituents has proven to be quite a challenge. The necessity of adding ethylenediamine

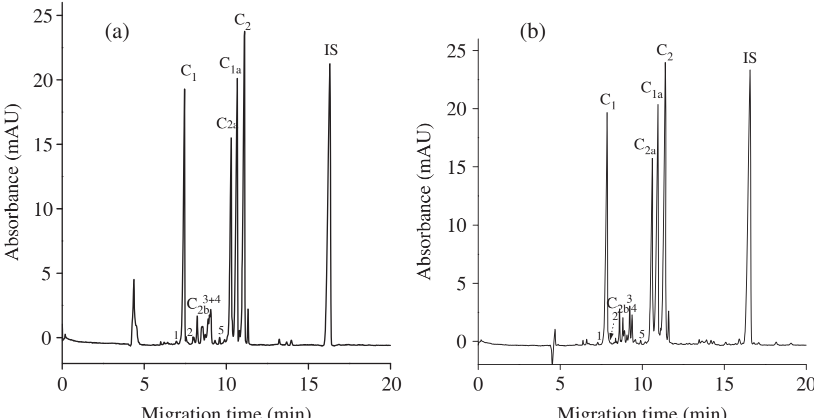


FIGURE 1 Electropherogram of a commercial sample of 2 mg/ml gentamicin sulfate: (a) EMMA and (b) pre-capillary derivatization. Derivatization solution: 30 mg/ml o-phthaldialdehyde, 25 µl/ml mercaptoacetic acid, and 10% (v/v) methanol in 30 mM potassium borate buffer, pH 10.4. Electrophoretic conditions: capillary: 40 cm total length (33.7 cm effective length), 50 µm ID; BGE: 30 mM sodium tetraborate, 7.5 mM β-cyclodextrin, and 15% (v/v) methanol, at pH 10.0; voltage: 19.5 kV; detection: 330 nm. Peaks: 1: garamine; 2: 2-deoxystreptamine; 3: reagent peak; 4: paromamine; 5: sisomicin; C_1, C_2, C_{1a}, C_{2a}, and C_{2b}: gentamicin C_1, C_2, C_{1a}, C_{2a}, and C_{2b}, respectively; IS: picric acid (internal standard). (Reprinted from reference 18, with permission.)

tetraacetic acid (EDTA) to the BGE was soon recognized, as it prevents metal–ion complexation of tetracyclines, the latter being detrimental to analysis. In 1992, the first study investigating CZE as an analytical tool for tetracyclines described baseline separation of tetracycline and several degradation products, using a phosphate buffer containing EDTA as an additive.[22] A CZE method was developed for the simultaneous determination of tetracycline and six fermentation and degradation impurities.[23] Employing sodium carbonate pH 9.0 containing 0.5% (v/v) methanol and 1 mM EDTA, most compounds could be separated and UV detected at 270 nm. Unfortunately, one impurity, 2-acetyl-2-decarboxamidotetracycline (ADTC), co-migrated with tetracycline. In a subsequent study, performed by the same authors, ADTC could be well resolved from tetracycline by means of a BGE consisting of a highly concentrated sodium carbonate buffer.[24] The applied voltage needed to be minimized in order to avoid excessive current and concomitant Joule heating. A majority of the studies dealing with CZE analysis of tetracycline antibiotics utilize an alkaline separation medium, which limits adsorption to the capillary wall as well as epimerization. Accordingly, the potential of high pH BGEs for the analysis of doxycycline has been explored.[25] Apart from 2-acetyl-2-decarboxamidodoxycycline (ADDOX), all five potential impurities could be separated in a 70 mM sodium carbonate buffer pH 10.5 containing 1 mM EDTA. Subsequently, an optimized CZE method for doxycycline analysis was developed by the same group, enabling separation of ADDOX from doxycycline.[26] The optimal separation buffer consisted of 145 mM sodium carbonate pH 10.3 containing 1 mM EDTA and methanol (11%, v/v). The method showed good selectivity, repeatability, linearity, and sensitivity. During quantitative analysis of six commercial samples, an impurity of unknown identity was detected in one sample. The results were similar to those found with the LC method of the European Pharmacopoeia. The applicability of “alkaline” CZE for the purity control of oxytetracycline (OTC) was further demonstrated by Van Schepdael et al.[27] A validated method including the use of 20 mM sodium carbonate pH 11.25 containing 1 mM EDTA at a

temperature of 10°C separated all the analytes involved. The development and validation of a CZE method for the determination of metacycline in the presence of its related substances was accomplished.[28] Addition of 13% (v/v) methanol to the sodium carbonate buffer was necessary for selectivity improvement. LOQs of 0.06% could be achieved and an unknown impurity was detected in all commercially available pharmaceutical substances. Since tetracyclines are furnished with a number of hydroxy groups, they are likely to interact with borate and such complexation could possibly improve selectivity and resolution. A 120 mM sodium tetraborate buffer pH 8.5 including 1 mM EDTA provided separation of chlortetracycline and related impurities.[29] The compounds were detected at 280 nm and the method was successfully validated. The same authors proposed an analogous method, employing a less concentrated BGE at higher pH, for impurity profiling of minocycline.[30] The performance of the CZE method was compared to that of an LC method. The speed of CZE, even including the washing procedure, was the most apparent difference. LC, though, performed better in quantitative analysis. Another study coupled the use of a borate buffer with fast on-line cyclic voltammetric detection with a mercury-film electrode.[31] Good resolution of tetracycline, chlortetracycline, and OTC was obtained. Few studies are found concerning the analysis of tetracyclines in neutral or acidic environment. The electrophoretic behavior of seven related tetracyclines has been examined by CZE in phosphate buffer solutions.[32] Under the predicted optimum conditions, pH 7.5 and 4.3 mM, the separation was satisfactory and all tetracyclines were readily identified. A mixture of five tetracyclines, including tetracycline, minocycline, demeclocycline, doxycycline, and sancycline, was separated by CZE within 6 min, utilizing a 25 mM phosphate buffer pH 2.3.[33] Moreover, each separate tetracycline was resolved from its main degradation product. The validated CZE method was applied to study the stability of the tetracyclines, dissolved in dimethyl sulfoxide (DMSO), and to analyze their content in three different pharmaceutical preparations. Seven tetracyclines were readily separated by CZE.[34] The BGE, 30 mM citric acid and 24.5 mM β-alanine, pH 3.0 containing 40% (v/v) methanol, allowed the separation of each tetracycline from its impurities and degradation products.

In the production of penicillin V by biosynthesis, several metabolites are observed in the medium and approximately 10–20% of the penicillin is degraded to by-products. Aqueous and non-aqueous electrolytes were investigated to determine their suitability for the separation of penicillin V from its impurities.[35] Fused-silica capillaries were pre-treated by flushing with 0.001% hexadimethrine bromide for EOF reversal. Separation of all analytes was achieved in less than 7 min, including separation of two isomers of phenoxymethylpenilloic acid. The aqueous system offered the best results in terms of selectivity and sensitivity. The applicability of this method in the analysis of a mixture representative of a real fermentation broth was demonstrated using both UV and electrospray ionization (ESI)/MS detection. A stability-indicating CZE assay was developed allowing selective determination of oxacillin in the presence of its degradation products.[36] Good separation was achieved using a 20 mM sodium phosphate buffer pH 6.5. Ro 23-9424 is an antibacterial agent consisting of a cephalosporin and a quinolone moiety, which are held together by a labile ester linkage. A CZE method, utilizing a borate buffer, was developed to separate the compound from its major degradation products.[37] The method was found to meet the acceptable criteria for precision, linearity, LOD, and LOQ. It was used to monitor the stability and measure the half-life of Ro 23-9424 in water and in a saline solution designed to mimic the drug delivery system. CZE was evaluated for the determination of cefotaxime in the presence of its major decomposition products.[38] Results were comparable to those of an LC method and comply with the requirements for drug quality control. The applicability of CZE in the analysis of cephalosporins in general is shown by two studies.[39,40] Complete separation of 12 cephalosporin antibiotics could be satisfactorily achieved with three buffer systems, namely, phosphate, citrate, and 2-(*N*-morpholino)ethanesulfonate.[40] Using a 25 mM phosphate buffer

pH 6.25, 14 cephalosporins could be separated within 20 min, although for some components baseline separation could not be achieved.[39]

Erythromycin, a macrolide antibiotic, lacks a significant chromophore. Detection sensitivity was enhanced by using a wavelength of 200 nm and selecting an injection solvent of lower conductivity than the BGE.[41] In order to facilitate the separation of erythromycin and its related substances, 35% (v/v) ethanol was incorporated into a 150 mM phosphate buffer pH 7.5. Resolution of all of the compounds was achieved in approximately 45 min. The method was employed as an assay method for erythromycin and for impurity determination. Peptide antibiotics, such as colistin and polymyxin, are mixtures of many closely related compounds. A validated CZE method for impurity analysis of polymyxin B was described, employing 130 mM triethanolamine–phosphate buffer at pH 2.5 to reduce the adsorption of analyte onto the capillary wall.[42] Methyl-β-cyclodextrin (M-β-CD) and 2-propanol were found to be necessary for selectivity enhancement. Using similar buffer additives, the same group developed and validated a method for colistin analysis.[43]

CZE with UV detection has been discussed in some detail for the separation, identification, and determination of ciprofloxacin and four of its impurities.[44] The separation buffer, sodium phosphate pH 6.0, was supplemented with 75 mM sodium pentane-1-sulfonate. The method was validated and fulfilled the requirements of the European Pharmacopoeia. The results obtained by CZE were compared with those obtained by LC and did not differ significantly. A CZE method operating at pH 1.5 was used for determining both drug content and levels of related impurities present in a quinolone antibacterial with limited solubility.[45] Acceptable levels of precision, linearity, LOD, and LOQ were achieved. Analytical techniques employing CZE and MEKC were developed for the analysis of five fluoroquinolone antibacterials and their major degradation products.[46] The theoretical determination of charge densities of the studied compounds allowed rapid development of optimal separation buffer conditions. The CZE method was applied to quantitatively follow the photostability of enrofloxacin with and without humic substances under natural sunlight conditions. CZE was used for the analytical separation of anthraquinone-1-sulfonate and its related impurities.[47] Optimal conditions implied the use of a borate solution at pH 10.0. The long-term stability of a commercial polyamine-coated capillary, eCAP™, was tested utilizing a validated method developed for the simultaneous determination of trimethoprim and four of its related impurities.[48] By reducing the capillary to a minimum length, assay of the trimethoprim mixture was achieved in less than 3 min, in a BGE consisting of 50 mM sodium acetate pH 4.2. Compared to an uncoated capillary, all components were clearly resolved in a shorter overall migration time and with higher peak efficiencies throughout. Chloramphenicol (CAP) is an antibiotic possessing an amide group. In aqueous solutions, it will hydrolyze to yield 2-amino-1-(4-nitrophenyl)-propane-1,3-diol (ANP). CZE with ECD at a carbon disk electrode was explored as analytical method for the determination of CAP and its hydrolytic impurity.[49] The method was validated and successfully applied to monitor CAP and ANP in commercially available eye-drops. ECD was also the detection mode of choice for CZE analysis of hydrazine, methylhydrazine, and isoniazid.[50] A 4-pyridyl hydroquinone self-assembled microdisk platinum electrode displayed a very high catalytic ability for the analytes.

2. Peptides and Proteins

In recent years, recombinant DNA (rDNA) technology has gained significant interest in the pharmaceutical sector and an increasing amount of biotechnology-derived pharmaceuticals is being developed and marketed. Biotechnological production processes involve a variety of host cells including bacteria, yeasts, and mammalian cells. Inherently, extraction from the fermentation medium and a number of purification steps have to be performed.

Multiple opportunities for heterogeneity exist, such as glycosylation, varying N-terminal sequence, amino acid modifications, and proteolytic clipping. Regulatory agencies require extensive characterization of the purified product and have provided guidelines to control and monitor product- and process-derived impurities and contaminants. Not only might these impurities have a profound effect on product quality and efficacy, they might also introduce unwanted and unknown side effects, even in trace amounts. With samples of this complexity no single technique yields the required depth of structural information to analyze the macromolecule. A wide range of analytical techniques with orthogonal selectivity, including UV and fluorescence spectroscopy, nuclear magnetic resonance (NMR), X-ray crystallography, N- and C-terminal amino acid analysis, biological activity tests, MS, LC, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, and CE, can be used simultaneously to determine protein integrity, by-products, and consistency of manufacture. The utility of CE as a tool for recombinant protein analysis has been discussed extensively in the past.[51–65] A selection of CZE applications in impurity profiling of recombinant proteins is presented below. Attention is mostly paid to those that are used therapeutically.

Human growth hormone (hGH) is one of the first-generation pharmaceutical products made by rDNA technology. Numerous studies on CZE analysis of recombinant hGH (rhGH), or somatropin, have been reported and illustrate the different approaches used in protein characterization and purity testing. Somatropin is produced recombinantly in *Escherichia coli* cells as an N-terminally extended negatively charged precursor. During the purification process, the N-terminal extension is specifically cleaved. Several related substances, e.g., deamidated rhGH, cleaved rhGH, oxidized rhGH, and oligomeric aggregates, are known to occur in the fermentation medium. One of the undesirable problems encountered in CZE separation of proteins is adsorption of the positively charged analyte onto the capillary wall, which can cause peak tailing and poor resolution. Shielding the silanol groups by chemical modification is one way to minimize protein–capillary wall interaction. Therefore, a CZE method, developed to quantitate the content of the rhGH precursor in the cytosol of *E. coli*, used hydrophobic C_{18}-coated capillaries and a 150 mM tricine buffer pH 7.55 containing 7.5% (v/v) methanol.[66] Precise determinations were possible as well as the evaluation of the presence of deamidated forms in cytosol samples. Utilizing a highly concentrated separation buffer is another strategy to suppress protein adsorption. CZE, employing phosphate-deactivated fused-silica capillaries and a BGE consisting of 250 mM phosphate pH 6.8 containing 1% (v/v) propylene glycol, resolved rhGH and its variants from crude mixtures of *E. coli* cell paste extract.[67] The validation of a CZE method for the assessment of the purity of rhGH has also been described.[68] A 100 mM ammonium phosphate buffer pH 6.0 separated hGH from its related impurities, namely, the cleaved form, the monodeamidated form, and two new compounds arising from post-translational modifications in *E. coli*. All compounds could be quantitated simultaneously in pharmaceutical preparations. The examples, mentioned heretofore, analyzed the intact protein. The most comprehensive information, however, comes from the analysis of peptide maps of the recombinant protein. Peptide mapping is an important requirement from a drug regulatory point of view to demonstrate structural equivalence with natural counterparts and is a key method for monitoring the amino acid sequence. This technique enables the detection of small changes in moderate size proteins, such as hGH, and its power has increased considerably at the time of appearance of commercially available mass spectrometers. In peptide mapping, intact protein is digested by a proteolytic enzyme resulting in a mixture of peptides due to the selectivity of cleavage. By separating these fragments, a fingerprint of the protein can be generated. CZE was applied to the separation of 19 peptide fragments produced by trypsin digestion of rhGH under non-reducing conditions.[69] Almost all of the fragments were resolved in less than 15 min. In a subsequent study by the same group, the electrolyte composition was optimized for separation

of the tryptic fragments.[70] Optimum conditions were found to be a 100 mM tricine buffer pH 8.1 including 20 mM morpholine. Eventually, all the efforts mentioned above led to the incorporation of a CZE method in the monographs of "somatropin," "somatropin bulk solution," and "somatropin for injection" in the European Pharmacopoeia.[71] CZE, employing a 13.2 g/l ammonium phosphate buffer pH 6.0 and UV detection at 195 nm, is used as a part of the identification procedure and in the test for charged variants, such as deamidated forms.

Insulin is a well-known biosynthetically produced protein hormone. The liability of insulin to degradation by dilute acid treatment was determined by CZE, native PAGE, and LC.[72] Two types of deamidated insulins were described as the main degradation products. Excellent correlation was observed between the different techniques. Addition of zwitterions and ACN to the running buffer was found to be necessary to attain a successful separation between insulin and its deamidation products.[73] In order to quantify insulin, both the degradation products and the commonly used preservative *m*-cresol have to be separated from the main compound. A validated CZE method for insulin assay in the presence of its degradation products and *m*-cresol was compared to two LC methods.[74] A 50 mM sodium acetate buffer containing 850 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid and 10% (v/v) ACN displayed higher separation efficiency and an up to four times shorter analysis time than that of the LC assays. Furthermore, if ACN evaporation was avoided, repeatability was even better than with the LC methods. A study conducted by Tagliaro et al.[75] to develop a CZE method for the separation of different calcitonins and calcitonin tryptic digests nicely exemplifies the influence of pH on protein adsorption to the capillary wall. A low pH decreases the charge of the capillary wall, thus weakening electrostatic interactions. Alternatively, working above the p*I* of the protein may induce electrostatic repulsions. A complete resolution of human calcitonins and salmon calcitonin was obtained in two buffer systems, namely, a 50 mM citrate buffer pH 2.5 and a 50 mM borate buffer pH 9.5. Moreover, separation of the four final trypsin cleavage fragments of salmon calcitonin and, at least tentatively, of the nine intermediate cleavage products was achieved in the citrate buffer.

A majority of rDNA-derived proteins are glycosylated, meaning carbohydrate groups being covalently attached at the polypeptidic chain through N- or O-linkage. The oligosaccharide structure of recombinant glycoproteins greatly depends on the system used for gene expression and on the culture conditions. Glycoproteins usually exist as heterogeneous populations of glycosylated variants (glycoforms) in which assemblies of different oligosaccharides are attached to each glycosylation site post-translationally. The carbohydrate moieties play a major role in their structure and function. Variations in the oligosaccharide structure can significantly affect many protein properties, such as solubility, specific activity, antigenicity, and thermal denaturation. When several glycosylated forms of the same protein exist in the same fermentation medium, a detailed analysis of these forms is compulsory. CZE is well suited for this purpose, as the variants can be separated on the basis of their charge differences mainly related to their various degrees of sialylation, sulfatation, or phosphorylation, as well as of differences in mass. The advances in CE of recombinant glycoproteins have been discussed elsewhere.[76–79] Recombinant human erythropoietin (rhEPO) is a prominent example of a biotechnologically produced glycoprotein. Pharmaceutical preparations of rhEPO are now available from several manufacturers around the world. A systematic approach was used to study the effect of pH, buffer type, and organic modifier on the CZE separation of the glycoforms of rhEPO.[80] Optimal resolution was obtained with a mixed buffer, 100 mM acetate-phosphate pH 4.0. The addition of 1,3-, 1,4-, or 1,5-diaminoalkanes to the separation buffer can improve the resolution of (glyco)proteins due to a binding of these additives to the capillary wall, thereby reducing the EOF and the interaction between proteins and the fused-silica wall. This beneficial effect has been employed to separate all major rhEPO glycoforms.[81] By incorporating 1,4-diaminobutane into the operating buffer, exquisite resolution was achieved. The presence of urea in the BGE further

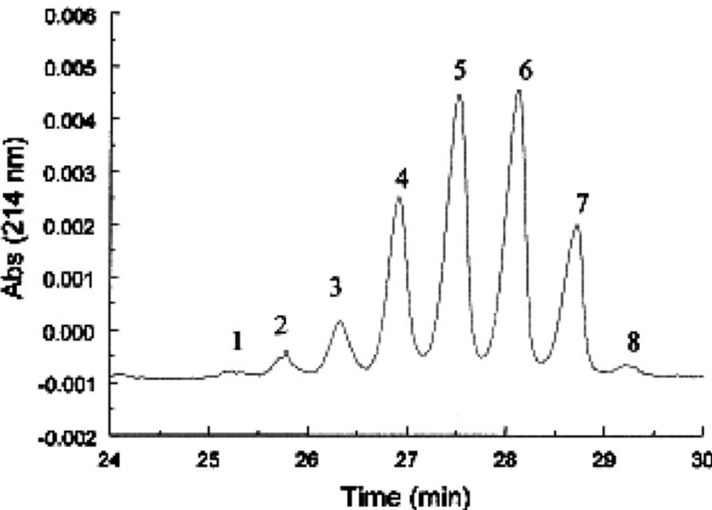


FIGURE 2 Electropherogram of recombinant human erythropoietin glycoforms. Electrophoretic conditions: capillary: 107 cm total length (100 cm effective length), 50 μm ID; BGE: 10 mM tricine, 10 mM NaCl, 10 mM sodium acetate, 7 M urea and 3.9 mM 1,4-diaminobutane at pH 5.50; voltage: 30 kV; detection: 214 nm. Peaks: 1–8: erythropoietin glycoforms. (Reprinted from reference 82, with permission.)

aided separation by suppressing protein aggregation. Results indicated that separation occurred in order of increasing number of sialic acids contained in the glycoforms. The aforementioned method was later improved by slightly modifying the running buffer and eight bands of rhEPO glycoforms were separated within reasonable analysis time (see Figure 2).[82] In order to make the method compatible with laser-induced fluorescence (LIF) detection, additional adaptations were performed including the replacement of 1,4-diaminobutane by morpholine.[82] Finally, a CZE/ESI/MS method was developed.[82] No buffer additives were used and protein adsorption was avoided by replacing the bare fused-silica capillary by a dynamically poly(ethylene imine)-coated capillary. In these conditions, however, baseline separation could not be obtained.

Analysis of recombinant proteins can be challenging because the amounts of active protein required for the therapeutic dose are often small compared to the large amounts of excipients added. Particular difficulties are encountered when the excipients are also proteins, such as human serum albumin (HSA), which is usually obtained from large pools of plasma and cannot be considered chemically homogeneous. An electrophoretic buffer consisting of 200 mM sodium phosphate buffer pH 4.0 and 1 mM nickel chloride allowed complete separation of rhEPO and HSA as well as separation of rhEPO into several glycoform populations.[83] Nickel was shown to interact selectively with HSA. Products from two manufacturers were analyzed and showed little qualitative but appreciable quantitative batch-to-batch variations in terms of units of biological activity. Peptide mapping is also useful to monitor changes in the positions and distributions of the carbohydrate attachments in glycoproteins. CZE has been applied to characterize the peptide map of rhEPO expressed from Chinese hamster ovary cells.[84] The methodology employed an ion-pairing agent, 100 mM heptanesulfonic acid, in 40 mM sodium phosphate buffer pH 2.5, in order to increase peptide resolution, decrease analyte–wall interactions, and evaluate glycopeptide microheterogeneity. The total tryptic map was segregated into two regions, non-glycosylated

and glycosylated peptides, and resulted in baseline separation of 16 tryptic peptides and one doublet peak composed of two peptides. An evaluation of CZE/MS as an analytical method for the characterization of endoproteinase V8 digested rhEPO has been performed.[85] The capillary was dynamically coated with polybrene in the presence of polyethylene glycol (PEG) and separations were carried out in 0.67 M formic acid. Glycosylation sites and carbohydrate branch patterns were easily assessed. CZE/ESI/MS, matrix-assisted laser desorption/ionization time of flight MS (MALDI/TOF/MS), and on-line LC/ESI/MS have been utilized simultaneously to unravel the structure of rhEPO.[86] A third analytical approach for glycoproteins, apart from analysis of the native protein and peptide mapping, is the release of the oligosaccharides by enzymatic or chemical cleavage and their subsequent separation, also called carbohydrate mapping. A database for N-linked glycans of sialylated complex type has been built employing an optimized buffer system containing 80 mM ammonium sulfate, 20 mM sodium phosphate, and 2.0 mM 1,5-diaminopentane.[87] The database was used to confirm the structure of oligosaccharides released from rhEPO.

Further, CZE has been examined as an alternative method to analyze carbohydrate-dependent microheterogeneity of recombinant tissue plasminogen activator (rt-PA), a serine protease that has potential applications in the area of thrombotic myocardial and cerebral infarctions.[88] High-resolution and repeatable separations of rt-PA glycoproteins carrying complex chains were obtained using either a 100 mM phosphate buffer pH 6.6 or a 100 mM tricine buffer pH 8.2 containing 1.25 mM of 1,4-diaminobutane. Application of the same additive proved to be essential for the CZE analysis of the various glycoforms of recombinant human factor VIIa.[89] Separation was primarily based on the different content of *N*-acetylneuraminic acid. Recombinant human bone morphogenetic protein 2 (rhBMP-2), a high-mannose glycoprotein, was separated into nine glycoforms by CZE, using a simple phosphate buffer pH 2.5.[90] The difference between any adjacent pair of peaks was only one mannose residue. A few years later, a CZE/ESI/MS method for the analysis of rhBMP-2 was established.[91] Combination of UV and MS data accounted for the determination of the identities of the glycoform peaks. Interleukins are glycoproteins too. CZE analysis of native recombinant human interleukin-2 (IL-2) expressed in bacteria demonstrated the existence of several recombinant species.[92] Resolution of the different glycosylated and non-glycosylated proteins was achieved with a 100 mM phosphate buffer pH 2.5. Run times of less than 10 min were sufficient. CZE and micro-LC have been applied to the analysis of recombinant human IL-4.[93] Separations for both the parent protein and its tryptic digest exhibited good repeatability. *Desmodus* salivary plasminogen activator is a large, complex protein with six sites for potential glycosylation and it has been analyzed by LC-UV, CZE-UV, LC/ESI/MS, and MALDI/TOF/MS.[94] Protein integrity was investigated at three specific levels of detail: the intact protein, proteolytic digests of the protein, and fractions from the proteolytic digest. Optimal CZE conditions implied the use of a phosphate-deactivated fused-silica capillary, a 100 mM sodium phosphate buffer pH 2.4 including 100 mM NaCl, and UV monitoring at 200 and 280 nm. For peptides, the injection was followed by a trailing electrolyte consisting of 1 mM phosphoric acid. This discontinuous buffer system served to sharpen the peaks. Finally, CZE with LIF detection is another option to address significant problems in impurity profiling of biopharmaceuticals as it has shown determination of impurities present in amounts less than 0.01% of the major component. The presence of trace impurities in purified recombinant human factor XIII and in a monoclonal antibody against human IL-2 receptor has been proven by CZE analysis of the proteins and their tryptic digests.[95] The BGE consisted of a 10 mM sodium tetraborate buffer pH 8.1 and electropherograms were considerably less complicated than their UV absorbance analogs. A drawback of CZE with native fluorescence detection (FD), though, is that peptides devoid of both tryptophan and tyrosine residues will escape detection.

The last part of this section briefly deals with CZE analysis of therapeutic peptides. More extensive papers on this topic can be found elsewhere.[96–101] Like proteins, peptides synthesized for therapeutic use must be rigorously tested for their purity. Synthetic peptides may contain closely related peptide impurities resulting from incomplete reactions or numerous side reactions. Pitfalls encountered in protein analysis, such as adsorption to the capillary wall, mostly apply to peptide analysis as well. The luteinizing hormone-releasing hormone analog goserelin is a nonapeptide and is often administered by parenteral injection. Aqueous parenteral peptide solutions are prone to all kinds of degradation such as hydrolysis, oxidation, and racemization. A CZE/MS system for goserelin analysis, using 10% (m/m) acetic acid pH 2.3 as running buffer, could separate the peptide and its degradation products.[102] As a result of the low pH, the EOF was minimal, assuring mainly electrophoretic movement and therefore relatively high resolution. The method was applied to a stability study of goserelin; samples degraded at pH 5 or 9 were subjected to analysis. Sensitivity-increasing field-amplified sample stacking (FASS) has been exploited in order to characterize the side compounds of the anti-cancer peptide drug buserelin.[103] FASS corresponds to electrokinetic injection from sample solutions of low conductivity into capillaries filled with highly conducting buffers. The amplification effect is much higher than that by using simple stacking after hydrodynamic injection. A CZE method with a BGE consisting of 60 mM potassium phosphate buffer pH 3.0 including 250 mM potassium sulfate was completely validated and found useful to study the behavior of buserelin implants under stress conditions. The increase in side components after sterilization using γ-radiation could be determined. The same impurities were detected in samples not treated with γ-rays but stored for several months. The high resolving power of CZE was demonstrated in purity testing of synthetic bradykinin.[104] Employment of a polyacrylamide-coated capillary and 50 mM potassium phosphate electrolytes of low pH conveniently eliminated sample adsorption and EOF. Protegrin IB-367 is a 17-amino-acid polycationic peptide developed to treat oral mucositis, a painful oral complication associated with aggressive cancer chemotherapy. The electrophoretic purity and levels of potential impurities of the drug substance were studied by CZE.[105] Separation and resolution between the peptide and truncated analogs showed to be much greater compared to LC methods. The method was validated and an internal standard was used for quantitation purpose. A sequential combination of LC/MS and CZE has been used to perform characterization of a multicomponent peptide mixture obtained during the synthesis of leuprolide.[106] Samples were first analyzed and fractionated by LC/MS and in a second stage the collected fractions were separated by CZE. Combination of the two techniques resulted in an enhancement of their individual selectivity characteristics. Later, the same authors established a CZE/ESI/MS method for separation and classification of a series of peptide hormones of pharmaceutical interest and wide therapeutical use including buserelin, bradykinin, and enkephalins.[107] The pH 2.85 running buffer contained 50 mM acetic acid and 50 mM formic acid. The main validation parameters of the proposed method were determined. Asparagine and aspartic acid are among the most unstable amino acids in peptides and proteins, being particularly susceptible to deamidation, isomerization, and enantiomerization reactions. CZE-tandem mass spectrometry (CZE/MS/MS), utilizing a 50 mM sodium phosphate buffer pH 3.0 or a 50 mM ammonium formate buffer pH 2.9 containing 10% (v/v) ACN, provided identification of degradation products of two aspartyl tripeptides, following incubation of the peptides in acidic and alkaline solution.[108] The method proved to be a strong tool to study the decomposition of peptides. Recently, the European Pharmacopoeia included CZE in the monograph of glutathione, i.e., in the test for related substances.[71] An uncoated fused-silica capillary, a BGE consisting of 6.00 g/l sodium phosphate pH 1.80, and UV detection at 200 nm are prescribed for the determination of specified and unspecified impurities.

Capillary isotachophoresis (CITP) is an electrophoretic separation technique in a discontinuous buffer system in which the analytes migrate according to their electrophoretic mobilities, forming a chain of adjacent zones moving with equal velocity between two solutions, leading and terminating electrolytes, bracketing the mobility range of the analytes. In the chemistry of peptides, CITP was most frequently used as a method to control the purity (electrophoretic homogeneity) of both natural and synthetic peptide preparations and also to monitor the peptide purity after individual steps in a purification procedure, thus providing information on the efficiency of the preparative separation method used.[109–112] It was demonstrated to be a rapid and sensitive method for peptide microanalysis in the nanomole and subnanomole range, giving both qualitative and quantitative information on peptide purity.[109–112] CITP enabled simultaneous monitoring of the reaction products and contaminating inorganic ions during synthesis and purification of the peptides.[109–111]

3. Miscellaneous Applications

In this last part concerning the use of CZE in impurity profiling, the widespread applicability of the technique is demonstrated by bringing forward numerous examples of structurally differing drugs being analyzed by CZE. As discussed before, even CZE without additives in the running buffer is capable of achieving high separation efficiency. One of the earliest papers showing the potential of CZE for the determination of drug-related impurities gives a survey of a few preliminary CZE applications.[113] Herein, trifluperidol and its six known impurities and decomposition products could be separated using a citrate-chloride buffer pH 4 and detected by UV at 250 nm. The same wavelength was set to identify domperidone and its four major known impurities following separation in a citrate-phosphate buffer pH 2. As a third example, a mixed borate-chloride buffer pH 11 containing 10 mM tetrabutylammonium hydrogensulfonate provided separation of diiodosalicylic acid and its six known, isomeric impurities. Small geometrical differences between codeine and its by-products were exploited for their simultaneous analysis by CZE.[114] Effective separation of codeine, thebaine, and 6-methylcodeine was achieved.

Ranitidine is an H_2-antagonist that is widely prescribed for the treatment of peptic ulcers. The drug is marketed in a variety of dosage forms including tablets, syrups and injectables. A range of synthetic and degradative impurities of ranitidine are known. CZE was evaluated for its performance in the determination of seven drug-related impurities in both ranitidine drug substance and various pharmaceutical formulations.[115] The data obtained clearly showed that the CZE method, employing a 190 mM sodium citrate buffer pH 2.6, was equivalent in terms of sensitivity and precision to an LC method used for a similar purpose and offered better selectivity. Bulk drug and injectable solutions were successfully profiled. The impurity levels of a related impurity of ranitidine were examined by the same group.[116] A solution of the impurity was positioned on the autosampler and analyzed sequentially in an electrolyte consisting of 50 mM sodium tetraborate pH 2.5. The extent of degradation was monitored by the loss of the main peak and the formation of two principal degradation products. It was found that after nine and a half hours only 2% of the original impurity remained. Enalapril and fosinopril are both antihypertensive agents belonging to the class of angiotensin-converting enzyme inhibitors and can undergo hydrolysis to form enalaprilat and fosinoprilat, respectively. A stability-indicating CZE method was developed for enalapril maleate.[117] A BGE containing 80 mM sodium borate at pH 9.7 separated the compound from two degradates, namely, enalaprilat and a cyclization product. Satisfactory resolution between the *cis* and *trans* rotamer of enalapril could only be achieved by MEKC. In fosinopril sodium tablet formulations containing magnesium stearate as a lubricant, two additional magnesium-catalyzed degradants have been identified. Fosinopril and its related substances have been resolved and quantified by a CZE method, utilizing a 50 mM sodium tetraborate buffer

pH 8.3.[118] Key validation parameters were determined and compared to an existing LC method. The CZE method displayed superior selectivity for several degradants of interest within a much shorter analysis time than did the LC method.

The analysis of a new generation of serotonergic anxiolytics including zalospirone, gepirone, ipsapirone, and buspirone, and their principal impurities was performed using CZE.[119] All compounds were present as cations in the pH 3 Tris-phosphate running buffer and were well separated. The beta-blocker drug atenolol is widely used in the management of hypertension, angina pectoris, and myocardial infarction. The commercial drug possibly contains four major related substances. A CZE method, combining a 20 mM phosphate-borate buffer pH 9.7 and UV detection at 226 nm, was described for the determination of atenolol in the presence of its impurities.[120] The method was fully validated and applied to the analysis of a commercial tablet preparation. Generally, positional isomers, such as the drug hydroxybutyridine and its *O*-alkylated impurity, are difficult to separate. Nevertheless, this was accomplished by CZE using a simple 10 mM tetraborate-phosphate buffer pH 9.5.[121] The optimum buffer pH was fixed by theoretical estimation of the pK_a. Acceptable values of validation parameters were obtained. *N*-Acetylcysteine (NAC) is a mucolytic drug extensively used in the treatment of respiratory diseases. The most important impurities are *N*,*N*-diacetylcystine, an oxidation product formed during storage of NAC, and cystine, a synthesis by-product. Wätzig et al.[122] showed the capability of CZE for the analysis of NAC. Another group applied CZE to the separation of NAC and its related impurities as well as auxiliaries such as methylparaben, propylparaben, and saccharin.[123] A 100 mM borate buffer pH 8.4 was used to determine the content and purity of NAC in commercial preparations.

The identification and quantitation of the main degradation product naphthylacetylethylene diamine in naphazoline hydrochloride bulk drug was accomplished by CZE employing a 100 mM phosphate buffer pH 3.[124] During validation, satisfactory specificity, linearity, precision, and accuracy results were attained. A CZE assay has been developed for the determination of isomeric aminopyridine and diaminopyridine analogs of the potassium channel blockers 3,4-diaminopyridine (DAP) and 4-aminopyridine (AP).[125] The compounds were separated using a 100 mM sodium acetate buffer pH 5.15 and UV detected at 240 nm (see Figure 3). As demonstrated for DAP, the assay allowed quantitation of the derivatives at the 0.1% level. In a subsequent study, a modified CZE method was presented for impurity profiling of DAP and AP.[126] Several isomeric and other related substances were separated in a 50 mM sodium phosphate buffer pH 2.5. The feasibility of the method was shown by analyzing a commercial sample of DAP. LAS 35917 is an orally administered drug intended for the treatment of erectile dysfunction. A CZE method was established in order to separate the chloromethylated, monomethylated, and hydroxylated impurities, arising from the synthesis route and degradation.[127] All three substances were separated from the parent drug, detected and quantified using a 60 mM tetraborate buffer pH 9.2. Stressed and non-stressed samples were subjected to analysis. To illustrate the utility of a coated eCAP™ capillary in CZE analysis, a two-component mixture of acyclovir and its major degradation product guanine was tested.[48] A clear improvement in resolution and peak efficiency over a conventional CZE method could be observed. The simple BGE used contained 50 mM sodium acetate at pH 4.2. Clodronate belongs to the bisphosphonate family and has been used in the treatment of Paget's disease, hypercalcaemia, and osteoporosis. Bisphosphonates lack strong UV chromophores impeding straightforward UV detection. However, CZE with direct UV detection at low wavelength and reversed polarity was applied to the separation and quantitation of bisphosphonate and phosphonate impurities in clodronate bulk material.[128] Polyacrylamide-coated capillaries were used to reduce the interactions between analytes and capillary wall, and to minimize EOF. A 40 mM sodium phosphate pH 7.4 running buffer enabled separation of all analytes within 15 min. The same authors proved CZE/ESI/MS to be an applicable technique for simple direct determination of bisphosphonates.[129] The use of a

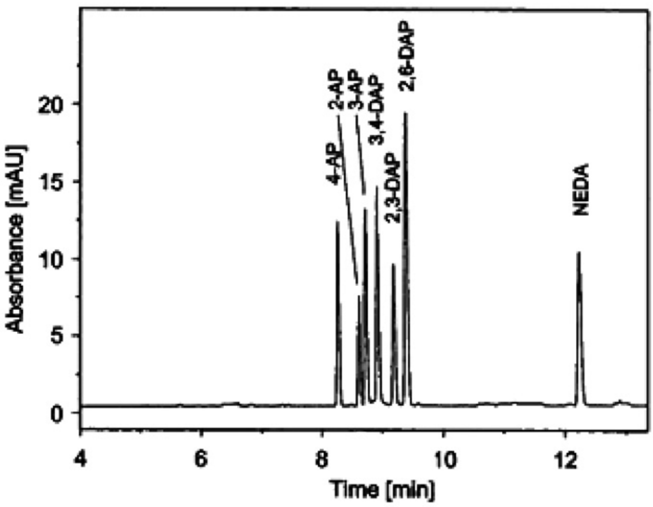


FIGURE 3 **Electropherogram of (di)aminopyridine standards. Electrophoretic conditions: capillary: 67 cm total length (60 cm effective length), 50 μm ID; BGE: 100 mM sodium acetate buffer, pH 5.15; voltage: 20 kV; detection: 240 nm. 4-AP: 4-aminopyridine; 2-AP: 2-aminopyridine; 3-AP: 3-aminopyridine; 3,4-DAP: 3,4-diaminopyridine; 2,3-DAP: 2,3-diaminopyridine; 2,6-DAP: 2,6-diaminopyridine; NEDA: *N*-(1-naphthyl)ethylenediamine (internal standard). (Reprinted from reference 125, with permission.)**

coaxial interface with optimized sheath liquid composition provided stable performance for routine analysis of clodronate and its common impurities. Inherently, a volatile electrolyte, 20 mM ammonium acetate buffer pH 8.0, was used.

A combination of a capillary coated with PEG and polyvinyl alcohol (PVA), a 10 mM ammonium acetate buffer pH 4.0, and MS detection significantly improved the separation of ephedrine and its related substances.[130] Clidinium bromide is a quaternary ammonium drug with anticholinergic and antispasmodic activity. A CZE method utilizing indirect UV detection at 205 nm was developed for the determination of a non-UV absorbing degradation product, Ro 5-5172, in clidinium bromide drug substance.[131] The electrophoresis buffer consisted of 10 mM sodium phosphate and 5 mM benzyltrimethylammonium bromide, a UV absorbing substance. Results obtained using the CZE method and a thin-layer chromatography (TLC) method were compared and found to be in agreement. The same detection mode was reported for the determination of bromide, chloride, and sulfate as impurities in calcium acamprosate, a drug which is used to maintain abstinence in alcohol-dependent patients.[132] Before each electrophoretic run, the capillary was coated with polybrene to reverse the EOF. Then, separation was carried out using an electrolyte containing 1 mM sodium borate and 10 mM potassium chromate, the latter being the visualization agent. The method was a putative replacement for the existing ion chromatography method. Homotaurine possesses no chromophore and is both a precursor of the synthesis and a potential degradation product of acamprosate. Derivatization with fluorescamine at ambient temperature followed by separation in a 40 mM borate buffer pH 9.2 and detection at 205 nm, allowed quantification of homotaurine down to the 0.01% level.[133]

The addition of an organic modifier to the electrophoretic medium can greatly improve separation, as shown by the examples cited below. A stability-indicating CZE assay for levothyroxine was developed, utilizing a BGE consisting of 100 mM phosphate buffer pH 2.5

and organic solvent.[134] Both ACN and methanol imparted better peak symmetry. Separation of levothyroxine from its possible deiodinated degradation products was also obtained under these operating conditions. TMT-NCS is a macrocyclic nonadentate metal chelator, which can be covalently conjugated to lysine residues on antibodies through an isothiocyanate moiety, allowing the targeted delivery of certain radionuclides for diagnostic as well as therapeutic applications. Due to a 13-step synthesis and high reactivity, the compound exhibits a complex impurity profile with the potential of over 30 impurities. The separation of TMT-NCS from its degradation products and synthetic intermediates was accomplished within 20 min in an alkaline medium containing 100 mM boric acid, 25 mM tricine, 0.5 mM EDTA, and 33% (v/v) ACN.[135] EDTA functioned as a competitive chelator, eliminating peak artifacts, while ACN improved peak symmetry. Sensitivity for low-level impurities was optimized using sample stacking. Specificity was established by examining stressed samples and evaluating peak purity with a DAD detector. CZE has been used for the quantitative determination of mirtazapine, an anti-depressant, and five structurally related substances in a tablet formulation.[136] It was found that the choice of extraction solvent was of great significance for the repeatability of both migration times and peak areas. 25% (v/v) methanol was added to the 70 mM sodium phosphate pH 2.0 running buffer. Validation data and batch analysis results were comparable to those of an LC method. The determination of residual amounts of bromide in a local anesthetic hydrochloride by CZE implied the incorporation of both 60% (v/v) ACN and 100 mM methanesulfonic acid in the running buffer.[137] In this way, selectivity was enhanced and electromigration dispersion minimized. The presence of a large excess of chloride puts great demands on the separation capacity. The method was validated in accordance with the ICH guidelines and proved to be suitable for its intended use. Loratadine, a tricyclic antihistamine, can include seven impurities in its raw material. As a complementary tool for undoubtful identification, a CZE method has been developed for measurement of these impurities in bulk drug and tablets.[138] The final separation buffer contained 100 mM sodium phosphate pH 2.5 and 10% (v/v) ACN. Adequate validation parameters for all the analytes were achieved permitting application to long-term stability and purity studies. Recently, a CZE method for impurity profiling of sodium cysteamine phosphate, a drug for the treatment of cystinosis, was described.[139] The two main decomposition products, cysteamine and cystamine, were separated in less than 4 min, using a 15 mM ammonium acetate buffer pH 8.85 containing 10% (v/v) methanol (see Figure 4).

Organic modifiers generally increase the volatility of the BGE facilitating hyphenation to MS. The application of CZE/ESI/MS to the impurity profiling of galantamine, the most recently approved cholinergic drug for the treatment of Alzheimer's disease and vascular dementia, has recently been discussed.[140] Extended release capsules were stressed at different storing conditions and subsequently analyzed in a 100 mM ammonium acetate buffer containing 25% (v/v) ACN and 25% (v/v) methanol. By combining an LC-UV, a chiral CZE-UV, and a CZE/ESI/MS method, the impurity profile was elucidated and nine related compounds, including the (+)-epimer of galantamine, were characterized. Three standardized CZE/ESI/MS methods were developed for the analysis of six drug candidates and their respective process-related impurities comprising a total of 22 analytes with a range of functional groups and lipophilicities.[141] The selected BGE conditions were found to be: a 10 mM ammonium formate buffer pH 3.5 containing 40% organic solvent; a 10 mM ammonium acetate buffer pH 7.0 containing 40% organic solvent; and a 10 mM piperidine buffer pH 10.5, where the organic solvent was methanol−ACN 50:50 (v/v). Factor analysis and informational theory were used to quantify the orthogonality of the methods and predict their complementarities. The three methods allowed the identification of 21 out of 22 of all the drug candidates and their process-related impurities, despite some of these not being fully resolved. These methodologies could form the basis of a generic approach to impurity

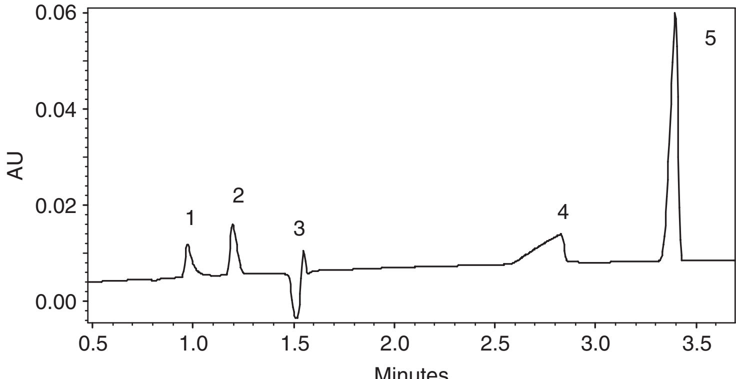


FIGURE 4 Electropherogram showing the separation of cysteamine phosphate and its impurities in a reference mixture. Electrophoretic conditions: capillary: 40 cm total length (30 cm effective length), 75 µm ID; BGE: 15 mM ammonium acetate, pH 8.85 with 10% (v/v) methanol; voltage: 28 kV; detection: 195 nm. Peaks: 1: cystamine; 2: cysteamine; 3: EOF; 4: cysteamine phosphate; 5: sodium benzoate (internal standard). (Reprinted from reference 139, with permission.)

profiling of pharmaceutical drug candidates and can be applied with little or no analytical method development, thereby offering significant resource and time savings.

CDs are inclusion complex-forming agents, which are well known to improve selectivity of some closely related compounds. They are mostly used as a buffer additive in chiral CE. Some studies, though, report their use in achiral CZE. A CZE method, employing an electrolyte solution containing 10 mM hydroxypropyl-β-CD (HP-β-CD), was developed for determination of the tricyclic anti-depressant drug dothiepin, which exists as a *cis* and *trans*-isomer, and two major related impurities, namely, an 11-oxo compound and a propanamine.[142] Several ranitidine-related impurities were resolved by a low pH CZE method.[143] The required selectivity was achieved by incorporation of 2 mM HP-β-CD in a 50 mM tetraborate buffer pH 2.5. A degraded syrup sample was analyzed. Atracurium is a highly selective neuromuscular blocking agent routinely used during anesthetic procedures. The commercial presentation of this drug is a mixture of positional isomers. Since they are all quaternary salts, CZE is highly suitable to evaluate the impurity profile. Complete resolution of all three isomers was accomplished in about 13 min using a 60 mM phosphate buffer pH 4 containing 20 mM β-CD and 4 M urea.[144] Laudanosine, a major metabolite of atracurium, was identified in two commercially available formulations. Its concentration increased considerably during storage of the product, even at low temperature. Another quaternary ammonium neuromuscular blocker, alcuronium, can enclose two main impurities, diallylcaracurine (DAC) and allyl-Wieland-Gumlich-aldehyde. Utilizing a 50 mM phosphate buffer pH 5.5 containing heptakis-(2,6-di-O-methyl)-β-CD or a 50 mM diethanolamine buffer pH 9.2 containing heptakis-(2,3-O-diacetyl-6-sulfo)-β-CD and ACN, the compounds were baseline separated and quantified.[145] In injection solutions of alcuronium that were stored at higher temperatures, three additional unidentified impurities were detected. The conversion of alcuronium to DAC under acidic conditions was monitored by means of the CZE method developed.

The utility of CZE for ximelagatran, a direct oral thrombin inhibitor, assay and related substances determination in both drug substance and tablets was confirmed.[146] Separation occurred in a 100 mM sodium phosphate buffer pH 1.9, to which 22% (v/v) ACN and 11 mM

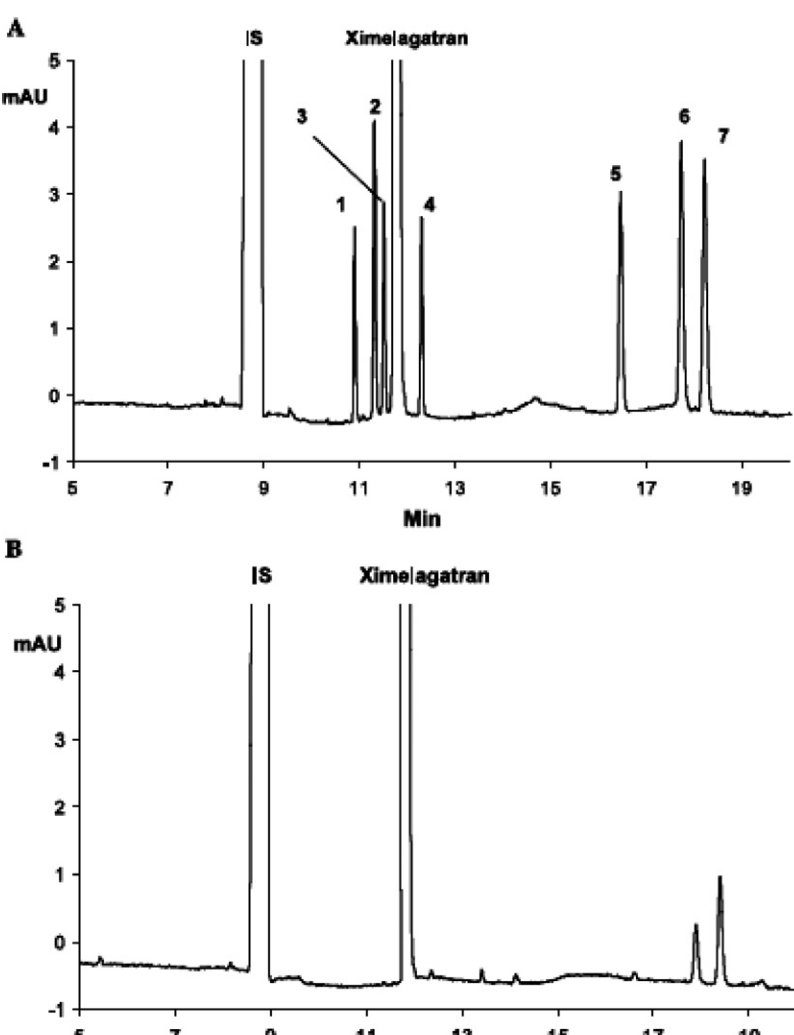


FIGURE 5 Electropherogram of (A) a ximelagatran solution where the seven known related substances are spiked and (B) ximelagatran tablets. Electrophoretic conditions: capillary: 64.5 cm total length (56.0 cm effective length), 75 μm ID; BGE: 100 mM sodium phosphate buffer pH 1.9, containing 22% (v/v) acetonitrile and 11 mM hydroxypropyl-β-cyclodextrin; voltage: 22.6 kV; detection: 230 nm. Peaks: 1–7: seven related substances of ximelagatran, respectively; IS: benzamidine (internal standard). (Reprinted from reference 146, with permission.)

HP-β-CD were added. All compounds were resolved in 20 min (see Figure 5). The data indicated that the validated method offered equivalent and complementary information, in terms of selectivity, sensitivity, accuracy, linearity, and precision, to that of an established gradient LC method employed for similar purposes. Ragaglitazar is a dual peroxisome proliferator-activated receptor α and γ agonist intended to restore insulin sensitivity and correct diabetic dyslipidemia. A chiral CZE method combining two CDs, sulfobutylether-β-CD and dimethyl-β-CD, lent itself to the analysis of ragaglitazar, its distomer (the (+)

enantiomer), and its counterion arginine in bulk samples and low-dose tablets.[147] The method was validated and proven to be reliable. Calcium levofolinate is used in rescue therapy of patients under treatment with methotrexate. A CZE method for the simultaneous determination of four related substances of calcium levofolinate has been developed and validated.[148] Optimized conditions corresponded to a 40 mM sodium tetraborate buffer pH 9.9 containing 20 mg/ml 2,6-dimethyl-β-CD. Quantitation of related impurities including the (6*R*,2′*S*) diastereomer of levofolinic acid was possible at the 0.05% level. In ursodeoxycholic acid (UDCA) raw material, other cholic acids, including the α-epimer of UDCA, can be present as impurities. Considering the low absorbance of UDCA and its related compounds, CZE with indirect UV detection was used for their analysis.[149] The capillary was filled with a BGE containing a UV absorbing ion, either benzoic acid or 5,5-diethylbarbituric acid. To enhance the selectivity of the assay, dimethyl-β-CD or trimethyl-β-CD was added to the running buffer together with methylcellulose or urea. All impurities and epimers were well resolved, with the exception of a neutral substance, methylursodeoxycholate. Coated fused-silica capillaries and inclusion of β-CD in a 100 mM sodium phosphate pH 6.9 migration buffer were exploited for the baseline separation of pilocarpine and its epimer, isopilocarpine.[150] The method was applied to the analysis of a commercial ophthalmic solution. Likewise, a dynamically C_8 tetraalkylammonium-coated capillary was used for determination of remoxipride-related impurities.[151] The shape of the overloaded principal peak was regulated by substituting tetraalkylammonium ions of different size for sodium ions, thereby altering the conductivity of the BGE. An adequate separation was obtained after addition of methyl-β-CD as structural selector in combination with a reversed EOF. Sensitivity was substantially improved for a fluorescent impurity by using an LIF detector.

Finally, the scope of CE even extends toward purity control of traditional herbal medicines and phytopharmaceuticals. The analysis of natural products by CE has been discussed previously.[152–154] A brief overview of some CZE applications is given below. The use of Chinese traditional medicine to maintain human health and to cure disease has a long and rich history. In particular, the merits of low toxicity and rare complications have led to considerable attention in various fields. In order to ameliorate symptoms quickly, it is not uncommon to see traditional Chinese medicine being illegally adulterated by the addition of synthetic chemical drugs. Sixteen of these drugs were studied by CZE with UV and ESI/MS detection.[155] Following separation in a 40 mM ammonium acetate buffer pH 9, only nine peaks were detected with UV, but on-line MS provided clear identification for most compounds. For co-eluting analytes, more reliable information was achieved by MS/MS. In a real sample, adulterants were identified by their migration times and protonated molecular ions. Chinese medicine preparations contaminated with coptisine, berberine, and palmatine were investigated by CE/ESI/MS/MS.[156] The volatile BGE consisted of 50 mM ammonium acetate pH 3.8 and analysis of the compounds in contaminated wuyoufun-13 was performed.

Alkaloids comprise one of the largest groups of secondary plant metabolites comprising over 10,000 different structures with often remarkable complexity. Many of them exhibit important biological and pharmacological activities and have prompted the development of broadly applied pharmaceuticals. The application of CE to alkaloid determinations has been reviewed by Stöckigt et al.[157] The same group suggested a general approach for CE analysis of four alkaloid classes.[158] Tropane alkaloids, such as atropine, or +/− hyoscyamine, and scopolamine, are obtained from various plants and are exploited for their anticholinergic characteristics. A number of analogs have been synthesized, the most common being homatropine, ipratropium, oxitropium, flutropium, and *N*-butylscopolamine. The simultaneous determination of these derivatives in pharmaceutical formulations was investigated by CZE employing low-wavelength UV detection.[159] The selected electrophoretic buffer consisted of a 80 mM sodium citrate buffer pH 2.5 containing 2.5 mM HP-β-CD. Satisfactory validation data were obtained. A CZE method for the separation of several tropane alkaloids,

including atropine, scopolamine, and synthetic derivatives, was developed by the same authors.[160] A 100 mM Tris-phosphate buffer pH 7 gave superior selectivity and was utilized to determine these compounds in ophthalmic solutions. The method was validated according to the ICH guidelines. Subsequently, CZE was interfaced with ESI/MS.[161] The original electrolyte was adjusted to a 40 mM ammonium acetate buffer pH 8.5 and found to be suitable for the analysis of atropine and scopolamine. The differentiation of atropine and its positional isomer littorine, commonly encountered in plant material, was also accomplished. The developed method was applied to the analysis of Belladonna leaf extract. Samples of *Hyoscyamus muticus* plants were examined by CZE, using a simple 40 mM phosphate buffer pH 7.8.[162] The main tropane alkaloids, atropine and scopolamine, as well as nor-scopolamine and tropic acid could be separated in less than 13 min. CZE was investigated for the analysis of by-products and impurities in illicit cocaine, another tropane alkaloid.[163] The use of β-cyclodextrin sulfobutylether IV (β-CD SBE IV) was necessary to achieve good separation of isomeric truxillines and similar impurities in less than 8 min. LIF detection using a krypton/fluorine laser provided greater selectivity and sensitivity, in comparison to UV detection.

In Chinese medicine, herbs are often treated before use. Since the preparations convert the original component into one or more products, characterization is usually complex and involves several compounds. Moreover, the hydrolysis products of, for example, aconitum alkaloids display weak UV absorbance. Hence, CZE/MS was applied to the determination of aconitum alkaloids in Maqianzi and Wutou during the preparation procedure.[164] Hydrolysis of the components in water and methanol was also studied. The active principal components in all tobacco products belong to the class of alkaloids as well. Nicotine is the major tobacco alkaloid and is the primary determinant of what constitutes a tobacco product from a regulatory standpoint. Nicotine is also present in pharmaceutical preparations such as chewing gum and patches used in the treatment of smoking cessation. Related impurities of nicotine can arise from synthesis, extraction procedures, or degradation in water and air. Preliminary CZE studies employing a 25 mM sodium phosphate buffer pH 2.5 and UV detection at 260 nm yielded promising results for monitoring of nicotine in regulated tobacco products.[165] At this low pH, the tobacco alkaloids were present in cationic form, showing high mobility and increased UV absorbance. An electrolyte containing a high concentration of citric acid to provide good buffer capacity at pH 3.6 was suitable for the analysis of nicotine and its related substances, nornicotine and anabasine, in single epidermal leaf cells of tobacco plants.[166] The migration behavior of nicotine and related tobacco alkaloids, including nornicotine, anabasine, myosmine, cotinine, and nicotine-1-oxide, was further investigated using three different CE modes.[167] Each technique resulted in different, orthogonal separation selectivity. The low-pH CZE method was applied to impurity profiling of nicotine lozenges due to its compatibility with an established lozenge extraction solvent. Ergot alkaloids, used in the treatment of migraine, are native fluorescent molecules. Under the influence of acid or base, they slowly decompose and isomerize. Qualitative and quantitative determination of ergot alkaloids and their epimers was carried out by CZE in a phosphate buffer pH 2.5 containing 20 mM β-CD, 8 mM γ-CD, 2 M urea, and 0.3% (m/m) PVA.[168] The sensitivity could be improved 30-fold when LIF detection was applied.

Morphine is an extremely powerful opiate analgesic drug and the principal active agent in opium. It has always been a challenge to perform accurate measurements of the opium alkaloids (normorphine, codeine, thebaine, papaverine, and noscapine) and especially of morphine in opium and pharmaceuticals. The main degradation impurities in crystalline morphine and its salts are oxidation products, namely, 10-*S*-hydroxymorphine, pseudomorphine, and morphine-*N*-oxide. CZE, using a Tris-borate buffer and untreated or PVA-coated capillaries, enabled separation of morphine and its degradants.[169] Addition of β-CD or its derivatives enhanced resolution. Impurity profiling of four different morphine drug products, including crude opium and a cough mixture, was realized employing CZE.[170] The use of

30 mM 2,6-di-*O*-methyl-*β*-CD as an additive to a 50 mM 6-aminocaproic acid buffer expedited separation from other alkaloids. In a subsequent study, the developed method was compared to a CE method, involving a non-aqueous electrophoresis medium, for the quantitative determination of morphine in pharmaceutical formulations.[171] Both CE methods were partially validated and also compared to an LC method. From an overall point of view, all three methods were equivalent in performance. Dynamically coated capillaries prepared using a commercially available reagent kit, CElixir™, gave rise to a relatively high and robust EOF at pH 2.5, allowing rapid and precise characterization of alkaloids in opium gum and opium latex samples.[172] In order to obtain sufficient selectivity, CDs were incorporated in the running buffer. Good agreement with the values of an LC method was observed.

Heroin, a morphine derivative, is one of the most abused drugs around the world. In terms of composition, clandestinely manufactured heroin preparations can be highly complex, reflecting the different quality of raw materials and reagents and non-standardized extraction and preparation procedures. Several excipients and adulterants might also be added. The aforementioned method using coated capillaries proved to be well suited for the analysis of heroin and its basic impurities and adulterants.[173] The determination of cations and anions down to the 0.001% level in illicit heroin was accomplished by the same group utilizing CZE with indirect UV detection.[174] Ammonium, calcium, potassium, magnesium, and sodium were analyzed in an acid Ion Phor™ BGE consisting of 4 mM copper sulfate, 4 mM formic acid, and 3 mM 18-crown-6; acetate, chloride, citrate, phosphate, sulfate, and tartrate analysis necessitated the use of another, proprietary buffer system, Anitron™ and a Micro-Coat™ capillary charge-reversal agent. A CZE method capable of rapid determination of heroin, secondary products and additives present in clandestine heroin samples, was described using 20 mM *β*-CD in a 100 mM potassium phosphate buffer pH 3.23.[175] Under these conditions, heroin, morphine, 6-monoacetylmorphine (MAM), acetylcodeine, papaverine, codeine, and noscapine were baseline resolved in less than 10 min. The good validation results obtained permitted application to real cases of seized controlled drug preparations. CZE with on-line permanganate chemiluminescence (CL) detection was established for the simultaneous determination of morphine, MAM, and heroin.[176] It was found that *β*-CDs could improve the separation efficiency and augment the CL signal. As confirmed by this study, CL should give lower detection limits than other emission procedures, as there is rarely any background emission or scatter to increase the noise level. Furthermore, because few compounds chemiluminesce, there should be minimal interference from endogenous compounds in complex matrices.

Coumarins are pharmacologically active and have been used in the treatment of a diverse range of diseases. The great diversity of coumarin structures and their wide range of polarities present special problems for their simultaneous analysis. The separation of seven closely related coumarins by CZE was studied.[177] Optimized conditions tallied with a 200 mM boric acid−50 mM tetraborate buffer pH 8.5 and were applied to the determination of coumarins in extracts from roots and aerial parts from the plant *Chrysanthemum segetum*. Baseline separation of six coumarins was achieved in 10 min.

B. Non-aqueous Capillary Electrophoresis

The use of non-aqueous media offered new attractive possibilities for the analysis of hydrophobic compounds, which are often difficult to be analyzed due to their low solubility in aqueous media. Selectivities that are difficult to be obtained in aqueous buffers can be easily obtained using non-aqueous systems, due to larger differences in the ionized−unionized equilibrium for two closely related substances in non-aqueous solvents compared to aqueous solvents. Organic solvents such as methanol, ACN, ethanol, formamide, dimethyl formamide,

or DMSO with dissolved salts are used as BGE in NACE. The use of organic solvents in the run buffer results in low currents and reduced joule heating, allowing the use of high electric field strengths. The low currents produced not only allow the use of higher electrolyte salt concentrations and higher electric field strengths, but also the sample load can be scaled-up by employing capillaries with wider inside diameter resulting in enhancement of overall detection sensitivity.[178] However, evaporation of the sample as well as electrophoresis medium has to be considered seriously during qualitative and quantitative measurements. So far, only a limited number of studies using NACE for impurity profiling have been reported.

NACE was found to be highly selective separating tetracycline hydrochloride from four known and several unknown impurities within 10 min (see Figure 6). The obtained selectivities are comparable or even better than the selectivities obtained in aqueous CE and LC. It was demonstrated to be a valuable supplement to LC in order to reveal unknown impurities.[179] Using a non-aqueous electrophoresis medium containing 500 mM magnesium acetate tetrahydrate in *N*-methyl formamide, selective separation was achieved for OTC and its related substances and degradation products extracted in a single step from an ointment.[180] The method was found to be advantageous over LC and aqueous CE, which require a tedious sample pre-treatment procedure consisting of several extraction steps. In a subsequent study, the separation of OTC and three of its impurities, tetracycline, 4-epioxytetracycline, and 4-epitetracycline, was achieved by NACE and it was successfully applied to the determination of the compounds in pig plasma by solid-phase extraction.[181] The potential of NACE with ECD

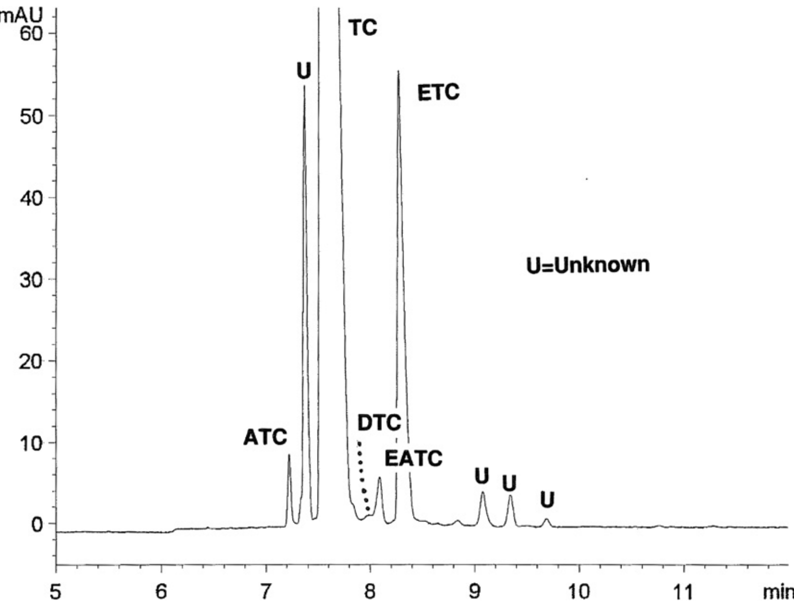


FIGURE 6 Electropherogram of tetracycline hydrochloride raw material 5.0 mg/ml. The sample was dissolved in MeOH−MeCN−DMF (45:49:6, v/v/v). Injection for 1 s at 50 mbar. Capillary: 64 cm × 50 μm ID; 55.5 cm to the detector. Electrophoresis medium: MeOH−MeCN−DMF (45:49:6, v/v/v) with 25 mM ammonium acetate, 10 mM citric acid, and 118 mM methanesulfonic acid added. Temperature: 25°C. Voltage: 30 kV resulting in a current of approximately 65 μA. Detection: 254 nm. ATC: anhydrotetracycline; U: unknown; TC: tetracycline; DTC: desmethyltetracycline; EATC: 4-epianhydrotetracycline; ETC: 4-epitetracycline. (Reprinted from reference 179, with permission.)

for impurity profiling was demonstrated by the determination of impurities in the poorly water-soluble drug trimethoprim. Samples containing 2 mM of trimethoprim spiked with different quantities of one impurity were analyzed and detection limits down to the 0.001% level could be obtained.[182] Both aqueous and non-aqueous systems were investigated for the separation of cimetidine from six of its impurities and a degradation product. With the aqueous system, two impurities were insoluble and significant peak tailing was observed for cimetidine and one of its impurities.[183] NACE was found to be beneficial over aqueous CE in separating the hydrophobic impurities with better peak shapes; however, all the eight components could not be separated in one experiment.

Three test mixtures containing structurally similar basic compounds such as BRL29060 and BRL57138A, SB202026A and BRL57259, and imipramine and desipramine were well separated within a short analysis time by NACE using sodium acetate−acetic acid in ACN/methanol mixture as running buffer for the first two test mixtures and sodium formate−formic acid in ACN/methanol mixture for the third mixture.[184] They were also analyzed by NACE/MS employing ammonium acetate in ACN/methanol mixture as running buffer and the results showed were compatible with detection of all the components, but there was insufficient separation of BRL29060 and BRL57138A. Therefore, the data have been used qualitatively for confirmation of peak identity and for peak tracking in method optimization but could not be used as quantitative tool because the MS parameters were not fully optimized. For an acidic test mixture containing hydrophobic, closely related naphthalene sulfonic acids, NACE employing short-end injection was found appropriate for their separation and three unknown impurities were detected.[184] When both aqueous and non-aqueous CE systems containing 20 mM ammonium acetate with both UV and MS detection were applied for the determination of penicillin V and its related substances, although the NACE offered some advantages, best results in terms of selectivity and sensitivity were obtained with aqueous CE.[35]

NACE was successfully applied to the analysis of the water-insoluble drug hexetidine and its hydrolytic (triamine and hexedine) and oxidative (dehydrohexetidine) degradation products and impurities in a commercial liquid pharmaceutical formulation. Using a short-length capillary (37 cm) with a wide bore (75 μm internal diameter) and BGE containing 50 mM sodium acetate and 70 mM acetic acid in methanol, good separation was achieved within 6 min.[185] Among a number of modes of CE (CZE, NACE, MEKC, and MEEKC) investigated for the separation of bromazepam and five of its impurities, NACE provided good separation of the impurities and was proved to be the best mode overcoming the disadvantage of the low solubility of benzodiazepines in water. The method showed good selectivity and sensitivity with LOQ for the impurities being 0.05% of the drug substance.[186]

1. Detection

UV detection, the commonly used method in aqueous CE is usually applicable to NACE as well. However, organic solvents exhibiting strong UV absorbance at the detection wavelength require the use of indirect UV or other alternate detection methods. These may provide better sensitivity than in aqueous systems, e.g., FD, electrochemical detection, and MS detection. The enhanced sensitivity with NACE-FD was attributed to lower quenching due to the low-molecular-weight oxygen present in organic solvents. Drug purity testing seems to be a promising area in NACE-ECD because the number of electrochemically detected compounds is larger than in aqueous systems and the high sensitivity of ECD can be exploited to ensure high-quality requirements.[182] However, not all impurities can be detected by FD and ECD and these selective detection modes are not suitable when full impurity profiling is required.[187] Replacement of water with organic solvents in CE/MS meets practically all the criteria required to obtain stable spray and high sensitivity, and the

formation of spray is favored by the low heat of vaporization and low surface tension of organic solvents. The use of NACE/MS for impurity profiling of drugs needs to be further exploited.

C. Micellar Electrokinetic Capillary Chromatography

MEKC was first introduced by Terabe in 1984 for the high-resolution separation of neutral analytes. Being a subclass of CE, MEKC also shows considerable promise for the analysis of both charged and neutral analytes. The presence of surfactants in the running buffer above the critical micellar concentration (CMC) creates a pseudostationary phase enabling high-efficiency chromatographic separations. The pseudostationary phases can be formed by using either charged micelles or covalently bonded polymerized charged assemblies. The surfactants can be categorized by the charge of the head group (as non-ionic, anionic, cationic, and zwitterionic surfactants) or by the variations in the nature of hydrophobic moiety (as hydrocarbon, bile salts, and fluorocarbon surfactants). The selectivity of the technique can be manipulated by controlling factors like temperature, choice of surfactant, modification of the micelle (mixed micelles), and modification of the aqueous phase. Additives such as CDs, ion-pair reagents, urea, organic solvents, and metal ions modify the aqueous phase. The micellar pseudostationary phase and the surrounding aqueous buffer phase migrate at a different velocity and the neutral solutes partition between the two phases and migrate at velocities between the electroosmotic velocity (V_{eof}) and velocity of the micelle (V_{mc}) and this interval is termed as migration time window.

Drug-related impurities tend to have similar charge-to-mass ratios to each other and to the main drug component and so are often difficult to be separated by CZE. In impurity profiling, the nature and physical properties of impurities are often at least partly unknown, and might contain both charged as well as neutral compounds. Therefore, MEKC is a more appropriate technique for impurity profiling than CZE, because neutral and charged components can be separated simultaneously with improved selectivity by a combination of charge-to-mass ratio, hydrophobicity, and charge interactions at the surface of the micelles. It can also be considered when simple mobility differences prove insufficient in CZE. The application of MEKC to the impurity profiling of drugs has been described elsewhere.[5,115,187–196]

Impurity profiling by MEKC was demonstrated for the first time in 1990 for the separation of diltiazem and its impurities. MEKC employing the bile salt, sodium cholate, as the micellar phase provided satisfactory separation of diltiazem and its impurities within 15 min.[197] Salicylamide and 12 of its impurities were separated within 6 min by MEKC with a separation buffer containing 20 mM phosphate buffer and 75 mM SDS. Trace impurities of salicylamide could be detected at 0.1% level.[198] MEKC allowed the determination of small quantities of caffeine and xanthine impurities present in purified pentoxiphylline when injected at a concentration of 20 mg/ml. By standard addition method the quantities of the impurities were estimated as 0.11 and 0.08% for caffeine and xanthine, respectively.[199] MEKC was found well suited for the quantitative determination of *p*-toluenesulfonic acid impurity present in BMS 180317-01, a pharmaceutical intermediate used in the synthesis of a novel anti-depressant drug candidate BMS 181101-02. The method offered good selectivity and the results obtained for several batches of the drug were comparable to those from an LC method.[200]

In 1990, the applicability of cyclodextrin-modified MEKC (CD-MEKC) was successfully explored for the first time by adding a neutral CD to the micellar solution for the separation of highly hydrophobic and closely related compounds such as chlorinated benzene congeners, polychlorinated biphenyl (PCB) congeners, and tetrachlorodibenzo-*p*-dioxin (TCDD)

isomers.[201] The separation of such substances is generally difficult, but in CD-MEKC the differential partition of the hydrophobic solutes between the CD and the micelle enables a separation to be achieved. CD-MEKC employing 15 mM HP-β-CD, 30 mM sodium cholate, and 25 mM phosphate-borate buffer at pH 6.3 provided satisfactory separation of ondansetron (DSI) from four of its drug-related impurities within 8 min with significant improvement in the peak shape for DSI over LC. The impurity levels determined by CD-MEKC were in good agreement to those obtained by LC but, as could be expected, the detection limit of the LC system was three orders of magnitude lower than the MEKC system.[202] CD-MEKC employing 2 mM β-CD and ACN as modifiers was used for the quantitation of the principal related impurities of bile acids present in UDCA and deoxycholic acid bulk drugs at 0.05−1.5% level.[203] The first time that a test for related substances by means of CE (CD-MEKC) was included in the European Pharmacopoeia was in the monograph "Levocabastine Hydrochloride".[71] The test includes the use of current gradient for 60 min and limits the total amount of impurities to be present (five specified and four detectable impurities) below 1%.

CZE allowed baseline separation of the anti-depressant GR50360A from a potential manufacturing impurity, the des-5-fluoro analog, which was difficult to achieve by LC.[204] However, CZE at low pH was unsuccessful to resolve the anti-depressant GR50360A from five closely related potential impurities including the des-5-fluoro analog and several *N*-alkyl derivatives. Only with the addition of SDS with organic modifier, it was possible to resolve all six compounds.[189] MEKC employing SDS as surfactant was tried for the analysis of samples of the anti-inflammatory steroid fluticasone propionate but was unable to resolve its potential impurity, the des-6α-fluoro analog. With the addition of 20% methanol as additive, baseline resolution of the desfluoro analog and an unknown impurity was possible.[189] MEKC using organic modifiers as additives was successfully applied to the separation and analysis of synthetic low-level diastereoisomer impurities present in both water-soluble and -insoluble chiral pharmaceutical compounds. The impurity profiles of nalbuphine obtained from three different MEKC systems containing sodium taurodeoxycholate (STDC), SDS, and cetyltrimethylammonium bromide (CTAB) as surfactants were compared and all of them were able to separate the impurities from the main compound with different selectivity for the anionic and cationic systems. Using a Z-cell, the diastereoisomeric impurities could be detected at 0.005% level, which allowed the sensitivity of the analysis to approach that of LC.[205] MEKC with ACN as organic modifier provided a sensitive and selective alternative to reversed-phase gradient LC to determine low levels of impurities ($<0.1\%$) in the drug substance SB-209247, a leukotriene B_4 antagonist. Seven potential impurities were separated with high resolution within 17 min using a separation buffer consisting of 50 mM sodium borate−ACN (65:35, v/v) containing 50 mM SDS.[206] Enhancement of separation efficiencies and method sensitivity was achieved by using a specific solvent system (20 mM borate pH 8.5−ACN; 80:20) to provide focusing effects for both charged and neutral analytes. The method demonstrated competitive detection limits and complementary selectivity in comparison with the LC method. Paclitaxel, the powerful anti-cancer drug obtained from extracts of the bark of *Taxus brevifolia* was separated by MEKC from 14 related taxanes, which are coextracted and can be found at low levels in the bulk drug and in injectable dosage form. A separation buffer containing 40 mM SDS, 25 mM Tris buffer at pH 9.0, 30% ACN, and 10 mM urea allowed resolution of the 15 taxanes from each other and from the principal matrix ingredient in the injectable dosage form of the drug (Cremophor EL; polyethoxylated castor oil) within 11.5 min. It was necessary to dissolve the samples in 10 mM SDS because unacceptably poor resolution of the taxanes was found when the sample was dissolved in methanol.[207]

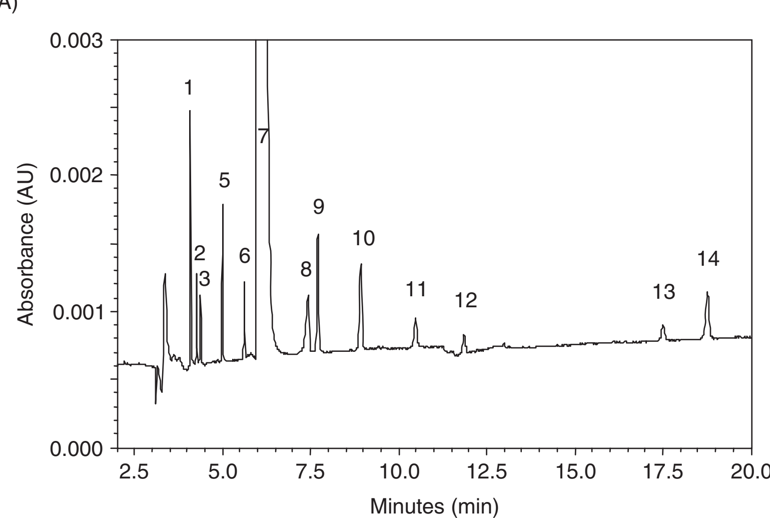
A mixed MEKC system employing zwitterionic and non-ionic surfactants was used for the investigation of impurities in imipramine-*N*-oxide. The non-ionic surfactant improved the resolution keeping the current as low as possible. With this system, four known impurities and

two unknown impurities were separated.[208] An MEKC system containing an ion-pairing reagent tetrabutylammonium bromide (TBAB) was successfully used for separation and quantification of mesalazine (5-aminosalicylic acid, 5-ASA) and its four major impurities 3-ASA, salicylic acid, sulfanilic acid, and 4-aminophenol at 0.1% (m/m) level within 6 min by using a short capillary.[209] Characterization of the impurity profile of amino acids at 0.1% level was accomplished by MEKC with prior derivatization of the compounds using 9-fluorenylmethyl chloroformate (FMOC) for UV detection at 254 nm. The method was sufficiently selective due to the fact that the reagent peaks did not interfere with the impurity peaks and it was successfully applied to phenylalanine samples of different manufacturers and tryptophan and serine samples.[210] In a subsequent study, using a similar MEKC system but with prior derivatization of the compounds with 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde (CBQCA) for LIF detection, determination of the potential impurities such as amino sugars, low-molecular-weight peptides, and amino acids was achieved at a level of 0.1% (w/w) with a broader spectrum of detectable substances compared to the method utilizing FMOC derivatization and UV detection.[211]

On-line coupling of MEKC to MS was accomplished by direct introduction of the BGE into an ESI source for the separation of mebeverine and its six related compounds. Although significant suppression of the analyte signal occurred and no signals were observed for the minor components in the total ion chromatogram (TIC), using selected ion monitoring (SIM) mode the detection limits for the related compounds were 10−50 ng/ml, which is quite favorable allowing the detection of specified impurities below 0.01% level. Direct coupling of MEKC to ion-trap MS with MS^n capabilities allowed the gain of structural information of unknown impurities.[212,213] Direct coupling of MEKC to MS with the use of MS-compatible surfactants such as perfluorooctanoic acid (PFOA) showed good separation performance without serious suppression of ionization, and quantitation of trace impurity present in terbutaline sample was possible at 0.01% level in a full-scan experiment and at 0.001% (25 ppb) level in SIM. By performing MS/MS experiments structural information of the impurity was successfully obtained.[214] A rapid and selective MEKC system was established for the simultaneous separation of the charged ketorolac tromethamine (KT) and three known potential neutral impurities at 0.1% (w/w) in both bulk drug and coated tablets. Taking into account the high difference in the expected concentrations and measured absorbances of KT and its impurities, two internal standards flufenamic acid and tolmetin, which have similar absorption characteristics, were selected to quantify the drug and its impurities, respectively. Using 73 mM SDS, 13 mM boric acid, and phosphoric acid adjusted to pH 9.1 with 1 M NaOH, all compounds were baseline resolved within 6 min.[215]

An MEKC system employing the anionic lithium dodecyl sulfate (LiDS) as the pseudostationary phase allowed selective separation of didanosine from 13 potential impurities within 20 min, which could not be separated by conventional CZE (see Figure 7).[216] The advantages of using LiDS as opposed to SDS include in this case higher separation efficiencies, shorter analysis time, lower operating current, better signal-to-noise (S/N) ratio, easier resolution optimization, and solubility higher than 1 mol/l. The method was successfully applied to the analysis of didanosine and its impurities in commercial bulk samples, and seven known and four unknown impurities were detected.[216] Purity determinations of alprostadil bulk drug were performed by MEKC, and with the aim of increasing signal sensitivity, two related procedures, FASS and extended path length detection (EPLD) through the use of a Z-cell capillary, were used. The drug was separated from eight process and degradation impurities and from the internal standard, except for 11-epi-PGE_1, which co-migrated with the alprostadil peak and appeared as a shoulder. Both techniques used to enhance signal response were found to be precise ($\pm$2% RSD) and when applied to the analysis of two batches of alprostadil samples, the results showed good agreement with those obtained by LC depicting the orthogonal means of using MEKC for purity testing of

(A)



(B)

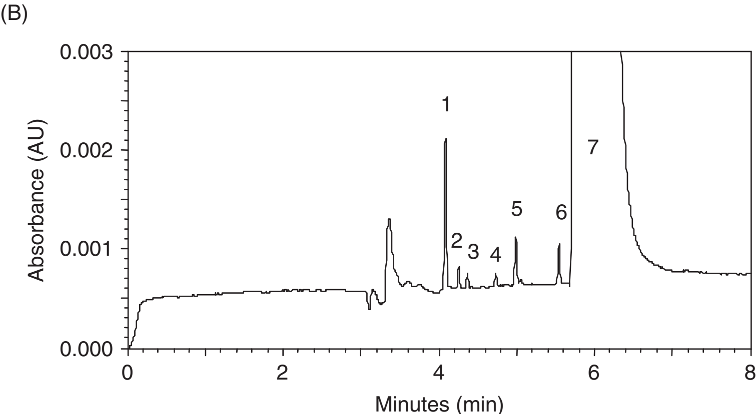


FIGURE 7 (A) A typical electropherogram of the separation of didanosine from 12 of its related substances by using an uncoated fused-silica capillary with a total length of 40 cm and an effective length of 30 cm (50 μm ID); a BGE containing 110 mM LiDS and 40 mM sodium tetraborate at pH 8.0; an applied voltage of 18.0 kV ($I = 61\ \mu A$) and the capillary temperature maintained at 15°C; UV detection at 248 nm; sample injection: 5.0 s × 3.5 kPa. (B) Separation of a profile standard mixture containing six of its impurities under the same conditions. Peaks: 1: hypoxanthine; 2: 3′-deoxyinosine; 3: 2′-deoxyinosine; 4: 2′,3′-anhydroinosine; 5: inosine; 6: 2′,3′-didehydrodidanosine; 7: didanosine; 8: 2′,3′-dideoxyadenosine; 9: olefine; 10: didanosine acetate; 11 and 12: two configurational isomers around the *N*-glycosidic bond; 13: isomer 1 of bromoester; 14: isomer 2 of bromoester. (Reprinted from reference 216, with permission.)

alprostadil.[217] Characterization, impurity profiling, and stability testing of a complex dendrimeric contrast agent Gadomer was performed by MEKC. Using 0.5 mM CTAB and 15 mM sodium phosphate, pH 6.3 as the BGE, the target substance Gadomer 24 was separated from four related dendrimers containing amino or carboxyl functions and from four impurities of lower molecular weight. The method was successfully applied to the purity and stability analysis of different Gadomer batches and to the injectable formulations.[218]

I. Stability-Indicating Assays

Impurity profiling is essential also in stability-indicating studies to separate and detect potential degradation products. The high peak capacities that can be obtained by MEKC are extremely useful in order to minimize the chance that impurities overlap and remain undetected. A number of stability-indicating CE methods were applied to both stored drug substance and various formulations. A stability-indicating assay for cefuroxime axetil suspension by MEKC was found to be advantageous over LC because the sample was simply dissolved in methanol prior to analysis by MEKC,[219] whereas the LC method required extensive sample pre-treatment because of the complex nature of the product. MEKC assay of pilocarpine and the three possible degradation products illustrated the potential utility of the technique for the separation of diastereoisomers that are difficult to be separated by other means.[220]

A mixed MEKC system employing the anionic detergent, SDS, and the non-ionic detergent, Brij 35, was advantageous over a reported CZE method for stability-indicating, quality control analysis of the antihypertensive drug enalapril maleate.[221] The CZE[117] method did allow the drug to be separated from its two degradates, enalaprilat and diketopiperazine (DKP), however, without the ability to quantitate DKP, which possibly co-migrated with other neutral components; whereas the mixed MEKC method allowed quantitation of the DKP degradate. An MEKC stability-indicating, quality control method was developed and validated for the diuretic drug substances hydrochlorothiazide (HCTZ) and chlorothiazide (CTZ).[222] Employing the anionic surfactant SDS, separation of HCTZ, CTZ, and the common impurity 4-amino-6-chloro-1,3-benzenedisulfonamide (DSA) was achieved, and for the quantitation of DSA, the use of 100 μm ID capillary was necessary to provide enough sensitivity.[222] MEKC parameters were optimized for stability measurements and investigations on hydrolysis of platinum anti-tumor drugs cisplatin, carboplatin, and lobaplatin in aqueous solutions. The optimized parameters allowed high-resolution separation of the intact drugs and hydrolyzed species within a short analysis time.[223] A stability-indicating assay by MEKC for a cholesterol-lowering drug BMS-188494 formulated in capsules has been accepted as part of a regulatory submission to the US Food and Drug Administration (FDA). The method offered an alternative selectivity to LC enabling both drug and its degradation products with differing polarity to be separated using "isocratic conditions," which do not require extensive column equilibration between analyses.[224] The analysis of rofecoxib and its photodegradation product in the presence of an internal standard was done by MEKC within 10 min. The method was successfully applied to the analysis of tablet formulations and the results were compatible with those from a voltammetric method.[225]

An MEKC method for the determination of ibuprofen, codeine phosphate hemihydrate, their nine potential degradation products, and impurities in a commercial tablet formulation was developed, optimized, and fully validated according to ICH guidelines and submitted to the regulatory authorities. The optimized system containing ACN as organic modifier allowed baseline separation of ibuprofen, codeine, and nine related substances within 12 min.[226,227] The concentration of the components in this system was slightly modified in order to develop and optimize a partial-filling MEKC/MS (PF-MEKC/MS) method for the separation of ibuprofen, codeine, and one of the degradation products.[228] PF-MEKC/MS is discussed more in detail in Section II.C.5. PF-MEKC allowed good separation but broader peaks were observed compared to conventional MEKC. However, the peak shapes and robustness improved markedly by injecting a small micelle zone after the sample. In a subsequent study, the borate buffer in the BGE was changed to the volatile ammonium acetate and the MS parameters were optimized for the separation and identification of degradation products of ibuprofen and codeine by varying the fragmentor voltage, composition of the sheath liquid,

and flow rate of the sheath liquid in an off-line mode. The optimized sheath liquid containing 50% methanol, water, and 1% ammonium acetate produced a high-intensity peak for ibuprofen but was not suitable for the PF-MEKC/MS system because it was not possible to detect codeine.[229] Therefore, an acidic sheath liquid was used for further study in order to detect codeine, despite not being optimal for ibuprofen, however, most intense ibuprofen and codeine peaks were obtained by using a flow rate of 8 μl/min. The precision of the system was acceptable although lower than in MEKC-UV, and in SIM mode, ibuprofen, codeine, and all degradation products gave an LOD of 10 pg.

2. Antibiotics and Other Antibacterials

The composition of antibiotics depends greatly on the fermentation conditions and the subsequent purification applied. Impurity profiling of different antibiotics by LC, LC/MS, and CE are described elsewhere.[7–9,230] The MEKC applications for impurity profiling of antibiotics are briefly discussed in this section. Amoxicillin and five of its degradation products, and clavulanic acid, an additional important component of the antibiotic Augmentin®, were separated by MEKC.[231] An MEKC system with ACN as organic modifier was found to be more selective in separating amoxicillin and its 16 potential impurities within 14 min, whereas LC analysis required a gradient elution of 40 min.[232] The selectivity of the method was better than LC; however, quantitative determination of impurities by MEKC was delicate because their migration depended very much on slight variations in the BGE. MEKC was successfully employed for impurity profiling of batches of β-lactam antibiotics [ampicillin, amoxicillin, and phenoxymethylpenicillin (pen V)] and erythromycin stearate samples obtained from different pharmaceutical suppliers. Although the identity of the impurities is unknown, the impurity profile in terms of number of impurities and their respective levels allowed distinguishing samples from different manufacturers and can therefore be used as a fingerprint for source verification in suspect/counterfeit cases.[233] MEKC permitted the separation of pen V[234] as well as benzylpenicillin (PG)[235] from their eight potential impurities. There was no significant difference between the results obtained by both MEKC and LC analysis of pen V and PG. In a subsequent study, a more rapid MEKC system using methanol as organic modifier was accomplished for the separation of pen V and its eight potential impurities and the results correlated well with those obtained by LC.[236] CZE and MEKC were employed for the separation of ampicillin and 15 related substances. Although both techniques allowed separation of ampicillin from its four oligomers, which are related to the allergenic effect of penicillins, MEKC was more suitable for the separation of other degradation products.[237] In a subsequent study, another MEKC system with voltage gradient was applied to the separation of ampicillin and nine related substances. The method was applied to ampicillin formulations and the impurity profile was in good agreement with that determined by LC.[238]

Baseline separation of the cephalosporin antibiotic cephradine, its main impurity cephalexin, and other related impurities was achieved by MEKC.[239] The method was validated in compliance with the USP XXII analytical performance parameters and the results were comparable with a validated LC method, depicting CE to be a valuable alternative technique to LC in pharmaceutical quality control. In most cases, the amount of impurities relative to the main compound measured by MEKC is similar to that obtained by LC. However, some reports reveal that there are differences in number and amount of impurities between MEKC and LC analysis. MEKC permitted the determination of seven known and three unknown impurities in cefotaxime and the results were in good agreement with those of LC.[240] MEKC yielded a higher amount of the cefotaxime dimer but a lower amount of an unidentified impurity with respect to LC. The differences may be due to the easier formation of the dimer in the aqueous sample solvent used in MEKC compared to the hydroorganic

mobile phase used in LC. Ceftazidime and its two major related impurities, pyridine and Δ^2-isomer, could be resolved by MEKC.[241] The method was successfully used to obtain stability-indicating data of reconstituted solutions of ceftazidime for injections in different storage conditions and the results were comparable to LC.[242] MEKC was shown to be suitable for the determination of 10 related substances in cefadroxil as well as in cefalexin with better selectivity and speed compared to LC.[243,244]

The separation of tetracycline and its degradation products anhydrotetracycline (ATC), 4-epitetracycline, and 4-epianhydrotetracycline (EATC) was accomplished by a mixed MEKC system employing two neutral surfactants, Triton X-100 and Brij 35. However, the ADTC was not separated and the peak shapes of ATC and EATC were poor.[245] MEKC employing a non-ionic surfactant Triton X-100 as the pseudostationary phase, satisfactorily separated demeclocycline, a member of the tetracyclines, from four related impurities.[246] However, the 2-acetyl-2-decarboxamidodemeclocycline (ADDMCTC) was not separated. Therefore, the method was modified by adding M-β-CD to the MEKC buffer system, and selective separation of all the impurities including ADDMCTC was achieved.[24] MEKC employing Triton X-100 allowed complete separation of OTC and its eight related substances within 15 min and the quantitative results were comparable with those of LC and CZE.[24,247]

The two main advantages of MEKC over LC for purity screening of compound families are (1) efficiencies often 10-fold that of isocratic LC methodology and (2) assurance of detection of all soluble material in a sample. MEKC employing SDS was found to be a generalized alternative to LC for purity determination of a class of investigational antibacterial drugs.[248] The purity data of a large number of oxazolidinone antibacterials (linezolid and some of its congeners) showed average impurity levels about twice that shown by LC (0.2% versus 0.1%). Five compounds out of 15 gave lower purity results by MEKC than by LC and this difference might be due to the fact that the higher efficiency and finite migration window of MEKC allows observation of impurities not seen by LC. MEKC employing the cationic surfactant trimethyltetradecylammonium bromide (TTAB) allowed the determination of residual quantity of streptomycin present in bulk dihydrostreptomycin and also some unknown components present in dihydrostreptomycin standard.[12] When both CZE and MEKC were employed for the analysis of fluoroquinolone carboxylic acids and their major photodegradation products, MEKC was found to be less selective and was not used for quantitation, but only used to compare and confirm the presence of some degradation products.[46]

A mixed micellar system employing the bile salt sodium cholate and cationic CTAB was applied to the analysis of the macrolide antibiotics, tylosin and erythromycin.[249] The method permitted the separation of tylosin from eight closely related substances and erythromycin from its important related substances; however, detection sensitivity and resolution were not sufficient for the analysis of erythromycin-related substances in commercial samples. A somewhat similar mixed micelle system was applied to separate spiramycin I from six related substances.[250] Although the method allowed separation of impurities in short analysis time, it was found to be less selective than the reported LC method. Clindamycin and related impurities have been selectively separated using a mixed micellar system containing anionic SDS and non-ionic Brij 35 within 23 min.[251] The MEKC analysis of two commercial bulk samples demonstrated the presence of five known and three unknown impurities of clindamycin. A mixed micellar system containing a zwitterionic surfactant 3-(*N*,*N*-dimethylhexadecylammonium)propane sulfonate (PAPS) and Brij 35 in acidic solution allowed selective separation of bacitracin from more than 50 peaks within 30 min.[252] The presence of the non-ionic surfactant Brij 35 in the mixed micellar system is important for good peak shape of the main components clindamycin[251] and bacitracin.[252] MEKC employing the cationic cetyltrimethylammonium chloride (CTAC) resolved vancomycin from more than

20 impurities within 8 min and was found to be highly selective and superior compared to the reported LC methods.[253]

Aminoglycoside antibiotics always contain in their structure a variety of structurally related amino sugars having neither a chromophore nor a fluorophore. CD-MEKC employing 20 mM sodium deoxycholate, 15 mM β-CD, and 100 mM sodium tetraborate buffer at pH 10 as BGE was able to separate the major and minor components of gentamicin as well as its known impurities garamine, 2-deoxystreptamine, and paromamine and several unknown impurities with prior derivatization of the compounds with OPA and MAA.[254] This system was successfully applied to the investigation of 46 bulk samples of gentamicin from different manufacturers and pharmaceutical companies, and almost all the samples met the requirements of the Ph.Eur. and USP. The results were supported by NMR spectroscopy, which was found to be a useful primary method orthogonal and complementary to MEKC for purity assessment of aminoglycoside antibiotics.[255]

3. Illicit Drug Seizures

Chemical characterization of illegal drugs can contribute to the success of the control of the illicit drug market. Comprehensive analysis of drug seizures involves qualitative and, in some instances, quantitative analyses of the controlled substances. For strategic and tactical intelligence, a more in-depth analysis of illicit drugs is usually necessary, which includes the determination of impurities, adulterants, and diluents.[173] The impurities present in illicit drugs can be biosynthetic if the drug is of natural origin and the number and quantity of these compounds depend strongly on the origin and the age of the drug-producing plant. Impurities of synthetic origin include manufacturing by-products and residual reactants as well as decomposition products due to storage. Moreover, often adulterants are added to illicit drugs as cutting agents and can form significant part of the sample, for example, pharmaceuticals such as quinine and phenobarbital are added to mimic heroin, and diluents such as starch and sugars are added merely to dilute the drug.[173,187] Strategic intelligence typically involves the determination of ratios of impurities present and these ratios are indicative of the originally manufactured drug since the absolute values can change if adulterants or diluents are subsequently added. For tactical intelligence the determination of ratios of adulterants to heroin are useful. MEKC is a promising analytical technique for the impurity profiling of illicit drug seizures and can be used as a complementary technique to LC and gas chromatography (GC). Applications of CE to the impurity profiling of illicit drugs have been described elsewhere.[187,193,194,196,256] The MEKC applications for impurity profiling of illicit drugs are briefly discussed in this section.

MEKC was found to be advantageous over LC for illicit drug screening. It resolved approximately twice as many peaks for a complex mixture consisting of acidic and neutral impurities present in an illicit heroin seizure sample compared to LC.[257] The MEKC analysis of bulk heroin, its basic impurities, and the common adulterants phenobarbital and methaqualone was performed in short analysis time with superior resolution; and the analysis of illicit cocaine and its basic impurities showed better peak symmetry for cocaine compared to LC.[257] In a subsequent study, a rapid MEKC system with a shorter capillary and a reduced micelle concentration was used to resolve heroin from accompanying impurities and adulterants in less than 3 min.[258] A similar system was used to separate heroin and amphetamine from structurally related compounds and commonly occurring adulterants in drug seizures in about 10 min.[259] The anionic SDS used by Weinberger and Lurie[257] was replaced with the cationic surfactant CTAB as the micellar phase in order to improve repeatability of the migration times and to achieve shorter run time for the quantitation of the complex heroin seized by law, and the quantitative results were comparable with those obtained by LC.[260] The same method was applied for quantitation of cocaine and related

substances at a different UV detector wavelength and the results were comparable to a fully validated GC method. The method was proved rugged and reliable for both heroin and cocaine in a number of inter-laboratory proficiency studies.[261] A similar system was also tried for the analysis of illicit amphetamine seizures, however, a totally new[262] MEKC buffer system employing CTAB, ethanolamine, and DMSO had to be used to enable reliable routine quantitation of the wide range of amphetamines and associated impurities. The use of DMSO as organic solvent improved the peak shapes; however, due to its high UV cutoff, sensitivity of the method was limited. In order to detect more cocaine constituents an MEKC system containing 75 mM SDS and 17.5% methanol was developed for the separation of cocaine from its common impurities and several degradation products, and the system was successfully applied to the analysis of various cocaine seizures.[263]

Improved separation of acidic and neutral impurities in illicit heroin was obtained using CD-MEKC.[264] The presence of anionic β-CD SBE IV in the system significantly improved the resolution of the late migrating peaks by increasing the migration window, and the developed methodology was applicable to both crude and refined heroin. The separation of a panel of forensic substances including heroin, heroin metabolites, impurities, and adulterants and other illicit drugs by means of MEKC and CZE was compared to infer the degree of mutual correlation. Although most of the compounds were separated by CZE, MEKC was able to resolve all 20 compounds.[265] An acidic MEKC system containing 50 mM 6-aminocaproic acid, 50 mM zwitterionic methylmyristylammoniopropanesulfonate (MAPS), and 5 mM 1-heptanesulfonic acid with 10% ACN as organic modifier was used for the separation of heroin from its by-products and adulterants.[266] The decomposition rate of morphine derivatives was monitored by MEKC and significant hydrolysis was found at $pH > 8$. Therefore, for the analysis of these derivatives, another MEKC system was designed to keep in situ hydrolysis rate negligible for every compound.[267] MEKC with dynamically coated capillaries provided an excellent separation of the neutral, acidic, and weakly basic adulterants of heroin within short analysis time compared to conventional MEKC, and the results were in good agreement with those obtained by LC. The dynamic coatings give rise to a relatively high and robust EOF at $pH < 7$ and therefore allow for rapid, precise, and repeatable separations.[173,268]

4. MEKC Strategies for Impurity Profiling

Generic strategies for impurity profiling of drugs by MEKC have been described elsewhere.[187] An MEKC system containing SDS with a buffer of pH 8−10 is a good starting point to achieve an initial separation, but does not always provide sufficient separation to resolve all the impurities from the main component. The resolution can be further enhanced by widening the migration time window through the addition of organic modifiers to the buffer system.[187] The potential of MEKC for impurity profiling was demonstrated by using both SDS and CTAB systems for the separation of fluvoxamine from its basic, neutral, and acidic impurities.[269] The elution order of the compounds in both systems was different indicating selectivity differences of anionic and cationic MEKC systems. With the SDS system, complete separation of all impurities required the addition of 15% methanol or 10% ACN to the buffer system, whereas the CTAB system allowed complete resolution of all the impurities without the addition of organic modifier. Using the SDS and CTAB systems operating at the same pH for the analysis of the same sample, it should be possible to detect every impurity present because all components of a sample pass the detector in at least one of the systems. Subsequently, this strategy was further optimized based on requirements such as the analysis time of both systems to be below 20 min and the migration window to be above 3 or 2 for the SDS and CTAB system.[270] The optimized strategy involves the combined use of an SDS system containing 10 mM phosphate pH 7.5, 60 mM SDS, and 10% ACN; and a CTAB system

containing 25 mM phosphate pH 7.5, 10 mM CTAB, and 10% ACN. The potential of this strategy for impurity profiling was demonstrated by testing multiple charged compounds (negative and positive) and by the analysis of fluvoxamine and some of its impurities at 0.1% level. Impurities that cannot be separated in one system can be baseline separated in another (see Figure 8). Such a strategy including a combination of an SDS and a CTAB system can be used for initial impurity profiling separation, after which, if necessary, further optimization can be performed using an MEKC optimization strategy developed by Terabe.[271]

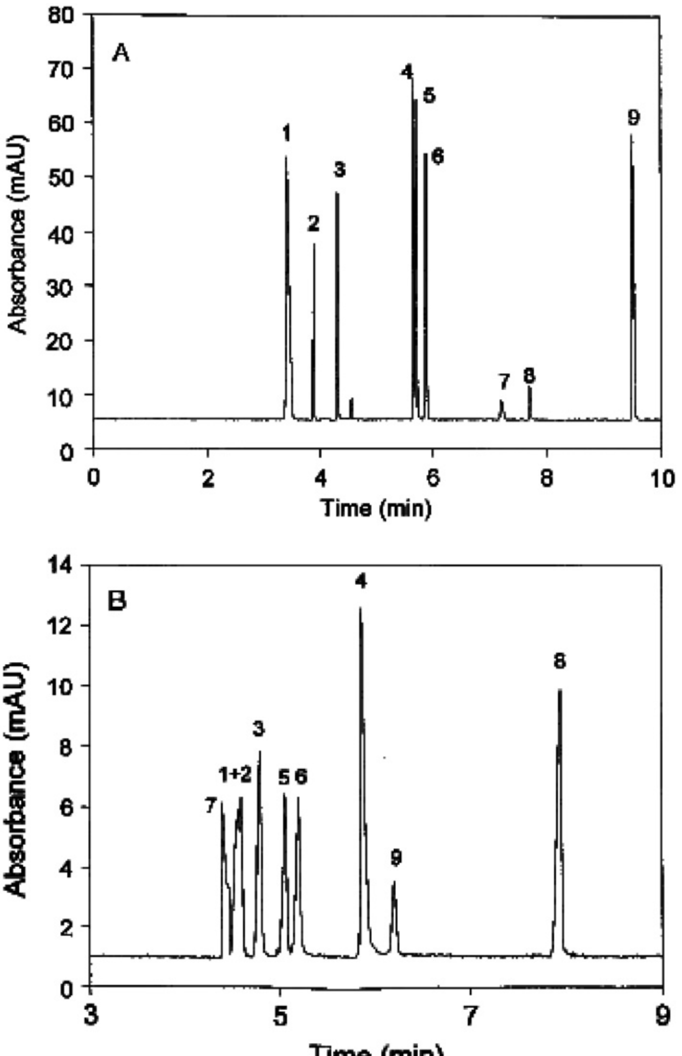


FIGURE 8 **Analysis of the test mixture with (A) the SDS–MEKC system containing 10 mM phosphate buffer (pH 7.5), 60 mM SDS, and 10% acetonitrile and (B) the CTAB–MEKC system containing 25 mM phosphate buffer (pH 7.5), 10 mM CTAB, and 10% acetonitrile. Peaks: 1: formamide; 2: pyridine; 3: aniline; 4: *meta*-cresol; 5: phenyl acetate; 6: nitrobenzene; 7: benzoic acid; 8: thiamine; 9: ethyl benzoate. (Reprinted from reference 270, with permission.)**

5. Detection

The detection modes employed are similar to those of CZE, which include on-column detection via UV absorbance (down to 185 nm) and LIF, or end-column detection by MS and ECD. To quantify impurities at low level or determine the structure of unknown impurities, it is necessary to interface MEKC with MS. As discussed earlier, the BGE containing SDS, sodium phosphate buffer, and organic modifier was directly introduced into an ESI source for the impurity profiling of mebeverine and no signals were observed for the impurities in the TIC due to significant suppression of the analyte signal but with SIM mode detection limits of 10−50 ng/ml could be achieved.[212,213] However, this coupling is very troublesome due to the presence of non-volatile surfactants and buffers in the BGE, which suppress ionization, forming clusters in the mass spectrum and eventually blocking the ion source. In order to circumvent this problem different approaches have been followed such as the use of either high-molecular-weight surfactants with zero CMC[272] or PF-MEKC[273] or by using sweeping-MEKC-atmospheric pressure chemical ionization (APCI)/MS[274] or MEKC-atmospheric pressure photoionization (APPI)/MS[213] or by using MS compatible surfactants such as PFOA[214]. PF-MEKC/MS was the most common approach in which only a part of the capillary is filled with BGE containing anodically migrating surfactants and the analytes separate as they pass the micelle plug and then continue in CZE until they reach the MS detector. This means that the analytes reach the mass spectrometer before the micelles and the voltage is switched off to prevent the micelles from reaching the MS, making both MEKC separation and MS identification possible. However, limitations such as reduced efficiency due to extra band broadening at the micelle zone buffer boundary and partly due to a smaller migration window are observed relative to conventional MEKC. Although PF-MEKC/MS prevented the surfactants from reaching the MS detector, the use of MS compatible volatile monomeric surfactants was found to be a more straightforward approach with the advantages of MEKC such as higher efficiencies with long-term stability and no memory effects.

The use of high and low injection volumes in CE offers great improvements for quantitative determination of drug-related impurities such as increases in both the total number and the total level of impurities detected, improved LOD, and improved precision when quantifying individual impurities.[143] Since the amount of sample hydrodynamically introduced into the capillary depends on the sampling time (seconds), a short injection time can be used to obtain on-scale analysis and a longer sampling time for measurements of impurity levels. The advantage of high−low CE is that the sample dilutions often required in high−low chromatography can be avoided as variable sample loadings can be achieved by employing different CE sampling times for the same solution. Another approach prescribed for sample preparation for purity testing is using either relatively high concentrations or to preconcentrate the samples to detect less than 0.1% of related substances, because of the relatively low concentration sensitivity of CE instruments.[275] Injecting high concentrations of the main compound with UV detection leads to overloaded main peaks and will cause a subsequent loss in resolution; however, the peak shape can be regulated by substitution of co-ions in the BGE by ions that have a matching mobility compared to the sample ions.[276] An alternative technique for monitoring drug purity without overloaded main peaks is LIF detection, which results in improved LOQ of the impurities exhibiting native fluorescence. CD-MEKC with LIF detection provided adequate resolution of the fluorescent NXX-066 and its impurities. However, with FD only native fluorescent impurities could be analyzed and derivatization with fluorescent labels is not a feasible option because some impurities might not be derivatized.[187] The nature of the solvent used to dissolve the sample has a great impact on the peak shape and separation efficiency. Therefore, the choice of an appropriate sample matrix is very important, particularly during impurity determination as it may affect the actual quantitation.[202,206,207]

D. Microemulsion Electrokinetic Capillary Chromatography

MEEKC employing oil-in-water (o/w) microemulsions was introduced in 1997 by Watarai[277] as an alternative means to MEKC for separating neutral analytes. Microemulsions are solutions containing nanometer-sized oil droplets suspended in an aqueous buffer and the surface tension between the oil and water components is reduced by covering the oil droplet with an anionic surfactant such as SDS and a co-surfactant such as short-chain alcohol allowing an emulsion to form. The separations are based on both partitioning and electrophoretic migration offering the possibility of highly efficient separation of both neutral and charged drug-related compounds covering a wide range of solubilities. To date, there have been only a limited number of MEEKC applications reported for impurity profiling of drugs. However, it can viewed as an alternative to CZE or MEKC due to the distinct advantages offered in the separation of hydrophobic neutral compounds, which penetrate the surface of the droplet more easily than the surface of a micelle which is much more rigid, providing a larger migration window.

MEEKC employing a single set of operating conditions was successfully accomplished for the analysis of drug-related impurities in compounds such as ranitidine and tolbutamide. The impurities could be detected at 0.1% level.[278] It was demonstrated to be a highly efficient technique for the separation of complex mixtures of drug-related impurities by establishing and applying a standard set of MEEKC operating parameters.[279] The method showed good selectivity for the separation of diastereoisomers of cefuroxime axetil and its principal degradation product cefuroxime in comparison to CEC.[280] MEEKC analysis of a heavily degraded sample of pen V provided adequate resolution of five charged and neutral impurities in approximately 5 min.[279] MEEKC allowed the separation of four impurities present in bumetanide within 11 min and this system was found to be very advantageous over LC, which normally requires gradient elution or ion-pair chromatography.[281] An MEEKC system was developed and optimized by mixture design for impurity profiling of KT, and by employing short-end injection, KT and its three potential impurities were separated within 3 min. The method was validated for both drug substance and drug product and was successfully applied to the quality control of KT in coated tablets.[282] The separation of KT and its related substances was compared in three different systems including MEKC,[215] CEC,[283] and MEEKC. Although CEC provided the highest selectivity, considering the analysis performances, MEEKC provided the lowest analysis time (3 min), which is about double and triple for MEKC and CEC, respectively. A generic MEEKC system was validated and found to be stability-indicating and quantitative for the determination of naproxen and rizatriptan benzoate and their degradation products. The method was able to monitor degradation impurities at 0.2% level.[284] MEEKC offered superior selectivity and separation efficiency for the separation of eight hydrophobic diastereoisomers of an HIV-protease inhibitor TMC114 compared to CZE, NACE, and MEKC (see Figure 9). However, eight enantiomers could not be separated.[285]

E. Capillary Gel Electrophoresis

CGE is employed to separate compounds with similar charge-to-mass ratios, such as oligonucleotides, nucleic acids, and dodecyl sulfate−protein complexes. The separations are based on differences in size and shape of the charged analytes and are performed in capillaries filled with gels (e.g., cross-linked polyacrylamide) or non-cross-linked, entangled hydrophilic polymers, including linear polyacrylamide (LPA), dextran, PEG, and hydroxyethyl cellulose (HEC). Although excellent repeatability of migration times and high efficiencies can be

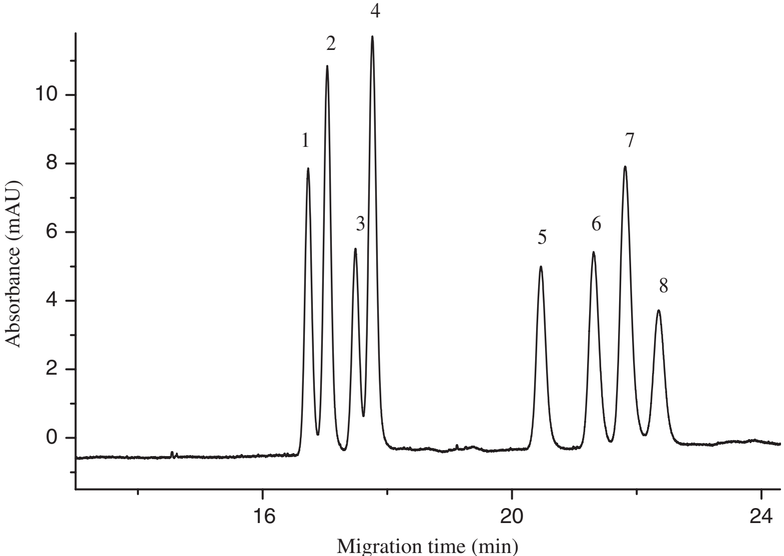


FIGURE 9 Typical electropherogram of TMC114 and diastereoisomers. Capillary: 75 μm ID × 40 cm (31.5 cm effective length); microemulsion buffer: 0.81% (w/w) *n*-octane, 6.61% (w/w) butanol, 3.31% (w/w) SDS, and 89.27% (w/w) 27.5 mM sodium tetraborate buffer; additive buffer: 0.5 mM diaza crown ether derivate and 10% (v/v) acetonitrile; voltage: 11 kV; temperature: 40°C; UV detection at 265 nm; sample injection: 0.4 kPa, 2 s. Peaks: 1: TMC181596; 2: TMC87344; 3: TMC181568; 4: TMC114; 5: TMC181603; 6: TMC181595; 7: TMC96606; 8: TMC96608. (Reprinted from reference 285, with permission.)

obtained when care has been taken for a repeatable production of gel-filled capillaries, the cross-linked polyacrylamide sieving network in the capillaries is heat sensitive and becomes unstable over time, giving the capillaries a limited lifetime. Furthermore, sample introduction can be done only by electrokinetic injection and the various hydrodynamic methods of injection cannot be applied because the capillary is essentially plugged with the gel.

On the other hand, viscous solutions containing entangled polymers are easily replaceable and the capillary can be refilled after each run to prolong the column life time. This technique is also referred to as capillary polymer sieving electrophoresis (CPSE), which uses a sieving medium (e.g., an entangled polymer network) in the BGE. With CPSE, the advantages of CZE such as the choice of injection methods, system automation, speed, and repeatability can be easily implemented. Furthermore, some of the polymers used have a high UV transparency, which is another important advantage compared to capillaries filled with polyacrylamide gel. CGE and CPSE have been majorly used in the impurity profiling of antisense oligodeoxynucleotides (ODNs). CPSE allows the separation of antisense ODNs differing by one nucleotide in length and this feature makes it suitable for checking the purity during chemical synthesis as well as stability in various biological matrices.

1. Purity and Stability Evaluations of Antisense Oligonucleotides

Antisense compounds are promising therapeutic agents for the treatment of viral infection and cancers. These drugs are synthetic single-stranded ODNs comprising 15−30 bases that have unnatural backbones and contain a sequence of bases that is complementary to that of the gene or mRNA of interest. When the drug binds strongly to genetic material,

transcription of the gene is halted, and when it binds to the mRNA, translation is stopped. A critical question in the evaluation of these molecules is their stability toward enzymatic breakdown by 3′- or 5′-exonucleases. In order to inhibit or at least limit the effect of these nucleases, chemically modified ODNs have been synthesized.

Synthetic antisense ODNs are normally manufactured with an automated synthesizer that builds the DNA chain, one nucleotide at a time, and this process yields small quantities of "failure sequences" or shorter pieces of DNA. Therefore, they rarely contain 100% of the full-length sequence due, in part, to the failure sequences produced during synthesis. The full-length ODN is referred to as the *n*-mer and failure sequences are categorized as $n-1$, $n-2$, etc., with the $n-1$ mer present as the largest impurity. Impurity profiling of the synthetic ODN is necessary and should be checked for their biological use. Impurity profiling includes the quantitation of the synthesis-related failure sequence impurities, which is straightforward since it involves a relative area% determination. LC is extensively utilized in the purification of the final product but cannot be used to analytically test the purity of the product because of the difficulty to achieve single-base resolution. Likewise, CZE and MEKC methods can only resolve nucleosides, nucleotides, and single-stranded oligomers of less than 10 bases in length. Larger ODNs have almost equal charge-to-mass ratios and thus require the sieving matrix of CGE. CGE offers best selectivity required for the evaluation of the final products and is therefore envisaged as an analytical tool for purity determination of chemically synthesized ODNs.

High-performance CGE with polyacrylamide gels provided baseline separation of picomole quantities of ODNs (polydeoxyadenylic acids, $(dA)_{40-60}$) in less than 8 min. Besides analytical separations, it has also been used to rapidly characterize synthesized ODNs and to isolate and collect purified fractions.[286] CGE permitted rapid separation of failure sequences of phosphorothioates (SODNs) from 1 to 50 bases within 65 min demonstrating its high resolving power.[287] Quantitative CGE was developed for the accurate quantitation of a 21-mer phosphorothioate ODN, ISIS 2922, and its degradation products in an intravitreal formulation. However, the method was suitable to quantitate the drug alone and was not selective enough to quantitate the degradation products.[288] CGE was successfully used for the stability measurements of antisense ODNs.[289]

CGE purity evaluations were proven to be more accurate than LC.[290,291] CGE in isoelectric buffers (histidine) at voltages as high as 800 V/cm allowed separation of an 18-mer ODN from the failed and truncated sequences (10- to 17-mer) within 4−5 min, with accurate purity evaluation of the product being 65%, whereas with LC results, the purity was thought to be >95% because of the poor resolution of the failed and truncated sequences.[291] The use of isoelectric buffers in CE allows application of very high voltage gradients (up to 1000 V/cm in relatively high bore capillary, e.g., 75−100 μm ID) permitting rapid separations with high resolution due to minimal diffusion-driven peak spreading.[292] CGE against a stationary pH gradient was tried for the separation of the 18-mer ODN contaminated by failed and truncated sequences. Unique analyte resolution was achieved within 12 min.[293]

CGE utilizing an entangled polymer gel matrix such as Micro-Gel allowed determination of purity of synthesized antisense ODNs and DNA analogs (specifically SODNs), and the method was found to be advantageous over LC due to its superior resolving power.[294] An entangled polymer solution system containing high concentration of a low-viscosity-grade HEC as the sieving agent in a coated capillary allowed separation of short-model homo-oligomeric deoxynucleotides ranging in length from 12- to 24-mer.[295] This system showed good separation of $p(dA)_{12-18}$ and $p(dA)_{19-24}$ with a difference of a single base unit in comparison with an MEKC system, which could only separate ODNs up to 13 bases in length.[296] In a subsequent work, a similar entangled polymer system was efficiently applied to check the purity of selected synthetic ODNs and the results showed purity ranges between 54.8 and 96.5%, depicting that the reversed-phase LC purification used after synthesis is

sometimes inadequate.[297,298] Enzymatic degradation of ODNs both in an "off-line" analysis (enzymatic reaction physically detached from the product separation step) as well as in an "in-line" analysis format (enzymatic reaction and product separation steps are integrated) was also studied. The "off-line" analysis showed nice degradation patterns and allowed the calculation of half-lives, and for the "in-line" analysis, "plug-plug" reaction in transient engagement EMMA was explored and the degradation patterns obtained were consistent with the off-line incubation and separation.[297,298] CPSE with similar entangled polymer system was also used to demonstrate the feasibility of in-line coupling (EMMA) of enzymatic degradation of short-model homo-oligomeric deoxynucleotides (dA_{21} and pdA_{21}) with phosphodiesterase I.[299] This automated procedure opened possibilities for screening larger libraries of synthetic antisense ODNs for stability against purified nucleases. To check the intra-cellular stability of the antisense ODNs against nucleases present in HBL 100ras cells, on-line combination of CITP and CPSE was used, however, with the failure to detect ODNs in intact cells.[300]

Determination of absolute concentration and purity of a 20-mer ODN by dynamic sieving CE was performed using a deoxynucleoside triphosphate as internal standard.[301] Likewise, the purity of another DNA analog, a chimeric ODN, which was purified by LC was estimated by dynamic polymer sieving electrophoresis.[302] The results were compared with those obtained with PAGE analysis, which did not allow a wide dynamic range of loadings and so could not be used to evaluate the relative impurity content of various LC fractions. Various kinds of polymers were investigated for CGE analysis of ODNs of less than 20 bases and 30% dextran was found to be effective by baseline separating ODNs $p(dT)_{11-20}$ within 35 min compared to other polymers, and this method can be used for the analysis of impurities in synthetic ODNs.[303] Excellent CGE separations of ODNs, principally dT_{12-18} and dT_{19-24}, were obtained in micellar liquid crystalline 18–30% solutions of Pluronic® F127. The Pluronic® F127 is an uncharged, non-cross-linked $[EO]_{106}[PO]_{70}[EO]_{106}$ triblock copolymer, convenient and easily replaceable medium especially well suited for high-performance CGE separation of ODNs in the size range of primers for DNA sequencing and polymerase chain reaction (PCR), and of oligonucleotide therapeutics.[304] 13% PEG-filled capillaries in 100 mM Tris-borate buffer pH 9.0 containing 30% formamide at 50°C successfully resolved different lengths of antisense phosphorothioates.[305] A CE-SDS non-gel sieving system was developed and validated for the analysis of therapeutic recombinant monoclonal antibodies (rMAbs) with prior derivatization of the rMAbs with 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA.SE). Using LIF detection, size-based separation of product-related variants, as well as non-product impurities (resulting from host cell proteins or cross-contamination from other products manufactured in the same facility), was possible at 0.05% level (w/w).[306]

F. Capillary Electrochromatography

CEC is a hybrid separation technique combining the stationary phase of LC with the electrically driven mobile phase transport of CE with which unique selectivities can be achieved. Solutes are separated according to their partitioning between both phases and, when charged, their electrophoretic mobility. CEC appears to be well suited for impurity profiling of drugs because of its advantages of high selectivity of LC and high efficiency of CE. Long columns with small particle sizes can be used to generate high peak efficiencies and high peak capacities, which is invaluable for impurity profiling.

CEC provided repeatable separation of isradepin and its six by-products within 1.6 min on a 143 mm column packed with 3 μm C_{18}.[307] CEC was found to be more selective compared to MEKC for the impurity profiling of pharmaceutical compounds such as fluticasone propionate and cefuroxime axetil.[280] With a homemade column packed with 3 μm

octadecylsiliyl (ODS) silica particles, the elution times for fluticasone propionate and its impurities ranged from 35 to 160 min.[280] However, by increasing both the pH and organic content of the mobile phase, the run time was drastically reduced to 10 min and an impurity peak previously undetected by LC was separated.[308] Using a similar CEC system, but with MS detection, characterization of the fluticasone impurities was possible. The CEC/MS setup required the use of a relatively long column (90 cm) with which high efficiencies could be achieved.[309] In a subsequent work, impurity profiling of fluticasone by CEC was performed on various stationary phases such as strong cation exchange (SCX) materials, mixed mode material containing both ODS and SCX phases, and a normal ODS column. The mixed mode and the ODS phases had the same selectivity in CEC, whereas the SCX phases had a quite different selectivity resulting in reversal of elution order and they could not separate impurities of similar hydrophobicity.[310] Prostaglandin and its related impurities including benzamidophenol, the enone, and *trans*-isomer were selectively resolved using a column packed with 3 μm Spherisorb ODS1.[280] CEC employing a C_{18} capillary was explored for the separation of various tetracycline mixtures and better separations and peak shapes were found for tetracycline, OTC, and their impurities compared to CZE separations; however, profiling of doxycycline and metacycline was not achieved.[34] A pressurized CEC system at an applied voltage of 30 kV provided baseline separation of an *N*-methylated C- and N-protected tetrapeptide from its non-methylated analog.[311]

Baseline separation of tipredane and its five related substances including the C-17 diastereoisomer, previously unresolved by LC, was accomplished by simply replacing the ammonium acetate buffer used for LC with Tris buffer.[311,312] CEC has been used as a method development tool for the LC separation of 2-phenylmethyl-1-naphthol and its three related substances. A rapid and efficient CEC method could be developed and then transferred to the more rugged LC for routine application, and significant parallels in retention behavior of the compounds were found between LC and CEC separations on the same column type.[313] Therefore, employing similar columns, it is possible to transfer methods from CEC to LC and possibly vice versa; however, in evaluating this approach, there is need to recognize those factors that influence either of the techniques individually.[313] Based on an existing LC method, CEC conditions were optimized and validated for the separation and quantitation of structurally related impurities of a non-steroidal analgesic drug.[314] The optimized system allowed the separation of the drug from its six structurally related impurities within 22 min, whereas the LC separation required a total cycle time of 75 min.

LC, CZE, and CEC techniques were compared for the analysis of tetracycline and its related substances and it was concluded that CEC might be the ideal technique, which combines the best of LC and CE techniques.[315] CEC was found to be a complementary separation technique to LC, CZE, and MEKC for the determination of drug-related impurities in Lilly compound LY3000164 and the drug has been selectively separated from its three potential impurities within 6 min, whereas LC separation required a long analysis time and CZE could not resolve the neutral impurities, which could be separated by MEKC, but with poor efficiencies.[316]

CEC using a binary solvent composition containing ACN and tetrahydrofuran provided rapid and reliable separation of norgestimate and its four degradation impurities within approximately half the analysis time of LC. The system could be effectively used to quantitate the degradation impurities at 0.1% level.[317] CEC employing a Spherisorb ODS I column and a hexylamine containing mobile phase allowed efficient separation of the basic compounds fluvoxamine and its stereoisomer (a potential impurity) demonstrating the potential of CEC for impurity profiling. The presence of the masking amine in the mobile phase (hexylamine) is important for good peak shapes of the basic compounds.[318] With LIF detection and 1.5 μm particle size ODS columns, greatly improved efficiencies were obtained for impurity profiling of illicit methamphetamine with higher peak number compared to LC. Good sensitivity in the

low ng/ml range was obtained for the impurities, which contain a highly fluorescent naphthalene chromophore.[319] Likewise, CEC with LIF detection resolved significantly higher number of peaks for the impurity profiling of heroin compared to LC and MEKC.[320] Using a positively charged stationary phase [monodisperse poly(glycidyl methacrylate-divinylbenzene) microspheres, derivatized with quaternary ammonium octadecyl groups] and a mobile phase containing 45% ACN and 10% ethanol in 30 mM phosphate buffer, pH 8.0, baseline separation of erythromycin A from several of its impurities was achieved, reducing the adverse electrostatic interactions between the macrolides and the silica-based stationary phases (see Figure 10).[321] CEC can be used as an alternative technique to LC for the quality control of ibuprofen. Using a RP-18 packed capillary and a mobile phase containing ammonium formate buffer at pH 2.5 and ACN, separation of ibuprofen and its four impurities could be achieved, however with a long run time of 32 min compared to LC (20 min).[322] On the contrary, CEC separation of KT, its three known related impurities, and internal standard flufenamic acid was achieved in less than 9 min using RP-18 packed capillary and a mobile phase containing 50 mM ammonium formate pH 3.5, water, and ACN (10:20:70, v/v/v).[283]

1. Detection

The detection modes employed are similar to CZE and MEKC. Due to the use of organic solvents, CEC is more suitable for interfacing to MS, which has been widely demonstrated,[323]

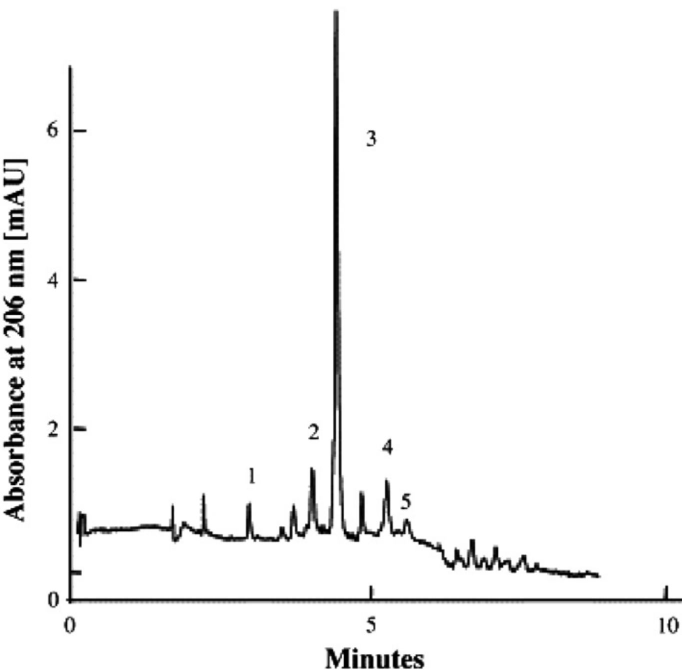


FIGURE 10 Isocratic CEC of erythromycin A and its impurities. Cationic column: 30 cm (effective length 20 cm) × 50 μm ID; mobile phase: 25% (v/v) acetonitrile and 25% (v/v) ethanol in 30 mM phosphate buffer, pH 8.0; applied voltage: −15 kV; detection, 206 nm. Sample: (1) *N*-demethylerythromycin A, (2) erythromycin C, (3) erythromycin A, (4) erythromycin B, (5) erythromycin enol ether. Mobility of EOF measured with DMSO, $\mu_{eof} = 3.33 \times 10^{-8}$ m^2/s V. (Reprinted from reference 321, with permission.)

as compared to MEKC. The sensitivity can be enhanced in CEC by sample enrichment, which can be achieved by simply dissolving the sample in a solvent containing less organic modifier than the mobile phase. Therefore, the analytes are retained and focused in the beginning of the column during injection, allowing larger injection times to be employed without loosing efficiency.[187] A similar strategy has been applied for the profiling of desogestrel, tibolone, and their impurities, which cannot be detected at low level due to their low UV absorbance. Dissolving the analytes in a solvent with significantly less ACN than the mobile phase allowed injection times up to 60 s without affecting the plate numbers.[324] Electrokinetic injection is less useful in impurity profiling due to its potential discrimination between analytes, however, in CEC, effective hydrodynamic injection is often not possible because of the minimum injection pressure allowed by the equipment to overcome the flow resistance of the CEC column.[187] This problem needs to be solved for quantitative impurity profiling by CEC.

III. CONCLUDING REMARKS

It is clear from the applications described above that the strength of CE in the field of impurity profiling lies in the various selectivities that can be applied. Not only can one take advantage of electrophoretic phenomena but also of the principle of chromatographic separation and partitioning that is present in techniques such as MEKC and MEEKC. The resolution of substances based on size differences as in CGE is also very useful in the field of biopharmaceuticals. CE can be seen as a complementary technique to other commonly used separation techniques as LC, and in view of its capacity to separate large numbers of compounds, it should certainly always be kept in mind when an analytical problem has to be solved.

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13

ION ANALYSIS USING CAPILLARY ELECTROPHORESIS

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ABSTRACT

Most drugs are charged molecules, which are weak bases or acids with a counterion. Basic drugs may have an inorganic anion or organic acid as counterion, and acidic drugs a cation. Regulatory agencies (the US FDA, European Pharmacopoeia, etc.) require that pharmaceutical products be tested for their

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identity, composition, strength, quality, and purity both, for the active as well the inactive ingredients. This means that determination of the counterion is an important part of determination of the purity of the drug. During the 15th International Symposium on Microscale Separations and Analysis HPCE meeting in Stockholm in 2002, a discussion group stated that one of the major applications for use of capillary electrophoresis (CE) in pharmaceutical companies is the determination and quantification of drugs counterions. Another application is determination of impurities to check for remaining traces due to the production process.

One of the characteristics of many of these ions is that they are UV transparent, while most CE instruments are equipped with a UV detector. For this reason, a special technique called indirect UV detection is often applied.

We will first review the principles of indirect detection, how it works, and what are its requirements. Thereafter, we will focus on considerations concerning buffer composition and sample preparation. The goal is to develop tools that can be used today with commercially available instruments present in many pharmaceutical companies. We will also review available publications and, in an addendum, describe a complete assay for phosphate as a counterion and an impurity.

I. INTRODUCTION

Ions are highly charged species by nature and lend themselves well to analysis by capillary electrophoresis (CE). In pharmaceutical analyses, we usually deal with small organic and inorganic anions and small cations or aliphatic amines.

This technique is often used as an alternative to ion chromatography (IC). However, CE offers a number of advantages over IC: It is associated with simplicity, great separation efficiency, and unique selectivity. Galli[1] describes the main features of CE as the ability to separate small molecules from complex matrixes, the possibility to measure absorbance at 200 nm or below, and a low volume of reagents. Galli[1] also mentions the requirement for limited sample pre-treatment: For many applications one dilution, centrifugation, or filtration step is enough. Altria et al.[2] points out that the advantages of CE for pharmaceutical analysis are the speed and cost of analysis, the reduction in solvent consumption and disposals, and the possibility of rapid method development. According to many authors, detectability is not as low with CE as with IC or high performance liquid chromatography (HPLC). This arises from the small dimension of the capillaries, typically with an inner diameter of between 20 and 75 μm and a length of 30−100 cm, which limits both the optical pathlength and the volume of sample injected. The optical pathlength may be increased by using larger capillaries or capillaries with extended pathlength. However, the improvement in sensitivity is affected by a loss in separation efficiency. Sensitivity may be an issue for some applications, but for most pharmaceutical applications this is not the case. When compared with IC, CE is also characterized by a smaller dynamic range; this is rarely a problem for pharmaceutical applications because the target value is often well defined. Some authors report the method's lack of reproducibility and robustness. In our experience, this may be overcome by using a dedicated buffer system and adequate methods, and by providing training for the operator. We believe that an additional advantage of CE is that different applications may be run on the same instrument just by changing buffers, method, and, sometimes, the capillary.

Many applications for ion analysis use a UV detector with indirect detection, though other electrochemical, laser-induced fluorescence (LIF), or mass spectrometry detectors have been described. The main advantage of UV detection is its availability on commercial instruments and that both UV-absorbing and non-UV-absorbing analytes may be detected. Nowadays, electrochemical detectors are also available; specific background electrolytes (BGEs) must be used and the detector has to be adapted to existing CE instruments.

Analysis of small ions has been published for many applications other than pharmaceutical applications, and has a growing impact in industrial, environmental, biomedical, clinical, and forensic laboratories. Sample matrices range from simple tap water to Kraft black liquor, including river and seawater, beer and wine, environmental water, and nuclear plant water, but also body fluids such as serum, urine, plasma, cerebrospinal fluid, and many others. Those topics alone would require a separate book.

For more information, we refer to some excellent reviews by Paull,[3] and king Galli et al.[1] and Baena et al.[4] and a more recent update from Timerbaev.[5] Pacáková et al.[6] reviews the importance of CE, capillary electrochromatography, and IC. Metal ions have been reviewed by Timerbaev[7] and shipgin and by Macka[8] and Haddad.

II. GENERAL PRINCIPLE

Separation is performed using free-zone electrophoresis, where the capillary is filled with a separating buffer at a defined pH and molarity. This buffer is also called a BGE. During separation, the polarity is set to cathodic or anodic mode, also called normal and reverse mode, depending on the charge of the molecule: cation or anion. For anions, the capillary is usually dynamically coated with an electroosmotic flow (EOF) modifier to reverse the EOF and separate the analytes in the co-electroosmotic mode.

As already stated, most anions, organic acids, cations, or aliphatic amines of interest, but not all, are characterized by poor UV absorption.

In most cases, indirect UV detection is used because existing commercial instruments are generally equipped with such detector. However, many of these ions may be detected at 185 or 200 nm, provided their concentration is high enough (above 100 µg/mL).

Several publications describe electrochemical detection techniques, and some authors report indirect fluorescence detection and mass spectrometry detection.[1,3,9]

An interesting review from Padarauskas[10] describes derivatization of inorganic ions before the analysis with CE. This technique is essentially used for the analysis of metal ions and their speciation.

A. Indirect UV Detection

Hjertén et al.[11] and Foret et al.[12] were the first to publish articles on indirect UV detection where the analyte ion modifies the concentration of BGE, a phenomenon also known as the displacement effect. The advantage of indirect detection is that all UV-absorbing and non-UV-absorbing analytes can be detected. The disadvantage is that identification of the analyte is only based on the migration time and that co-migrating peaks may occur. For this reason, it is recommended to use highly reproducible migration time or corrected migration time.

An absorbing co-ion is used for detection. It must have the same charge sign as the analyte. This co-ion is also called an absorbing ion or probe and is present in the BGE. The degree of displacement of this co-ion by the analyte is called the transfer ratio (R), which is the number of moles of the probe displaced by one mole of analyte ion (Figure 1). This means that a single-charged analyte ion displaces a single-charged probe ion and a double-charged analyte ion displaces two single-charged probe ions.[13]

The transfer ratio is not only dependent on the charge of the probe and analyte ions, but also on their electrophoretic mobilities. This is due to Kohlrausch's regulating function (KRF),

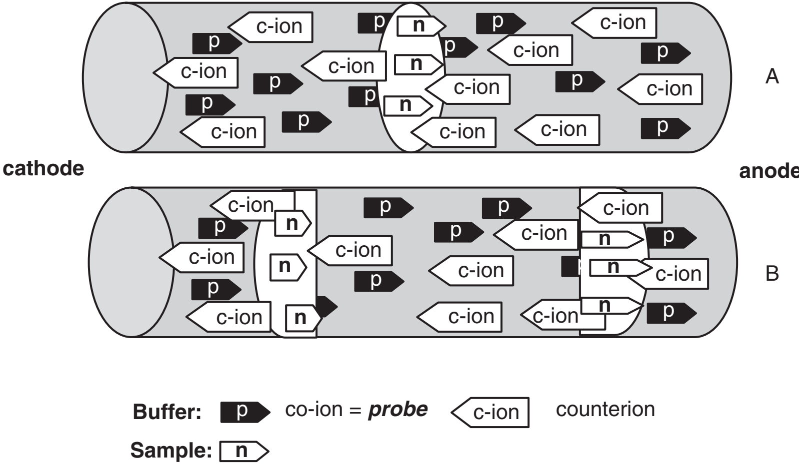

FIGURE 1 Diagram of a capillary filled with an UV-absorbing BGE. A non-absorbing analyte will displace the UV-absorbing probe, resulting in a negative peak due to displacement of the probe (A). When the mobilities of the analyte and the probe are different, a non-symmetric peak will be observed (B).

which must remain constant during the separation process:

$$\omega = \sum_i \frac{c_i z_i}{\mu_i} = \text{constant} \tag{1}$$

where c_i, z_i, and μ_i represent the ionic concentration, the absolute value of the charge, and the absolute value of the effective mobility of each ionic constituent.

The maximum transfer ratio (R) occurs when the probe and analyte have similar mobilities. When the separation is performed on a testmix of dicarboxylic organic acids, each at the same concentration of 0.2 mM with a buffer consisting of 30 mM pyridinedicarboxylic (PDC) acid at pH 8.2 (Figure 2, trace A), we obtain a separation based on their molecular weight while both the analyte and the probe bear two negative charges. To correct for the mobility of each analyte when migrating through the detector window, we use a time-corrected area for quantitation. This area is specific not only to their concentration, but also to their ability to displace the probe; in this case, the PDC acid.

At pH 5.4 (Figure 2, trace B) with this same probe and the above-described mix of dicarboxylic organic acids, the migration times of the analytes are longer. At pH 5.4, the diacids are not fully ionized and thus the migration times reflect the weighted average of all mobilities associated with the different ionized forms present at this pH, including the probe itself that is not totally ionized. This seems to be an intricate situation. It can be simplified if one considers that it is the ions bearing two charges that displace the probe ions bearing two charges as well. In fact, the corrected areas at pH values 8.2 and 5.4 correlate well.

The electrophoretic mobilities of the probe and the analyte will determine the shape of the peak as a result of electromigration dispersion.[14] We will observe a typical sawtooth peak (Figure 3) for all analytes migrating away from the position where the probe itself would migrate.

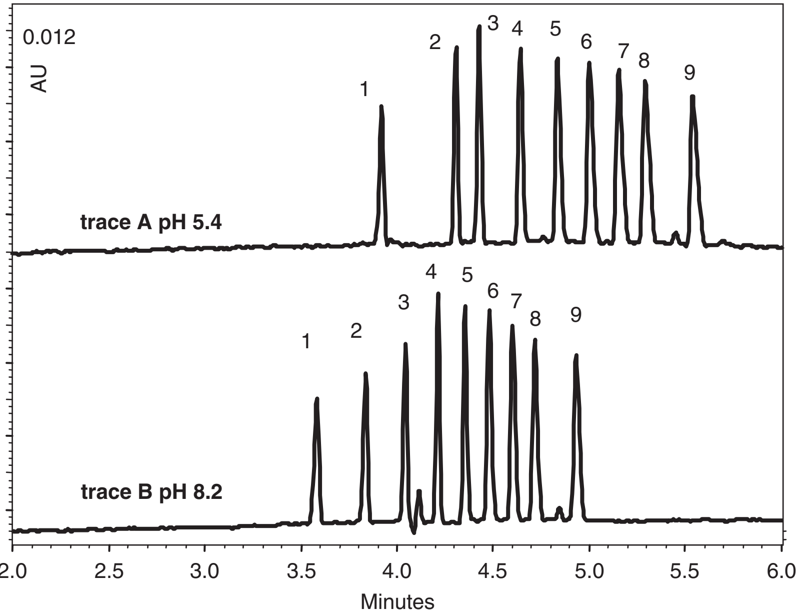

FIGURE 2 Separation of 0.2 mM dicarboxylic acids with 30 mM PDC buffer at pH 5.4 (trace A) and pH 8.2 (trace B). On trace B at 4.1 min, a carbonate peak is observed. 1, Malonic acid; 2, succinic acid; 3, glutaric acid; 4, adipic acid; 5, pimelic acid; 6, suberic acid; 7, azelaic acid; 8, sebacic acid; 9, dodecandioic acid.

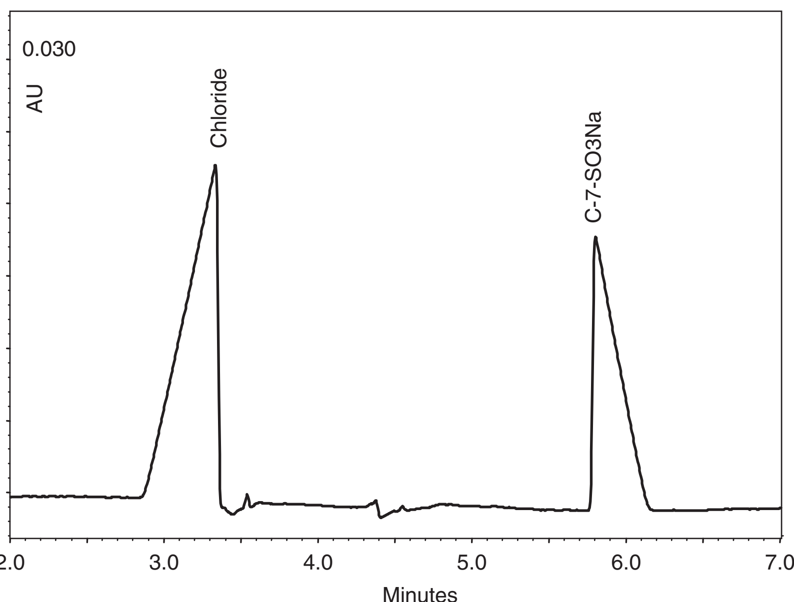

FIGURE 3 Typical "sawtooth skewed peaks" when the mobility of the analyte and probe differ. The signal was inverted to obtain positive peaks.

TABLE 1 Peak Characteristics at pH 8.2 and 5.4.

Name	Migration time	Height	Theoretical plates (USP)	Asymmetry (10%)	Corrected area
30 mM Pyridinedicarboxylic acid at pH 8.2					
Malonic	3.58	3410	89937	0.843	1372
Succinic	3.84	4050	126094	0.873	1390
Glutaric	4.05	4833	172796	0.879	1426
Adipic	4.21	6232	204729	1.061	1643
Pimelic	4.35	5902	183794	1.065	1683
Suberic	4.48	5759	163400	1.213	1812
Azelaic	4.60	5332	148349	1.282	1733
Sebacic	4.72	4942	138318	1.234	1632
30 mM Pyridinedicarboxylic acid at pH 5.4					
Malonic	3.92	3693	126242	0.862	1243
Succinic	4.31	5312	208462	0.943	1396
Glutaric	4.43	5860	201055	0.979	1593
Adipic	4.64	5231	162278	1.150	1557
Pimelic	4.84	4947	139431	1.183	1612
Suberic	5.00	4865	117851	1.471	1699
Azelaic	5.15	4702	108670	1.357	1691
Sebacic	5.29	4361	104527	1.497	1591

This is illustrated in Table 1 related to Figure 2, where the analytes with the mobility closest to the probe will have an asymmetry value close to 1 and will also have the highest theoretical plate number. Analytes with higher mobility will have an asymmetry value below 1 and analytes with a lower mobility will have an asymmetry value above 1, both with a lower theoretical plate number.

Zone broadening or electromigrational dispersion[14] also depends on the concentration of the BGE and the ionic strength of the sample.[15] This means that one should ideally look not only for a probe with mobility close to the analyte, but also for a BGE with a high concentration and a sample volume that is as low as possible.

The detector will observe negative peaks. Most instruments can cope with negative peaks by reversing the signal (Figure 4) or subtracting a reference channel so that the peaks appear as positive or at least are integrated as positive peaks.

The absorptivity and concentration of the probe should ideally be as high as possible, though they must be in the linear range of the detector. These parameters also have an impact on the noise.

Noise consists mainly of two elements: instrumental noise, which includes the lamp and its age, and noise due to the high background absorptivity. As this is an important parameter, Yeung[16] has proposed the term the dynamic reserve (D_r) for this:

$$D_r = \frac{S}{\sigma} \tag{2}$$

where S stands for the signal of the detector; and σ the standard deviation of the signal.

In some cases, a less optimal wavelength is used. The absorption observed will be sufficient to stay in the dynamic range of the detector. This is documented by the example of the nitrate ion that still absorbs at 230 nm, but almost not at all at 254 nm. When the analysis is run at 254 nm instead of 233 nm, it results in an overall higher signal (Figure 5).

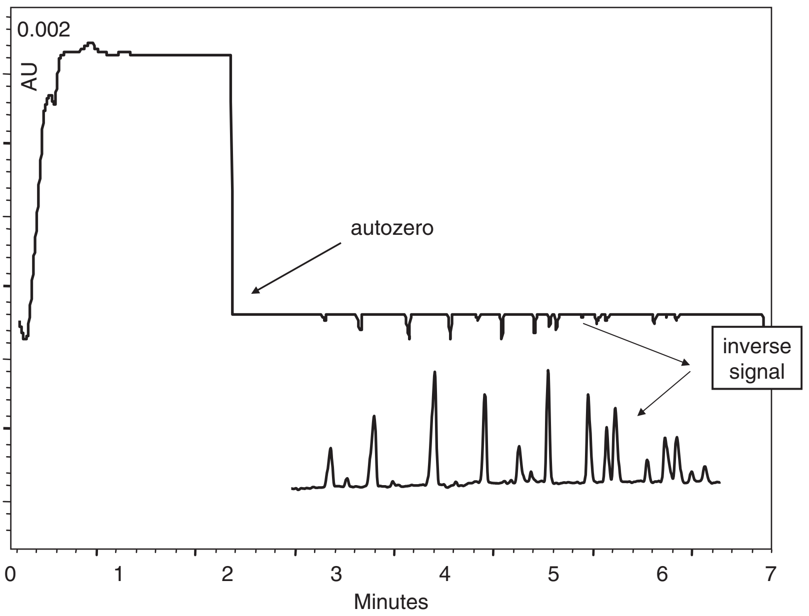

FIGURE 4 When inverting the signal, a positive electropherogram will be observed.

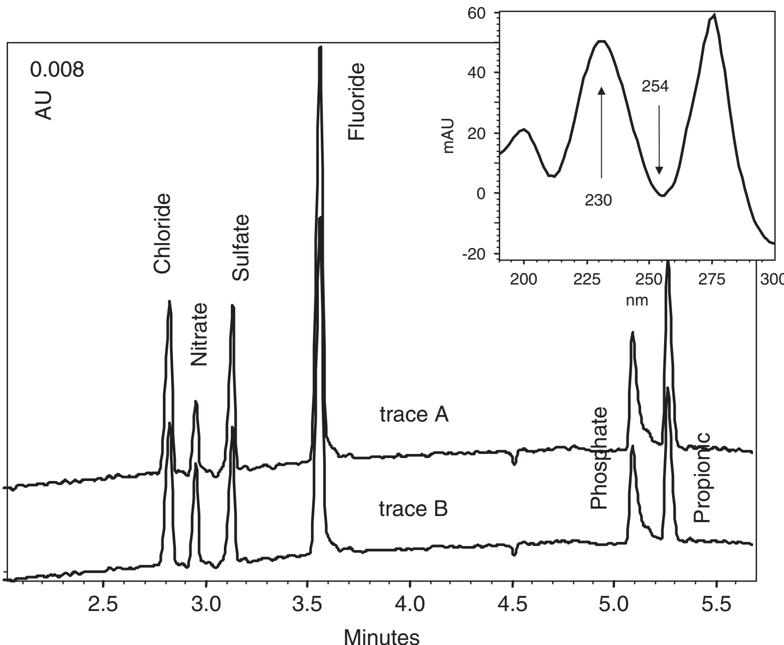

FIGURE 5 Running at 230 nm (trace A) or at 254 nm (trace B) will not give a difference in detection for a buffer composed of PDC, unless the analyte absorbs at that wavelength, for example, the nitrate ion. Here to have a maximum signal, a wavelength should be chosen at which the analyte does not absorb. Enclosed is the spectrum of the PDC buffer.

In theory, the limit of detection (LOD) may be lower with indirect detection than with direct detection. Macka[17] and Haddad describes the LOD with the equation:

$$C_{LOD} = \frac{C_p}{RD_r} = \frac{N_{BL}}{R\varepsilon I} \quad (3)$$

where C_{LOD} is the concentration at the LOD, C_p is the concentration of the probe, R is the transfer ratio, D_r is the dynamic reserve, N_{BL} is the baseline noise, ε is the molar absorptivity of the probe, and I is the detection cell pathlength.

From Equation (3), we learn that increasing the absorptivity of the probe and the detector pathlength will lower the LOD, while the noise should be as low as possible. However, some of these factors are interdependent: lowering the concentration of the probe will decrease the dynamic reserve and increasing the detector pathlength will increase the baseline noise.[18]

When dealing with noise, one should consider use of a peak detection algorithm optimized for CE.[19] This algorithm, which is readily available on some CE systems, will allow a much lower signal-to-noise ratio and an improvement of reproducibility by a factor 1.5−5, and thereby, of the LOD.

B. Direct UV Detection

Several ions such as bromide, nitrate, nitrite, iodide, iodate, etc. will absorb enough in the UV region to be detected directly as described by Paull[3] and king Timerbaev et al.[20] gives an example of analysis of inorganic anions in seawater. Gáspár et al.[21] performed direct UV analysis of nitrite and nitrate in saliva, and Pascali et al.[22] determined bromide in human serum. Several applications with direct UV detection of carboxylic acids were described by Baena et al.[4] in body fluids (carboxylic acids with exclusion of an aromatic moiety or double bond). Jariego[23] and Hernanz analyzed several organic acids in urine at 185 nm, and Mato et al.[24] in honey.

Figure 6 is an example of organic acids and anions analyzed with a 70 mM phosphate buffer at pH 2.5 and 200 nm.

C. Electrochemical Detection

Most CE applications are based on a UV detector using direct and indirect detection, though other detectors have been developed.

One of these is electrochemical detection, which can be used with traditional CE as well as with the microchip design. Electrochemical detection generally provides good sensitivity and bulk property response (conductivity, potentiometry), and can be selectively tuned to a certain class of compounds (amperometry).[25]

Potentiometric detection is based on selective transfer of an ion in the solution into a lipophilic membrane phase, which generates a potential difference between the internal filling solution of the sensor and the sample solution.[25] The electrode is generally placed at the outlet of the detector, and the membrane does present ion-selective characteristics.[26,27]

Amperometric detection is based on transfer of an electron to or from an electroactive compound at a solid electrode under the influence of externally applied DC voltage. Amperometric detection is limited to a certain type of compounds and is quite difficult to mount on a CE unit.[25]

For conductivity detection, an electrical potential is applied across two electrodes that are in contact with the electrolyte. The potential induces a force on the ions in solution in a

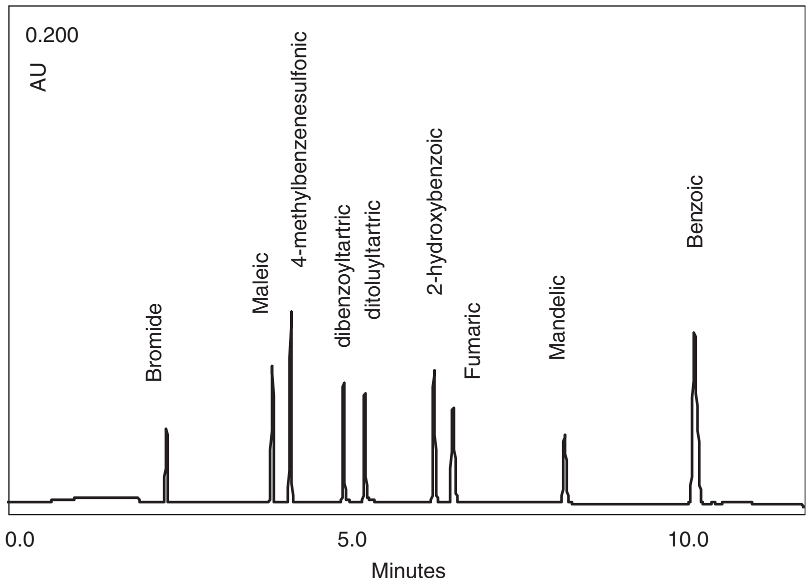

FIGURE 6 Analysis of UV-absorbing anions and organic acid is possible with a 70 mM phosphate buffer at pH 2.5.

direction opposite to their charge. The resulting net movement of the ions will represent an electric current whose strength is proportional to the conductivity of the solution. The conductivity is related to the flow of charge in response to the electric field, and its magnitude is dependent on concentration, mobility, ionic charge, and the temperature.[25]

Two types of conductivity detectors exist: the "contact conductivity detector," where the electrodes are in direct contact with the electrolyte,[28] and the "contactless coupled conductivity detector" (C^4D also called oscillometric detector). With this detector, two stainless-steel tubes that act as electrodes are mounted on a capillary at a certain distance from each other. By applying an oscillation frequency, a capacitive transition occurs between the actuator electrode and the liquid inside the capillary. After having passed the detection gap between the electrodes, a second capacitive transition between the electrolyte and the pickup electrode occurs (see Figures 7 and 8 which is an example of separation of cations). In different reviews, Zemann[29] and Kubáň[30] and Hauser discuss the advantages of this technique which include rather simple mechanical parts and electronics, and Kubáň et al.[31] compared several C^4D detectors. This technique has also been used as a detector for analysis by microchip CE.[32] C^4D detectors are available to be mounted on existing CE instruments.

During electrophoretic separation, the analyte ions displace background co-ions equivalent to their charge. The difference in the conductivity between the analytes and the BGE co-ions induces a signal recorded by the C^4D detector. It is, therefore, important for optimal sensitivity that the difference in conductance between the analyte and the electrolyte be as high as possible. On the other hand, optimal efficiency of separation is attained when the μ_{BGE} and μ_{analyte} are matched. However, the μ and the equivalent conductance λ_{equiv} are linked:[28]

$$\mu = \frac{\lambda_{\mathrm{equiv}}}{F} \tag{4}$$

where F is Faraday constant.

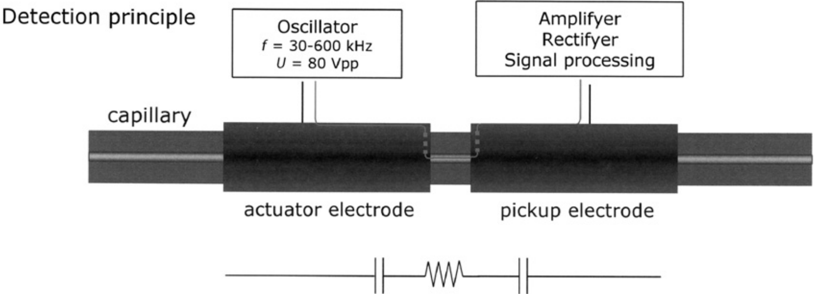

FIGURE 7 **Contactless coupled conductivity detection (C^4D) is based on two cylindrical metal electrodes, actuator, and pickup electrode, which are placed on the separation capillary. Schematically, it represents a series of a capacitor, an ohmic resistor, and a second capacitor (from Innovative Sensor Technologies GmbH).**

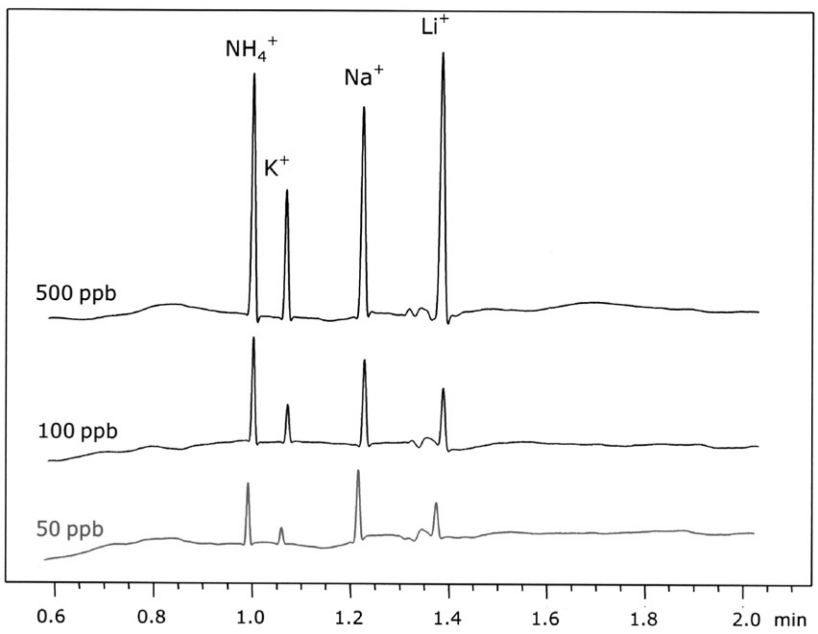

FIGURE 8 **Separation of inorganic cations with capillary electrophoresis and TraceDec® C^4D detector. Capillary 50 µm ID × 51 cm total length (41 cm effective length). Buffer 25 mM MES, 25 mM His, 2 mM 18-crown-6, pH 6.1. Separation at 30 kV (from Innovative Sensor Technologies GmbH).**

It is also favorable to have a BGE with high ionic strength. The compromise for conductivity detection is to use a BGE based on amphoteric buffers because they present low conductivity and can therefore be used at relatively high concentrations.

Typical buffers are 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 20 mM histidine (His) for the direct conductivity detection of both inorganic cations and inorganic anions. An alkaline sorbate/arginine (7.5/15 mM) buffer is suitable for direct conductivity

detection of inorganic anions. For organic anions, the equivalent conductivity is too low and does not differ sufficiently from the BGE. In this case, indirect conductivity detection may be used with, for example, chromate present in the electrolyte.

D. Background Electrolyte and Buffers

Several parameters, such as the physicochemical properties of the analytes, the variables of the BGE, the capillary dimensions (diameter, total length, and length to the detector), and operational parameters (electric field strength, temperature, injection, and detection zone length), play an essential role in separation. The BGE or the buffer is an essential part of the method, which includes the rinsing solutions, the EOF flow modifier, or the dynamic coating technique. The conditioning method for a new capillary or after a long shutdown and the rinsing method between two separations must be designed carefully. They are important for the reproducibility and robustness of the test and play a role in the transferability of the method.

The main role of the BGE, which is present inside the capillary, is to conduct the electric current and separate the analytes of interest. According to Ohm's law, the BGE is responsible for producing Joule heating and has also an effect on the EOF. Together with the diameter and the length of the capillary, the composition of the BGE and its concentration are very important. In order for the method to be reproducible and robust, the BGE must also have good buffering capacity.[33] This will avoid alteration of pH due to electrolysis or contamination of the buffer. Even small changes in pH will cause a non-reproducible migration time, alteration of the peak area, and disturbance of the baseline.

The ionic mobility[34] of an ion can be described according to the following equation:

$$\mu_i = \frac{\lambda_0}{F} = \frac{q_i}{6\pi\eta r_i} \tag{5}$$

where μ_i is the ionic mobility, λ_0 the ionic conductivity, F the Faraday constant, q_i the charge of the hydrated ion, η the dynamic viscosity of the electrolyte, and r_i the radius of the hydrated ion. The hydrated radius is usually, but not always, a function of the molecular weight of the ion.

The effective mobility of an ion is also a function of the degree of dissociation of this ion.[33] For a weak monovalent acid (HA), it can be represented by Equation (6), and for a weak acid base (B) by Equation (7):

$$\mu_{\mathrm{eff,A}} = \alpha_{\mathrm{A}}\mu_{\mathrm{A}} \tag{6}$$

$$\mu_{\mathrm{eff,B}} = (1\alpha_{\mathrm{A}})\mu_{\mathrm{B}} = \alpha_{\mathrm{B}}\mu_{\mathrm{B}} \tag{7}$$

where α_A and α_B represent the degree of dissociation of HA and BH^+, and μ_A and μ_B the ionic mobility of A^- and BH^+.

This means that the effective mobility will depend on their pK in relation to the pH of the BGE. For weak bases, the analyte will be practically fully protonated (BH^+) at $pH < pK_B - 2$, partially protonated at $pK_B - 2 > pH < pK_B + 2$, and practically neutral at $pH > pK_B + 2$. The same happens with a weak acid which will be practically non-ionized at $pH < pK_A - 2$, partially ionized at $pK_A - 2 < pH > pK_A + 2$, and practically fully ionized at $pH > pK_A + 2$. The pH of the BGE is thus of key importance for optimization of the selectivity of separation.[33]

Inside the capillary, the apparent mobility of ion $\mu_{app,A}$ is the sum of the mobility of the EOF and the effective mobility of the ion:

$$\mu_{app,A} = \mu_{eff,EOF} + \mu_{eff,A} \tag{8}$$

From the above equations, it could be easily understood that the key properties of the analytes are the hydration radius, the pK_A, the diffusion coefficient, and the mass fraction. They will play a role in the separation. Another important factor is the μ_{eff} of the ion and the μ_{eff} of the EOF in accordance with Equation (8). For $\mu_{app,A} \neq 0$, they need to have the same sign or the μ_{eff} of the ion must be smaller than the effective mobility of the EOF.

Because of the high mobility of most ions of interest, separation needs to be performed in the "co-electroosmotic mode." For anions, the anode must be on the detector side, and a reversed polarity or anodic mode must be used. The EOF should be reversed by using an EOF modifier, also called a cationic surfactant. If the capillary is rinsed with long-chain aliphatic quaternary ammonium surfactants or with a polycation, the inside wall of the capillary will be positively charged and the EOF generated when the current is applied will move toward the anode. The EOF modifier is usually present in the buffer and the coating is performed dynamically by rinsing the capillary between the two separations. The cationic analytes are separated in the normal polarity mode or cathodic mode. In most cases, a fused silica capillary is used without coating, while other methods may use a double-coating technique.

When the effective mobility of the analyte is smaller than the effective mobility of the EOF, we may separate anions in the normal mode or cathodic mode. This may be very interesting for body fluids to avoid interfering ions such as chloride, which are present in high concentrations, while several organic acids will be observed migrating after the EOF.

The UV-absorbing co-ion or probe may be the co-ion of the buffer or a specific co-ion that is added to the buffer. As already described in the previous paragraph, the probe should ideally show mobility close to that of the analyte of interest. The absorptivity of the probe is a parameter of secondary importance. Both Pacáková et al.[34] and Macka et al.[35] propose an extensive table matching the ionic mobilities of analytes and probes. Others use the same probe for a large range of analytes.

The ionic strength, pH, and viscosity of the BGE will influence the EOF and the apparent mobility of the analytes. On one hand, high-ionic-strength BGE will provide high buffering capacity and analytes will have a good peak shape, but, on the other, it will generate Joule heating. In their original research, Mikkers et al.[15] found that the ideal ionic strength of the BGE should be 10 times higher than that of the sample. BGEs as high as 500 mM have been employed, but this is exceptional and requires an adequate cooling system.

When selecting a buffer, Beckers and Boček[33] described a series of golden rules for the composition of a BGE. Some of them should be stressed. (a) The BGE should contain enough ions to conduct the electric current, but excessive ion concentration will induce Joule heating. (b) If the current is too high, the BGE concentration, the applied voltage, or the diameter of the capillary can be decreased. Alternatively, the length of the capillary may be increased. (c) The use of multiple co-ions should be avoided because they will give system zones (SZ), or disturbance peaks, with mobility in between the mobilities of the co-ions.

One should consider that one or a small number of generic buffers may solve a great deal of analytical problems. When problems still arise, some minor modifications of an existing generic buffer may provide the solution. The overall advantage is that validation of the buffer is strongly reduced.

1. Buffers for Anions and Organic Acids with Indirect UV Detection

We have seen that a BGE for analysis of anions and organic acids needs to have (a) a pH above the pK_A −2 of the analyte, (b) a sufficient buffering effect, and (c) a co-ion as a probe for indirect UV detection, with mobility close to that of the analytes of interest. Furthermore, care should be taken to reverse the EOF of the capillary and to work in the anodic or reverse mode.

A review by Galli et al.[1] describes several buffer-absorbing chromophores as co-ions. These include phthalate, PDC (2,6-pyridinedicarboxylic acid), PMA (1,2,4,5-benzenetetracarboxylic acid or pyromellitic acid), TMA (trimellitic acid), MES, 2,4-dihydrobenzoic acid with ε-aminocaproic acid, *p*-hydroxybenzoate, *p*-anisate, 3,5-dinitrobenzoic acid, salicylic acid with TRIS, benzoic acid with tris (hydroxymethyl)aminomethane (TRIS), and many others. On the other hand, some inorganic chromophores such as chromate (Figure 9) or molybdate may be added to a buffer. A BGE-containing chromate should have a pH above 8, because it precipitates below this value. The advantage of a TRIS buffer or buffers at around pH 6 is that carbonate will not interfere with the separation because it is not soluble in TRIS or at lower pHs.

A wide choice of cationic surfactants such as CTAB (cetyltrimethylammonium bromide), CTAH (cetyltrimethylammonium hydroxide), TTAB (tetradecyltrimethylammonium bromide), TTAOH (tetradecyltrimethylammonium hydroxide), MTAB (myristyltrimethylammonium bromide), OFM (OFM Anion-BT, Waters, Milford, MA, USA), HDB (hexadimethrine bromide), and many others may be used to reverse the EOF. CTAH and TTAOH should be preferred to CTAB and TTAB to avoid interference from bromate contamination. The capillary coating is performed just by rinsing with the BGE containing this flow modifier or even with an additional rinse step with a solution containing this flow modifier.

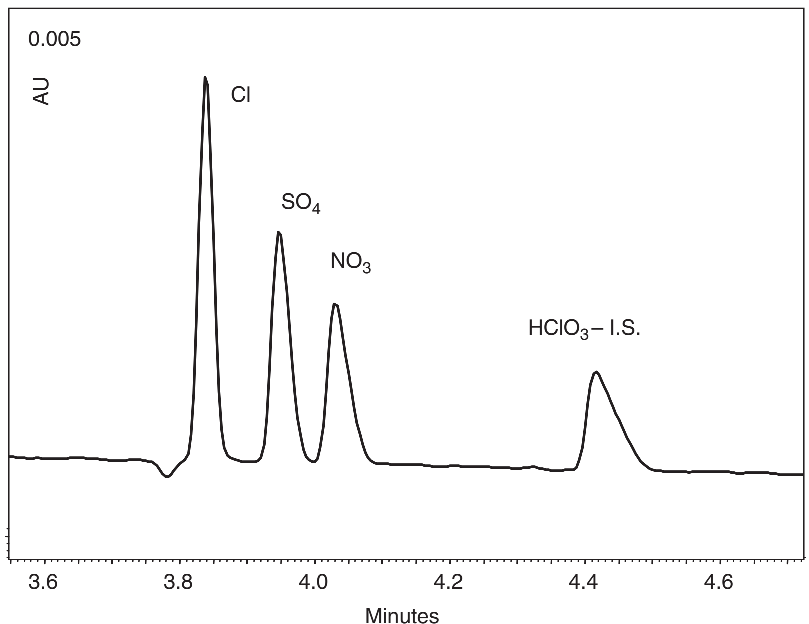

FIGURE 9 Separation of chloride, sulfate, and nitrate at 40 µg/mL with 5 mM chromate, 0.5 mM TTA-OH, 1.0 mM borate as BGE. Chlorate is used as internal standard. Linearity between 1 and 4 µg/mL with an $R^2 = 0.999$ for all analytes. Indirect UV detection at 254 nm (10 nm bandwidth).

In many cases, it is more efficient to modify an existing and validated buffer rather than to design a new one. Modification can be as easy as changing the temperature of the capillary during separation. This will influence pH, viscosity, and radius of the hydrated ion. Also, using a longer capillary and/or a capillary with a smaller diameter will influence the current as well as the heat generated during separation. Some anions interact with α-cyclodextrin, so separation of nitrite and nitrate may be improved, but also sulfate from nitrate or iodide.[5,36] To manipulate selectivity, calcium or other alkali-earth metals have been used or ethylenediaminetetraacetic acid (EDTA) has been added to improve peak shape of citrate when calcium is present, for example. Organic solvents such as methanol (5−60%) or acetonitrile (5−30%) may be used.

2. Buffers for Anions and Organic Acids with Direct UV Detection

Non-absorbing BGEs such as tetraborate at pH 9.3 and phosphate at pH values 10.2, 7.5, 6.5, 6.25, 6, or 2.5 may be used. Low pH buffers are also useful for the separation of nitrite from nitrate, taking advantage of their respective pK_A of 3.29 and −1.3. Special applications are CE−MS-compatible buffers where ammonium bicarbonate or ammonium acetate may be used.

3. Buffers for Cations and Aliphatic Amines

The buffers for cations are usually composed of an absorbing co-ion such as imidazole (Figure 10), phenylethylamine, benzylamine, 4-methylbenzylamine, nicotinamide, or amino-pyridine (Figure 11). Beckers[33] and Boček recommends histamine, His, or imidazole as they

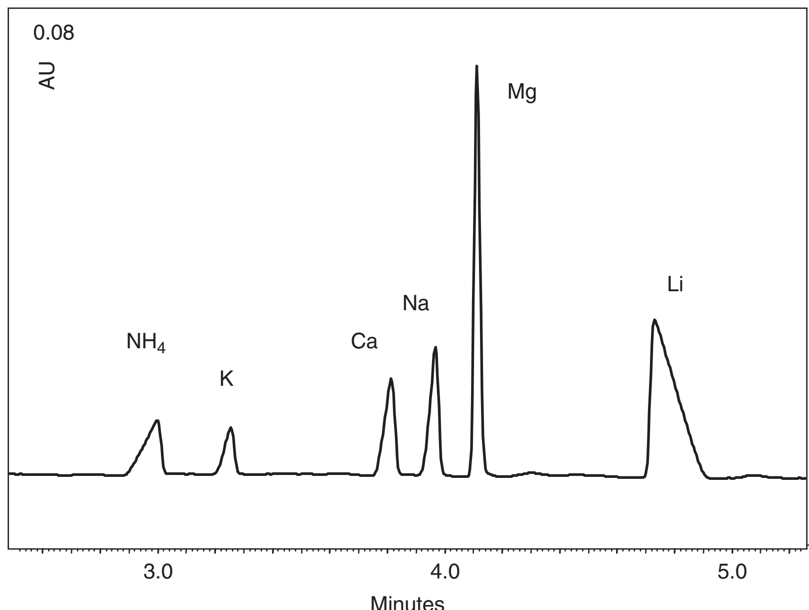

FIGURE 10 **Separation of ammonium, potassium, calcium, sodium, magnesium, and lithium at 20 µg/mL with 15 mM imidazole, 2 mM 18-crown-6 and 33 mM acetic acid as BGE. Linearity between 2 and 20 mg/L with an $R^2 > 0.999$ for all analytes. Indirect UV detection at 205 nm (10 nm bandwidth).**

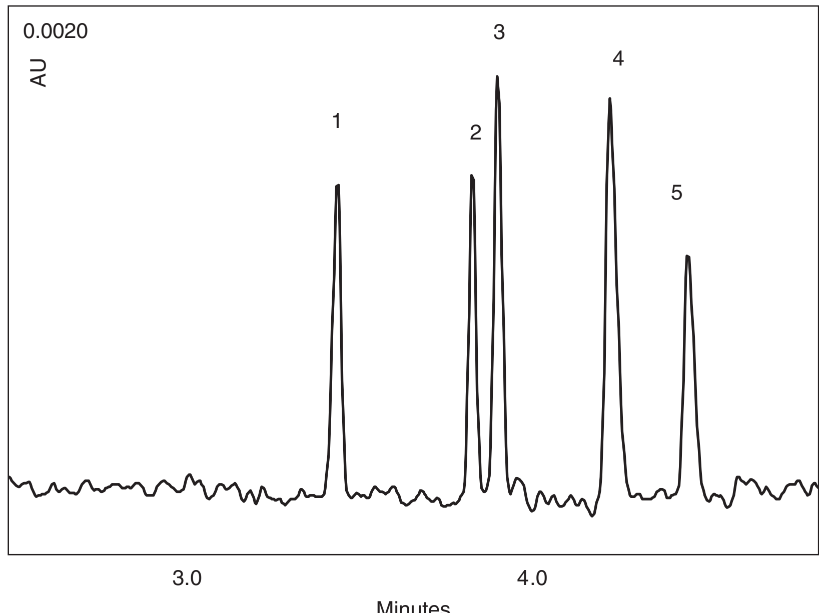

FIGURE 11 Separation of (1) trimethylamine, (2) morpholine, (3) isopropylamine, (4) tertbutylamine, and (5) cyclohexylamine with 20 mM aminopyridine, 17 mM malic acid, 17 mM 18-crown-6 as BGE. The capillary was first rinsed with a polyanion solution, followed by a polycation solution to coat the capillary. Indirect UV detection at 200 nm (filter).

are weak bases and have good buffering capacity. The pH of the buffer is in the range 4–5 and the molarity between 5 and 20 mM.

The effect of the hydration radius of these cations is very important, and mobilities are sometimes very close or the same as for potassium and ammonium. For this reason, a complexing agent is added to the buffer. Several complexing agents such as α-hydroxyisobutyric acid (HIBA), 18-crown-6, phthalic, malonic, tartaric, lactic, citric, oxalic, or glycolic acid may be used.

Fançois et al.[37] studied the optimization of lactic acid and 18-crown-6 as complexing agents. She found that 0.5 mM 18-crown-6 in a 10 mM imidazole buffer (pH 4.5) could resolve sodium from lead as well as ammonium from potassium cations.

The cations are separated in the normal polarity or cathodic mode. At around pHs 4–5, an EOF is still present and a double coating may help to improve reproducibility.

A common problem of cation analysis is the quality of the water used for rinsing the capillary. Sodium is a common contaminant in purified water, but it can also come from improper handling.

The same buffer may be used for separation of aliphatic amines such as methyl-, dimethyl-, trimethyl-, ethyl-, propyl-, diethyl-, triethylamine, morpholine, etc.

4. Setup of the Instrument

We should not forget that the capillary plays a key role in the separation. Even if a fused silica capillary is usually used, the choice of diameter and capillary length has a major impact. Decreasing the capillary diameter or increasing the capillary length will lower the current and

thus the Joule heating produced. These two parameters also have an impact on the quantity of sample injected:

$$Q = \pi r^2 \left[\frac{\Delta P r^2 t_{\mathrm{inj}}}{8\eta L}\right] C_i \tag{9}$$

where Q is the quantity of injected sample, ΔP the pressure difference, r the capillary internal radius, t_{inj} the injection time, η the viscosity of the sample solution, L the total length of the capillary, and C_i the concentration of the ion.

Ideally, one should inject between 1% and 2% of the total volume of the capillary. For example, a 0.5 psi (34.5 mbar) injection for 8 s (Table 2) in a capillary with 75 μm internal diameter and 60 cm total length represents 2.02% of capillary volume to the window or 44.7 nL of injected volume. For a capillary with a diameter of 50 μm and under the same conditions, it represents 0.9% and 8.83 nL of injected volume, which is five times less. For a 75 μm capillary of 120 cm, the same conditions produce a sample plug of 0.39% and 0.63 nL.

To obtain a sample plug of 2% of the total capillary volume at 0.5psi, the injection time should be adapted as shown in (Table 3). The injected volume is increased by a factor 2 when using 75μm × 60cm capillary instead of 50 μm × 60cm and by a factor 5 when using a 120 cm × 75μm. A free software program, "CE expert," is available from Beckman Coulter to help in the calculation of these parameters.

Before the first run with a new capillary or after a shutdown of the instrument, the capillary must be initialized. We recommend a rinse with sodium hydroxide (0.1 M), followed by incubation (for example, for 4 min) with the sodium hydroxide inside the capillary, and

TABLE 2 Injected volume at 0.5 psi/8s

Pressure of 0.5 psi/8 s					
Capillary	**ID (μm)**	**Lt (cm)**	**Percentage[a]**	**Vol (nL)**	**Ratio**
1	75	60	2.02	44.7	
2	50	60	0.9	8.83	1/5
3	75	120	0.39	0.63	1/20

ID, internal diameter; Lt, total length of capillary; Vol, volume injected; Ratio, volume injected/volume injected with capillary 1.
[a]Percentage of injected plug volume to capillary volume to the window.

TABLE 3 Injected volume at 0.5 psi and a 2% injected plug volume

Pressure of 0.5 psi and a 2% injected plug volume					
Capillary	**ID (μm)**	**Lt (cm)**	**Injection time (s)**	**Vol (nL)**	**Ratio**
1	50	60	18	19.86	
2	75	60	8	44.70	2 ×
3	75	120	35	97.8	5 ×

ID, internal diameter; Lt, total length of capillary; Vol, volume injected; Ratio, volume injected/volume injected with capillary 1.

finally, two rinses first with sodium hydroxide and another with water (20 psi, 30 s). The first run may also be discarded for optimal performance.

When necessary, coating of the capillary should preferably be performed before each run with a dedicated vial of buffer containing the flow modifier. This buffer may be the same as the separation buffer, but should be kept in a different vial than the vial used during the separation. Separation buffer may be contaminated by carryover from the sample, altered by electrolysis, or its composition could be changed from analysis to analysis (pH, molarity, etc.). After separation, the capillary may be rinsed, for example, with sodium hydroxide and ultrapure water. For some applications, rinsing with hydrochloric acid (0.1 M) may be useful. The sodium hydroxide will ionize the silanol groups and the hydrochloric acid will protonate those groups, thus removing impurities on the capillary wall.

After addition of the sample, a 0.5% water plug is injected to avoid contact of the sample with the separation buffer, which means less contamination of the buffer, no loss of sample during vial change, no loss of sample when applying current, and some stacking effect. It is also recommended to apply adequate voltage ramping (for example, over 1 min). This will avoid local heating and is associated with enhanced separation efficiency.[38]

Temperature should be constant during separation and Joule heating should be removed adequately. One can also take advantage of modifying the temperature, as this will influence the pH and viscosity of the buffer and also hydration of the ion.

III. METHOD DEVELOPMENT AND OPTIMIZATION

Before setting up a method, it is recommended to document each of the species to be analyzed: (a) the mobility (if not available, estimation of the mobility as a function of charge and molecular weight), (b) the pK_A of each analyte, (c) the expected concentration range of the analyte, (d) the solubility of the sample, and (e) the absorbance or the lack of a chromophore.

An interesting tool to document some of these parameters and to evaluate the separation is a computer freeware program available from Gaš et al.[39,40] The PeakMaster software allows prediction of the behavior of BGEs and analytes. This program contains a large database of products with their pK_A and mobility. Though some data may be inaccurate, it affords a global view of the separation. Nevertheless, real separation will provide the final answer on the feasibility of a separation.

A. Sample Preparation

Most samples may be prepared by dissolution in water. The final concentration should be optimized according to the aim of the analysis, counterion or impurity analysis. For the control of impurities, the main counterion may be fairly overloaded. This may have an impact on the ionic strength of the sample and will produce a disturbed peak profile for the main compound. When solubility problems are encountered, up to 30% of methanol, ethanol, or acetonitrile may be added to improve solubility. However, the presence of too much organic solvent may produce an instrumental error, because the conductivity of the sample plug will differ too much from BGE conductivity, leading to current leakage. Or, when the sample is insoluble in water, it may be suspended, vortexed, and then centrifuged. The analysis is then performed on the supernatant as the ions are water soluble.

It is common sense that the analytes in solution are ionized. However, in some cases, the pH of the solution is too low for anions or too high for cations. The pH of the sample solution may be corrected by adding HCl, H_2SO_4, NaOH, or NH_4OH accordingly (Figure 12).

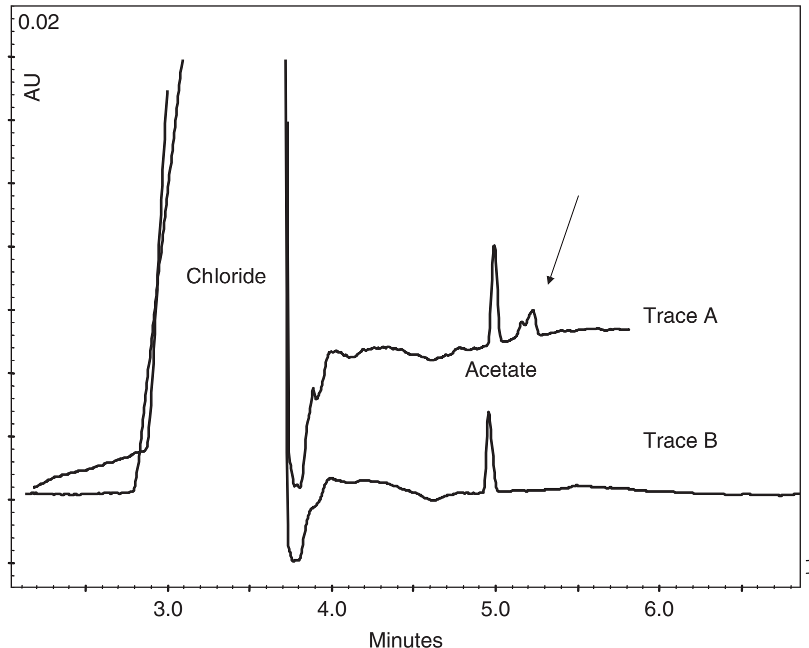

FIGURE 12 Analysis of acetate as impurity of an HCl salt with 30 mM PDC buffer. Trace A shows acetate peaks migrating at different migration times. By adding 20 μL of NH_4OH (28%) to 400 μL of sample solution, its pH increases and acetate is totally ionized, and thus migrates immediately, showing a single peak (trace B).

Filtration of the sample solution is usually not necessary; when particles are present, a simple centrifugation step may be performed.

B. Calibration

When external calibration is used for quantitative purposes, Pauli and King[3] recommends a range of five standards analyzed in duplicate ranging from 50% to 150% of the analyte concentration in the sample. The expected linearity correlation R^2 should be greater than 0.999. Linearity generally exists over two to three orders of magnitude. Special care should be taken at the low and high ends of the calibration range where the response might not be linear. When working with complex matrices, standard addition calibration may be more accurate to obtain a calibration curve. At least three standard additions should be carried out. One may also compare the slope obtained after standard addition with the slope obtained with external calibration. A difference in the slope is an indication that a matrix effect has occurred.

Most authors recommend the use of an internal standard to improve reproducibility in migration time and, more importantly, in peak area. The internal standard should not be a potential analyte of sample and should be resolved well from the analyte of interest. or example, Altria[73] reports better quantification and precision of the method when using an internal standard. He describes an improvement of migration time with RSD from 0.6% to 0.11%, and an improvement of peak area for chloride with RSD from 4.19% to 0.52%.

Standard addition should be carried out to assess recovery. In doing so, the variation in concentration due to other phenomena is avoided. As an example, Guan[41] reports 51% recovery for nitrite in water due to rapid oxidation of the analyte.

The accuracy of the method should be evaluated by comparing the value obtained with a known reference or with an alternative analytical technique.

C. Selectivity

Most determinations may be performed using standard buffers without any changes. However, in some cases when analytes co-migrate, modification may be required.

One is to modify the temperature of the separation. Changing the temperature will modify the pH and the viscosity of the buffer. For example, acetic and acrylic acids are not separated with a PDC buffer at 25°C and at a pH of 5.55. However, at 35°C, the pH will be 5.44 and baseline separation will be obtained (Figure 13). By slightly changing the pH of the buffer, we take advantage of the difference in ionization due to a slight difference in pK_A (4.756 versus 4.258). Bromide and chloride may also be difficult to separate with the same buffer at 25°C. However, by analyzing at 15°C, good separation will be observed. This is mainly due to a change in viscosity of the buffer and, possibly, the hydration radius of the analyte. In this case, a longer capillary (total length: 110 cm instead of 60 cm) also improves the separation (Figure 14).

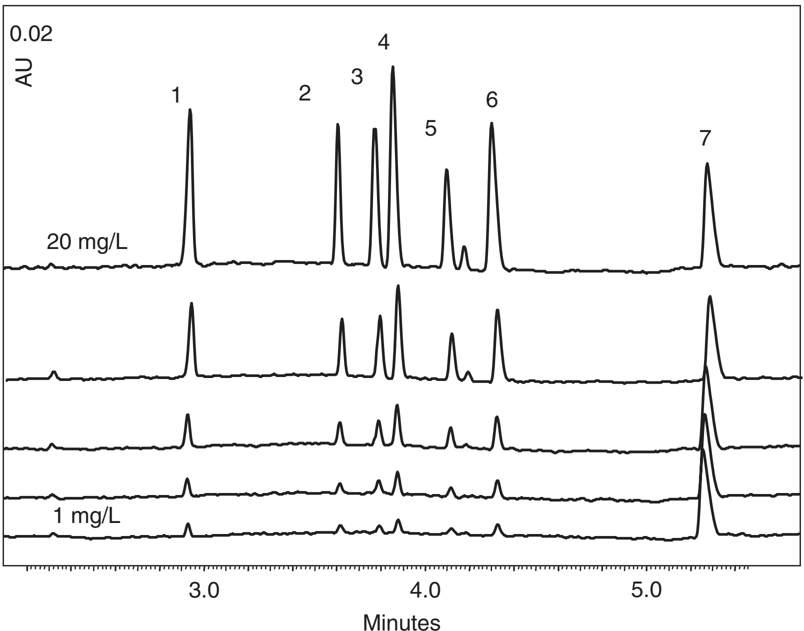

FIGURE 13 Analysis of (1) formic, (2) chloroacetic, (3) acrylic, (4) acetic, (5) methacrylic, (6) propionic acids, between 1 and 20 μg/mL, with 30 mM PDC buffer at pH 5.4. The separation temperature is set at 35°C to lower the pH by 0.02 units, which is sufficient to allow separation of acrylic and acetic acids which otherwise will co-migrate. Octanoic acid (7) is used as internal standard.

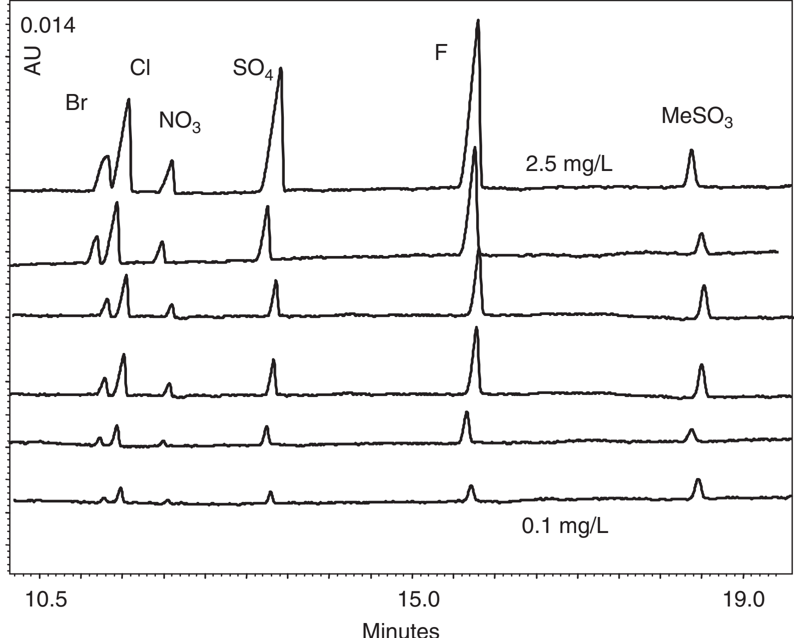

FIGURE 14 Analysis of several anions with 30 mM PDC buffer at pH 8.2. To improve resolution between bromide and chloride, the temperature was set at 15°C and a 110 cm capillary was used instead of a 60 cm capillary. For a symmetric fluoride peak, the capillary was rinsed with a 0.1 M methanesulfonate solution.

A second way to improve resolution is the modification of mobility by complexation of the analyte. Many buffers for analysis of cations use HIBA or 18-crown-6 to improve the resolution between sodium, potassium, calcium, magnesium, etc. as well as some aliphatic amines. By diluting an existing validated buffer, one can change the concentration of the complexation agent and thus also the selectivity of the system.

Galli[1] mentioned the addition of alkali-earth metals (mainly calcium) to the BGE to improve separation of organic acids. Calcium ions interact through the formation of complexes with different stabilities giving different mobilities. EDTA may also be used to form different complexes. Alternatively, EDTA may remove interfering compounds.

Some authors have studied the addition of organic solvents to the BGE. Solvents such as methanol, acetonitrile, ethylene glycol will change the selectivity of the analysis.[6] We observe changes in ion hydration and a decrease of EOF by increasing the viscosity. Furthermore, the pK_A of the analyte as well as the silanol groups of the capillary wall are changed. For example, Diress and Lucy[42] studied the effect of methanol up to 60% (v/v) in a 15 mM phosphate buffer containing Cetyltrimethylammonium chloride (CTAC) at pH 8.0.

Tindall et al.[43] has used cyclodextrin to fine-tune ion mobility. The addition of β-cyclodextrin up to 8 mM in a 1 mM 2,6-naphthalene dicarboxylate pH 9 buffer increases the dynamic range of the analyte from 0.025 to 100 μg/mL. The analytes described were butyric, propionic, and acetic acids.

Stathakis and Cassidy[44], on his part, used α-, γ- (0−40 mM/L), or β-cyclodextrin (0−10 mM/L) to separate a mix containing iodide, nitrate, perchlorate, thiocyanate, bromate, iodate, ethanesulfonate, pentanesulfonate, and octanesulfonate. The separation of nitrate and nitrite can be improved by the addition of 3% α-cyclodextrin in a 30 mM PDC buffer at pH 5.4 (Figure 15).

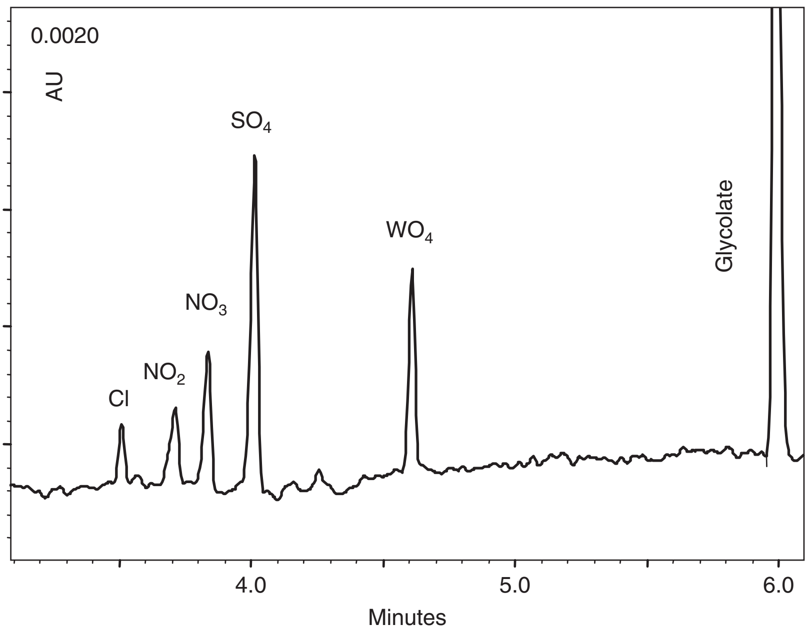

FIGURE 15 **Analysis of several anions with 30 mM PDC buffer at pH 5.4. Addition of 3% α-CD improves the separation of nitrate and nitrite, which otherwise will co-migrate.**

D. Improving Sensitivity

For analysis of a major compound that is a counterion, sensitivity is not an issue. As a general rule, one should not inject more than 2% of the capillary volume; however, one may explore to inject up to 8% to decrease the LOD. Alternatively, a capillary with a larger diameter and/or greater length may be used. This allows injection of more samples. An extended pathlength may also be considered.

1. Electrokinetic Injection

With this technique, the sample is injected by applying a range of a few volts to the sample for a certain period. The ions that are ionized will migrate into the capillary as a function of their mobility, as will a small amount of sample mobilized by the EOF that is generated due to the voltage applied. Jackson and Haddad[45] was able to analyze low levels (ng/mL) of anions in samples of moderate ionic strength. For quantification, he recommended the use of one or two internal standards or a standard addition.

Electrokinetic injection requires a few special considerations. The material used must be very pure. Classic glass vials may give unknown peaks. The water must be very pure and fresh as contamination may be picked up from the air. During the run, the different buffer and rinse vials may be contaminated and need to be replaced more frequently. The vial used for post-injection water must be replaced for every run.

When analyzing very low conductivity samples, it is necessary to add some ions with slow mobility to allow the tension applied to generate current in the sample vial. For example, we may add heptanesulfonate (CAS 22767-50-6 at a level of 1.2 mM in the sample) in the sample to thus generate current and EOF.

The linearity range is usually smaller than with a pressure injection alternatively a quadratic calibration curve may be used for a larger range.

2. Electrophoretic Concentration

Electrophoretic concentration techniques are based on the difference in mobility of an analyte in two separate zones, mainly due to differences in electric field strength between the zones.[46] The main advantage of this type of concentration is that no modification of the basic instrument is required.

The most straightforward technique is field-amplified sample stacking (FASS), which was already discussed by Mikkers et al.[15] in 1979. When injecting a sample in a capillary, the sample zone should have an ionic strength lower than that of the buffer by a factor 10. When the voltage is applied, the electric field in the sample zone is higher due to lower conductivity of this zone. Analytes will migrate very quickly until they reach the boundary with the buffer zone where the conductivity is higher. They will also build up in this boundary zone, giving high and sharp peaks. FASS is the standard method used when performing CE.

Different pre-concentration techniques based on stacking have been described by Breadmore and Haddad.[46] Timerbaev[5] describes transient ITP (tITP) as the most viable option. Isotachophoresis or ITP occurs when an analyte ion is placed between a leading and a terminating electrolyte. The leading electrolyte contains a high concentration of a high-mobility ion with the same charge as the analyte of interest. The terminating electrolyte contains an ion with lower mobility.

Complex matrices such as serum contain anionic components of low and high concentration, which may act as leading and terminating electrolytes in the sample zone. In this case, we may speak of sample self-stacking. In 2005, Křivánková et al.[47,48] used this approach to determine ethyl glucuronide in serum using chloride from the serum as the leading ion. A buffer composed of 10 mM nicotinic acid and ε-aminocaproic acid was demonstrated to provide an LOD of 0.079 μg/mL for ethyl glucuronide.

IV. APPLICATIONS

From 1993 to the present, several publications have described anion and cation analysis for determination of pharmaceutical counterions (Figure 16). and impurity profiling. For these applications, several buffers were used on different CE instruments. In most cases, detection was indirect or direct UV detection for analytes such as bromide that do absorb at 200 nm. Two papers describe use of a conductivity detector.

The main characteristics of these applications are good linearity, with squared correlation coefficient above 0.999, and repeatability with RSD below 2% when no internal standard is used and below 1% with an internal standard. Depending on the buffer and injection optimization, the different authors found an LOD and limit of quantitation (LOQ) close or below 1 μg/mL. Using a conductivity detector, the LOD and LOQ decreased by a factor 100. Many authors also report the accuracy and recovery of the method.

Noteworthy are the articles from Altria et al.[66–68] and Assi et al.[75] describing the robustness and validation of the determination of potassium as a counterion. An inter-company cross-validation of the determination of sodium in an acidic drug salt was also published.

In an overview on CE for pharmaceutical applications, Altria[73] describes the analysis of ionic salts, organic acids, and also water purity, which may have deleterious effects on synthetic processes. In her review, Natishan[74] also included indirect UV detection when performing pharmacokinetic studies.

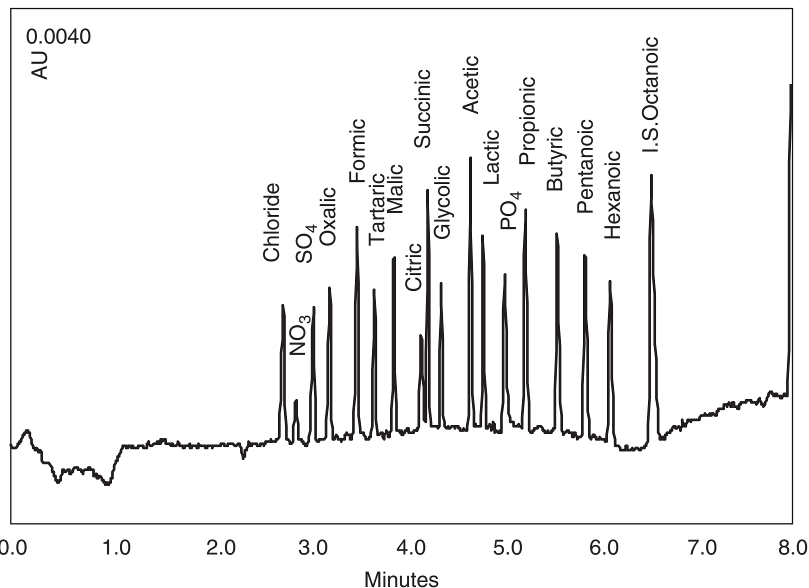

FIGURE 16 **Analysis of different counterions at 20 μg/mL level with 30 mM PDC buffer.**

When the counterion is a UV-absorbing compound, two articles describe simultaneous determination of the drug with its counterion. This was done for denatonium benzoate by Assi et al.[75] and ragaglitazar arginine by Jamali and Lehmann.[76]

The US Drug Enforcement Administration also validated two methods for illegal drugs. In the first, Lurie et al.[77] screened for carbohydrates, inorganic and organic anions, and acidic adulterants in confiscated heroin. This application is based on a modified basic anions buffer kit (Agilent, Waldbronn, Germany), adjusting the pH from 11.9 to 12.1 with sodium hydroxide. The other method is described by Knops et al.[78] He analyzed phosphorous species in methamphetamine to trace back the clandestine laboratories' production process. For his application, he used the CElixir OA™ 8.2 buffer (MicroSolv, Eatontown, NJ, USA).

Table 4 gives a non-exhaustive list of counterions that may be analyzed by indirect and/or direct UV detection using a limited number of buffers, while Table 5 shows a list of applications found in the literature.

CASE STUDY I: EXAMPLE OF COUNTERION DETERMINATION

A. Introduction

How to analyze the phosphate counterion of a known active pharmaceutical compound (API), primaquine diphosphate, is shown as an example. A simplified method was used to demonstrate the feasibility of the method. For this purpose, we prepared three sample solutions and three standard solutions each at 70%, 100%, and 130% of the expected value. Octanoic acid was chosen as internal standard.

Identification was based on addition of a standard phosphate solution and then on the relative migration time of the peak of interest. It is noted that dihydrogeno- and hydrogenophosphate ion migrate with the same mobility. The reproducibility of the relative

TABLE 4 Pharmaceutical Counterions

	Reversed polarity and Indirect UV	Reversed polarity and Direct UV	Normal polarity and Indirect UV
Organic acid			
Acetate	x		
Benzoate		x	
Citrate	x		
Camphorsulfonate	x		
Dibenzoyl-tartrate		x	
Di-*p*-toluoyl-tartrate		x	
Fumarate		x	
2-Hydroxybenzoate		x	
Hydroxybutanedioate	x		
2-Hydroxypropanoate	x		
Maleate		x	
Malonate	x		
Mandelate		x	
Methanesulfonate	x		
4-Methylbenzenesulfonate		x	
Oxalate	x	x	
Propanoate	x		
Succinate	x		
Tartrate	x		
Inorganic anions			
Nitrate	x	x	
Phosphate	x		
Sulfate	x		
Bromide	x	x	
Cations			
Ammonium			x
Calcium			x
Potassium			x
Magnesium			x
Sodium			x

migration time of the phosphate peak should be below 1.5% RSD, and linearity is expected to have an R^2 above 0.999. Accuracy was assessed by comparing the obtained result with the calculated value for a known sample; limits are set at 97.0% and 103.0%.

B. Materials and Methods

The instrument is a P/ACE MDQ (Beckman Coulter, Fullerton, CA, USA) equipped with a UV detector and 230 nm filter. Polarity was set in the reversed polarity mode and the detection used was indirect UV. The capillary had an internal diameter of 75 μm, was made of fused silica (Polymicro, Phoenix, AZ, USA), and was cut at 60 cm (50 cm to the detector).

The buffer used was CEofix™ Anions 5 (Analis Suarlée, Belgium), consisting of a PDC acid at pH 5.6 and a polycation for capillary coating. The method was based on the standard

TABLE 5 Determination of Pharmaceutical Counterions and Impurities

Application	Method	Result	Reference
Pharmaceutical anions analysis			
Screening of anionic impurities in bulk drugs and intermediates: bromide, chloride, nitrite, citrate, fluoride, phosphate, acetate	Chromate with diluted sulfuric acid and OFM Anion-BT (Waters, Milford, MA, USA).	R^2 between 0.999 and 0.987	49
	Indirect UV detection at 254 nm	Range 1−100 μg/mL	
		LOD/LOQ: 0.5 and 1.0 μg/mL	
		Detection at 0.1% w/w	
Quantitative analysis of anions from a prenatal vitamin formulation chloride, sulfate, nitrate, citrate, fumarate, phosphate, carbonate, acetate (see also cations)	5 mM Chromate 0.4 mM CIA-Pak OFM BHT at pH 8.0	$R^2 = 0.998-1$	50
	Indirect UV detection at 254 nm	Range up to 100 μg/mL	
		LOD/LOQ of sulfate: 0.157 and 0.523 μg/mL, nitrate: 0.210 and 0.700 μg/mL	
		%RSD repeatability N=10: sulfate 1.90, nitrate 1.58	
Quantitative determination of drug counterions stoichiometry chloride, sulfate	5 mM Chromate with 0.5 mM TTAB.	$R^2 = 0.9999$ for chloride	51
	Indirect UV detection at 254 nm	%RSD 0.5−2.1	
Organic acid counterions determination: succinate	0.5 mM TTAB, 5.0 mM phthalate, 50 mM MES at pH 5.2.	R^2 0.9991−0.9995 for a range between 50% and 150% of the nominal succinate level (50 μg/mL)	52
	Indirect UV detection at 254 nm	LOD/LOQ: 1 and 2 μg/mL	
		%RSD repeatability of 2.10 for succinate and 0.50 when using IS (citrate)	

TABLE 5 (Cont.)

Application	Method	Result	Reference
Acetate counterion determination	LiOH, 20 mM 4-hydroxybenzoic acid, 15% MeOH, OFM Anion-BT at pH 6.0.	R^2 > 0.999 for a range between 0.9 and 46 µg/mL	53
	Indirect UV detection at 220 nm (DAD 450 nm with reference at 220 nm)	LOQ: 0.9 µg/mL	
		Method precision %RSD: 1.5	
Sodium acetate in antisense oligonucleotides	5 mM Phthalate with 2.5% v/v CIA-Pak OFM Anion-BT at pH 5.6.	R^2 0.994 for a range of 0.06−240 µg/mL	54
	Indirect UV detection at 254 nm	Estimated LOD: 0.06 µg/mL, which is 0.0012% w/w acetate	
		%RSD repeatability: 1.88	
Bromide, nitrite, sulfate, fluoride, phosphate as impurities and acetate as counterion	(a) 50 mM 2-(N-Cyclohexylamino) ethane sulfonic acid (CHES), 20 mM LiOH monohydrate, 0.03% Triton X-100.	(a) Impurities %RSD repeatability between 0.63 and 2.29 for 2 µg/mL. LOD: 0.050 µg/mL	55
	Flush with 1 mM CTAB		
	(b) 60 mM MES, 60 mM His, 0.7 mM TTAOH, 0.03% Triton X-100.	(b) Acetate %RSD repeatability: 1.25. LOD: 0.040 µg/mL.	
	Conductivity detector	Linearity up to 80 µg/mL	
Determination of acetic acid and trifluoroacetic acid (TFA) as impurity in synthetic peptide	5 mM Sodium phthalate, 5 mM CTAB at pH 5.85.	Acetate: $R^2 = 0.9986$ for a range between 2 and 14 µg/mL	56
	Indirect UV detection at 200 nm	TFA: $R^2 = 0.9993$ for a range between 10 and 70 µg/mL	
		%RSD repeatability (N=5) between 1.5 and 0.9 with	

TABLE 5 (Cont.)

Application	Method	Result	Reference
		glutamic acid as internal standard (IS)	
Acetic acid determination as counterion of bradykinin	5 mM Sodium phthalate, 5 mM CTAB at pH 5.85.	$R^2 = 0.9992$ for a range between 25 and 108 µg/mL	57
	Indirect UV detection at 200 nm	l-glutamic acid was used as IS	
Bromide, chloride, and sulfate as impurities	10 mM Potassium chromate, 1 mM Sodium borate at pH 9.15. Flush with 0.2% polybrene.	$R^2 > 0.9993$ for a range between 1 and 200 µg/mL	58
	Indirect UV detection at 254 nm	Mean relative error Br < 2%, Cl < 7%, and SO4 < 3% %RSD repeatability < 4% for 10 µg/mL LOD between 4 and 10 µg/mL, LOQ between 8 and 20 µg/mL	
TFA content in biphalin	5 mM Phthalate, 5 mM CTAB at pH 5.8.	$R^2 = 0.999$ for a range up to 300 µg/mL	59
	Indirect UV detection at 200 nm		
Bromide in anesthetic hydrochloride	100 mM Methanesulfonic acid adjusted to pH 1.3 with triethanolamine and 60% acetonitrile.	$R^2 = 0.9996$ for a range between 13 and 167 µM/mL	60
	Direct UV detection at 200 nm	%RSD reproducibility of 1.4% at 0.3% (w/w) bromide level	
Hydrochloric acid and trifluoroacetate as counterion in drug discovery	For TFA: 5 mM phthalate, 50 mM MES, 0.5 mM TTAB at pH 5.0.	$R^2 = 0.9999$ for TFA for a range between 11 and 800 µg/mL.	61
	For chloride method from.	$R^2 = 0.9995$ for chloride for a range between 5 and 20 µg/mL	
	Indirect UV detection at 214 nm	LOD/LOQ of TFA 4 and 11 µg/mL,	

TABLE 5 (Cont.)

Application	Method	Result	Reference
		chloride 2 and 0.500 μg/mL	
Sulfate in aminoglycoside antibiotics	15 mM Chromic acid, 40 mM TRIS, 0.2 mM CTAB at pH 8.1.	$R^2 = 0.9999$ or 0.9995 for range between 60 and 170 μg/mL for without and with IS (nitrate), respectively	62
	Indirect UV detection at 276 nm	LOD/LOQ: estimated at 2 and 6 μg/mL %RSD repeatability ($N = 10$) with IS of 0.6 at 100 μg/mL	
Sulfate in Indinavir	10 mM Ammonium molybdate, 0.15 mM CTAH at pH 7.5.	$R^2 = 0.999$ for a range between 10.1 and 79.8 μg/mL	63
	Indirect UV detection at 230 nm	LOD/LOQ: 0.34 and 1.13 μg/mL %RSD repeatability ($N = 10$) with perchlorate as IS: 0.87 Intraday: 2.8%	
TFA as counterion during drug discovery	5 mM Potassium hydrogen phthalate, 50 mM MES, 0.5 mM TTAB at pH 5.0.	$R^2 = 0.999$ between 12 and 800 μg/mL	64
	Indirect UV detection at 254 nm	LOD/LOQ for TFA: 3.3 and 11.3 μg/mL %RSD reproducibility (intraday and interday for $N = 5$) of 2.7% at 200 μg/mL. When using IS (pentafluoro-propionic acid), 1.3% for interday and 2.7% for intraday	
Pharmaceutical cations analysis			
Alkali and alkaline-earth cations in solutions for parenteral use: sodium, potassium, magnesium, and calcium	5 mM UV-Cat-1 (Waters, Milford, MA, USA) from 6.5 to 40 mM α-hydroxyisobutyric acid (HIBA) at pH 4.4.	R^2 ranging from 0.997 to 0.9997 between 5 and 80 μg/mL	65

TABLE 5 (Cont.)

Application	Method	Result	Reference
	Indirect UV detection at 214 nm		
Quantitative determination of inorganic ions in vitamin formulation: calcium, zinc, iron	5 mM UV-Cat-1, 6.5 mM 2-hydroxyisobutyric acid at pH 4.4. Indirect UV detection at 185 nm	$R^2 = 0.998-1$ Range up to 100 μg/mL LOD/LOQ of calcium: 0.274 and 0.913 μg/mL, zinc: 0.313 and 1.04 μg/mL, iron: 0.326 and 1.09 μg/mL %RSD repeatability $N = 10$: calcium 2.23, zinc 2.06, iron 1.77	50
Determination of potassium in an acidic drug salt: robustness testing	6 mM Imidazole, 4 mM formic acid at pH 4.5. Indirect UV detection at 214 nm		66
Validation of potassium in acidic drug salt	Same as above	$R^2 = 0.9974$ for a range between 5 and 150 μg/mL %RSD repeatability $N = 20$: 0.65 with sodium as IS at 5 μg/mL	67
Inter-company cross-validation of sodium in acidic drug salt	Same as above	Six different companies %RSD repeatability ranges from 0.5 to 2.6 with potassium as IS Average result was 5.45%, which represents 99.0% of theoretical sodium content	68
Counterions of glycosaminoglycans: calcium and sodium	40 mM 4-Aminopyridine at pH 9.1. Indirect UV detection at 254 nm	$R^2 = 0.97$ for sodium and calcium heparin	69

TABLE 5 (Cont.)

Application	Method	Result	Reference
Residual quaternary amine in bulk drug; tetra-*n*-butylammonium	25% v/v THF (tetrahydrofuran), 3 mM sodium acetate, 12 mM quinine at pH 4.5. Indirect UV detection at 335 nm	$R^2 = 0.9998$ for a range between 1.08 and 1.08 mg/mL LOD/LOQ: 1.08 and 2.17 µg/mL %RSD repeatability ($N = 3$) for TBA at 1.08 mg/mL: 1.6	70
Calcium in calcium acamprosate	10 mM Imidazole, 1 mM tetrabutyl-ammonium sulfate at pH 4.5 adjusted with sulfuric acid. Indirect UV detection at 214 nm	$R^2 = 0.9999$ for a range between 5 and 15 µg/mL LOD/LOQ: 0.7 and 2.5 µg/mL %RSD repeatability ($N = 10$) with IS (magnesium) of 0.26	71
Potassium counterion and inorganic impurities: ammonium, potassium, sodium, trimethylammonium hydroxide (TMAH), lithium	30 mM Creatinine, 30 mM acetic acid at pH 4.5, and 18-crown-6. Conductivity detector	$R^2 = 0.9997$ for a range between 0.020 and 50 µg/mL LOD is approximately 0.020 µg/mL %RSD repeatability ($N = 6$) at 20 µg/mL was below 2.0. Very high %RSD for potassium and sodium at 1 µg/mL	72

method of the kit and included a rinse of the capillary for 30 s at 20 psi with a first buffer containing a dynamic coating, followed by a second rinse of the capillary for 30 s at 20 psi with a buffer solution, then the sample was injected for 8 s at 0.5 psi. A post-injection of water was foreseen for 10 s at 0.1 psi. Separation was performed for 8 min at −30 kV. The current was applied with a 1 min ramping time. After separation, the capillary was rinsed with NaOH 0.1 M for 30 s at 20 psi and with water for 30 s at 20 psi. This last rinse was adapted as described below.

Primaquine diphosphate was obtained from Aldrich (CAS 63-45-6) and had a purity of 98% (data from the manufacturer). Sodium phosphate (CAS 10039-32-4) and octanoic acid

(CAS 124-07-2) were used to prepare the standards, and bi-distilled water was produced in our laboratory.

C. Method Development

When running a testmix containing chloride, nitrate, sulfate, fluoride, and phosphate, we observed a peak tailing for fluoride and phosphate. This may indicate interaction of the analyte with the capillary wall. By replacing the rinse step with water by a rinse step with HCl 0.1 M, there were no more tailings (Figure 17) (see also reference 78). Rinsing with HCl may give an additional chloride peak, which did not interfere with our analysis.

D. Analysis and Results

Primaquine diphosphate was dissolved to obtain 200 µg/mL of phosphate. Previous experience indicated that a concentration of 100−200 µg/mL allows good reproducibility. Addition of a standard phosphate solution confirmed that the peak observed was due to the phosphate ions.

Phosphate standards were prepared at 140, 200, and 260 µg/mL to have 70%, 100%, and 130% of the nominal value. Octanoic acid was added as an internal standard (Figure 18).

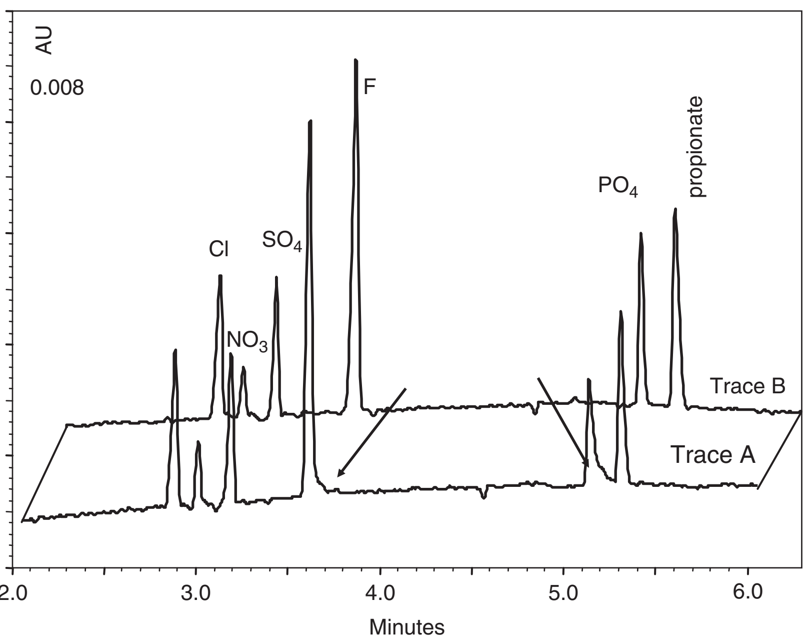

FIGURE 17 Analysis of a testmix composed of chloride, nitrate, sulfate, fluoride, phosphate, and propionate with a 30 mM PDC buffer at pH 5.4. Fluoride and phosphate show a peak tailing (arrows on trace A). By rinsing the capillary, between each sample, with HCl 0.1 M, the peak tailings disappear. Trace B is shifted intentionally to show disappearance of tailings.

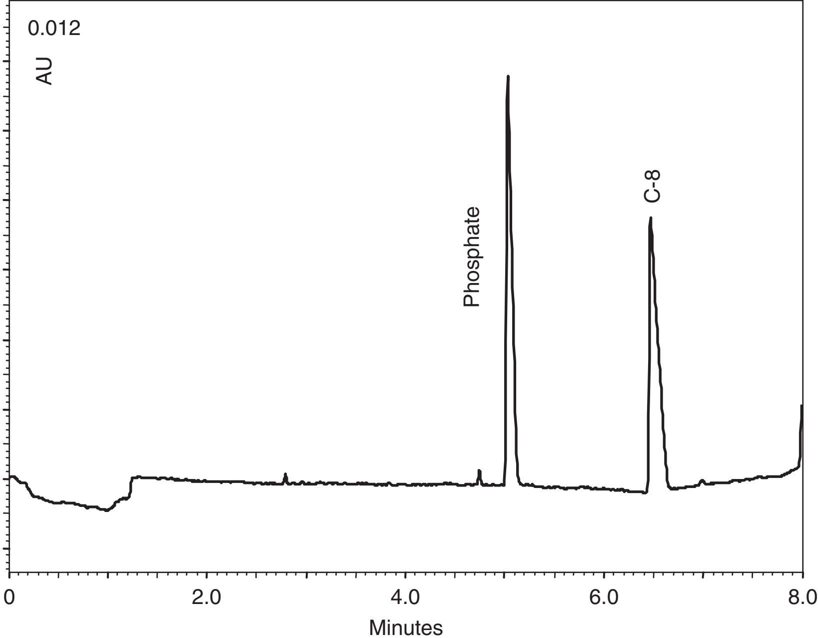

FIGURE 18 Analysis of phosphate from primaquine diphosphate using a 30 mM PDC buffer. Octanoic acid (C-8) is used as internal standard.

A sequence was set up alternating analytes three times with the standards followed by a control sample giving a total of 12 runs. Previous experience had demonstrated that the buffers needed to be changed every 20 runs for optimal conditions.

The overall relative migration time of the phosphate peak showed an RSD of 0.30%. The linearity correlation R^2 was found to be 0.999. The samples gave values of 99.82%, 99.62%, and 99.75% (RSD of 0.11%). These values were well within the expected range of the analysis.

CASE STUDY 2: EXAMPLE OF A FEASIBILITY STUDY FOR IMPURITY PROFILING

A. Introduction

We studied the feasibility of phosphate determination in a low water-soluble API hydrochloride salt. The aim of the work was quantification of phosphate below 0.1% and evaluation of the effect of the high chloride counterion on the determination of phosphate.

B. Materials and Methods

The same instrument and materials as described in Appendix A were used. However, instead of CEofix Anions 5, CEofix Anions 8 (PDC acid at pH 8.1 containing a polycation) was used.

C. Method Development

The API was dissolved in methanol and further diluted to a 10% methanol solution (w/w), which corresponds roughly to 400 µg/mL of chloride. To obtain a maximum signal for phosphate, we used a buffer at pH 8.1 instead of 5.6. Indeed, at pH 5.6 the phosphate ion bears one negative charge, while pyridinedicarboxylate has two negative charges. As a result, two phosphate ions are needed to displace one pyridinedicarboxylate ion. At pH 8.1, the phosphate ion carries two negative charges, and thus one phosphate ion will displace one pyridinedicarboxylic ion. The result is a peak that is twice as high for the same phosphate ion concentration. The drawback of the buffer at pH 8.1 is the presence of a carbonate peak, which migrates close to the phosphate peak (Figure 19).

D. Feasibility

As it was not possible to obtain the API without phosphate, we prepared a test sample containing 400 µg/mL chloride and 0.4 µg/mL phosphate. We checked the effect of increasing the amount of chloride, between 0 and 400 µg/mL (Figure 20), to evaluate the de-stacking effect of the fast-moving ion. On the other hand, an increasing amount of heptanesulfonate, between 0 and 400 µg/mL (Figure 21), was added to evaluate the possible re-stacking effect of a slow-moving ion. This was done to evaluate for possible tITP optimization.

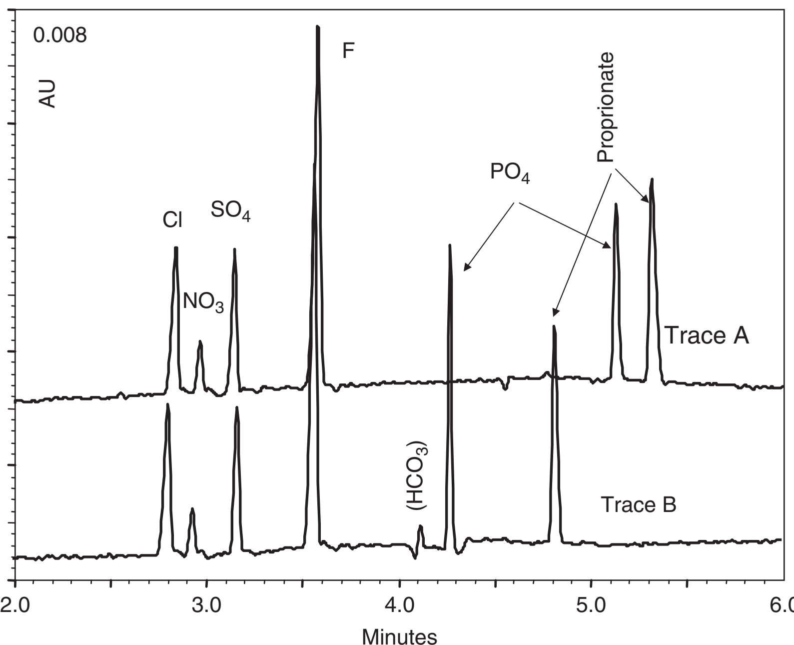

FIGURE 19 Analysis of a testmix composed of chloride, nitrate, sulfate, fluoride, phosphate, and propionate with a 30 mM PDC buffer at pH 5.4 (trace A) and with a 30 mM PDC buffer at pH 8.2. At pH 8.2, the mobilities of phosphate and propionate increase and the corrected area for phosphate is twice as high due to the presence of two negative charges compared with one negative charge at pH 5.4 (trace B).

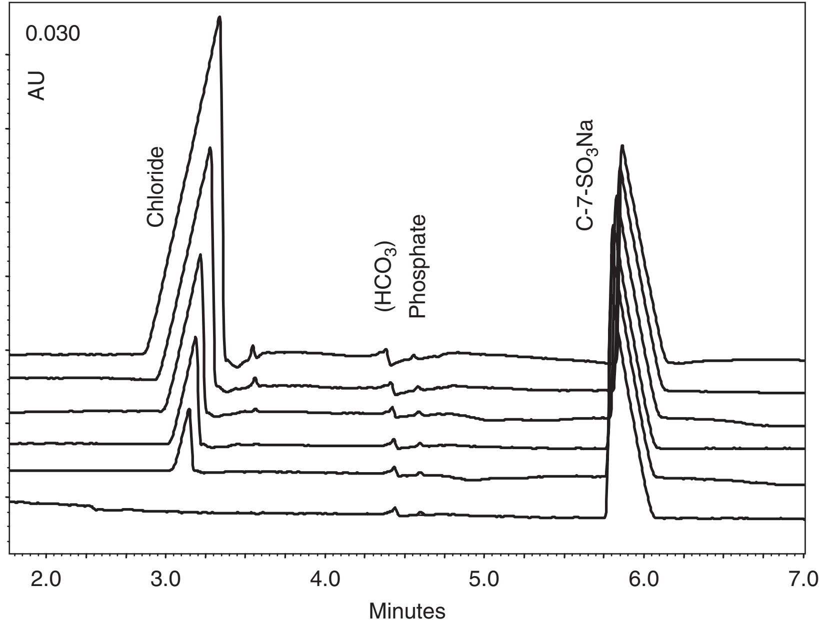

FIGURE 20 **Analysis of phosphate with a 30 mM PDC buffer at pH 8.2 in presence of 400 µg/mL heptanesulfonate (C-7-SO_3Na) and increasing concentration of chloride (between 0 and 400 µg/mL). Phosphate is at 0.1% level.**

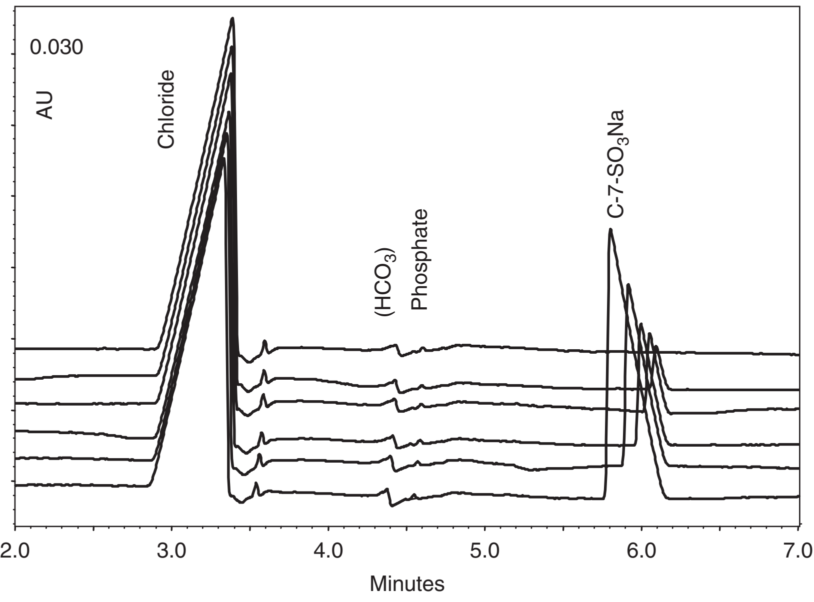

FIGURE 21 **Analysis of phosphate with a 30 mM PDC buffer at pH 8.2 in presence of 400 µg/mL chloride and increasing concentration of heptanesulfonate (C-7-SO_3Na) (between 0 and 400 µg/mL). Phosphate is at 0.1% level.**

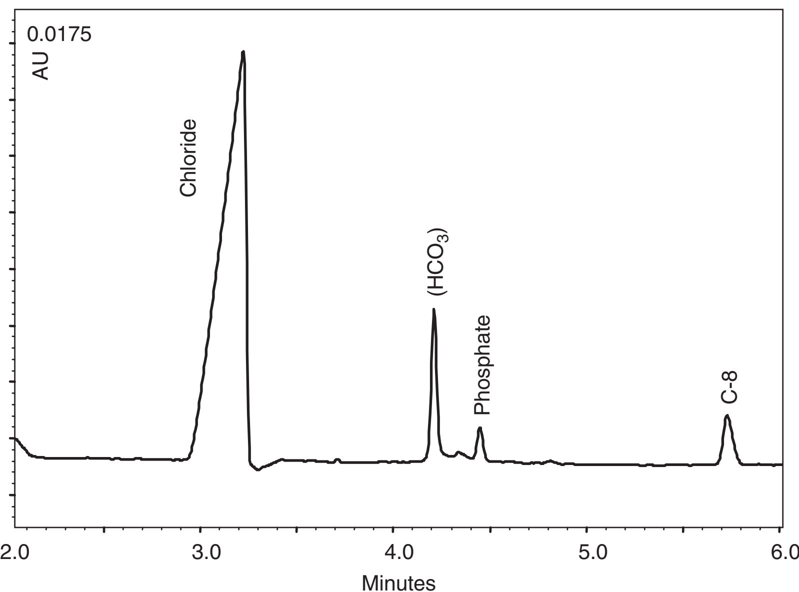

FIGURE 22 **Analysis of API with chloride at 400 μg/mL and quantitation of phosphate with 30 mM PDC buffer at pH 8.2.**

E. Results

The highest theoretical plate number was obtained with 400 μg/mL chloride and 400 μg/mL of heptanesulfonate. However, when looking at the peak area, we obtained an RSD value of 11.74%, which indicated that stacking and de-stacking only had a minimal effect. Further development was performed without searching for tITP stacking (Figure 22).

V. CONCLUSIONS

Existing commercial CE instruments are able to analyze anions, organic acids, cations, and amines that do not absorb in the UV by using indirect UV or C^4D conductivity detection.

The methods used are easy and robust for the analysis of ions in the pharmaceutical laboratory. They can be used for counterion as well as impurity analyses. Other applications are water and raw material analyses.

It is possible with a few buffer systems to solve a large number of analytical problems, and, when needed, small modifications can be made to existing buffers. Method development is fast and results are robust and easily transferable.

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14

ROLE OF CE IN BIOPHARMACEUTICAL DEVELOPMENT AND QUALITY CONTROL

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ABSTRACT

This chapter demonstrates the essential role of capillary electrophoresis (CE) in biopharmaceutical research, development, manufacturing, and release of therapeutic biomolecules. Three main separation techniques – CE-SDS, cIEF, and CZE – are discussed. Detailed method development examples based on four analytical release methods commonly used in the biotech industry are illustrated for the determination of product purity, identity, and consistency. Considerations for method qualification, transfer, and validation are also discussed. Practical examples, for troubleshooting including abnormal electropherograms, are presented from experience gained during method development and transfer. Although CE methods are relatively new to the highly regulated quality control (QC) environment, our experience demonstrates that they can be adapted to this environment with proper analyst training, detailed standard operating procedures, and specific system suitability and sample acceptance criteria. The superior performance of CE over traditional slab-gel methods demonstrates a great advancement for the overall quality release of biopharmaceutical molecules.

I. INTRODUCTION

Over the past 40 years, capillary electrophoresis (CE) has advanced significantly as a technique for biomolecular characterization. It has not only passed the transition from a laboratory curiosity to a mature instrumental-based method for micro-scale separation,[1–4] but also emerged as an indispensable tool in the biotech and pharmaceutical industries.[5–15] CE has become a method of choice in research and development (R&D) for molecular characterization, and in quality control (QC) for the release of the therapeutic biomolecules.[16–20] In the biopharmaceutical industry, more and more CE methods have been validated to meet International Conference on Harmonization (ICH) requirements.[21–24] In this chapter, we present real industrial examples to demonstrate the role of CE in R&D of pharmaceutical products. The focus in this chapter is on method development analytical control for manufacturing and release of therapeutic proteins and antibodies.

II. METHOD DEVELOPMENT AND QUALIFICATION

Different types of CE methods are used in the biotech/pharmaceutical industry for the analysis of biomolecules: capillary electrophoresis-sodium dodecyl sulfate (CE-SDS), capillary isoelectric focusing (cIEF), and capillary zone electrophoresis (CZE) for oligosaccharide and protein analysis. The CE-SDS method separates proteins based on their hydrodynamic size under denatured conditions and is often used for purity determination. The cIEF and CZE methods separate proteins based on their charge and are used as identity and purity methods. CZE oligosaccharide analysis determines the composition of oligosaccharide structure upon enzymatic release of glycans from glycoprotein and is often used as a quality assay to monitor consistency of the manufacturing process. The following sections provide detailed examples of method development and qualification for CE-based methods such as purity and identity assays for biopharmaceuticals.

Method qualification is based on ICH method validation guidelines.[24] Method type (purity or identity) will dictate the level of qualification testing necessary. Several strategies for method qualification and validation exist and are based on needs, resources available, and the project timeframe. One approach is to perform minimal development and qualification, which may be necessary for projects with shorter timelines, but it may place more burden and risk on future validation activities for robustness testing, and can result in failure. As discussed in Chapter 4, an alternate approach would invest more time and resources into method development, followed by extensive qualification and robustness testing to determine if further development is

required. This strategy requires more time and resources early in the process, but lessens the amount of workload and risk level involved during validation. The purpose of method qualification is to define its intended use with respect to the sample type. Method qualification also provides an assessment of the method used to establish the acceptance criteria for method validation. For a purity determination method, the following eight parameters/properties are studied during method qualification: range, linearity, limit of detection/limit of quantification (LOD/LOQ), precision/repeatability, intermediate precision, specificity, stability-indicating capabilities, and robustness. For an identity determination method, only specificity is required by ICH. Table 1 lists method qualification/validation parameters based on ICH guidelines.

A. Capillary Electrophoresis-Sodium Dodecyl Sulfate

For many years, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) methods have been used as an essential tool to determine the hydrodynamic size, monitor product purity, detect minor product or process-related impurities, and confirm batch-to-batch consistency of protein and antibody products. However, gel-based techniques have several limitations, such as lack of automation, varying reproducibility, and a limited linear range. SDS-PAGE is also labor-intensive and generates large volume of toxic waste. Most importantly, the technique does not provide quantitative results for purity and impurity determination of proteins and antibodies.

The evolution of micro-scale separation technologies has allowed capillary-based techniques such as CE-SDS to replace SDS-PAGE for determining the hydrodynamic size of proteins and antibodies. CE-SDS utilizes a capillary filled with replaceable polymer gel matrix and the application of high voltage for separation. The instrument generally contains an online auto-sampler, power supply, and a UV/photo diode array (PDA) detection system to allow automation in a controlled environment. Compared with SDS-PAGE, CE-SDS offers several advantages such as improved resolution, greater linear range, and extremely low chemical waste, and most importantly, the on-capillary absorbance detection provides

TABLE 1 Interpretation of ICH Guidelines for Method Qualification/Validation

Parameters	Examples
Specificity	Test for interference from all possible sources (i.e., sample matrix, UF/DF artifacts, etc.)
Linearity/range of injection	Range of 50−150% of the nominal load. Data may also be used for determining LOD/LOQ
Linearity/range of sample concentration	Range of 50−150% of the nominal load. Data may also be used for determining LOD/LOQ
Accuracy	Use of an orthogonal technique, spike recovery or inferred once specificity, linearity, and precision established
Precision	Multiple instruments, capillaries, runs, analysts, and days to calculate repeatability (intra-assay), intermediate precision (inter-assay), and reproducibility (overall) variability of method
Detection limit/quantitation limit (LOD/LOQ)	Appropriate ICH-recommended methodology
Robustness	Evaluate effects of deliberate perturbations of system (e.g., pH, sample stability, temperature, buffer composition, etc.)
System suitability	Use of appropriate assay controls to determine if a particular run is valid. May also include assay fit-for-purpose evaluations, such as stability indicating methods, etc.

quantitative analysis. The entire process including capillary conditioning, sample injection, separation, integration, and report generation can be fully automated.

CE-SDS methods for protein separation are generally developed under both reduced (often using a reducing agent such as β-mercapto ethanol, BME) and non-reduced conditions (often using alkylating reagents such as iodoacetamide (IAM) or N-ethylmaleimide (NEM). Like SDS-PAGE, CE-SDS denatures molecules using SDS sample buffer at elevated temperature. Development of a CE-SDS method includes the following five steps: (1) determination of sample preparation conditions to ensure proteins are fully denatured and/or reduced, (2) determination of assay linear range, (3) optimization of separation parameters such as separation voltage and temperature to maximize resolution, as well as capillary inner diameter (ID) and length (L) studies, (4) determination of the best type of detectors for maximizing sensitivity, and (5) development of a data processing method for data analysis.

1. Sample Preparation

A typical sample preparation approach in biopharmaceutical analysis follows: To a protein or antibody aliquot of 150 μg, add 3 μL internal standard (IS, 10 kDa molecular weight marker, SDS-MW Analysis Kit, Beckman Coulter, Fullerton, CA), 10 μL neat BME for the reduced CE-SDS or 10 μL of 250 mM IAM for the non-reduced CE-SDS (nrCE-SDS), and QS with SDS sample buffer (SDS-MW Analysis Kit, Beckman Coulter, Fullerton, CA) to a total volume of 150 μL. IAM is usually prepared fresh. Vortex the mixture and centrifuge briefly to bring the liquid to the bottom of the tube. Heat the tube using a water bath at 75°C for 10 min. Cool the solution to room temperature, centrifuge briefly to bring the liquid to the bottom of the tube, and vortex again to mix the solution. The solution is now ready for injection. A sample preparation flow diagram is shown in Figure 1.

The heating temperature and time needed for denaturation are dependent on the properties of the molecule, and therefore should be optimized for each biomolecule.

2. Separation Conditions in CE-SDS

Separation conditions can be optimized with respect to the capillary length (L), capillary ID, separation voltage (V), and separation temperature (T).

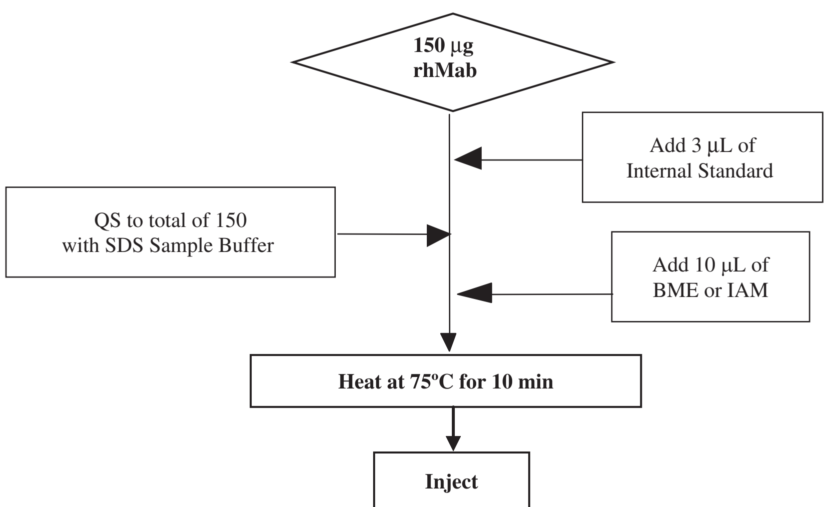

FIGURE 1 Sample preparation for the CE-SDS analysis.

Figure 2 shows the results of CE-SDS separation of a protein using a short capillary (33.0 cm total) and a long capillary (48.5 cm total). The analysis using the long capillary takes more time and shows decreased sensitivity. The short capillary is generally preferred, since it requires less analysis time and produces better sensitivity.

Separation voltage is another important parameter to optimize for resolution during method development. Figure 3 shows an example of the electropherograms (e-grams) obtained by applying different voltages for separation. Higher separation voltage produces a faster separation with better resolution; however, a fluctuated baseline was observed. In this case, −15 kV was chosen for suitable baseline stability and profile resolution.

The effect of capillary temperature, which can also have a significant impact on molecule separation, is shown in Figure 4. A higher temperature provides faster migration; however, it also causes baseline instability. Our recommendation is to use 20−25°C.

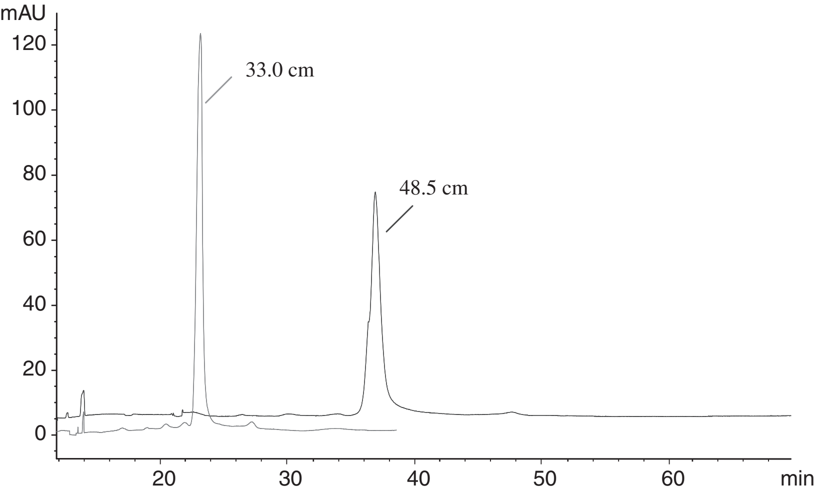

FIGURE 2 **Effect of capillary length on CE-SDS separation of a protein.**

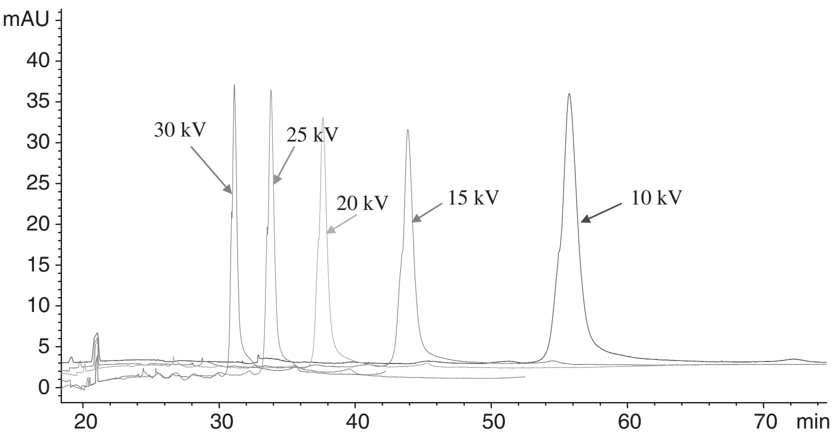

FIGURE 3 **Effect of separation voltage on CE-SDS separation of a protein.**

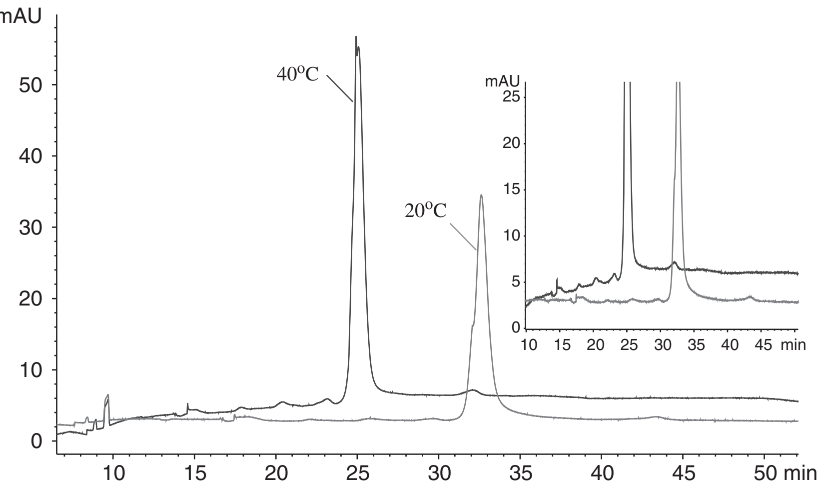

FIGURE 4 **Effect of capillary temperature on CE-SDS separation of a protein.**

Hydrodynamic injection was compared with electrokinetic injection (data not shown). The two injection modes gave comparable percent peak areas. Electrokinetic injection gave slightly higher resolution compared to hydrodynamic injection. For the CE-SDS method, electrokinetic injection is generally recommended.

Generally the recommended CE-SDS separation conditions for proteins and antibodies are as follows:

Capillary length	10–30 cm
Capillary inner diameter (ID)	50 μm, bare fused silica capillary
Separation voltage	Negative 10–15 kV
Capillary temperature	~20–25°C
Injection voltage	−5 kV
Injection time	~20 s

3. Data Analysis in CE-SDS

Integration of the electropherogram for an antibody to quantify main protein species percent light chain and heavy chain (%LC and %HC) and minor species such as percent non-main and percent high molecular weight species (%non-main species and %HMW) is shown in Figure 5. Corrected peak areas were used for quantification. The % corrected peak area is defined as[25]

$$\%\ \text{Corrected Area} = \frac{\text{Corrected Area (Peak or Peak Group)}}{\text{Corrected Area (Total Peaks)}} \times 100\%$$

4. CE-SDS Qualification

(a) Injection Linearity:

As mentioned earlier, the injection conditions can be optimized by varying injection time and injection voltage. Figure 6A shows the linearity study plot of peak area versus injection

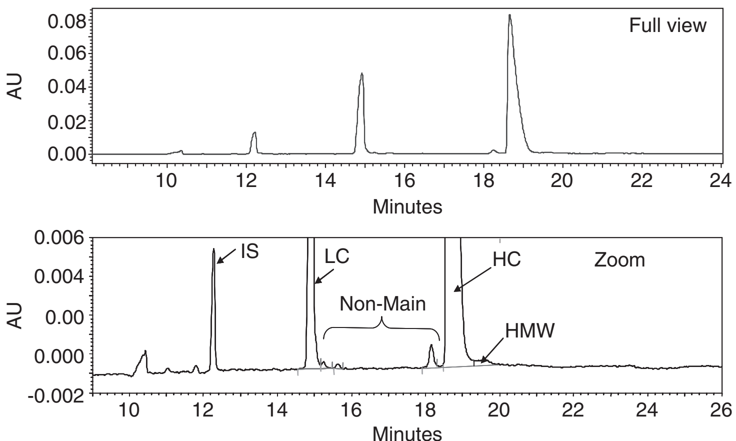

FIGURE 5 **Integration of an electropherogram of an antibody. Note: heavy chain (HC), light chain (LC), high molecular weight (HMW), non-main peaks (Non-main).**

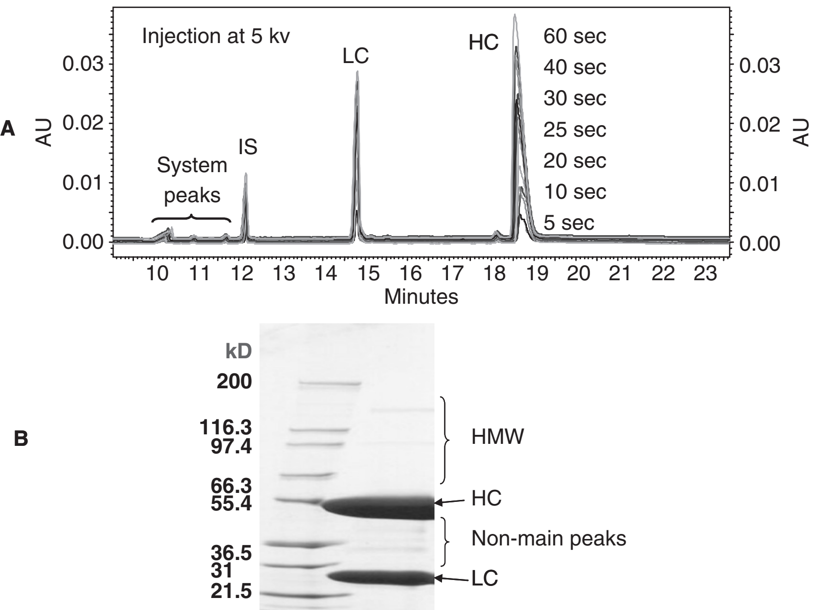

FIGURE 6 **Injection time linearity of an antibody molecule. Panel A: e-gram for injection linearity. Panel B: reduced SDS-PAGE Novex (8–16% Tris-Glycine gel, Invitrogen, carlsbad, CA). (See color plate 2.)**

time at an injection voltage of −5 kV for a reduced CE-SDS method. The advantage of performing an injection linearity study is to optimize and qualify the method for ability to quantitate major and minor protein species within assay linearity range. The internal standard (IS), the LC and HC were baseline resolved in Figures 5 and 6A. This method provides higher resolution of these major species than the SDS-PAGE method (Figure 6B).

Figure 7 shows the results for the corrected peak area plotted against injection time for the HC, LC, and non-HC and LC species called non-main species. The corrected peak area values are used because they compensate for velocity differences between species of different molecular size as they pass through the detector. These results demonstrate that the reduced CE-SDS method has a wide injection linearity range (5–40 s at −5 kV) for the HC, LC, and non-main species.

To further confirm the ability of the reduced CE-SDS method to be used for determination of purity, percent corrected peak area (%HC, %LC, and %non-main) was calculated for each component with respect to the total corrected peak area. Figure 8 presents the relationship between %HC, %LC, %non-main species, and injection time.

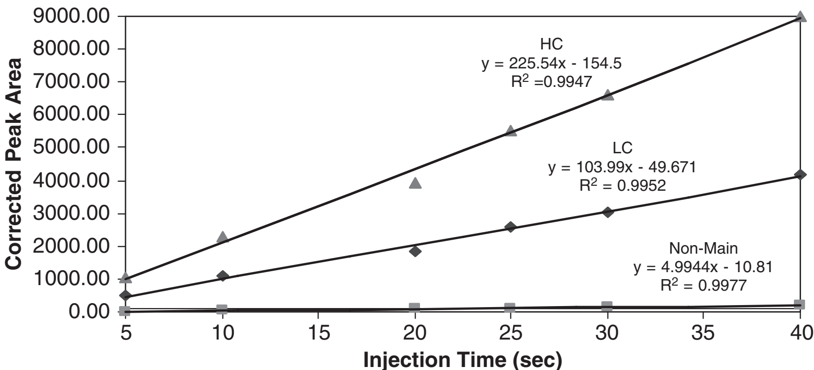

FIGURE 7 **Linear relationship between corrected peak area and injection time.**

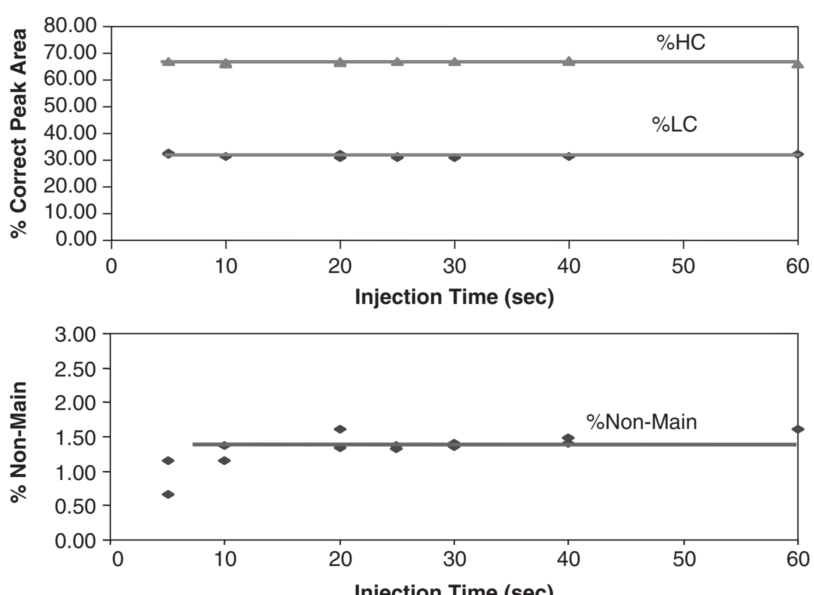

FIGURE 8 **% Corrected peak areas for HC, LC, and non-main versus injection time. Panel A: %HC and %LC species. Panel B: %non-main species.**

TABLE 2 % Corrected Peak Areas Obtained from the Injection Time Linearity Study

Injection time (s)	%Heavy chain	%Light chain	%Non-main	%HMW
5	66.73	32.10	1.16	0.00
5	66.89	32.45	0.66	0.00
10	66.62	31.20	1.37	0.82
10	66.22	31.19	1.15	1.44
20	**66.25**	**32.15**	**1.61**	**0.00**
20	**66.86**	**30.69**	**1.34**	**1.12**
25	**66.92**	**30.84**	**1.38**	**0.86**
25	**66.81**	**31.13**	**1.33**	**0.73**
30	**66.91**	**30.89**	**1.41**	**0.79**
30	**66.92**	**31.07**	**1.35**	**0.65**
40	**67.43**	**31.13**	**1.40**	**0.00**
40	**66.77**	**31.17**	**1.48**	**0.57**
60	**65.84**	**32.00**	**1.61**	**0.56**
Average	66.75	31.23	1.43	0.59
SD	0.45	0.51	0.11	0.37
%RSD	0.68	1.62	7.61	63.62

The qualified linearity range is indicated in bold.

The % corrected peak area values are shown in Table 2. When an injection time of 20 to 40 sec is used, the assay generated reasonable precision. The overall percent relative standard deviation (%RSD) are 0.7, 1.6, and 7.6 for the HC, LC, and non-main species, respectively. The %RSD of HMW species is relatively high due to poor resolution between the HMW and HC peaks. In this case, we recommend that the HMW species not be integrated separately from the HC component unless there is a clear graphic valley point for integration.

(b) Sample Concentration Linearity:

Sample concentration linearity was also evaluated at a fixed injection voltage of −5 kV and injection time of 25 s. Figure 9 shows the e-grams obtained from the linearity study.

The corrected peak area was plotted against antibody concentration for each component, and the results are shown in Figure 10. The % corrected peak areas for each component (HC, LC, and non-main) are linear over a wide protein concentration range, with coefficient of determination (R^2) values greater than 0.99.

The % corrected peak areas for HC, LC, and non-main species were plotted against protein sample concentrations and are presented in Figure 11. The % corrected peak area values are shown in Table 3. These results show that the assay is linear for sample concentrations above 0.5 mg/mL (Table 3), and that the assay is capable of quantitating all three components with overall %RSDs of 0.8, 1.0, and 11.7% for the HC, LC, and non-main species, respectively. The recommended final protein concentration is 1 mg/mL.

(c) Range and LOD/LOQ:

The range of the CE-SDS method depends on each individual protein molecule. Generally the method is linear from 0.5 to 2.0 mg/mL for both UV/PDA and laser-induced fluorescent (LIF) methods.[11,19] Although protein concentrations as high as 3.0 mg/mL can be used for protein quantification, considering the SDS to protein ratio required, high protein concentrations are not recommended. LOD/LOQ values are different for UV/PDA versus LIF detection. For LIF detection with 5-carboxytetramethylrhodamine succinimidyl

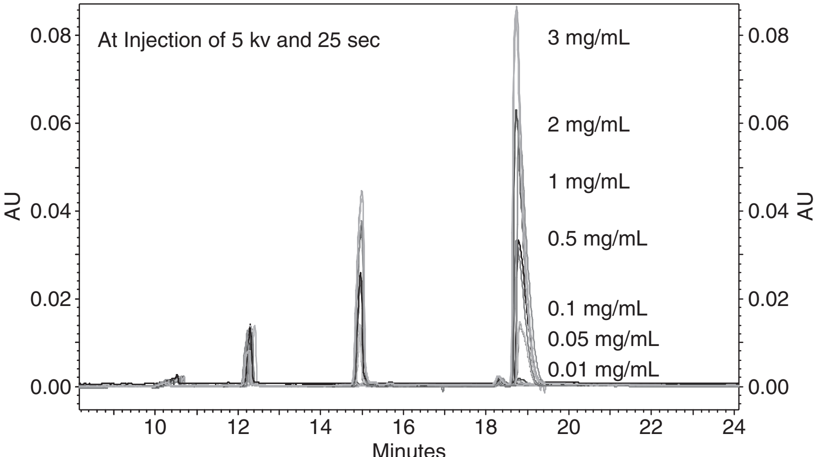

FIGURE 9 **Concentration linearity of an antibody. (See color plate 3.)**

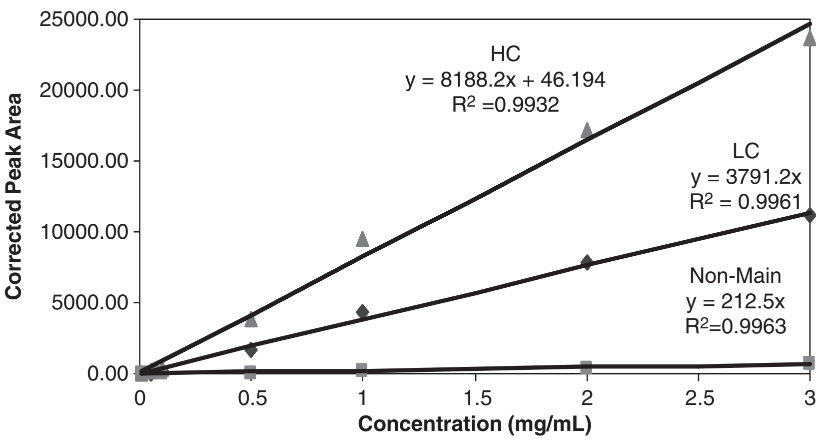

FIGURE 10 **Linear relationship between corrected peak area and sample concentration.**

ester (5-TAMRA-SE) and 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQCA) labeled protein, the reported LOD was 0.01–0.05% (w/w).[11,19] For UV/PDA detection, the LOD is generally 0.1% (w/w), with a LOQ of ~0.3–0.5% (w/w).

(d) Repeatability/Precision/Intermediate Precision:

Precision of the reduced CE-SDS method was studied via repeatability (intra-assay) and intermediate precision (inter-assay) studies. Figure 12 shows electropherogram of the intra-assay repeatability data, and Table 4 demonstrates the precision of the assay (%RSDs of 0.3, 0.6, and 3.7% for the HC, LC, and non-main species, respectively).

(e) Detection:

Two types of detectors were evaluated: UV/PDA and LIF detector. The use of the UV/PDA detector is relatively simple, as no sample pre-labeling derivatization with fluorophore is

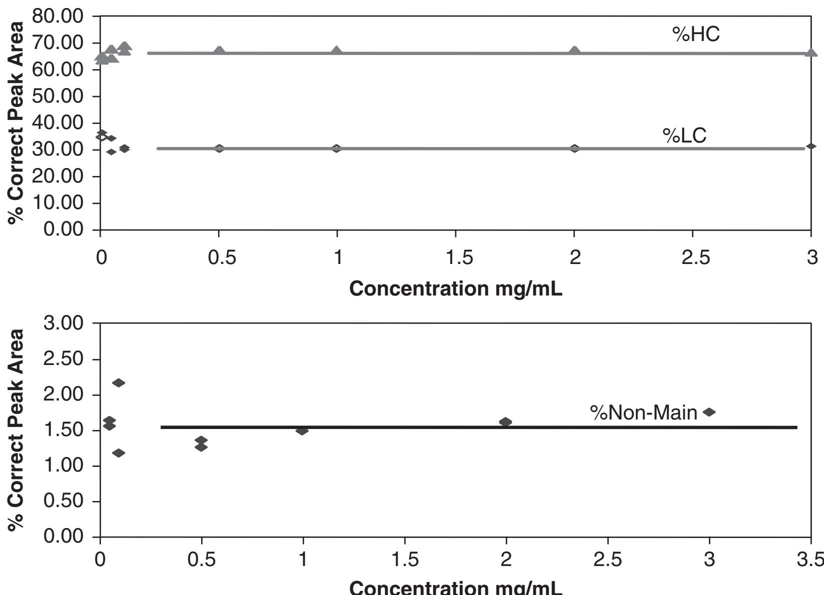

FIGURE 11 **% Corrected peak areas for HC, LC, and non-main versus sample concentration. Panel A: %HC and %LC. Panel B: %non-main.**

TABLE 3 **Integration Data for Concentration Linearity**

Concentration (mg/mL)	%Heavy chain	%Light chain	%Non-main	%HMW
0.01	65.22	34.78	0.00	0.00
0.01	63.69	36.31	0.00	0.00
0.05	67.53	29.17	1.58	1.72
0.05	64.27	34.08	1.66	0.00
0.1	66.80	31.03	2.17	0.00
0.1	68.86	29.94	1.21	0.00
0.5	**67.30**	**30.79**	**1.39**	**0.52**
0.5	**67.44**	**30.66**	**1.30**	**0.60**
1.0	**67.33**	**30.37**	**1.51**	**0.79**
1.0	**67.38**	**30.47**	**1.51**	**0.65**
2.0	**67.46**	**30.38**	**1.62**	**0.55**
2.0	**67.40**	**30.37**	**1.64**	**0.59**
3.0	**66.39**	**31.12**	**1.77**	**0.71**
3.0	**66.05**	**30.97**	**1.85**	**0.51**
Average	67.09	30.64	1.57	0.62
SD	0.55	0.29	0.18	0.1
%RSD	0.82	0.96	11.72	15.79

The qualified linearity range is indicated in bold.

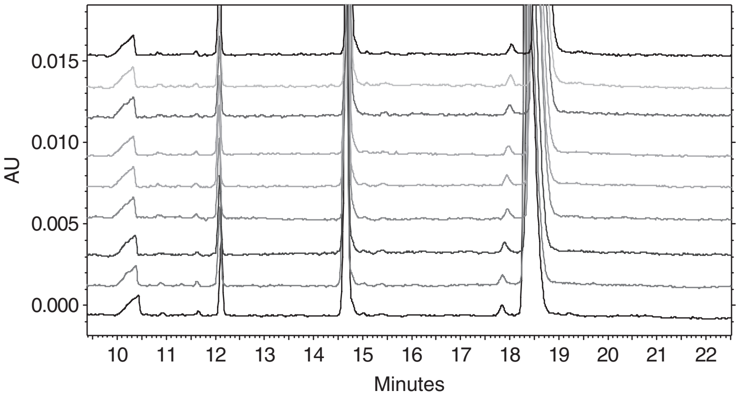

FIGURE 12 Repeatability of reduced CE-SDS of an antibody.

TABLE 4 Repeatability of Reduced CE-SDS of an Antibody

Run#	%Heavy chain	%Light chain	%Non-main	%HMW
002-1	67.24	30.41	1.46	0.90
002-2	66.69	31.00	1.39	0.92
002-3	67.28	30.69	1.38	0.65
002-4	67.19	30.54	1.43	0.83
002-5	66.92	30.58	1.49	1.01
002-6	67.24	30.69	1.33	0.74
005-1	67.16	30.72	1.43	0.70
005-2	66.68	30.90	1.45	0.97
005-3	67.30	30.73	1.43	0.54
005-4	67.11	30.61	1.43	0.86
005-5	67.22	30.80	1.40	0.59
005-6	66.81	30.95	1.55	0.69
008-1	66.94	30.74	1.44	0.88
008-2	67.14	30.88	1.43	0.54
008-3	67.05	30.81	1.52	0.62
008-4	67.13	30.95	1.47	0.45
008-5	66.89	30.89	1.49	0.74
008-6	66.88	31.10	1.45	0.58
Average	67.05	30.78	1.43	0.73
SD	0.2	0.18	0.05	0.17
%RSD	0.3	0.58	3.74	22.51

required. The sensitivity is comparable to Coomassie blue stained SDS-PAGE gel. With the LIF detector, sensitivity is improved significantly by labeling the protein molecule with a fluorophore. Additionally, the baseline noise decreases significantly. However, the labeling procedure adds an additional step in sample preparation. The sensitivity of LIF detection is comparable to silver-stained SDS-PAGE.[19] Figure 13 compares the reduced CE-SDS profile for an antibody using UV/PDA versus LIF detection. The fluorescent label used in this example was 5-TAMRA-SE.[19] These results show that the LIF detector provides much higher sensitivity than the UV/PDA detector.

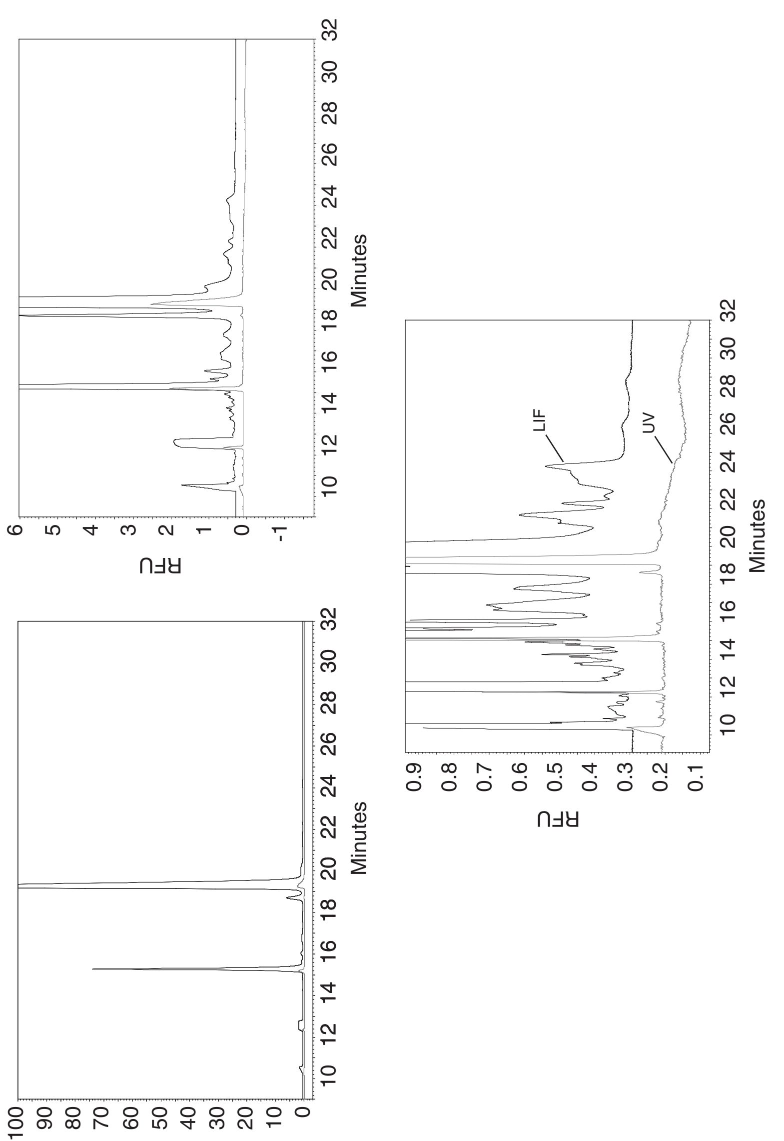

FIGURE 13 Comparison of the UV/PDA and LIF detectors.

TABLE 5 Precision of the Reduced CE-SDS Method

	%Heavy chain	%Light chain	%Non-main	%HMW
Day-to-Day ($n = 6$)				
Day 1	67.1±0.2	30.7±0.2	1.4±0.1	0.8±0.1
Day 2	67.3±0.3	30.8±0.2	1.4±0.1	0.5±0.1
Capillary-to-capillary ($n = 6$)				
Capillary 1	67.1±0.2	30.7±0.2	1.4±0.1	0.6±0.1
Capillary 2	67.2±0.2	30.0±0.3	1.5±0.1	0.5±0.1
Instrument-to-instrument ($n = 6$)				
Instrument 1	67.1±0.2	30.7±0.2	1.4±0.1	0.8±0.1
Instrument 2	68.4±0.1	29.6±0.1	1.5±0.1	0.5±0.1

Another fluorescent dye for use with LIF detection,[9–11] 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQCA), has been recently developed for the CE-SDS method. It is a fluorogenic dye. Sample preparation is simpler than other traditional fluorescent labeling procedures, and the assay is easy to perform.[11]

A third type of detector is the intrinsic or native fluorescence detector that utilizes native fluorescence properties of amino acids. The sensitivity of this detector is between UV/PDA and LIF detection. The advantage of this technique over pre-labeling is that there is no pre-labeling step required; therefore, the sample preparation is relatively simple, and the sensitivity is improved over UV/LIF. However, the intrinsic fluorescence detection relies on the presence of Tryptophan (Try), Tyrosine (Tyr), Phenylalanine (Phe),[12] and this detector has just become commercially available.

(f) Specificity:

The CE-SDS method is a size-based separation technique generally applicable to proteins from 10 to ~200−300 kDa. The specificity is generally tested against the formulation buffer and any other possible contaminant proteins. There is usually no interference from the formulation buffer with the assay. For samples that contain contaminant proteins with a hydrodynamic size of 10−200 kDa, the method is not specific.

(g) Precision:

Precision of the assay was evaluated for day-to-day, capillary-to-capillary, instrument-to-instrument, and the results are shown in Table 5.

5. Stability-Indicating Properties in CE-SDS

The ability of reduced CE-SDS to be used as a stability-indicating method was studied using an antibody stressed at pH 4 and 37°C for up to four weeks. The e-grams show that additional peaks were generated which increased with time, demonstrating that the rCE-SDS is a stability-indicating method (Figure 14).

6. CE Instrument Comparison (CE-SDS)

In the US market, there are two major brands of commercially available CE instruments: Agilent (HP^{3D} CE System) and Beckman (PA 800). Agilent uses a PDA detector, while the Beckman instrument uses a UV, PDA, or LIF detector. For CE-SDS, we have compared CE

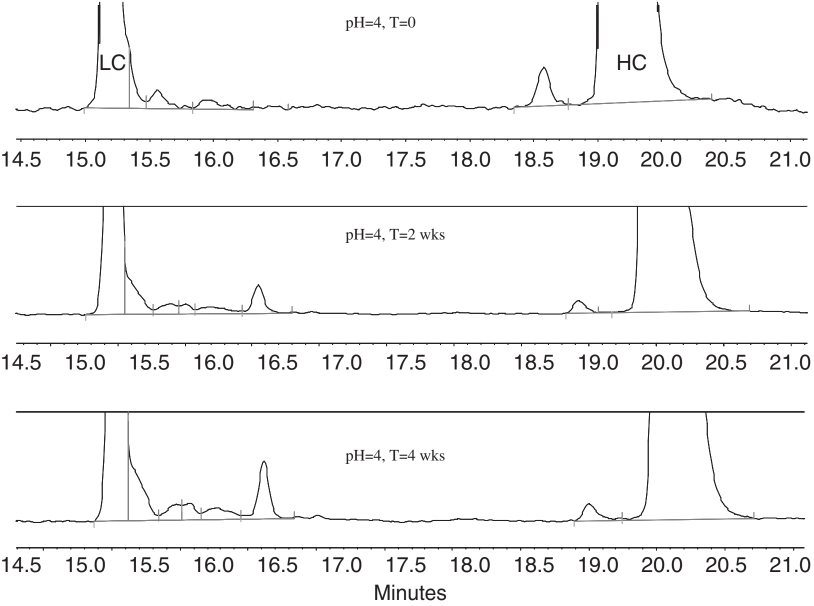

FIGURE 14 **Electropherogram of pH and temperature stressed antibody.**

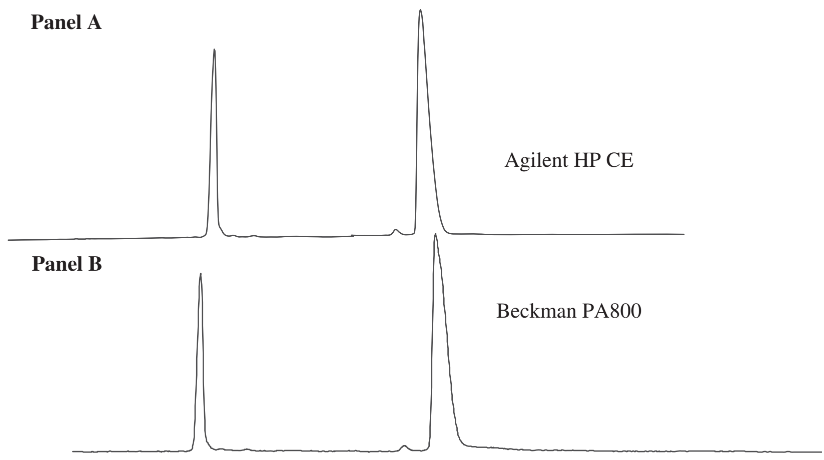

FIGURE 15 **Comparison of the CE instruments.**

instruments from Agilent and Beckman, and they appear to provide very similar performance. Figure 15 shows e-grams of an antibody analyzed using both an Agilent (Panel A) and a Beckman instrument (Panel B). Beckman's older model MDQ is not appropriate for the CE-SDS analysis due to issues of baseline stability caused by the viscosity of the gel buffer, as well as other instrumentation reasons (data not shown).

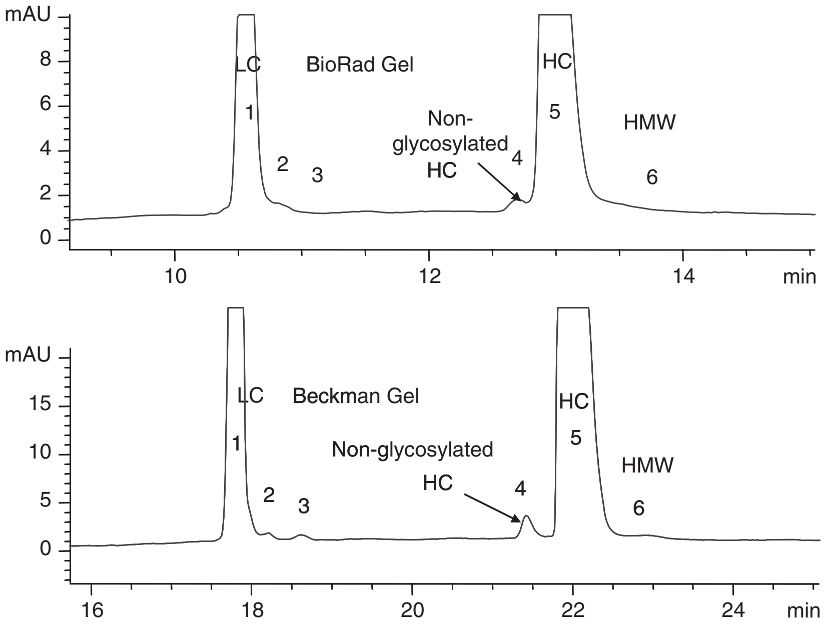

FIGURE 16 **Comparison of the Beckman and the Bio-Rad gel buffers.**

7. CE-SDS Gel Buffer Comparison

CE-SDS gel buffers manufactured by two different vendors, Beckman and Bio-Rad, were compared. The results are shown in Figure 16. The Beckman SDS gel buffer showed better resolution than the Bio-Rad gel buffer.

8. Lab Chip Method for In-process Samples

The Lab Chip instruments/methods currently commercially available generally have less resolution and are less sensitive compared to conventional CE-SDS methods. However, the high throughput of the Lab Chip technique makes it attractive for in-process monitoring during process development. The Lab Chip method is generally used for process development activities such as clone selection, cell culture process development, and optimization of the downstream purification process.

B. Capillary Isoelectric Focusing

As with SDS-PAGE gel methods, gel-based isoelectric focusing (IEF) methods have been used for decades to determine isoelectric point (p*I*), which is an intrinsic property of protein molecules. Some complex proteins have multiple charge isoforms with multiple isoelectric points. These isoforms are separated as multiple bands in the IEF gel method. However, like other gel method, the IEF gel has limitations: it is not automated, not reproducible, and not quantitative for p*I* determination. It is also labor intensive and requires large volumes of toxic reagents for staining.

cIEF methods offer several advantages over the IEF slab-gel method for protein analysis:[20,26] high resolution, wide linear range, extremely low chemical waste, and most importantly, the on-capillary absorbance detection provides quantitative analysis. The entire cIEF process (including capillary conditioning, sample injection, separation, integration, and report generation) can be fully automated. cIEF methods are typically used for monitoring the charge distribution of a protein molecule and isoform p*I* values. cIEF provides a fingerprint of charge isoforms and measures the intrinsic property of p*I;* it is a good identity method for proteins and antibodies.

cIEF method development includes optimization of the ampholyte pH range and concentration, selection of p*I* markers, optimization of protein concentration, selection of injection parameters, and selection of separation conditions (including focus time and voltage), as well as mobilization of the focused proteins toward the detector.

As an identity (ID) test, per ICH guidelines, only selectivity is required in method qualification and validation. Repeatability and intermediate precision are often included to ensure reliability of p*I* determinations. Additionally, method robustness should be tested to assure that the assay performance is suitable for QC environment. Quantitative parameters such as LOD/LOQ are not required for an ID assay. If a cIEF method is used for purity determination, then all the purity parameters shown in Section 4 should be qualified. The following sections illustrate an example of method development and qualification procedures for cIEF.

1. cIEF Sample Preparation

Proteins or antibodies (36 µg) were mixed with ampholine pH 3.5–9.5 (final concentration of 5%, Amersham Biosciences, distributed by GE Healthcare, Uppsala, Sweden), p*I* markers (Bio-Rad, Hercules, CA), and hydroxypropyl methyl cellulose (final concentration of 0.2% HPMC, Sigma-Aldrich, St. Louis, MO). The final protein concentration was 0.3 mg/mL. Figure 17 shows a schematic of the sample preparation. The mixture was mixed thoroughly and was introduced to the capillary (eCAP neutral-coated, 50 micron × 30 cm, Beckman, Fullerton, CA) by hydrodynamic injection. Injections were performed using 20 psi for 99 s. The solution was then separated under an electric field of 25 kV for 10 min. The focused protein was then "pushed/pulled" out of the capillary through a mobilization process using the cathodic mobilizer (Bio-Rad, Hercules, CA).

2. Optimization of Protein Concentration in cIEF

Protein sample concentration can have a significant impact on cIEF peak resolution. Figure 18 shows the results of an experiment in which the antibody concentration was varied

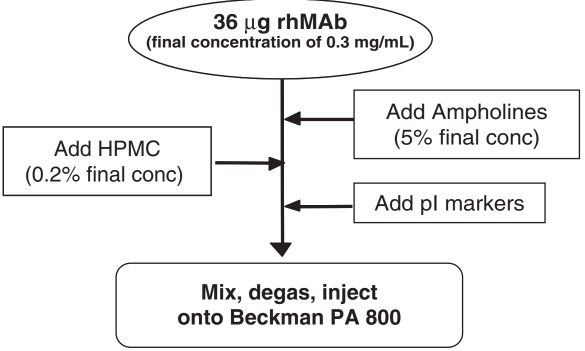

FIGURE 17 **Sample preparation for cIEF analysis of proteins and antibodies.**

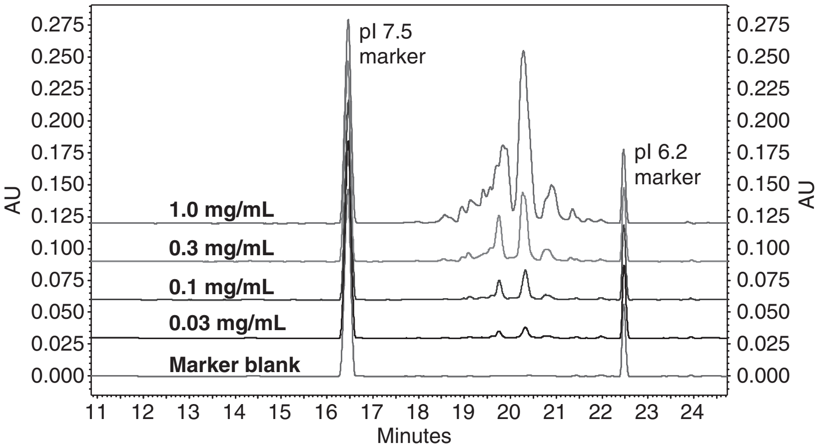

FIGURE 18 **Optimization of protein concentration.**

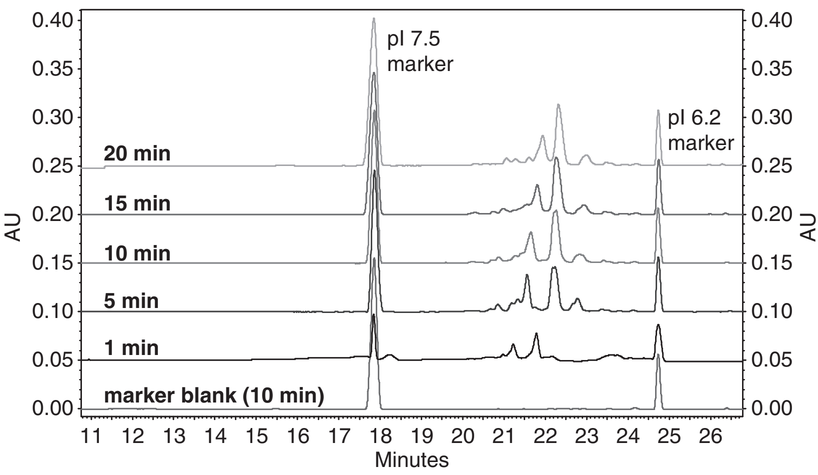

FIGURE 19 **Effect of focusing time on cIEF separation.**

from 0.03 to 1.0 mg/mL. The results show that, at lower protein concentrations, the assay is less sensitive; however at higher protein concentrations, antibody resolution was lost. A protein concentration of 0.3 mg/mL was chosen to balance sensitivity and resolution. It is important to minimize the amount of salt in the final sample solution for reduction of assay interference.

3. Optimization of Separation Conditions in cIEF

The separation conditions were subsequently optimized using the same antibody molecule. Focusing times of 1, 5, 10, 15, and 20 min were studied using a separation voltage of 25 kV. The optimized focusing time for sufficient separation was determined to be 10 min (Figure 19).

Separation voltages of 5, 10, 15, 20, 25, and 30 kV were evaluated for the same molecule using a 10 min focusing time. The results are shown in Figure 20. The data demonstrate that 25 kV is an appropriate separation voltage for this molecule.

4. Optimization of Mobilization Step

After the proteins are focused in the capillary, the isoforms are mobilized past the detector for UV detection. The mobilization step utilizes either hydrodynamic pressure or chemical means. Chemical mobilization can be performed using either ionic or zwitterionic compounds. The general consensus is that hydrodynamic mobilization results in reduced resolution.[27] Bio-Rad (Hercules, CA) zwitterion cathodic mobilizer with chemical mobilization provides superior resolution (Figure 21).

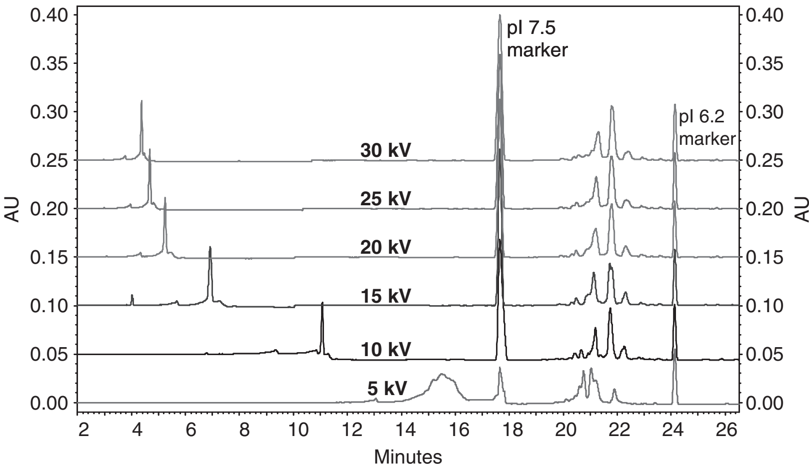

FIGURE 20 **Effect of focusing voltage on cIEF separation.**

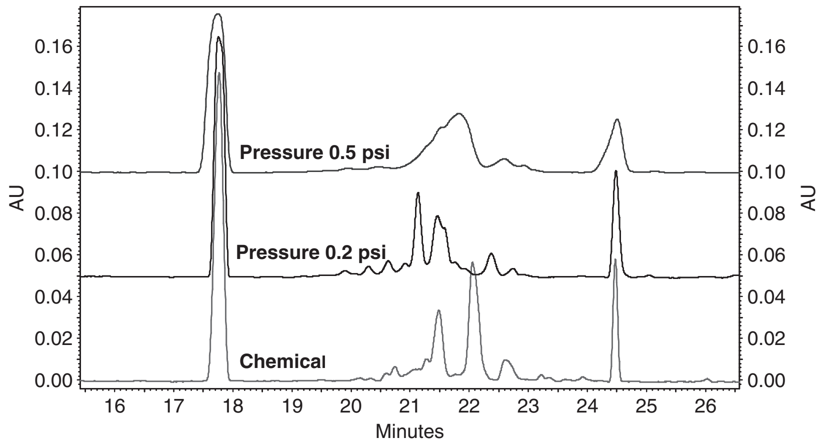

FIGURE 21 **cIEF profile of an antibody using chemical and hydrodynamic mobilization.**

TABLE 6 Selection of the p*I* Markers

Source	p*I*	Product number	Concentration (mg/mL)	Marker type
Bio-Rad	6.2	148-2103	1.0	Small molecule
	7.5	148-2110	1.0	Small molecule
Beckman	5.1	477482	Solid, 0.64 mg	*β*-lactoglobulin
	5.9	477494	Solid, 0.32 mg	Carbonic anhydride II
	9.45	477481	Solid, 3.84 mg	RNase A
Convergent	5.85	102225	~2.0	Peptide
	6.14	102220	~2.0	Peptide
	7.40	102227	~2.0	Peptide
	7.96	102228	~2.0	Peptide
Elphotech	5.9	P1-05901	Not indicated	Small molecule
	7.4	P1-07401	Not indicated	Small molecule
Fluka (from Sigma)	5.5	77866	3.0	Peptide
	6.2	73938	1.0	Peptide
	7.6	89952	1.0	Peptide
	8.1	75734	3.0	Peptide
Synthetic peptides	5.31	N/A	Solid, 10+mg	Peptide (WDDHH)
(produced in-house)	5.91	N/A	Solid, 10+mg	Peptide (WER)
	7.27	N/A	Solid, 10+mg	Peptide (WEHHHR)

5. Selection of p*I* Markers in cIEF

The selection considerations for appropriate p*I* markers for cIEF with proteins/antibodies included purity and stability of the p*I* markers, p*I* values of the protein analytes, and potential protein–p*I* marker interactions. High purity, stable p*I* markers that give reliable p*I* values with no protein–p*I* marker interaction are desirable. Table 6 lists sets of p*I* markers used for optimization. The antibody of interest had a p*I* range of approximately 6.3 to 7.0. In this case, six different vendor sources were evaluated. These p*I* markers vary in nature, from proteins and peptides to small molecules. The e-grams obtained using these markers with the antibody of interest are shown in Figure 22. Although the nature of the p*I* markers and exact p*I* marker values were different, the cIEF profiles of the antibody were the same.

The recommended cIEF separation conditions for proteins and antibodies are as follows:

Capillary length	30 cm
Capillary inner diameter (ID)	50 μm, neutral-coated capillary
Separation voltage	25 kV
Separation temperature	20–25°C
Focusing time	~10 min

6. Data Analysis in cIEF

An example of cIEF e-gram showing antibody peak integration is shown in Figure 23. The p*I* values of the two main peaks (main peak 1 and 2) were recorded. As an identity assay, a reference standard is always included in the same run sequence. The cIEF profile of the sample is compared with the profile of the reference standard. The criterion for an identity test is usually that the sample be "comparable to reference standard," as measured by a comparison of the p*I* values of the sample and the reference standard, with "no new peaks" visible.

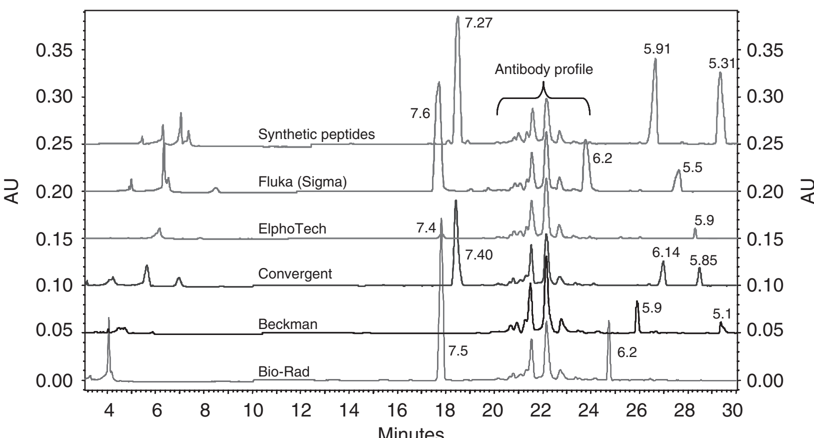

FIGURE 22 **cIEF electropherograms of a protein using different vendors of p*I* markers (aligned using the dominant antibody peak).**

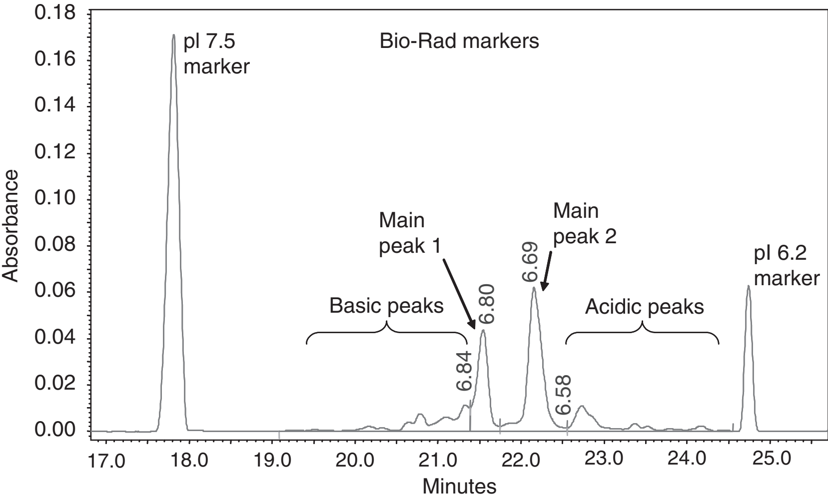

FIGURE 23 **Integration of an electropherogram of an antibody analyzed by cIEF.**

7. cIEF Qualification as an Identity Method

(a) Specificity:

The purpose of the specificity test is to differentiate a particular molecule from other molecules that have potentially similar profiles and also to demonstrate there are no interfering peaks introduced from the sample matrix. Figure 24 shows the cIEF profiles of four different monoclonal antibodies analyzed using the same cIEF method. The profiles are significantly different. Since cIEF provides high resolution, it typically provides results that are molecule specific, and is useful as a "fingerprint" method. In the case where two molecules have similar p*I* values and profiles, an alternative identity method is required.

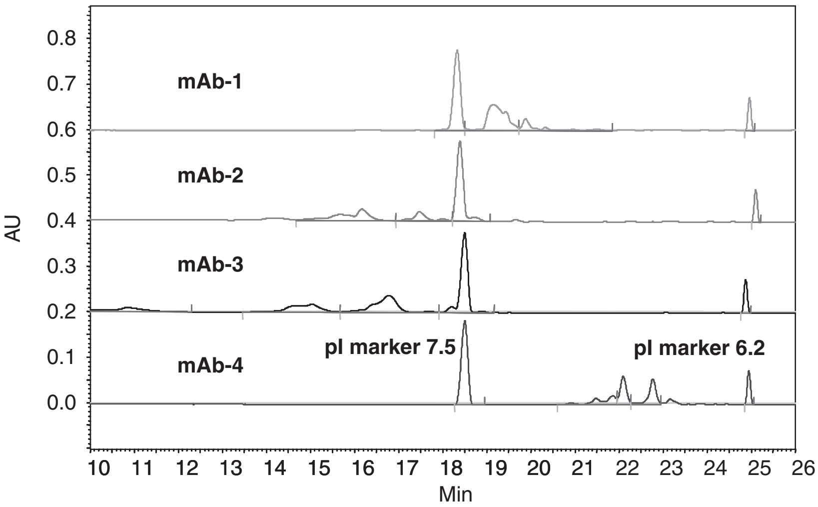

FIGURE 24 **Specificity of a given cIEF method for mAbs.**

(b) Reproducibility:

Figure 25 shows the e-grams of a monoclonal antibody run by multiple labs at multiple sites. The p*I* values are consistent from site to site (Table 7).

8. CE Instrument Comparison (cIEF)

A comparability study using two Beckman CE instrument models (MDQ vs. PA 800) was performed to assess instrumental comparability. In this study, p*I* values were determined for an antibody using each model. As shown in Table 8, the two instruments produced comparable p*I* values and comparable % corrected areas for each group of peaks.

9. Imaged cIEF

Real-time monitoring of the focusing process, without the mobilization step, is defined as image cIEF (i-cIEF). The elimination of the mobilization step significantly simplifies the procedure for use in a QC environment. A comparison study of conventional cIEF (using a Beckman PA 800) and imaged cIEF (using a Convergent Biosciences iCE) for a monoclonal antibody was conducted and the e-grams are shown in Figure 26. Panel A shows an e-gram of an antibody analyzed using the conventional cIEF method, and Panel B shows an e-gram using the imaged cIEF method. The mobilization step in conventional cIEF reverses the e-gram resulting in species A and B, the acidic species, migrating after the main peak whereas the imaged cIEF uses CCD camera to capture the real-time focusing profile resulting in the corresponding species A and B appear prior to the main peak. Overall, the two techniques gave similar results with a slightly higher resolution for the conventional cIEF.

The CFR 21 part 11 compliant software newly released by Convergent Bioscience makes imaged cIEF a viable technique for use in QC environments for release testing of therapeutic proteins or antibody molecules. i-cIEF has also been used heavily for characterization of protein modifications such as deamidation and isomerization, as well as oligosaccharide structure analysis.[7,28–31]

C. Capillary Zone Electrophoresis for Oligosaccharide Analysis

Carbohydrate chains in glycoprotein pharmaceuticals have important roles for their biological activities. Analysis of oligosaccharide structure provides essential information for molecular characterization. Many methods have been reported for characterizing oligosaccharide structures with good resolution and sensitivity.[32–35] Recent advances in CE with laser-induced fluorescence (CE-LIF) detection have provided a rapid, high-resolution, and high-sensitivity analysis of a mixture of fluorescent-labeled oligosaccharides.[36]

Glycoprotein was digested using the peptide N-Glycosidase F (PNGase F) to release N-linked glycan, then labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS). The detection used LIF with a argon-ion laser.

Oligosaccharide analysis using CE method provides faster run time compared to the HPLC method. Figure 27 shows a comparison of the oligosaccharide analysis using the HPLC and the CE method.

The main challenge for glycan analysis is linked to the lack of chromophore or ionizable groups on the carbohydrate molecules. These challenges are addressed by two approaches: (1) increase the analyte mobility by either operating at high pH, or formation of borate or charged

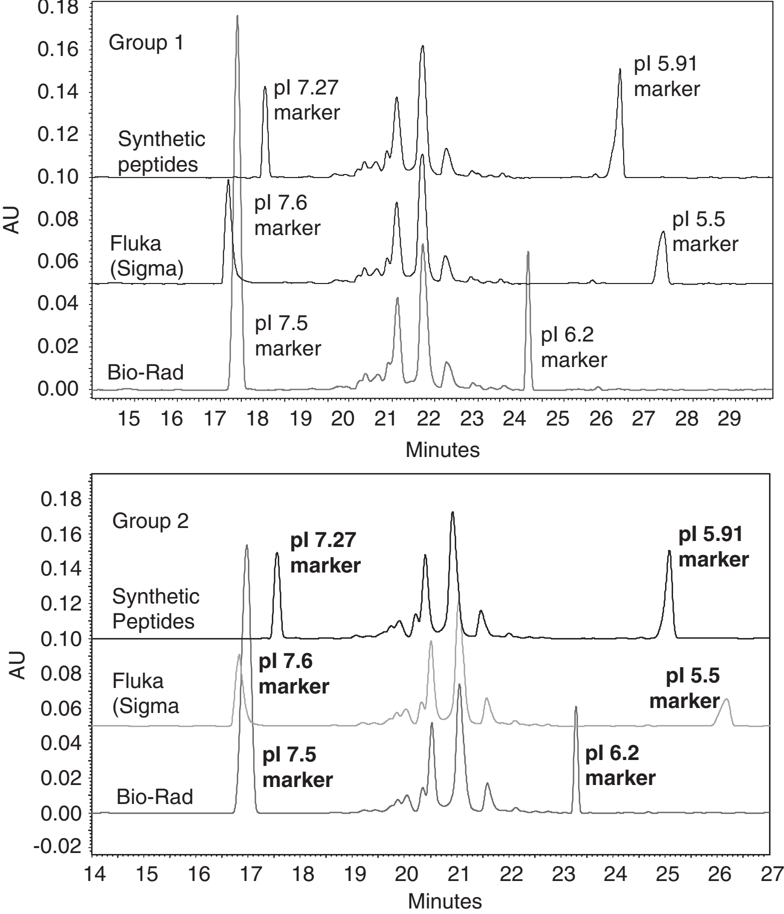

FIGURE 25 **cIEF profile of a monoclonal antibody run by various groups.**

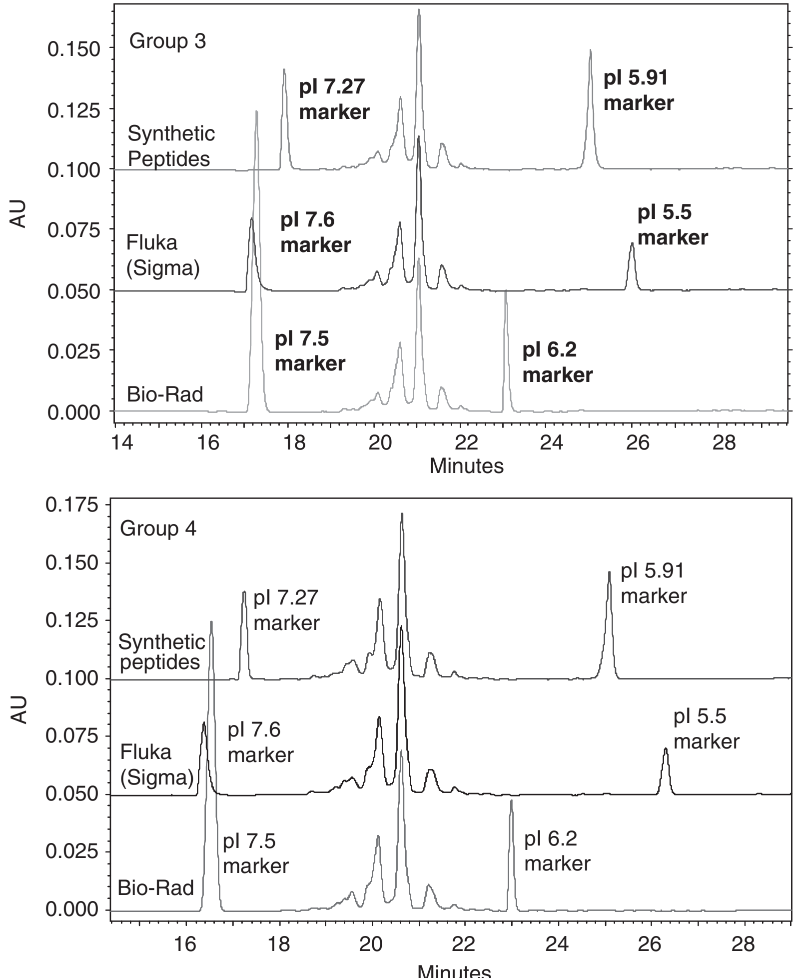

FIGURE 25 **(Cont.)**

complexes; and (2) increase sensitivity by pre-column derivatization (chromophores or fluorophores) to glycan molecules. Reductive amination with APTS is one of the commonly used techniques for carbohydrate analysis by CE. The chemical derivatization is shown in Figure 28. APTS attached to the carbohydrate molecules provides mobility for separation and sensitivity for fluorescence detection. In this case, LIF detection requires laser wavelength of 488 nm.

CE oligosaccharide analysis with APTS labeling has been used in biotech industry for many years and has demonstrated reproducibility. It is now routinely used in GMP environment for release of therapeutic proteins and antibodies. Figure 29 shows the reproducibility of the analysis.

D. Capillary Zone Electrophoresis for Protein Analysis

As with cIEF, CZE separates proteins based on charge and size differences and can also be effective as a purity or identity test. CZE differs from CE-SDS and cIEF in one

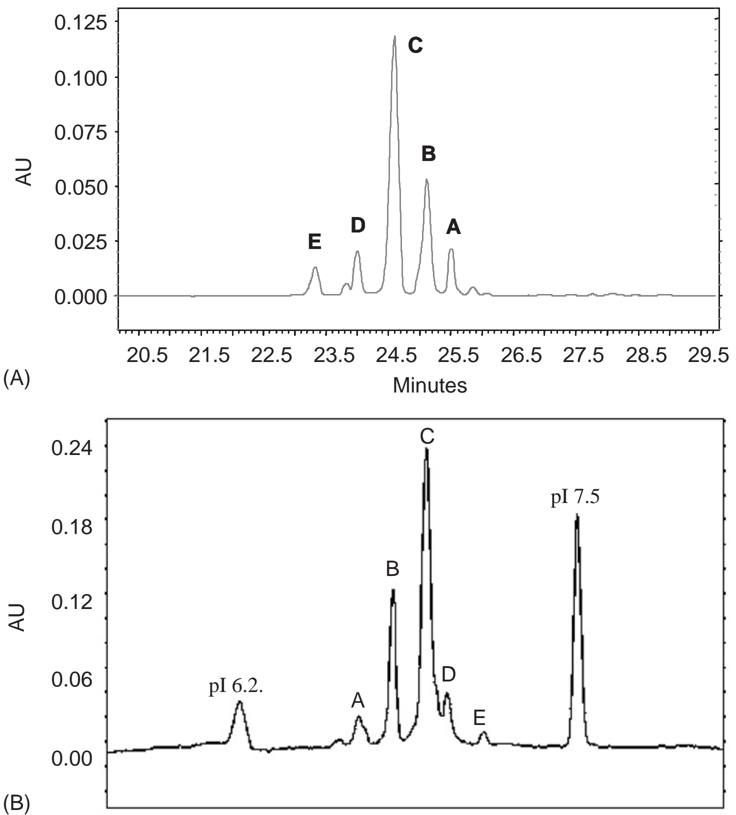

FIGURE 26 Comparison of electropherograms produced using conventional cIEF and imaged cIEF. Panel A: Beckman PA 800. Panel B: Convergent i-cIEF.

important way: it uses free solution (zone) chemistry to selectively move proteins past the detector based on electrophoretic mobility. Selectivity of a CZE method depends on a number of variables that affect protein mobility with respect to electroosmotic flow (EOF). Careful optimization of buffer type, buffer salt concentration, pH, separation voltage and time, capillary temperature, and the use of coated capillaries can maximize protein separation. Method development activities include optimizing background electrolytes (BGE), protein concentration, sample preparation (desalting), injection type and parameters, type of capillary, capillary ID, and detection wavelength and/or mode.

Operating parameters for CZE can vary widely depending on the protein of interest. The discussion below contains a specific example of the use of CZE for analysis of a glycoprotein.

1. Sample Preparation in CZE

Most CZE separations are very sensitive to conductivity (e.g., salt concentration) in the run buffer. Therefore, to avoid introducing a high amount of salt from the sample injection, samples should be buffer exchanged with an appropriate low salt buffer prior to analysis. Centrifugal UF/DF devices are ideal for this purpose, as they are typically very reproducible and allow the analyst greater flexibility in controlling the final sample concentration.

TABLE 7 **p*I* Values of a Monoclonal Antibody Run by Various Groups**

Marker source	Upper p*I*	Lower p*I*	Group	Average antibody isoform p*I* values			
				Basic	Main 1	Main 2	Acidic
Bio-Rad ($n = 2$ each group except group 1 $n = 7$)	7.5	6.2	1	6.84	6.80	6.68	6.58
			2	6.81	6.77	6.66	6.55
			3	6.80	6.75	6.66	6.54
			4	6.82	6.78	6.68	6.56
			Average	6.82	6.77	6.67	6.56
			Std dev	0.02	0.02	0.01	0.02
			%RSD	0.23%	0.28%	0.17%	0.23%
Fluka ($n = 2$ each group except group 1 $n = 6$)	7.6	5.5	1	6.85	6.80	6.68	6.57
			2	6.81	6.77	6.65	6.53
			3	6.83	6.78	6.68	6.55
			4	6.86	6.81	6.71	6.58
			Average	6.84	6.79	6.68	6.56
			Std dev	0.02	0.02	0.02	0.02
			%RSD	0.32%	0.28%	0.37%	0.34%
Synthetic peptides ($n = 2$ each group except group 1 $n = 6$)	7.27	5.91	1	6.80	6.77	6.66	6.58
			2	6.79	6.76	6.66	6.56
			3	6.79	6.75	6.67	6.57
			4	6.80	6.77	6.68	6.59
			Average	6.80	6.76	6.67	6.57
			Std dev	0.01	0.01	0.01	0.01
			%RSD	0.09%	0.13%	0.13%	0.19%

TABLE 8 **Comparison of Beckman MDQ versus PA 800**

	MDQ Average	PA 800 Average	Difference
p*I* Value (Basic)	6.81	6.81	0.00
p*I* Value (Main peak 1)	6.77	6.77	0.00
p*I* Value (Main peak 2)	6.64	6.64	0.00
p*I* Value (Acidic)	6.55	6.54	0.01
% Corrected area (Basic)	17.87	17.76	0.11
% Corrected area (Main peak 1)	26.09	26.55	0.46
% Corrected area (Main peak 2)	43.87	43.27	0.60
% Corrected area (Acidic)	12.18	12.43	0.25

Optimizing protein concentration (i.e., amount of protein injected) entails balancing resolution versus sensitivity. Indeed, injecting more protein can result in greater sensitivity for small peaks, but separation of poorly resolved peaks can be negatively affected. The example in Figure 30 demonstrates this effect. Optimization of the ideal loading conditions should be performed during linearity testing for qualification.

Other aspects of sample preparation to consider, depending on the objective of the CZE method, are sample denaturation, reduction and/or derivatization, and the use of an IS. IS are widely used in CE separations to aid in normalization of slight inter-assay variability and in method troubleshooting.

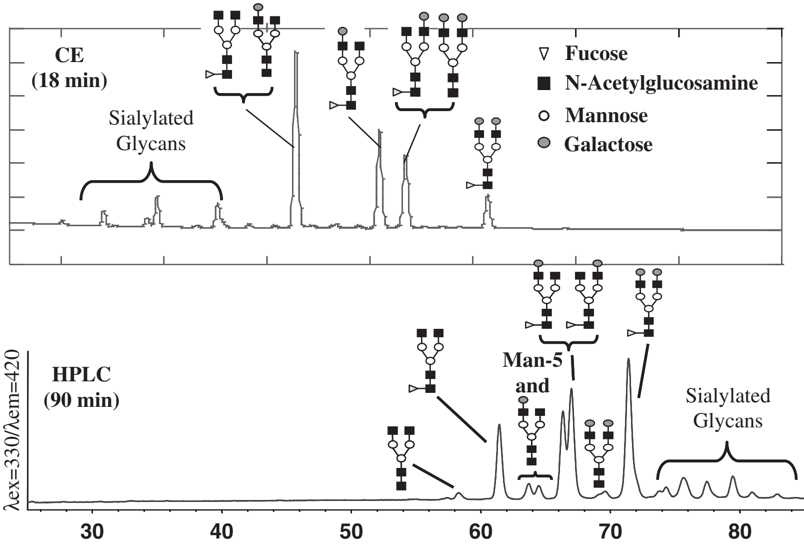

FIGURE 27 **Comparison of the HPLC and the CE method for oligosaccharide analysis. Panel A: analysis using the CE method. Panel B: analysis using the HPLC method.**

APTS

HOAc/NaBH$_3$CN

FIGURE 28 **Reductive amination with APTS.**

2. Separation Conditions in CZE

Separation conditions in general refer to the entire system, including sample, instrument, detection, capillary, buffer, separation voltage, time, and polarity. Again, due to the flexibility in development that CZE methods afford, special attention to each of these variables may be necessary. Once the method has been optimized, qualification testing

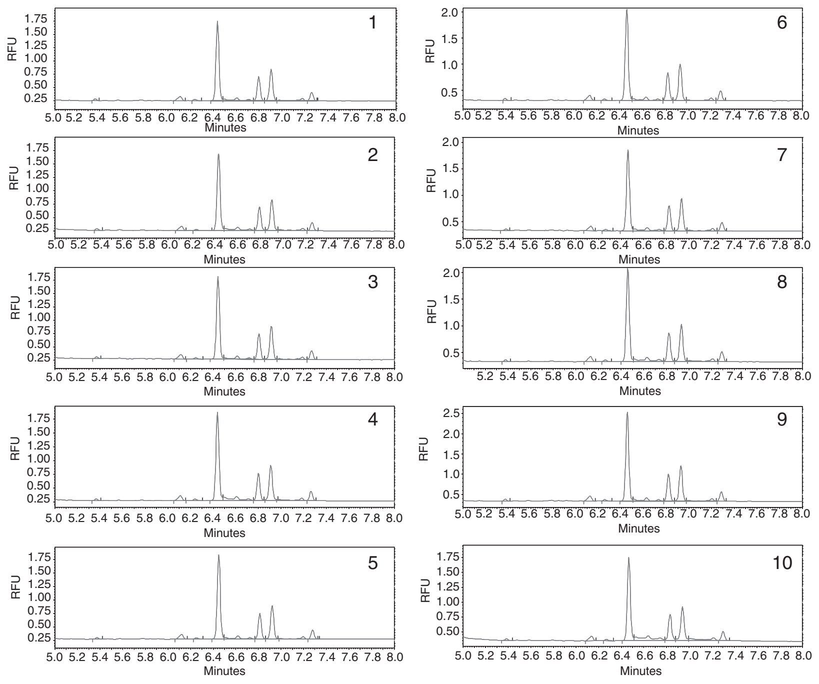

FIGURE 29 Reproducibility of the CE oligosaccharide analysis.

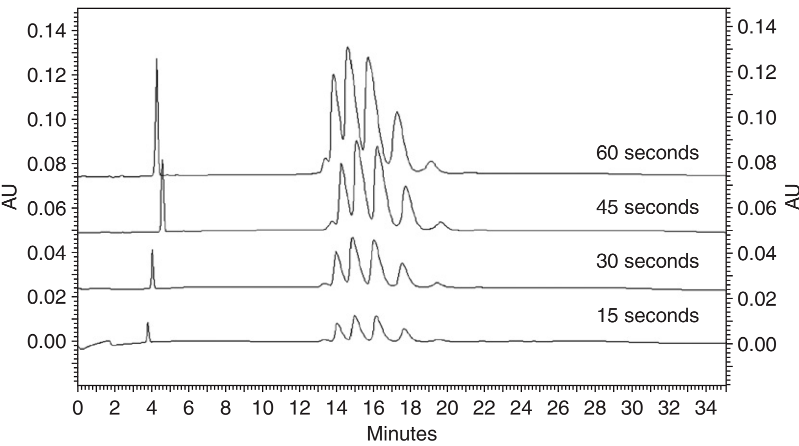

FIGURE 30 Injection optimization (constant pressure).

may indicate certain aspects require further refinement. As with most analytical methods used routinely in a cGMP or QC environment, a dynamic life cycle exists in which the assays are routinely evaluated for improvement in terms of new technology, sensitivity, and robustness.

3. Run Buffer in CZE

Minor changes to run buffer can have a significant impact on the antibody profile, these impacts are shown below. Figure 31 illustrates the effects on separation and migration time when the pH is changed by only 0.2 units.

Note that the migration time of the IS does not change when the run buffer pH is varied; however, shifts in mobility and resolution of the glycoprotein isoforms become apparent.

As shown in Figure 32, changes to the run buffer concentration can also affect migration time, resolution, and detector response (e.g., an increased background due to the use of non-UV transparent salt results in lower peak response and greater baseline drift).

Finally, the choice of run buffer salt can also significantly affect separation, as shown in Figure 33.

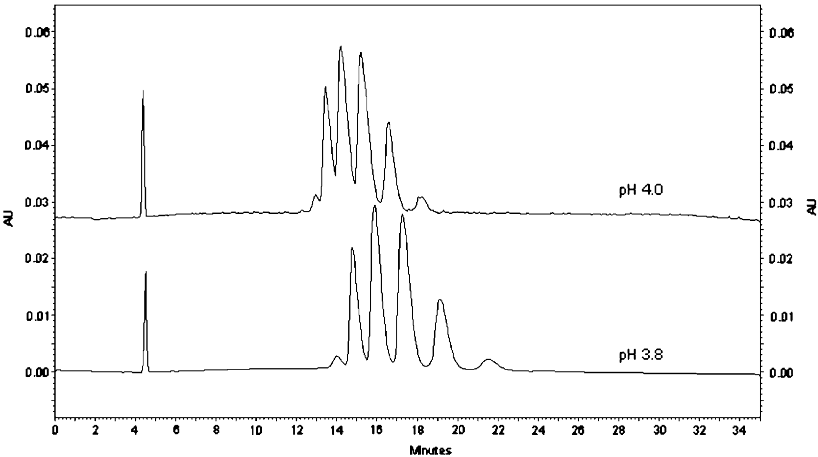

FIGURE 31 **Effects of pH on e-gram of a protein molecule.**

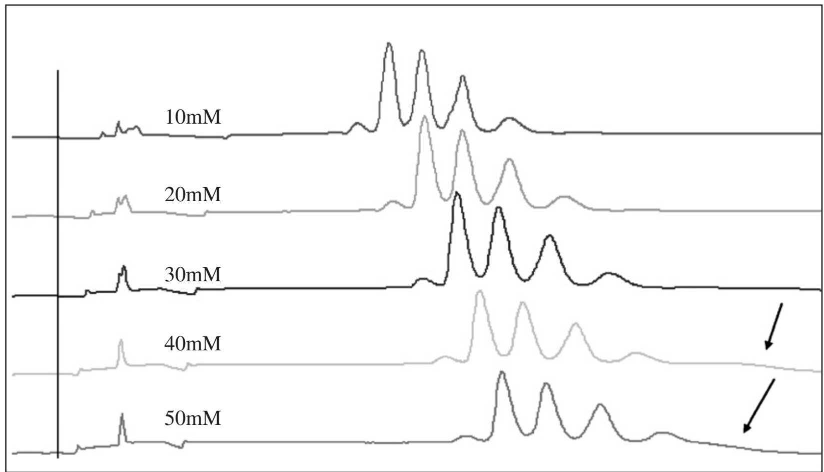

FIGURE 32 **Effects of buffer salt concentration. (Note: Baseline deflections as indicated by the arrows.)**

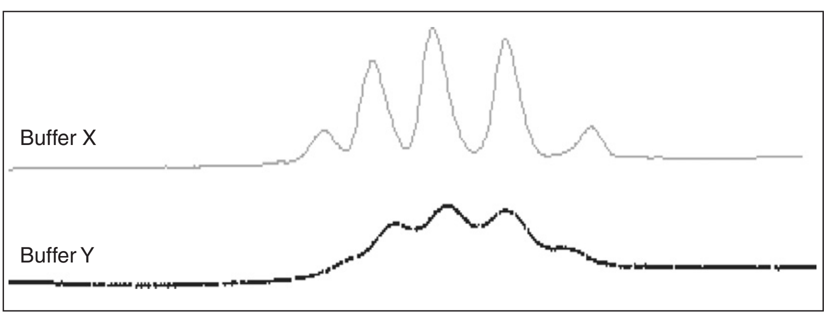

FIGURE 33 Effects of buffer salt type at pH 4.

Based on the data presented above, the final run buffer conditions selected for this example were 30 mM Buffer X at pH 3.8.

4. Data Analysis in CZE

In addition to developing robust CZE separation chemistry, the data analysis must be equally robust if the method is to be implemented within a cGMP or QC environment. Therefore, the method should be optimized to be free of excessive baseline fluctuations (e.g., noise and/or drift), with peak integration parameters developed to take into account minor changes in inter-assay migration times. Based on migration of the IS, the QC analyst should only need to make slight adjustments to peak integration parameters to automatically process a batch run.

5. CZE Method Qualification

(a) Specificity:

As discussed in Chapters 9 and 10, all sample types to be analyzed (drug substance, drug product, in-process, stability) should have the appropriate buffer matrix evaluated to determine whether there are interfering peaks. Figure 34 illustrates an example of an in-process sample compared to the corresponding buffer matrix, demonstrating that no interfering peaks occur in the region of interest.

(b) Linearity and Range:

As indicated in Table 1, linearity and range can be tested in a number of ways, including linearity of injection and linearity of sample concentration. Typically, a range of 50−150% of the desired injection parameter is tested (all sample types will be analyzed using the method evaluated). Figure 35 represents results from an injection linearity assessment, showing 50−150% of the desired injection time plotted against total peak area for replicate injections. The data demonstrate a wide range of linearity between total peak area and injection time.

Further analysis of linearity data typically involves inspection of residuals for fit in the linear regression form and to verify that the distribution of data points around the line is random. Random distribution of residuals is ideal; however, non-random patterns may exist. Depending on the distribution of the pattern seen in a plot of residuals, the results may uncover non-ideal conditions within the separation that may then help define the range of the method or indicate areas in which further development is required. An example of residual plot is shown in Figure 36. There was no apparent trend across injection linearity range.

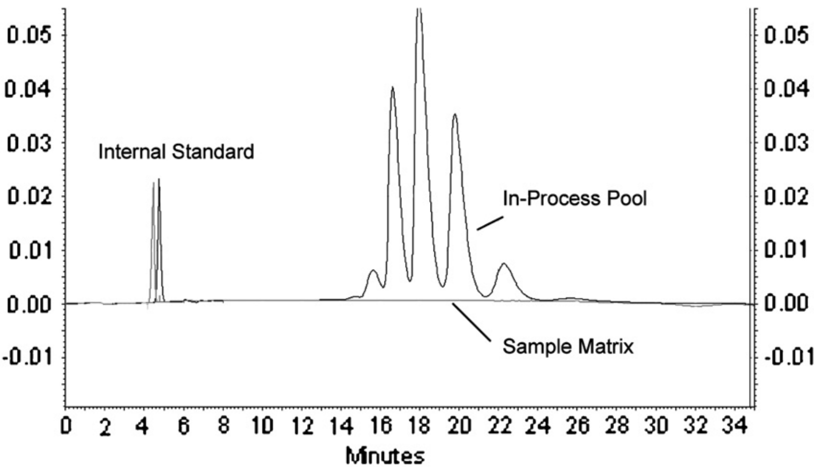

FIGURE 34 **Specificity testing of an in-process sample.**

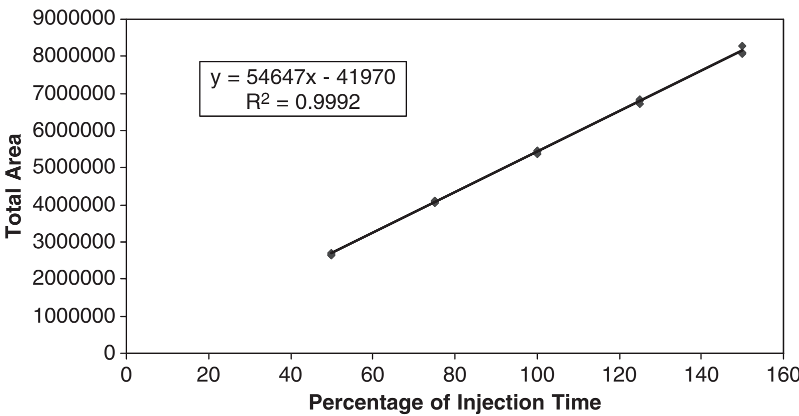

FIGURE 35 **Linearity of injection time.**

(c) Accuracy:

The accuracy of a method is defined as the closeness of the value obtained to known or accepted values. Accuracy can be determined in a number of ways, depending on the nature of the CZE method and availability of orthogonal techniques to compare results. If practical, spike recovery studies (i.e., testing to determine whether recovery matches the amount of a known analyte or impurity spiked) are good alternatives to orthogonal assay comparisons. ICH guidelines also allow method accuracy to be inferred, once specificity, linearity, and precision are established.

(d) Precision:

Method precision refers to the variability in measurement of the same sample. There are three main components of method precision: repeatability (also known as system or intra-assay precision), intermediate precision (also known as inter-assay or intra-laboratory precision), and reproducibility precision (also known as ruggedness, overall or inter-laboratory

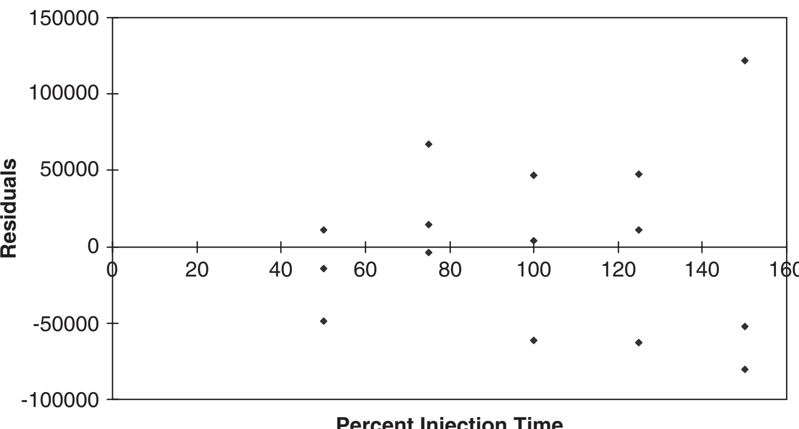

FIGURE 36 Plot of residuals versus injection time.

TABLE 9 Precision Testing Matrix

Run #	Instrument	Analyst	Capillary
1	1	1	1
2	1	1	2
3[a]	1	2	1
4	1	2	2
5	2	1	1
6	2	1	2
7	2	2	1
8	2	2	2

[a]Run designated for repeatability.

precision). During method qualification, only repeatability and intermediate precision are typically addressed in the originating laboratory. Upon transfer of the method to another laboratory, such as a cGMP lab or QC, additional data are generated from which method reproducibility can be calculated. Assay precision for all sample types to be analyzed is generally determined with this method.

Precision studies can be planned in a full factorial matrix-type format involving multiple analysts, days, capillaries, buffer preparations, instruments, etc. Table 9 displays an example matrix for intermediate precision testing involving two analysts, two instruments, and two capillaries over a multiple day period. Typically, qualification targets for acceptable precision will be pre-defined based on method type, capability, and intent. Acceptable targets can also be mathematically determined by the method of Horwitz.[37]

A single run performed during intermediate precision testing can also be designated in advance for repeatability testing (see run 3 in Table 9), resulting in less total runs.

Once precision data have been generated, they should be summarily compiled to determine the average, standard, and %RSD or coefficient of variation (CV) values. Intermediate precision data can also be used to initially populate assay trending databases if the method will be used routinely in the future.

(e) LOD/LOQ:

Depending on method type or intent, the LOD or LOQ may need to be determined. ICH guidelines describe several approaches and allow alternative approaches, if scientifically justifiable.[24] Suggested approaches include calculation based on: signal-to-noise ratio (typically set at 3:1 for LOD and 10:1 for LOQ); standard deviation of the response and slope; standard deviation of the response of a blank, or a calibration curve.

(f) Robustness:

Robustness testing of a CZE method for biopharmaceutical compounds should typically focus on three main areas: the sample, the capillary, and the run buffer. Over the course of assay development, certain perturbations made for optimization will likely result in changes to migration time and resolution. Each variable can be isolated and tested to make more definitive conclusions regarding the effects of each perturbation. In recent years, greater availability of experimental design software such as JMP or Cornerstone has enabled analysts to more easily analyze data from partial factorial experimental designs created to test multiple variables at one time. Method development experience aids in the design of robustness testing experiments. These results are valuable in determining whether operating ranges specified in the method are acceptable and can also help with method troubleshooting.

6. System Suitability

System suitability (see chapters 9 and 10) is a set of previously defined acceptance criteria used to determine whether data produced in a particular assay run are valid. Following method qualification, a standard operating procedure (SOP) for the method is generally issued that contains the qualified parameters and determined limits. At this point, system suitability requirements set forth for method qualification are revised and further developed in order to be placed in the SOP. For example, conclusions from linearity and range, precision, LOD/LOQ, and robustness testing are used to evaluate and revise system suitability, or to make further definitions. These new revisions or definitions of system suitability should become semi-permanent and governed by a change control process, especially in a cGMP or QC environment. The control samples, such as reference standards or appropriate samples, are most commonly used in monitoring system suitability in a routine testing mode. For CZE, system suitability criteria such as assay trending (results from controls), migration times, and electrophoretic profile (electropherogram) can be used. If the system does not pass the suitability requirements, the data are rejected and the assay must be repeated.

III. METHOD TRANSFER

Method transfer is a critical step required to move CE methods from the R&D stage to a QC environment. Developing a robust CE method is essential (see Chapters 9 and 10); the success is measured by fluidity in transferring and adopting the CE methodology to a QC environment for release testing. As global demand increases, biopharmaceutical companies are further looking into global technology transfer to multiple sites, contract manufacturing (CMOs) and partners which will require the transfer of QC methods for drug substance and drug product release at the site.

Method transfer from a method development laboratory to a QC laboratory, or transfer from one QC site to multiple QC sites, often require an approved transfer protocol and the SOPs. The transfer requirements are often different depending on the stage of the assay (i.e., whether or not it is a validated assay). In the case of a method that has not yet been validated, demonstrating equivalency between the two laboratories is generally required.

The acceptance criteria should be included in the protocol and are usually based on assay capability (i.e., intermediate precision expressed as standard deviation (SD)). It is recommended that the results fall within 2–3 times the intermediate precision SD for the transfer to be considered successful. If greater windows of variation are needed, scientific justification should be provided. When transferring a validated assay from one QC site to another QC site, the transfer criteria should be more stringent. ICH requirements should be demonstrated in both laboratories. In the assay transfer process, sample selection is important. In general, the sample should be representative and the stability-indicating capabilities should be considered. For an impurity test such as CE-SDS, a spiking experiment using an impurity having a known concentration is recommended.

The roles and responsibilities of the transfer lab and the receiving lab as well as an outline describing deliverables and timelines should be defined clearly at the initiation of the assay transfer process. Once a transfer protocol is defined, any deviation from the protocol during execution should be discussed in the transfer report. Explanations as to why the deviation is acceptable should be included in the report. The transfer report should also be approved by relevant functional departments. Due to the complexity of multi-site involvement, having a good change control system in place is important for making SOP modifications or updates.

Experience with CE method transfer in the biotech/pharmaceutical industry over the past 10–20 years has demonstrated that training is a key element that requires special attention for CE methods. A training video with troubleshooting examples can be very useful. Tips and hints should also be shared during the method transfer process. Other key elements for a successful transfer include selection of the proper testing strategy and assay acceptance criteria.

A. System Suitability

Appropriate system suitability is very important for CE assays in a QC environment. It is preferable to define CE-specific system suitability criteria rather than directly adopt system suitability based on HPLC methods. Earlier data from biotech companies showed a high assay failure rate due to inappropriate system suitability criteria. When appropriate criteria were applied in the QC environment, the assay failure rate improved.

Following are few key suggestions for setting system suitability of a purity assay: (1) select an appropriate reference control and set the corrected peak area percent of the main component as purity criterion for quantification; (2) ensure that the total corrected peak area is within linear range; (3) tracking migration time is not absolutely necessary; however, it can be referenced to confirm consistency between runs; and (4) bracketing with a reference control at the beginning and the end of the run can be useful to ensure the system is suitable. For an identity assay such as cIEF, p*I* values of the reference control can be an important system suitability parameter.

B. Sample/Assay Acceptance Criteria

The purpose of sample acceptance criteria is to confirm that the run for a test article is suitable for quantification. The corrected peak area of the sample should be within linear range of the assay; the baseline should be suitable for integration; the corrected peak percentage should be in a reasonable range. Again, for a purity assay, migration time is usually not a critical parameter.

C. Typical Steps for Assay Transfer

The following paragraph summarizes the typical steps for an assay transfer:

- Establish a transfer strategy
- Define roles and responsibilities of the transfer and receiving labs, ensure management oversight of the transfer
- Write an assay transfer protocol with acceptance criteria based on development experiences
- Establish an SOP with the system suitability criteria
- Select the appropriate samples for transfer
- Establish the proper training procedures and training materials
- Execute the protocol
- Write a transfer report, review and audit all data including raw data
- Justify assay transfer parameters as needed
- Address/justify variances when necessary
- Make conclusions, noting any limitations of the method and how the SOP will be revised
- Maintain a log and document all the transfer activities.

IV. METHOD PERFORMANCE IN QC ENVIRONMENT

The robustness of an assay becomes critical when evaluating its performance in a QC environment for the release of therapeutic proteins and antibodies. Over the past 5−10 years of product release experience in the biotech industry, assay failure rate is in the range of 5−30% depending on the method type and system suitability criteria. The types of assay failure are mainly as follows: technical error (including analyst error), equipment error, and system suitability/assay acceptance errors. A periodic review of an assay's performance in the QC labs and timely feedback to the development labs are crucial to minimize the assay failure rate. A concerted effort in working with vendor is also helpful to ensure that instruments are in good condition to minimize the assay failure rate.

A. Common Operator/Instrumental Errors

Technical errors may be related to a number of different causes: incorrect reference standard preparation; vial misposition can lead to pressure failure errors; incorrect sequence setup or incorrect capillary installation can cause other errors such as x, y, and z error and bent electrodes; a cracked capillary at the detection window or a misaligned window can produce a PDA light low error; an overfilled waste vial can cause back flow of the gel to the pressure loop, causing abnormal e-grams; leaving the PDA light on when not in use can increase the lamp hours and result in a noisy baseline; a rusty electrode can cause baseline noise and an abnormal e-gram; improper degassing can result in a current out-of-limit error and stop the run. Equipment errors also include computer issues such as a frozen computer during a run and data transfer issues. One buffer vial stacked on top of another has caused problems due to stretching of the spring holding the vial ejector. Current leakage, XYZ errors, and coolant leakage issues have also been observed. Many of these issues can be improved through a vigorous training program and frequent instrument maintenance.

B. System Suitability/Assay Acceptance Errors

System suitability errors often include profiles that are not consistent with historical data such as (1) excessive peak tailing, poor resolution of critical components or noisy baseline; (2) peak spikes due to micro bubbles or electric shock; and (3) integration parameters such as percent main peak area out of range for the assay reference control sample.

V. LESSONS LEARNED

The following points describe lessons learned from over a decade of experience with CE method development, qualification, and transfer to quality labs in the biotech and pharmaceutical industries. These lessons may be beneficial to other companies on their journey to implement CE methods for characterization and release of therapeutic products.

- A close and efficient interaction between the development lab and the release lab is essential.
- A SOP should include detailed procedures.
- Training should be thorough, and training material should include theory and a video that can be easily followed.
- Troubleshooting examples as part of the assay transfer material is very helpful.
- Instrumental SOPs should include lamp hour maxima and cleaning procedures.
- Change control procedures should be in place when multiple sites are involved for release.
- Management oversight is very important.

VI. CONCLUSIONS

The successful application of CE technology has resulted in dramatic growth of CE as an essential tool for protein characterization, R&D, and QC of therapeutic biomolecules. CE methods have clearly been shown to be superior over traditional slab-gel methods. Many biopharmaceutical companies have adopted CE techniques in QC environments for determination of product purity, identity and consistency needed for the release of protein products. The success of validation per ICH guidelines has moved CE technology to a position of greater prominence and ensures the quality release of therapeutic proteins and antibodies.

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APPENDIX: TROUBLESHOOTING EXAMPLES

A. CE-SDS Assay (see Figures A to J)

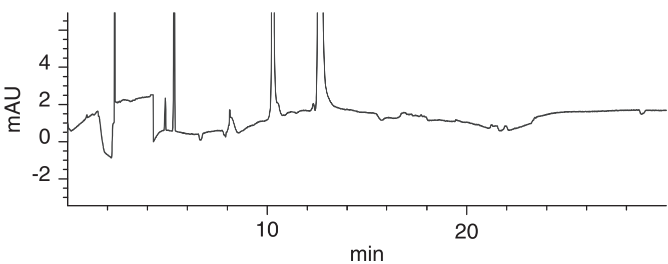

FIGURE A **Power stability due to deficient HV cable.**

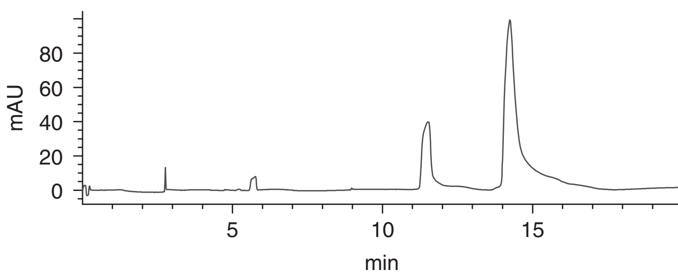

FIGURE B **Peak tailing due to expired gel or gel degradation.**

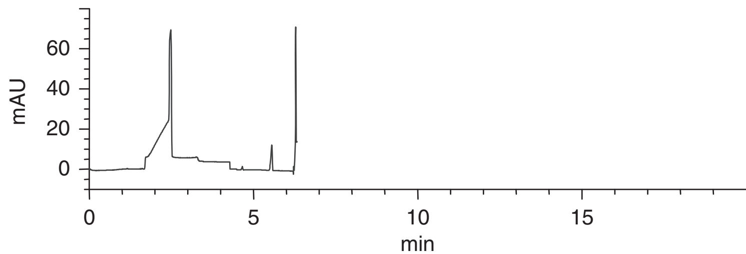

FIGURE C **Prematurely stopped run due to micro bubbles.**

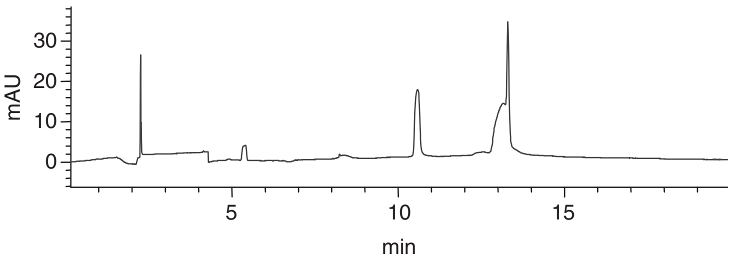

FIGURE D **Atypical peak shape due to buffer composition.**

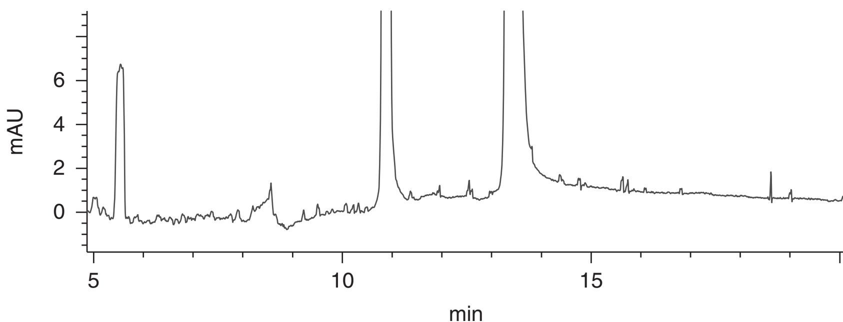

FIGURE E **Unstable baseline caused by a bad lamp.**

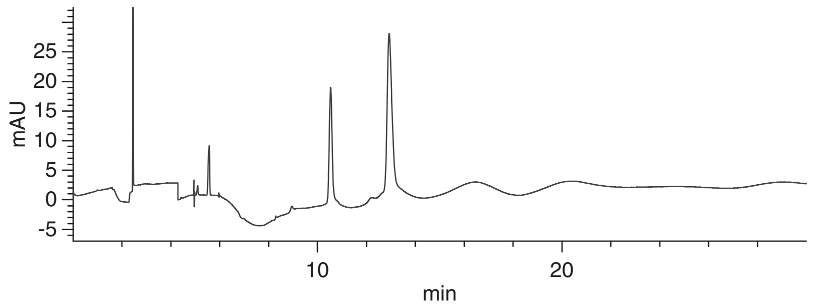

FIGURE F **Insufficient pre-conditioning due to an overfilled waste vial.**

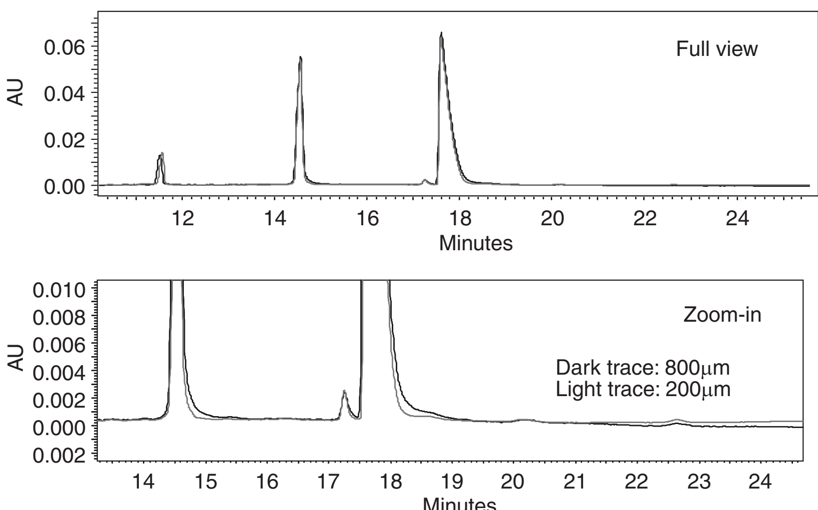

FIGURE G Peak tailing due to improper selection of the aperture 200 µm vs. 800 µm.

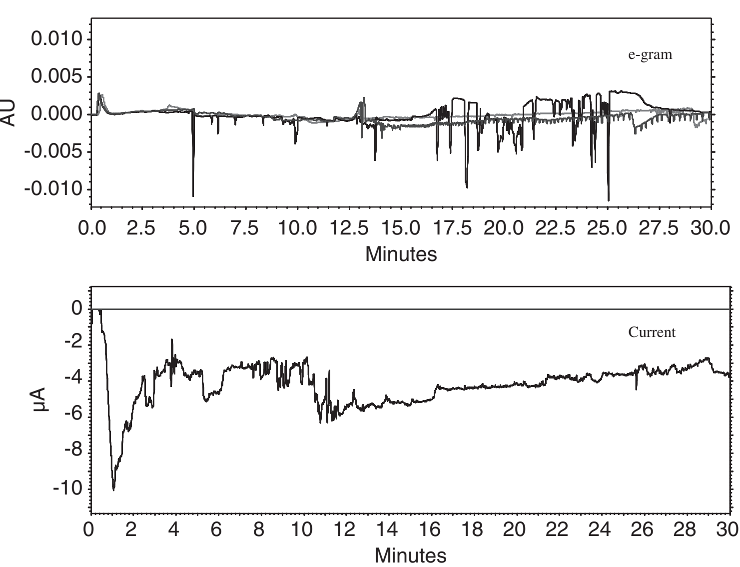

FIGURE H No SDS gel buffer in the outlet buffer vial.

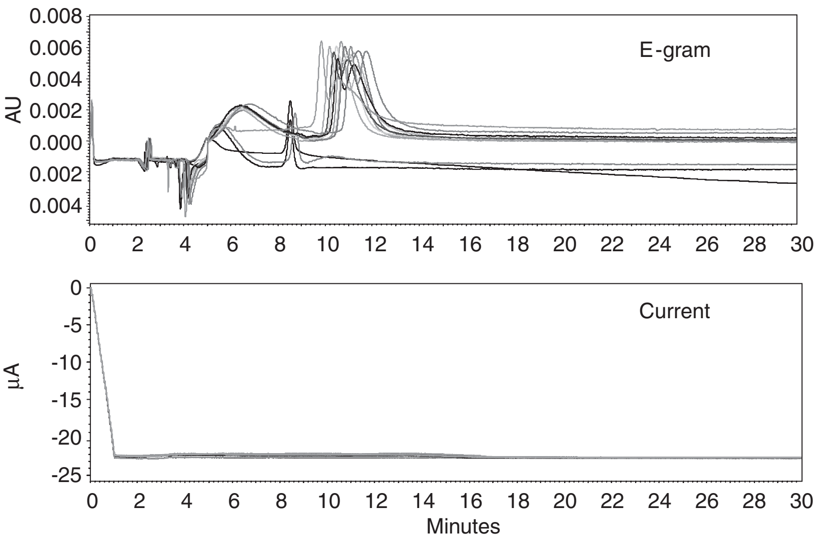

FIGURE I **Abnormal e-gram due to pressure imbalance at anode and cathode.**

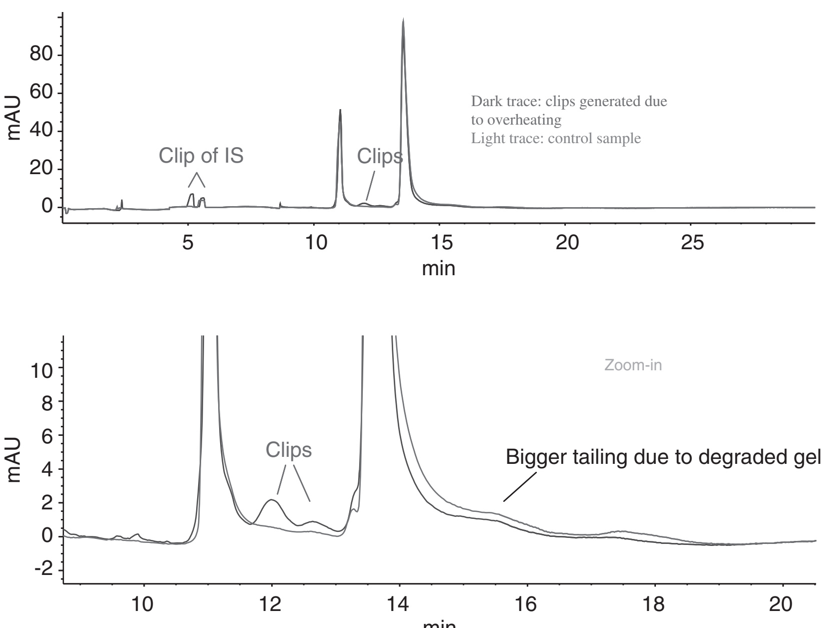

FIGURE J **Overheating sample due to non-functional water bath.**

B. Capillary Isoelectric Focusing (see Figures K to N)

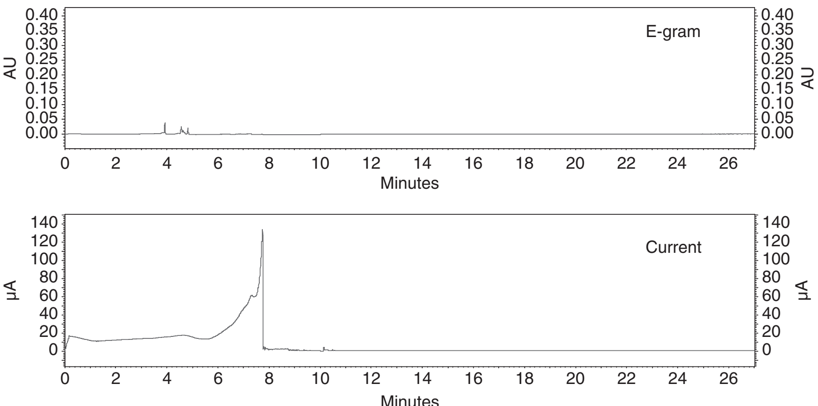

FIGURE K **Broken capillary.**

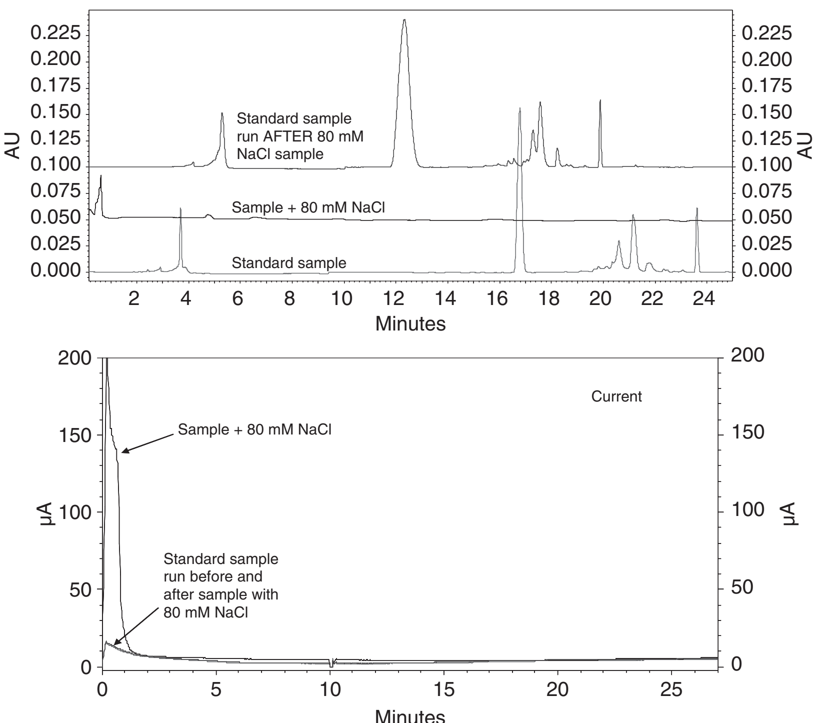

FIGURE L **High salt in the sample solution.**

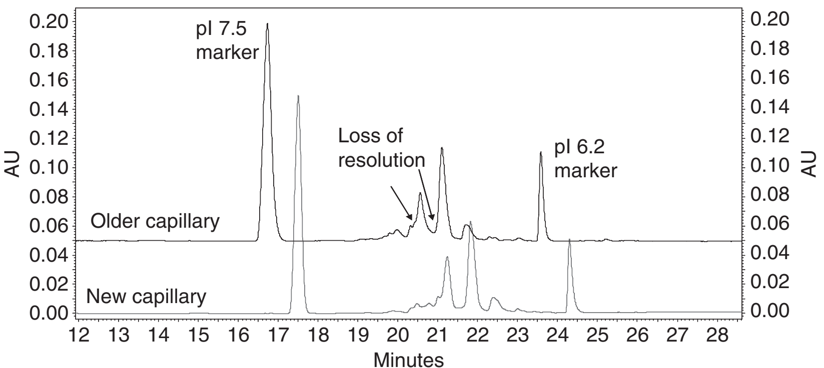

FIGURE M New versus older capillary: profile shifted and loss of resolution.

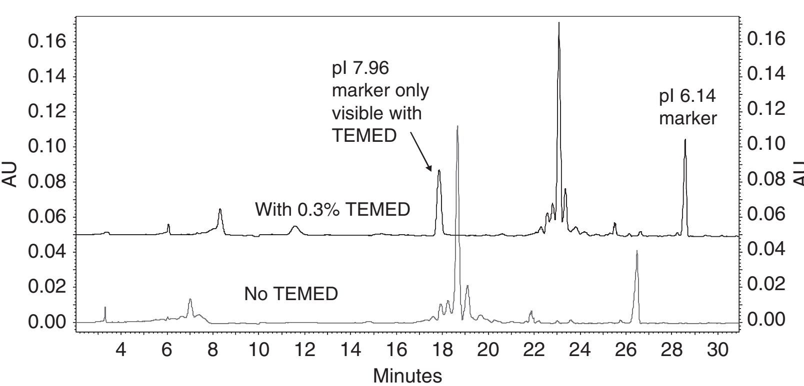

FIGURE N Lack of cathodic blocker.

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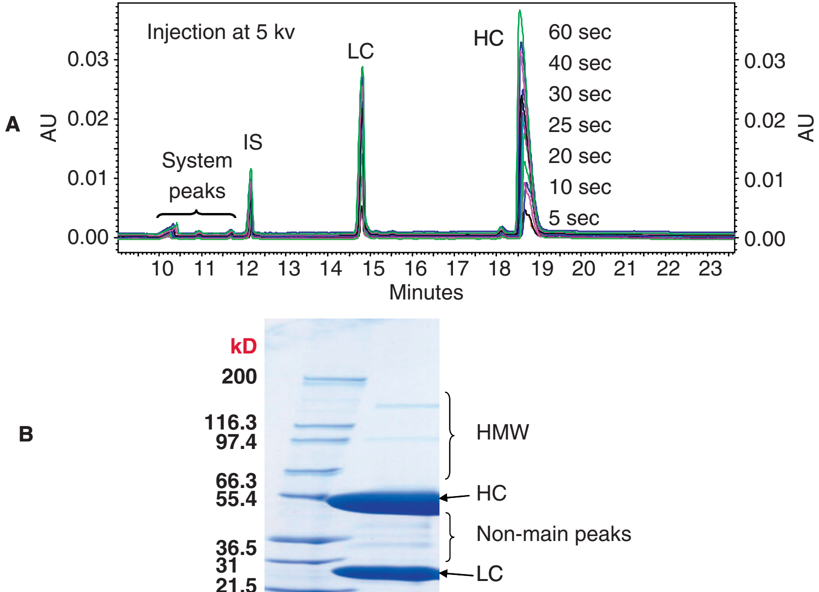

PLATE 2 **Injection time linearity of an antibody molecule. Panel A: e-gram for injection linearity. Panel B: reduced SDS-PAGE Novex (8–16% Tris-Glycine gel, Invitrogen, carlsbad, CA).**

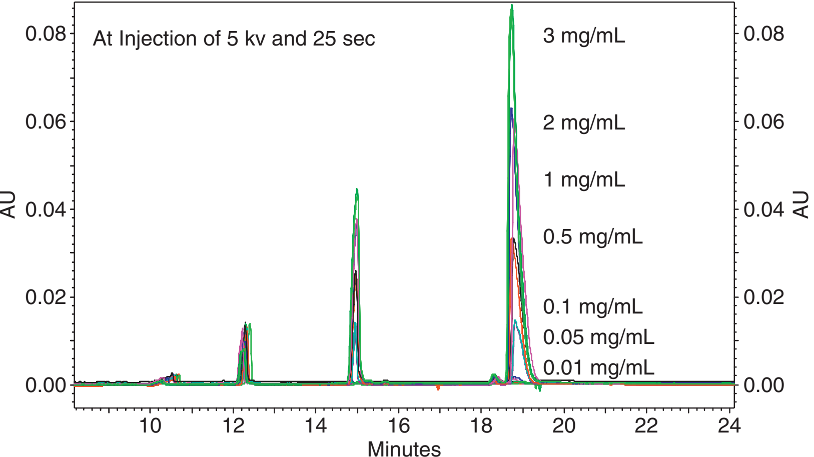

PLATE 3 **Concentration linearity of an antibody.**

15

CAPILLARY ELECTROPHORESIS AND BIOANALYSIS

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ABSTRACT
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ABSTRACT

The use of capillary electrophoresis (CE) for the analysis of therapeutic proteins produced by recombinant DNA technology has significantly increased over the past several years. In this chapter, a summary of the most important CE applications implemented at Genentech, Inc. is presented. The applications reviewed in this work are divided into the following areas: (i) capillary electrophoresis sodium dodecyl sulfate as a replacement for traditional SDS-PAGE, (ii) CE to monitor charge heterogeneity by capillary zone electrophoresis and capillary isoelectric focusing, and (iii) analysis of

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N-linked oligosaccharides release from an rMAb by CE. Finally, an overview of the implementation of CE in the quality control of therapeutic proteins is described.

I. INTRODUCTION

The development of recombinant therapeutic proteins such as monoclonal antibodies (rMAbs) used to treat unmet medical needs has significantly increased in the biopharmaceutical industry. During production and shelf life of therapeutic proteins, several post-translational modifications can occur such as deamidation, oxidation, isoaspartate isomerization, and proteolytic cleavages.[1–4] It is important to characterize and monitor these product-related substances to gain a better understanding of the resulting changes on the bioactivity and safety of a biopharmaceutical product. Detailed product characterization supports the suitability of the manufacturing process controls and helps to measure the effect that process changes have on the identity, strength, quality, and purity of a drug, as these factors relate to the drug's safety and efficacy.[5,6] Analytical techniques are therefore required to assess physicochemical properties of these therapeutic proteins such as charge, size, and hydrophobicity.

One of the techniques that has gained increasing acceptance is capillary electrophoresis (CE). As the name implies, CE separates analytes within the lumen of a small bore capillary filled with an electrolyte. The separation mechanism is based on differential migration of analytes when subject to an electric field. Since its first inception in the late 1960s, CE techniques analogous to most conventional electrophoretic methodologies have been demonstrated: zone electrophoresis, isoelectric focusing (IEF), isotachophoresis, and molecular sieving separations. CE offers many advantages over traditional electrophoretic techniques as a result of the modern instrumentation design, including capabilities of automation and the online concentration-sensitive detectors such as absorbance or fluorescence, which eliminates the need for staining and destaining. Data presentation and analysis are similar to high-performance liquid chromatography (HPLC); the output (peaks or baseline) can be displayed as an electropherogram and integrated to obtain quantitative information in the form of peak area or height. Compared to conventional electrophoresis, CE is characterized by high resolving power and decreased analysis time since the narrow-bore capillaries with excellent heat dissipation properties enable the use of very high electric fields. Because of its many advantages, CE shows great promise as an analytical tool in the characterization of biotechnology products and is often used in conjunction with existing techniques, which provides different separation selectivity, improved quantification, and automated analysis.

This chapter provides an overview of the applications of CE for the analysis of therapeutic proteins produced using recombinant DNA technology with a focus on three main areas: (i) CE-SDS as a replacement for the traditional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and IEF methodologies, (ii) capillary zone electrophoresis (CZE) as an orthogonal separation technique to ion-exchange liquid chromatography to determine charge heterogeneity of proteins, and (iii) CE for analysis of release of N-linked oligosaccharides from rMAbs. In the final section of this chapter, an overview of the application of the CE methods mentioned above in a quality control laboratory is presented.

II. CAPILLARY ELECTROPHORESIS SODIUM DODECYL SULFATE (CE-SDS)

SDS-PAGE has traditionally been used as the primary method for size-based protein separations.[7] SDS binds to polypeptide chains so that similar charge densities and constant mass-to-charge ratios of proteins are obtained. Then, electrophoretic separation of SDS–protein complexes based on size is achieved in a sieving medium.[8] Detection of the separated

proteins by SDS-PAGE is generally accomplished by staining with either Coomassie Brilliant Blue,[9] or the more sensitive silver stain dyes.[10] As the logarithm of the molecular mass of a protein is linear with electrophoretic mobility, the molecular weight of a given protein can be estimated from a series of protein standards. In addition to acting as a tool to determine the apparent molecular weight of proteins, SDS-PAGE is also used to evaluate the size heterogeneity, purity, and manufacture consistency of biologics. The major drawbacks of conventional SDS-PAGE are its inconvenience and irreproducibility associated with the staining/destaining steps used in analyte detection, the use of toxic reagents, and high intra- and inter-gel effective mobility variability.[11]

In an effort to overcome these obstacles, CE has emerged and shown many advantages over classical SDS-PAGE including on-column direct UV or fluorescence detection, automation, enhanced resolution and reproducibility, as well as accurate quantification of proteins and determination of their molecular weight.[12–19] Currently, linear or slightly branched polymers such as linear polyacrylamide, polyethylene oxide,[20] polyethylene glycol, dextran, and pullulan are often used as the sieving matrix for capillary electrophoresis sodium dodecyl sulfate (CE-SDS).[13,21–24] In comparison to cross-linked polyacrylamide gel matrix,[25] these polymers add great flexibility to CE-SDS since they are water-soluble and replaceable after each CE analysis, resulting in enhanced overall precision and robustness.[26] There are, however, disadvantages such as poor mass sensitivity with UV detection and inability to simultaneously separate multiple samples. Nevertheless, high-speed separation, efficiency, and ease of use of CE-SDS outweigh those limitations.[11,27–29]

Several groups have reported the use of CE-SDS with UV detection for both qualitative and quantitative analysis of antibodies. Bennett et al.[30] used this technique to analyze and quantify bovine immunoglobulin G (IgG). The molecular weights determined by CE-SDS were comparable to those measured by SDS-PAGE with Coomassie Blue staining and published values. Reproducibility of protein quantitation was achieved, resulting in a relative standard deviation (RSD) of approximately 13%. Another study showed the use of CE-SDS to detect fragments of a chimeric (mouse–human) monoclonal antibody conjugates with the anti-cancer drug doxorubicin.[31] Hunt et al.[29] detected aggregates and fragments of the antibodies in their study. The intra- and inter-assay precision of migration time was approximately 2% and precision values of normalized peak areas for smaller peaks were between 3 and 8%. Lee observed that high ionic strength and/or high pH conditions plus heat treatment of the samples also enhanced the fragmentation of SDS–rMAb complexes.[27]

Hunt and Nashabeh developed a CE-SDS method with pre-column labeling and laser-induced fluorescence (LIF) detection of rMAbs to replace silver-stained SDS-PAGE for detection of low-level impurities. The analysis of trace-level rMAb variants and process impurities at levels as low as 50 ppm was reported.[26] The CE-SDS profiles of both reduced and non-reduced rhodamine-labeled rMAb samples prepared using Hunt and Nashabeh's method are shown in Figure 1. For the non-reduced sample, the major peak observed is the intact antibody. Other smaller peaks of various rMAb fragments and aggregates or higher molecular weight (HMW) species are also shown. Upon treatment with DTT, rMAb is mainly reduced to LC, NGHC, and HC. The CE-SDS profile of the reduced sample also shows a smaller peak immediately after the LC and other small peaks migrating at the position of the incompletely reduced antibody. This method was validated according to the guidelines of the International Conference on Harmonisation (ICH) for use as part of the control system for the release of an rMAb pharmaceutical.

A. Glycosylation Occupancy of an rMAb by CE-SDS

It is known that carbohydrates have an essential and central role in a range of biological phenomena.[32] For glycoprotein-based therapeutics such as rMAbs, the oligosaccharides

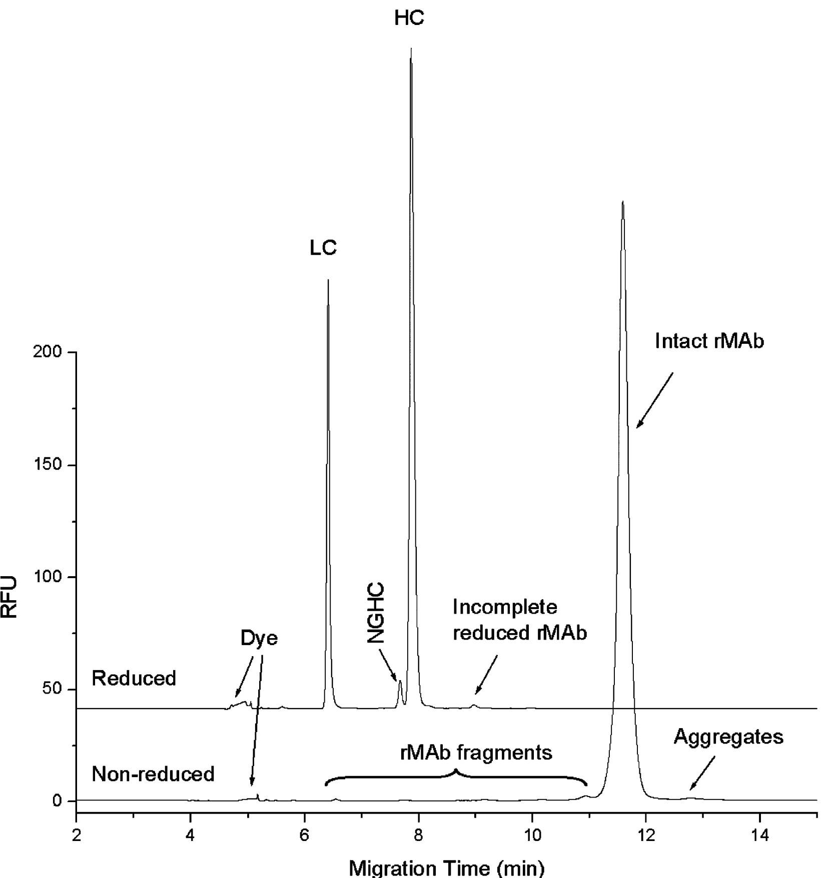


FIGURE 1 CE-SDS separations of non-reduced and reduced preparations of a 5-TAMRA SE-labeled rMAb sample. Electrophoretic conditions were as follows: Bio-Rad Biofocus 3000 instrument with LIF detection, effective length 19.4 cm, total length 30 cm, 50-μm ID, 375-μm OD uncoated fused-silica capillary; both anode and cathode buffers were the Bio-Rad CE-SDS running buffer. The samples were injected at a constant electric field of 417 V/cm for 20 s and electrophoresed at 625 V/cm (21.2 μA) and 20°C.

present on the heavy chain of the protein could affect their in vitro biological activity. In addition, it was demonstrated that glycosylation is necessary for the antibody-dependent cell-mediated and complement-dependent cytotoxic activities of monoclonal antibodies.[33] In these cases, it is imperative to have analytical methods in place to measure the extent of glycosylation occupancy during the drug development phase, as well as after regulatory approval for the market. In CE-SDS, the separation is based on the molecular or hydrodynamic size of the protein–SDS complexes. Since carbohydrates are relatively large in their hydrodynamic size in comparison to proteins, they are expected to have an impact on the migration time in CE-SDS. To demonstrate the effects of glycosylation on the mobility of antibodies in CE-SDS, an rMAb was deglycosylated by PNGase F enzyme digestion and then analyzed by CE-SDS with UV detection. For both the reduced and the non-reduced rMAb samples (Figure 2), the peaks of the two deglycosylated forms shifted to earlier migration times due to the decrease in hydrodynamic size upon the removal of the carbohydrates on

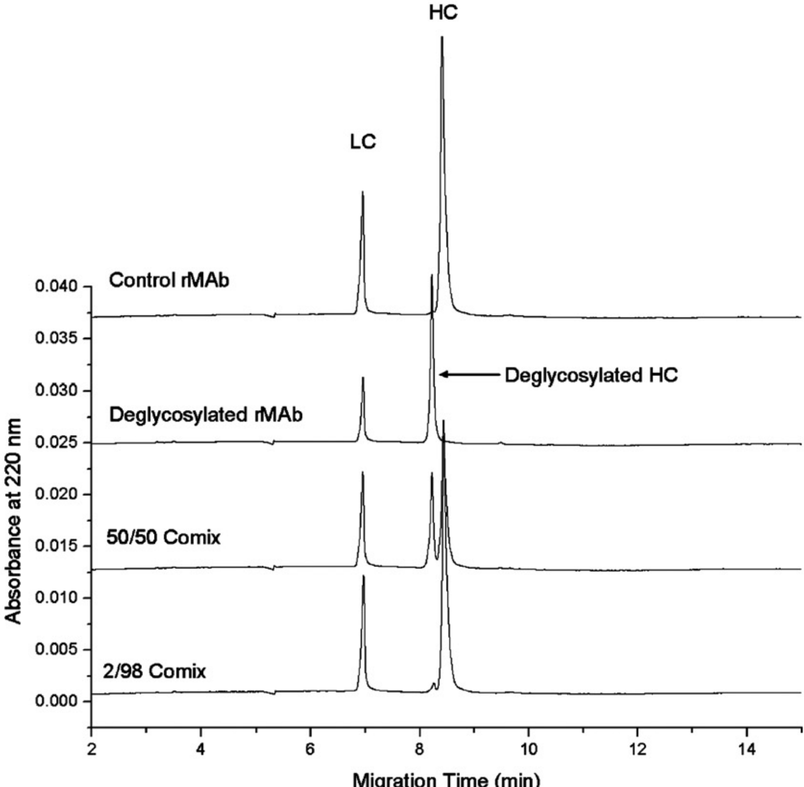


FIGURE 2 **Application of CE-SDS for the analysis of glycosylation occupancy of an rMAb: (top) non-reduced samples and (bottom) reduced samples. (Reprinted from reference 40, with permission.)**

either the intact antibody or the heavy chain. Co-mixtures of 50% (w/w) and 2% (w/w) of PNGase F-treated rMAbs with the glycosylated material were also analyzed to evaluate the resolving power of CE-SDS. As shown in Figure 2, the deglycosylated peak could only be resolved in the non-reduced samples containing a 50% (w/w) mixture of the deglycosylated and glycosylated rMAb. On the other hand, the non-glycosylated heavy chain (NGHC) peak was baseline resolved in both the 50% and 2% (w/w) co-mixtures. The improved resolution of the reduced samples facilitates more reliable, accurate, and reproducible quantification of the glycosylation occupancy even at low levels. This is an important advantage that CE-SDS offers over SDS-PAGE when monitoring consistency of manufacture during production of therapeutic rMAbs.

B. Low-Level Impurity Detection

Evaluation of the CE-SDS method for the purpose of detecting impurities that are non-product related was performed.[11] The selectivity of the method was demonstrated through the analysis of a rejected sample contaminated by microbial growth during the cell culture process. The electropherograms from the CE-SDS analysis of both reduced and non-reduced samples are shown in Figure 3. The presence of impurities was evident and noted by the presence of two additional peaks in the contaminated sample compared to the control rMAb. This example clearly illustrates a practical application of the CE-SDS methodology for detecting manufacture inconsistencies during the process development and manufacture of therapeutic rMAbs.

III. EFFECT OF SAMPLE PREPARATION ON CE-SDS AND SDS-PAGE ANALYSIS OF RMABS

Traditional sample preparation conditions to form SDS–protein complexes prior to electrophoretic analysis included heat treatment at elevated temperatures (e.g., 90°C).[11,27,28,34,35] In the case of non-reduced rMAbs, this could lead to sample preparation artifacts in the form of thermally induced fragmentation attributed to disulfide reduction and exchange reactions.[11] Moreover, it was reported that high pH (>9.0) also enhanced the

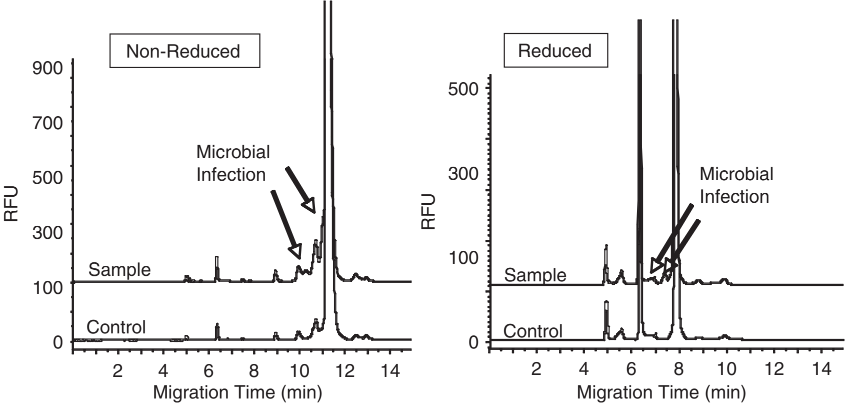


FIGURE 3 CE-SDS separations of non-reduced and reduced preparations of rMAb control samples labeled with 5-TAMRA SE, and a sample that exhibited evidence of a microbial infection during cell culture fermentation. The arrows indicate the appearance of new peaks in the infected rMAb samples. (Reprinted from reference 11, with permission.)

fragmentation of antibodies due to the same disulfide reshuffling.[28] As expected, these artifacts can significantly alter the true representation of an rMAb sample and also increase the variability of the assay. Reduction of disulfide linkages by nearby –SH groups of cysteine residues and exchange reactions were proposed as an explanation for the presence of free light-chain (LC) and heavy-chain (HC) fragments in addition to other subunits of a monoclonal antibody.[36,37] On the other hand, several reagents proved to be useful for protection of thiols prior to analysis by Edman sequencing, mass spectrometry, and SDS-PAGE, including iodoacetamide (IAM) and iodoacetic acid (IAA).[38] As a result, an investigation of the effect of these alkylating agents on the thermally induced fragmentation of labeled non-reduced rMAb samples for CE-SDS analysis was performed. It was reported that alkylation of proteins with IAM or IAA is more efficient at pH levels higher than 8 since the cysteine reacts as a thiolate anion.[38] To systematically understand the effect of alkylation on rMAb fragmentation, the pH of sample solution was kept constant at 9.0. In addition, all the non-reduced samples were incubated at 90°C for 5 min in a water bath prior to CE-SDS analysis. Different amounts of IAM or IAA were dissolved in the SDS solution to final concentration value of 10, 40, 80, and 100 mM of the alkylating agent, respectively, which were similar to concentrations used to completely alkylate peptides during mapping procedures.[38] Non-reduced SDS–rMAb conjugates were prepared by mixing the labeled rMAb samples reconstituted in 100 mM Tris-HCl buffer (pH 9.0) with the SDS solutions containing IAM or IAA at the different concentrations cited above. The final SDS concentration in all the samples was 1% (w/w). The control sample was the labeled rMAb sample in SDS solution without the alkylating agent. As shown in Figure 4A, a significant

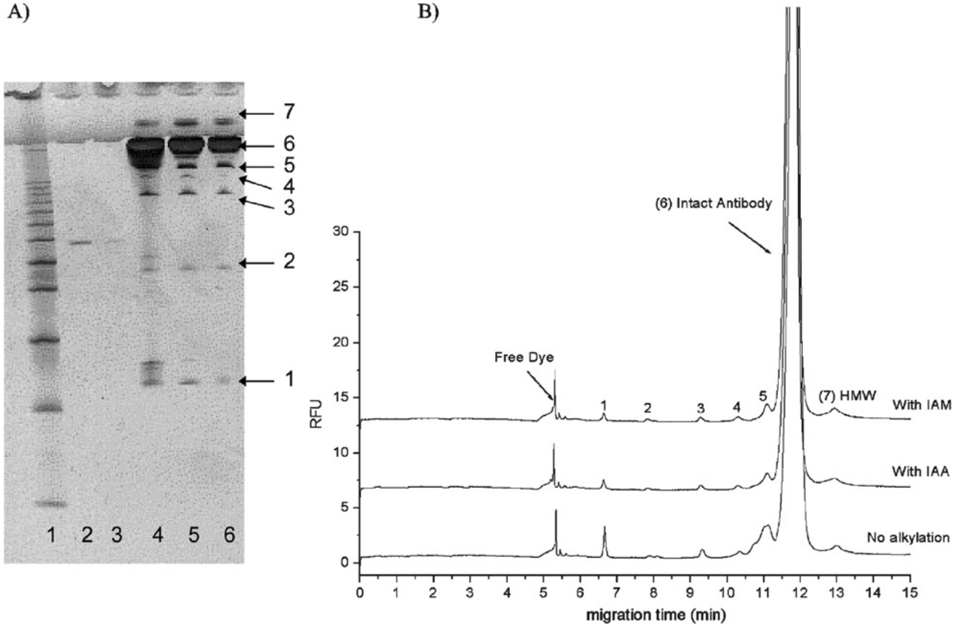


FIGURE 4 Effect of sample preparation on the fragmentation of an rMAb observed in (A) SDS-PAGE and (B) CE-SDS with LIF detection. SDS-PAGE lanes: (Lane 1) molecular weight standards; bovine serum albumin at (Lane 2) 8 ng and (Lane 3) 2 ng; (Lane 4) rMAb control; after alkylation with (Lane 5) iodoacetic acid and (Lane 6) iodoacetamide. (See color plate 4.)

decrease of the peak areas corresponding to the rMAb fragments was observed for the sample with 40 mM IAM in the solution compared to the control sample. The corrected percent peak area (%CPA) of the intact antibody increased from 90.0% in the non-alkylated sample to 97.0% in the sample containing 40 mM IAM. Similar results were obtained when IAA was used as the alkylating agent. However, IAM was the reagent of choice since solutions of IAA were less stable as indicated by the appearance of a yellow coloration after a few minutes. It was also determined that the optimum concentration of IAM in the sample was 40 mM. At concentrations lower than 40 mM IAM some induced fragmentation was still observed. On the other hand, the presence of IAM in the sample solutions at concentration higher than 40 mM did not further reduce fragmentation. The suppression of rMAb fragmentation upon alkylation implicates free-SH groups present in rMAbs as the mediators of the disulfide exchange reactions in the formation of antibody fragments. It is important to mention that the aggregation level of the alkylated labeled rMAb samples was similar to that of the non-alkylated control sample (0.2%).

Silver-stained SDS-PAGE profiles of the same rMAb before (lane 4) and after alkylation with either IAA (lane 5) or IAM (lane 6) are shown in Figure 4B. It can be seen that the number of bands decreases from 11 in the control samples (lane 4) to about 7 after alkylation with IAM (lane 6). Interestingly, the three light-chain bands seen in the 20–25 kDa region collapsed into a single band after alkylation with IAM. This further supports the hypothesis that these multiple bands often seen on the gels are indeed sample preparation artifacts. In addition, the intensity of some of the bands appeared to be significantly reduced.

A. Optimization of the ProteomeLab™ PA800 Instrument for Routine CE-SDS Analysis

As part of the validation studies, it is important to demonstrate that existing methods are compatible with alternative CE-SDS instrumentation and chemistry. Figure 5 shows the electropherograms of non-reduced and reduced rMAb samples using the replaceable polymer matrix introduced by Beckman Coulter, Inc (Fullerton, CA) and the ProteomeLab™ PA800 CE instrument. Both the signal and resolution of the rMAb species was comparable to those obtained with the Bio-Rad system and matrix (Figure 1). The main difference was that longer analysis times were required for the separation of the rMAb samples in the ProteomeLab™ PA800 instrument resulting from the use of more viscous CE-SDS polymer solution. Even though the feasibility of the ProteomeLab™ PA800 instrument and new separation matrix for CE-SDS analysis of rMAbs with precolumn labeling and LIF detection was demonstrated, several issues seriously limiting the instrument performance for routine analysis were observed.

1. Pressure Failure Errors

The ProteomeLab™ PA800 instrument was set up to analyze a sequence of five different labeled rMAb samples with a total of 10 duplicate injections. On many occasions, the electrophoretic analysis of approximately 60% of the injections did not take place due to "pressure failures" in the instrument. Upon closer inspection, some of red rubber caps of the gel matrix-containing vials were missing, electrodes were bent, and capillaries were broken. Additionally, it was observed that the interface block was coated with a sticky film of gel matrix. It was apparent that the build-up of gel buffer was causing the red rubber caps to remain stuck to the interface block and interfere with the contact of vials in subsequent steps. To improve the removal of gel buffer, the interface block and the surrounding areas were carefully flushed with deionized water. Then, cotton swabs were used to wipe the grooves on

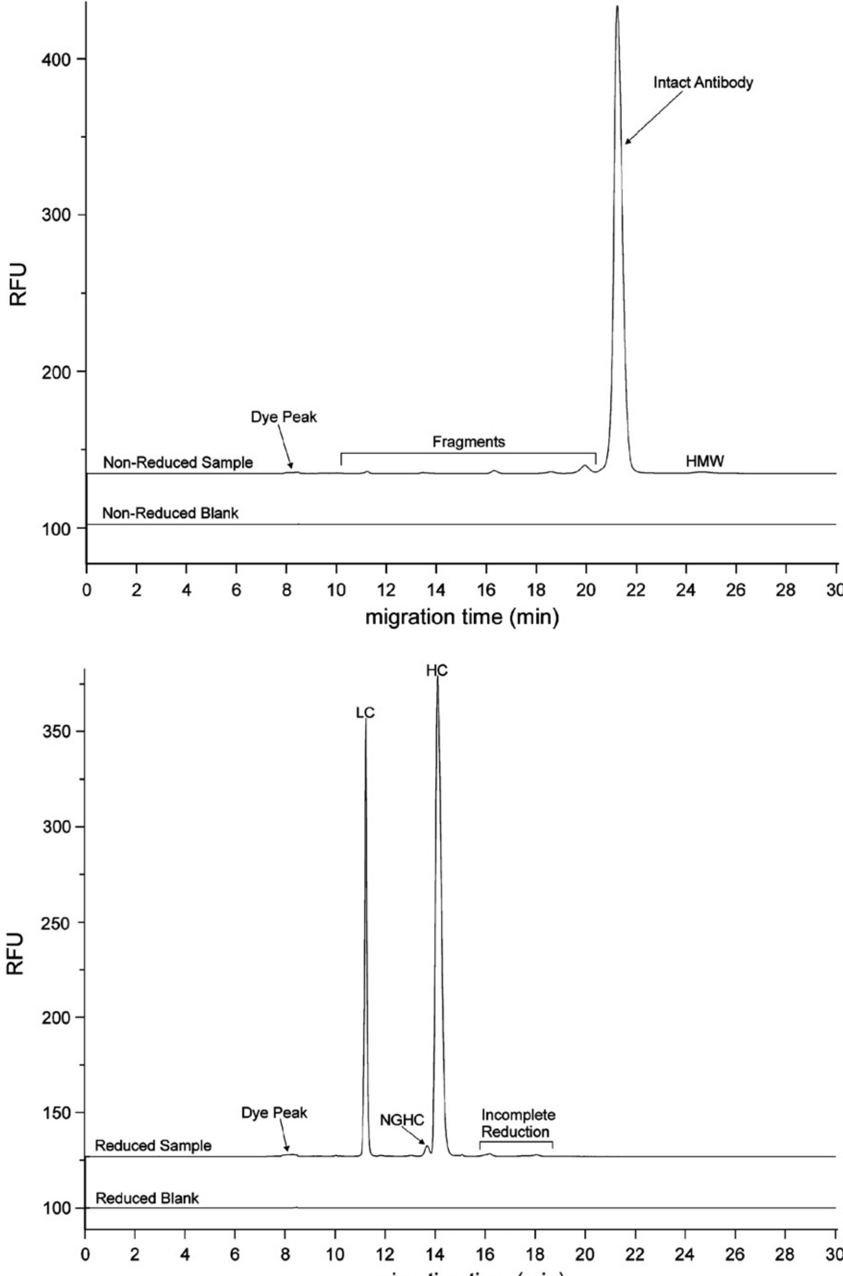


FIGURE 5 **CE-SDS separations of (top) non-reduced and (bottom) reduced preparations of a 5-TAMRA SE-labeled rMAb sample. Electrophoretic conditions were as follows: ProteomeLab™ PA800 instrument with LIF detection, effective length 21.2 cm, total length 31.2 cm, 50-μm ID, 375-μm OD uncoated fused-silica capillary; both anode and cathode buffers were the Beckman CE-SDS polymer solution. The samples were injected at a constant electric field of 160 V/cm for 20 s and electrophoresed at 480 V/cm (32.5 μA) and 40°C.**

the underside of the interface block. Finally, a red-capped buffer vial covered with a Kimwipe™ was used to remove the residual water from the grooves by rotating the vial. The cleaning procedure was repeated at least three times. Diligent cleaning of the interface block prior the analysis of 10 consecutive injections resulted in the elimination of the issues described above.

2. Peak Tailing Issues

Another issue greatly reducing analysis throughput was peak tailing. Peak tailing impairs the ability to accurately quantify the peaks observed in the electropherograms. Figure 6 shows the CE-SDS electropherograms with LIF detection of six consecutive injections of the same non-reduced rhodamine-labeled rMAb sample. Peak tailing of the monomer was observed during the analysis of the second and fifth injections. This problem was observed in ~40% of the electropherograms from the analysis of a particular sequence of 10 sample injections. In addition, this phenomenon was seen during the analysis of rMAb samples using either LIF or UV detection and under both non-reducing and reducing conditions. A possible source of peak tailing is carry-over, possibly caused by incomplete removal of sample left in the outside of the capillary and electrode surfaces after a sample injection. The method used to generate the data shown in Figure 6 already included a water dip step to remove any residual sample in the outside of the capillaries. Since the peak tailing issue still remained we investigated the liquid levels in contact with the capillary and electrode during the sample injection and subsequent water dip steps. As shown in Figure 7, the distance between the bottom of the sample vial and the meniscus of the 100 μL sample in the PCR tube placed inside the sample vial was ~19 mm. On the other hand, the distance between the bottom of the 2.0 mL vial and the meniscus of 1.3 mL of deionized water added to the vial for the dip step was ~15 mm. This is the water volume recommended by the manufacturer. As a result, there is about ~6 mm of the capillary and electrode exposed to the sample during the injection that are not rinsed in the water dip step. This phenomenon is more pronounced if sample volumes larger than 100 μL are used. To improve the rinse of the capillary and electrode outside surfaces, the

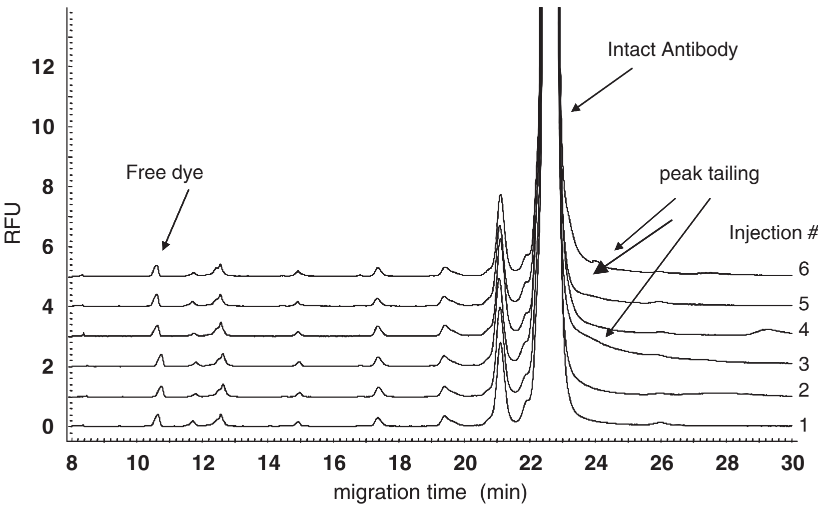


FIGURE 6 CE-SDS separations under non-reduced conditions of a 5-TAMRA SE-labeled rMAb sample showing tailing of the main peak. Electrophoretic conditions as in Figure 5.

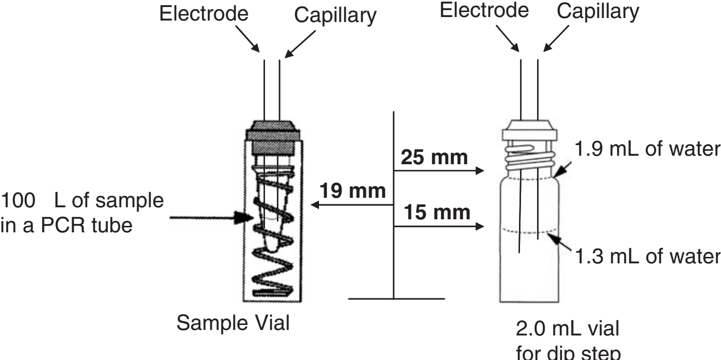


FIGURE 7 **Sample injection and water dip configurations in the ProteomeLab™ PA800 CE system.**

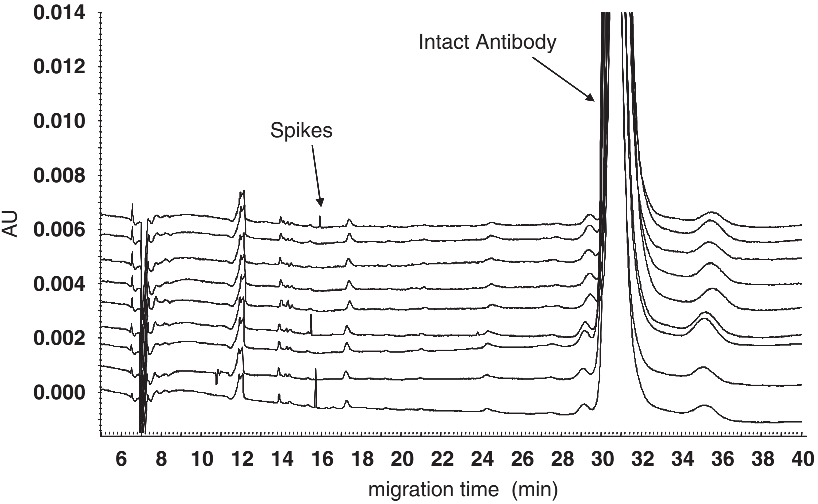


FIGURE 8 **CE-SDS separations of non-reduced rMAb samples in the presence of spike peaks. Electrophoretic conditions were as follows: ProteomeLab™ PA800 instrument with PDA detection, effective length 21.2 cm, total length 31.2 cm, 50-µm ID, 375-µm OD uncoated fused-silica capillary; both anode and cathode buffers were the Beckman CE-SDS polymer solution. The samples were injected at a constant electric field of 160 V/cm for 20 s and electrophoresed at 480 V/cm (25 µA) and 20°C.**

water volume in the 2.0 mL dip vial was increased to 1.9 mL and the sample volume for injection was reduced to 75 µL. Upon the implementation of these changes in the CE-SDS method, the issues observed in Figure 6 were eliminated.

3. Spike Peaks and Migration Time Variability

The final obstacles remaining were migration time shifts and/or spikes in approximately 20% of any 10 consecutive injections of the same rMAb sample. Figure 8 shows typical spike peaks observed in the electropherograms of injections 1, 4, and 9 of the same rMAb samples

prepared under non-reducing conditions. In addition, the RSD of the migration time for the monomer peak was ~2%. It is important to mention that these issues were observed also during the analysis of non-reduced rMAb samples. A probable source of migration time shifts and spike peaks was the introduction of bubbles at the inlet of the capillary. During the gel-loading phase for the analysis of a particular rMAb sample, the cartridge was removed from the instrument and the inlet of the capillary was inspected at 20 × magnification. A bubble was visually confirmed. The vendor recommends loading of the gel buffer into the capillary at 70 psi from a vial located on the inlet side (section of the instrument where the samples are injected). Gel loading from the outlet has proven a viable approach in CE methods for DNA sequencing using replaceable polymer solutions.[39] As expected, using a modified method with reverse gel loading eliminated spikes in the electropherograms. In addition, the variability of migration time for the monomer peak during the CE-SDS analysis of 10 consecutive injections of the same rMAb sample using the reverse gel loading was less than 0.5%.

IV. CAPILLARY ZONE ELECTROPHORESIS

In CZE, proteins are separated from each other based on the differences in their electrophoretic mobilities. The electrophoretic mobility is a function of the molecular charge and hydrodynamic size of a protein. In a given environment, the electrophoretic mobility is an intrinsic property of the protein, similar to the isoelectric point. Therefore, the mobility can be used to distinguish proteins from each other. This is the basis for using CZE as a simple identity test for final product and package labeling. As an example, the CZE profiles of six

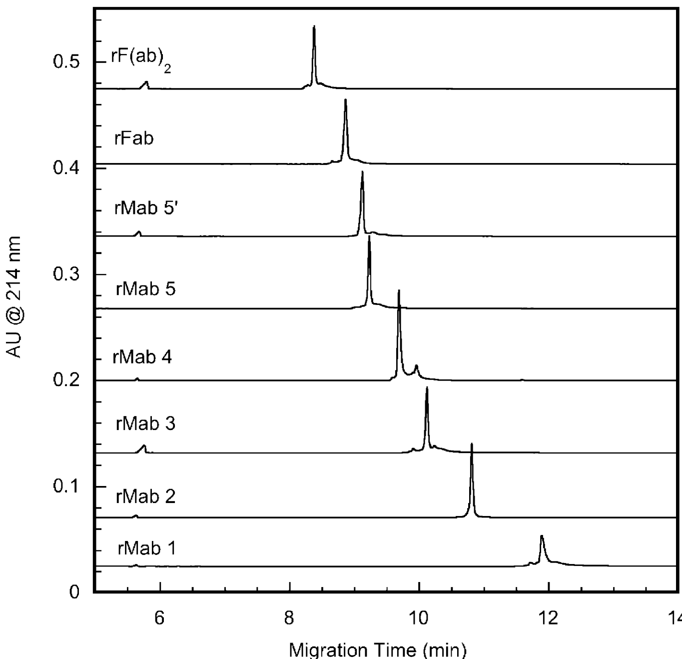


FIGURE 9 Capillary zone electrophoresis of eight recombinant human antibodies/antibody fragments. Capillary: BioCAP XL coated capillary (50 μm × 47 cm); 45 mM ε-amino-*n*-caproic acid/acetic acid, pH 4.5, 0.1% HPMC; voltage: 30 kV, normal polarity; capillary temperature 20°C; detection, UV at 214 nm.

rMAbs, one rFab, and one $rF(ab')_2$ are shown in Figure 9. The proteins used here are similar in terms of their p*I* values ranging from 7.5 to 9.3. In addition, the amino acid sequence homology between the rMAbs is greater than 90%, with 99% homology between rMAb 5 and rMAb 5′. The profiles of these therapeutic proteins show that multiple peaks related to the protein charge variants are resolved under one generic condition in less than 15 min. It is important to note that the migration times of the main peak for each molecule are clearly different from each other. As indicated by Ma and Mire-Sluis,[40] this unique migration time provides the specificity needed for an identity test.

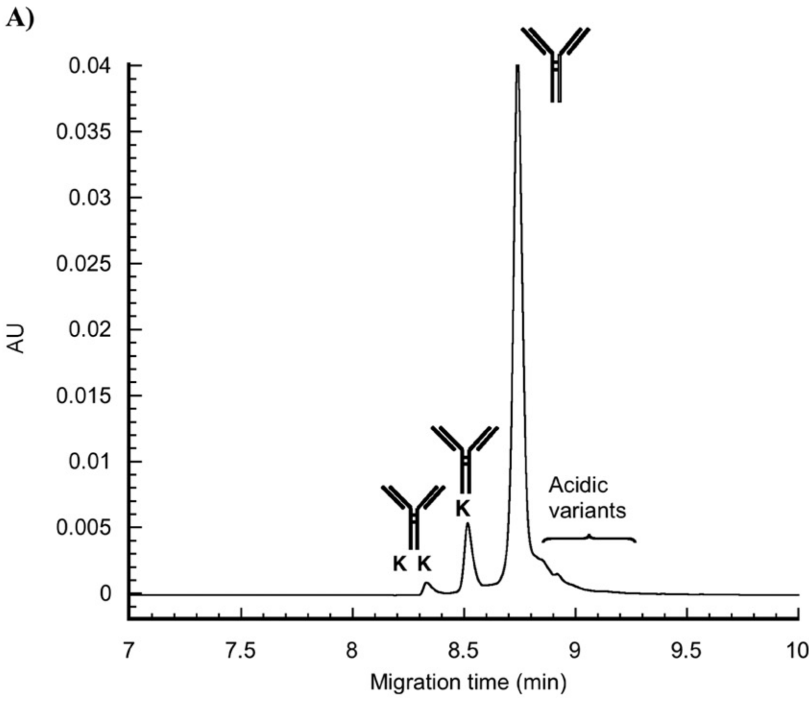
The potential of CZE to monitor charge heterogeneity was studied by further investigation of the various peaks observed in the profiles of the rMAb samples. An expanded view of the electropherogram of rMAb 1 is shown in Figure 10A. Treatment with carboxypeptidase B digestion confirmed that the three major peaks corresponded to the antibody forms with zero, one, and two C-terminal lysine[41] residues on the heavy chain. Under the CZE conditions used, the antibody is positively charged and migrates toward the cathode passing the detector. The 2-Lys form of the antibody has the highest number of net positive charges and therefore migrates fastest due to its high electrophoretic mobility. Consequently, the 1-Lys and 0-Lys forms of the antibody migrate in the order of decreasing mobility. In addition, the acidic variants of the antibody with even lower electrophoretic mobilities were observed migrating after the 0-Lys peak.

CZE is compared to the widely used ion-exchange chromatography (IEC) technique as a potential complementary tool for the analysis of charge heterogeneity. A typical chromatogram obtained from the IEC analysis of rMAb 1 is shown in Figure 10B. Similar to CZE, the three major species were the antibody forms with zero, one, and two C-terminal residues at the heavy chain. In this case, the elution order was opposite to that in CZE with the 0-Lys species eluting first because it was the most acidic species among the three forms; therefore, it had the weakest interaction with the negative surface of the cation-exchange column. The acidic variants were also observed eluting before the 0-Lys peak. The advantages of the CZE method for monitoring the charge heterogeneity of recombinant antibodies include the possibility of using one generic CZE conditions to analyze multiple products in rapid analysis times. This is an important attribute to satisfy the high-throughput analysis of rMAb samples generated during the cell culture and purification efforts of the process development. CZE could also be used as a practical technique in formulation development and monitoring stability of therapeutic rMAbs. The ability of the assay to detect changes in rMAb samples subjected to various forced degradation conditions was evaluated. Representative CZE profiles of forced degraded samples under various conditions are shown in Figure 11. These results demonstrated that the assay could detect changes of the charge heterogeneity profile of a control rMAb sample.

V. CAPILLARY ISOELECTRIC FOCUSING

The process development and analytical characterization of therapeutic proteins pose many challenges due to the complexity and heterogeneity associated with these biotechnology products. Previously, therapeutic rMAbs have been characterized using the qualitative, labor intense gel IEF methods.[42,43] Capillary isoelectric focusing (cIEF) combines the high resolving power of conventional gel IEF with the advantage of CE instrumentation.[44] Proteins are separated according to their isoelectric points (p*I*) in a pH gradient formed by carrier ampholytes when an electric field is applied to the capillary. A protein's p*I* (pH at which the protein has a net zero charge) is defined by its amino acid composition, sequence, 3-D structure, post-translational modifications, etc. In cIEF, online concentration-sensitive detectors such as LIF and UV are typically used. The detector in most commercial CE

A)



B)

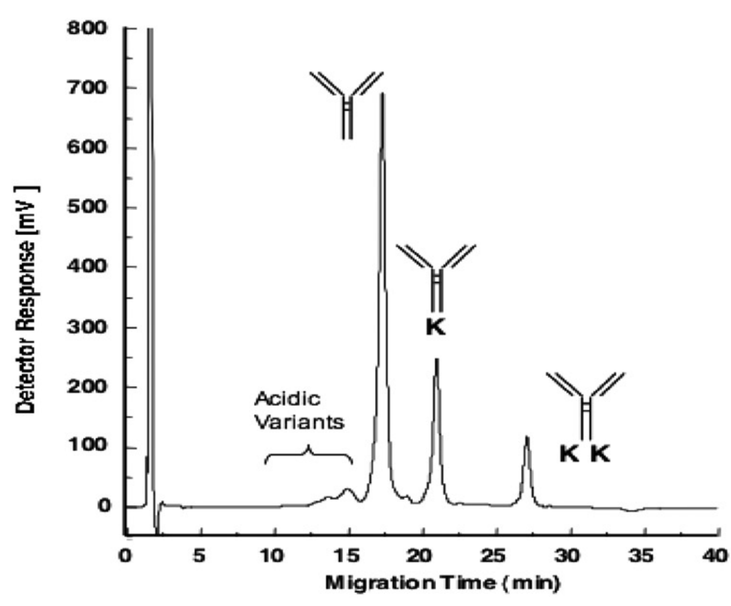


FIGURE 10 **Charge heterogeneity of an rMAb by (A) capillary zone electrophoresis (CZE) and (B) cation-exchange chromatography (CEC). (Reprinted from reference 40, with permission.)**

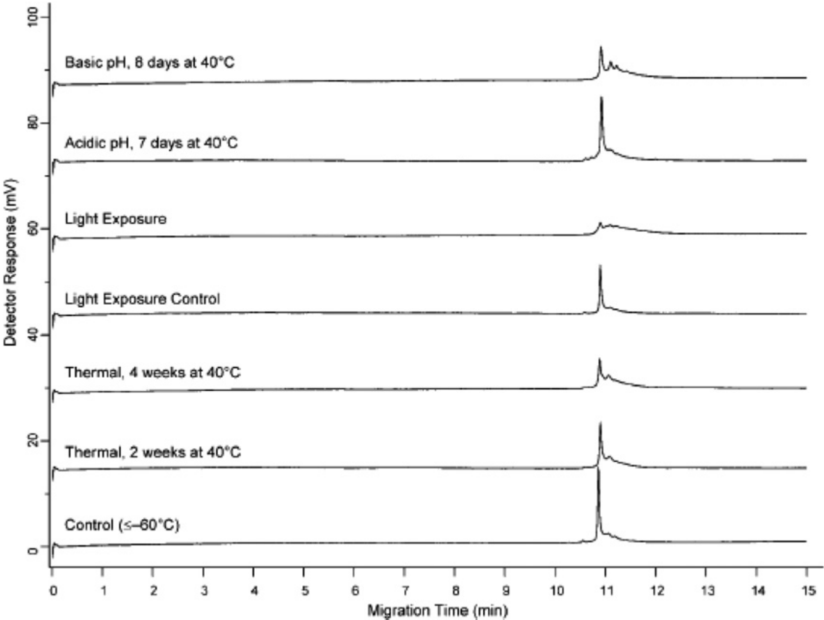


FIGURE 11 Capillary zone electrophoresis of forced degraded rMAb sample. Experimental conditions as in Figure 9.

instruments is at a fixed point toward the outlet of the capillary. As a result, the focused protein zones in the capillary must be mobilized to pass through the detector window.

A. Determination of Isoelectric Point

One important application of cIEF is the determination of a protein's isoelectric point. As in classical IEF, the cIEF method involves calibration with standard markers whose p*I*s are known. In cIEF, the commonly used markers are synthetic low-molecular-weight compounds with great stability and detection sensitivity in comparison to traditional protein standards. Typical cIEF profiles of an rMAb with unknown isoelectric point and p*I* markers are illustrated in Figure 12. Three major peaks were observed representing the three antibody forms with zero, one, and two lysine residues at the carboxyl-terminal of the heavy chains. A plot of the mobilization time versus the p*I* should exhibit linearity within a defined pH range. Such a calibration plot is shown in Figure 13 and the apparent p*I* values were calculated to be 9.0, 9.1, and 9.2 for the 0-Lys, 1-Lys, and 2-Lys antibody forms, respectively, for the determination of the p*I*s of components of an rMAb.

B. Monitoring rMAb Charge Heterogeneity

The potential of cIEF to assess the overall rMAb charge distribution is demonstrated in Figure 13. The electropherogram shows the various isoforms of the antibody between the p*I* markers 7.9 and 5.3. The main peak or 0-Lys form of the antibody appears at ~27 min. The more basic species with one and two C-terminal Lys residues at the heavy chain are baseline resolved from each other and the main peak. Their results are similar to those obtained by

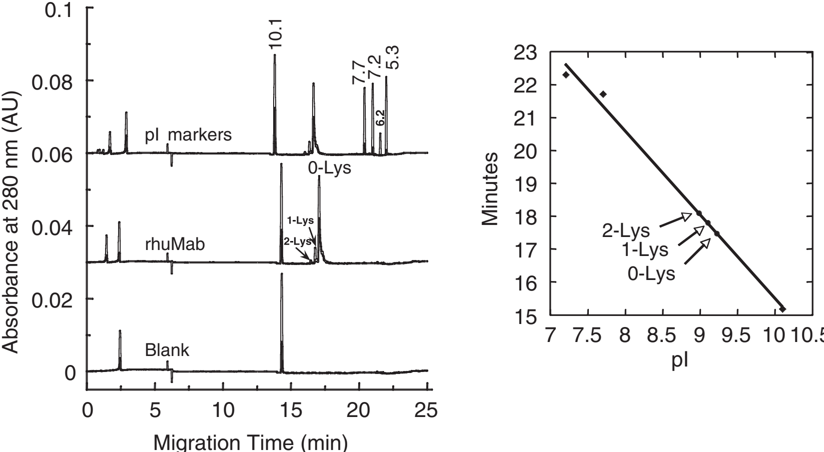


FIGURE 12 Application of capillary isoelectric focusing (cIEF) for the determination of apparent p*I* values of rMAb samples. Capillary: Bio-Rad Bio-CAP XL capillary (50 µm × 24 cm); ampholyte: 80% cIEF Bio-Lyte Ampholyte 3–10 (2% solution with 0.5% TEMED, 0.2% HPMC); anolyte: 20 mM phosphoric acid; catholyte: 40 mM sodium hydroxide; focusing: 15 kV (625 V/cm) for 5 min; mobilization: 20 kV (833 V/cm) for 25 min with zwitterions (cathodic mobilizer from Bio-Rad); capillary temperature: 25°C. (Reprinted from reference 40, with permission.)

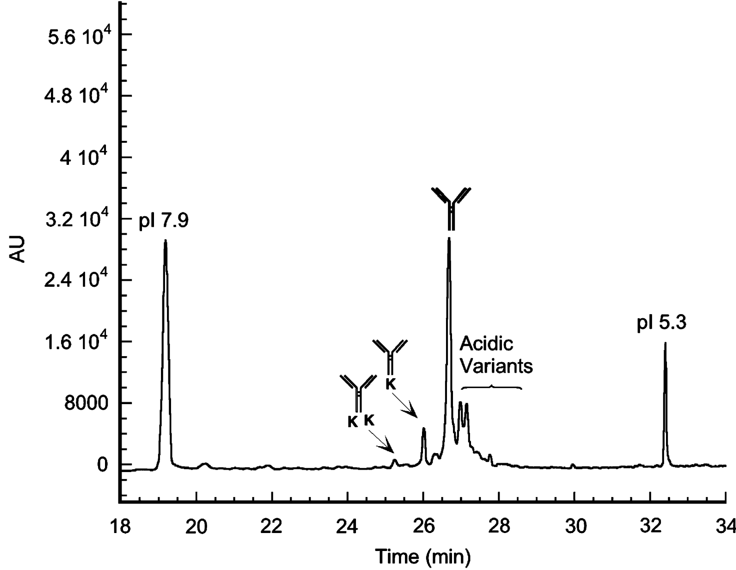


FIGURE 13 Capillary isoelectric focusing (cIEF) of an rMAb. Experimental conditions as in Figure 12.

CZE and IEC, as illustrated in Figure 10. On the other hand, the acidic variants are better separated from the 0-Lys form by cIEF, as shown in Figure 13, compared to the CZE profile in Figure 10. Taken together, these data demonstrate the feasibility of using cIEF as a complementary technique with IEC to monitor charge heterogeneity of therapeutic proteins.

VI. CARBOHYDRATE ANALYSIS BY CE-LIF

Carbohydrates are known to play a key role in the therapeutic use of recombinant proteins.[32] In recent years, in vitro studies have unequivocally established that essential effector functions are dependent on appropriate glycosylation of the antibody molecule.[45] Therefore, glycosylation has been a focus of attention for the biopharmaceutical industry, and regulatory agencies require that a consistent human-type glycosylation be maintained for rMAbs, independently of the system in which they are produced. As a result, high-performance analytical tools to accomplish both the structural characterization and routine analysis of carbohydrate are required. The routine profiling and quantitative analysis are mostly performed by chromatographic and planar electrophoretic techniques. Among them, the most widely used techniques are high-pH anion-exchange chromatography and pulsed amperometric detection,[46,47] liquid chromatography with pre- and/or post-column derivatization schemes,[48,49] and fluorophore-assisted carbohydrate electrophoresis.[50]

More recently, CE has emerged as a powerful tool in carbohydrate analysis.[51] However, the quantitative introduction of chromophores into the carbohydrates by chemical derivatization is required not only to facilitate their detection using absorption or fluorescence detector, but also to alter their physical properties such as hydrophobicity and electric charge. Therefore, the introduction of an electric charge makes the derivative amenable to electrophoretic separation. Most of the derivatization methods are based on reductive amination with various aromatic or heterocyclic amines, utilizing the reactivity of the amino group in the reagent and the hemiacetal group in the reducing carbohydrate. Reports included the use fluorophoric-tagging compounds such as 8-aminonaphtalene-1,3,6-trisulfonic acid (ANTS),[52] aminonaphtalene sulfonic acids,[53] and 8-aminopyrene-1,3-6-trisulfonic acid.[54] To date, the use of on-column LIF is the most sensitive detection method employed by CE, with a limit of detection in the low nanomolar range.[55]

One of the applications of CE for carbohydrate analysis that has shown increase acceptance in the biotechnology industry is the relative distribution of the N-linked oligossacharides on the conserved asparagines residue on the Fc portion of rMAbs. The advantages of CE over traditional methods include enhanced separation efficiencies, shorter analysis time, and improved robustness. The oligosaccharides present on a monoclonal antibody are asialo-N-linked complex biantennary structures with a core fucose, typical of the structures produced in the Chinese hamster ovary (CHO) cells. The main forms share the fucosylated branched core structure but vary in their terminal galactose occupancy: (1) a degalactosylated glycan (GO), composed of eight sugar units with no terminal galactose; (2) a partially galactosylated glycan (G1), containing nine sugar units with one terminal galactose and hence two positional isomers; and (3) a fully galactosylated glycan (G2), containing 10 sugars units having galactose in both termini. During cell culture production, microheterogeneity of the oligosaccharides was observed even under a defined set of expression and culture conditions. To monitor the manufacturing consistency, a CE assay was developed to determine the relative distribution of these closely related N-linked oligosaccharides.[54] This method involves three steps. First, the oligosaccharides are enzymatically removed from the rMAb with a highly specific endoglycosidase, PNGase F. Second, the released oligosaccharides are subsequently derivatized with APTS at the reducing termini. Finally, without further purification the derivatization mixture containing the excess reagents and the APTS–oligosaccharide adducts are simply diluted with water and analyzed by CE with LIF detection. A typical electropherogram for the analysis of N-linked oligosaccharides of an rMAb is shown in Figure 14. The APTS–oligosaccharide adducts migrate between 3.0 and 4.5 min. Since the APTS–glycans carry about the same net charge, the separation is based on the differences in their apparent hydrodynamic sizes. Therefore, the adducts migrate in the order of

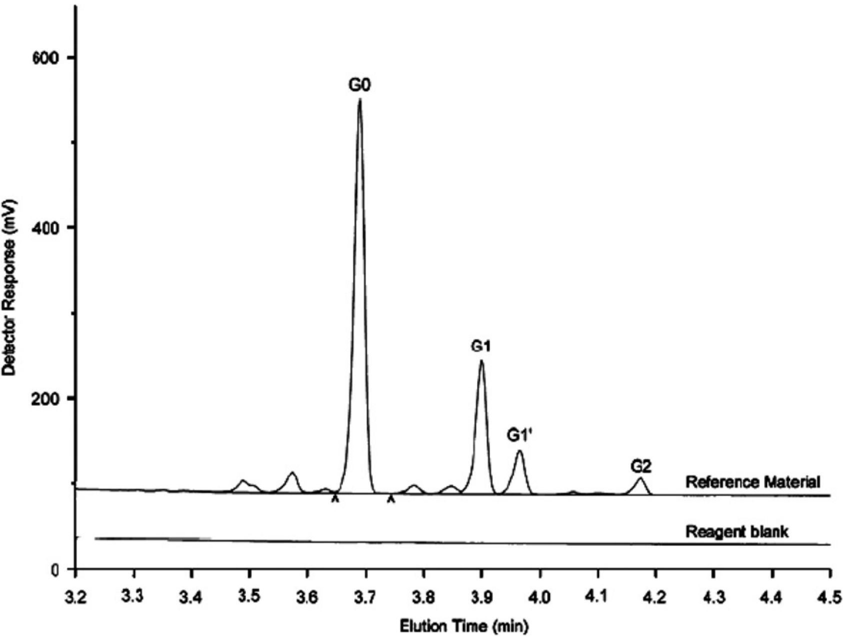


FIGURE 14 **Typical electropherogram of the relative distribution of N-linked oligosaccharides on an rMAb.**

increasing size (GO < G1 < G2) and are baseline resolved. Moreover, the two G1 positional isomers are also separated with baseline resolution with the first eluting peak as the G1 (α1,6).[54] The separation of the isomers is probably based on the differences in their apparent hydrodynamic sizes as they have the same net charges. The other minor peaks correspond to the high mannose structures (high mannose 5 and 6).[54] This assay was validated according to the ICH guidelines and found to be accurate, precise, and suitably robust for routine monitoring of the relative distribution of these oligosaccharides as a drug substance release test.

VII. CE AND QUALITY CONTROL TESTING OF THERAPEUTIC PROTEINS

With the more routine application of CE and the advances made in commercial reagents and instrumentation, CE has become a viable option for quality control release specifications. The specifications are an integral part of the release of a commercial drug substance/drug product. They are defined as a set of criteria to which a drug substance, drug product, or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. The specifications consist of a list of tests, with references to the appropriate test procedure and its respective set of acceptance criteria. The specifications generally contain methods to measure safety, identity, purity, and composition of a drug substance/product. SDS-PAGE and IEF slab gels have historically been part of biopharmaceutical control specifications, but as of late, CE methods such as CE-SDS, cIEF, and CZE have replaced the slab gel format for many applications in biotechnology. In the next few paragraphs, we will describe some common CE methods and their purpose in a quality control system.

A. Applications of CE in Quality Control

CE is applied to two major categories of quality release testing: identity and impurity testing. Identification assays are intended to ensure the unique identity of an analyte in the sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc.) to that of a reference standard. As shown in Figure 9, CZE can be used to determine identity for monoclonal antibodies and proteins based on their unique electrophoretic profile.

Purity assays are intended to accurately reflect the impurity/purity characteristics of the sample. These tests can be either quantitative or limit based. CE-SDS and CE glycan are classified as purity assays. For purity, CE-SDS is a reliable replacement for SDS-PAGE and can be applied as an orthogonal method to other sizing methods such as SEC. Compared to traditional SEC, CE-SDS offers the advantage of separation of fragments and additionally offers the possibility to control for process impurities. It therefore can be validated both as a quantitative product-related purity assay and as a limit test with respect to process-related impurities. Specifications can be set based on the percentage of main peak and/or the detection of a new peak. Another potential application of CE in quality is the quantitative determination of the relative glycan distribution on a recombinant humanized monoclonal antibody by CE with LIF detection as shown in Figure 14. Specifications can be set on a variety of glycoforms, depending on the biological significance of the glycosylation pattern.

B. Method Validation Principles

For a method to be considered as part of commercial specification, validation using ICH guidelines (Q2A and Q2B) is required (see also Chapters 9 and 10). Depending on method characteristics, different validation schemes may be used. The following parameters should be considered to ensure the method is valid and appropriate for its intended purpose: accuracy, precision (repeatability, intermediate precision), specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, and range.

Not all of the mentioned parameters will be required for validation of every method. Validation of some methods may require consideration of other parameters and should be justified. It should also be noted that robustness is not listed here but should be considered at an appropriate stage in the development of the analytical procedure.

1. Accuracy

It is required for quantitative purity assays, and it must be established across the specified range of the analytical procedure. This can be done, by establishing the recovery rate over the range of the method. Alternatively, a method comparison between a validated method and a new method can be performed. Accuracy can be determined by spiking degraded, aggregated, pure or impure material into a known amount of sample. A theoretical recovery would then be calculated and the spike material analyzed using the chosen method. The actual recovery should then be compared to the theoretical recovery to calculate the accuracy of the method. Accuracy in this case would be reported as percent recovery.

CE as a technology is not well adapted for fraction collection, thus the classical spike accuracy approach cannot be followed for CE-based purity methods. However, recovery may be evaluated through a sample recovery study by dilution, which must be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range. If one applies 15 determinations over five concentration levels covering the range, the data can be used as both accuracy and linearity.

2. Precision

Validation of methods for quantitative determination of impurities includes precision studies. Repeatability is generally assessed by analysis of the same sample or samples prepared by the same analyst in replicate assays within a short duration of time. Repeatability should be assessed using: (i) a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/three replicates each) or (ii) a minimum of six determinations at 100% of the test concentration. Repeatability is evaluated by averaging the mean results from replicate assays and calculating the standard deviation (SD) and RSD. Repeatability of the method can be stated as either SD or RSD values. If an instrument is required for assay performance, then the same instrument should be used for the replicate assays.

Intermediate precision generally incorporates test results from several assays both within a day and day-to-day over a time period (e.g., days, months, etc.). If appropriate, it includes replicates of multiple samples from various analysts and instruments. As in the repeatability studies, intermediate precision is determined by calculating mean, SD, and RSD values across analyst-to-analyst, instrument-to-instrument, and day-to-day studies. Intermediate precision can be reported as an SD or RSD value, depending on preference.

Reproducibility represents the precision of the method between two or more laboratories and it is typically assessed during method transfer between laboratories, but may be assessed during method validation when more than one laboratory will be performing the method. Reproducibility would also be reported as the SD or RSD value of the mean results between laboratories. These data are not part of the marketing authorization application.

3. Specificity

Specificity is a measure of the degree of interference (or absence thereof) in the analysis of the analyte of interest. Specificity must be demonstrated for identification tests as well as for impurity determination assays. For identity tests, the measurement of specificity must show a unique migration time or profile for the product of interest when compared to that of other products. In such cases, the product has to be evaluated against all other products manufactured within the same facility. Specificity is the main component of an identity validation and should be re-evaluated over time if new products are added to the manufacturing plant to ensure specificity is still valid. For quantitative methods, specificity is generally expressed as the degree of bias, enhancement, or inhibition of sample signal. This is determined by comparing the analysis of samples with added impurities, degradation products, related-chemical compounds, or placebo ingredients to that of samples without the added substances. Specificity studies are typically designed to determine the effect of the sample matrix (the formulation buffer, compounding buffer, drug product excipients, or process step elution buffer) on the accurate quantification of the component of interest. Studies can be designed to analyze the matrix alone or to spike known amounts of samples into different matrices. In all cases, the result is typically reported as either no response from the matrix (analysis of matrix alone) or acceptable recovery of the spike from the matrix (sample spike into matrix). Frequently, a comparison of the sample trace and the sample matrix trace is sufficient to establish specificity of an impurity assay.

4. Limit of Detection (LOD)

The LOD must be assessed for limit tests. In some cases, the LOD should also be defined for quantitative impurity/purity tests. The LOD is generally expressed as either the minimum level at which the analyte can be reliably detected or as a set amount above the SD from the repeated analysis of suitable sample, such as a blank or negative control sample.

The following approaches are considered to be acceptable to determine the detection limit of an analytical method.

(a) Visual Evaluation:

The minimum level at which a signal can be reliably detected is determined. This approach is followed mainly for non-instrumental methods but may also be used with instrumental methods.

(b) Signal-to-Noise Ratio:

The signal-to-noise ratio is determined by comparing measured signals of the analyte at low concentrations with the baseline noise of blank samples. In general, a signal-to-noise ratio between 3:1 or 2:1 is generally considered acceptable.

(c) Standard Deviation of the Response and the Slope:

The LOD can be expressed as $3.3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve.

The LOD and the method used for determining the LOD should be presented. If the LOD is determined based on visual evaluation or a signal-to-noise ratio, the presentation of relevant chromatograms is considered acceptable justification.

In cases where an estimated value for the LOD is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

5. Limit of Quantification (LOQ)

The LOQ should always be assessed for quantitative purity/impurity tests. The following approaches are considered to be acceptable to determine the LOQ of an analytical method.

(a) Visual Evaluation:

The minimum level at which a signal can be quantified with acceptable accuracy and precision is determined. This approach is followed mainly for non-instrumental methods but may also be used with instrumental methods.

(b) Signal-to-Noise Ratio:

The signal-to-noise ratio is determined by comparing measured signals of the analyte at low concentrations with the baseline noise of blank samples. In general, a signal-to-noise ratio of approximately 10:1 is considered acceptable.

(c) Standard Deviation of the Response and the Slope:

The LOQ can be expressed as $10\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. The LOQ and the method used for determining the LOQ should be presented. The limit should subsequently be validated by the analysis of a suitable number of samples known to be near or prepared at the LOQ.

6. Linearity

A linear relationship should be evaluated across the range. Physicochemical assays are usually linear over a wide range of concentrations.

Linearity studies are usually performed by analyzing different concentrations of the analyte of interest and then establishing the linear response by regression analysis and plots. A minimum of five concentrations that span the range of the expected product concentration

should be analyzed, and three determinations at each concentration are recommended. Linearity should be confirmed by visual inspection of a plot of signal as a function of analyte concentration. All data points should lie on or be equally distributed around the regression line. The correlation coefficient (r), the y-intercept, and the slope of the regression line should be calculated from the resulting line of the plots.

7. Range

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. This is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, and precision within the extremes of the specified range.

8. Robustness

The evaluation of robustness should be considered in the development of the assay and will depend on the type of procedure under development. It must show the reliability of a method with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement might be included in the procedure. One consequence of the evaluation of robustness may be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used. Typical parameters to be tested would be the following: sample concentration, sample stability, labeling variability (if applicable), injection variability, reagent lot-to-lot variability, and capillary vendor.

9. System Suitability Tests

The system suitability tests are performed to verify that the analytical system meets predefined acceptance criteria at the time of performance. System suitability parameters should be established based on the type of method being considered and before the validation of the method actually starts. A common method of system suitability will request bracketing reference injections, with measurable quantitative acceptance criteria, such a migration time and/or a range on the main peak area. The peak of interest can be the major peak but it can also be a secondary peak, which may give more control over the sample preparation (e.g., the HMW peaks in non-reduced CE-SDS or incomplete reduced in the case of reduced CE-SDS LIF).

VIII. CONCLUSIONS

CE plays an important role in the analysis of monoclonal antibodies in our laboratory, by providing complementary information of the physicochemical attributes of these proteins in terms of charge, hydrophobicity, and size. In particular, CE has been extremely useful in replacing traditional slab gel electrophoresis techniques such as CE-SDS as a replacement of SDS-PAGE and cIEF as a replacement of IEF gels. The advantages of CE over traditional slab gel electrophoresis including automation, reduced analysis times, online detection, and high separation efficiencies has made routine applications of CE in all stages of the product development life cycle possible, including process and formulation development, analytical characterization, comparability studies, and quality control lot-release testing.

CZE and cIEF are also used as complementary tools with traditional ion-exchange chromatography to monitor the charge heterogeneity of monoclonal antibodies. Advantages

of these CE techniques include minimum material requirement, faster analysis times, and generic experimental conditions suitable for high-throughput multi-product needs.

CE has recently emerged as a powerful tool in carbohydrate analysis with enhanced resolution for isobaric isomers, shorter analysis times, and high sensitivity with LIF detection, as well as better assay reproducibility and robustness over the traditional methods.

The CE applications described in this chapter have been validated according to the guidelines of the ICH, and are currently being used in routine lot-release testing and stability monitoring of selected marketed protein therapeutics.

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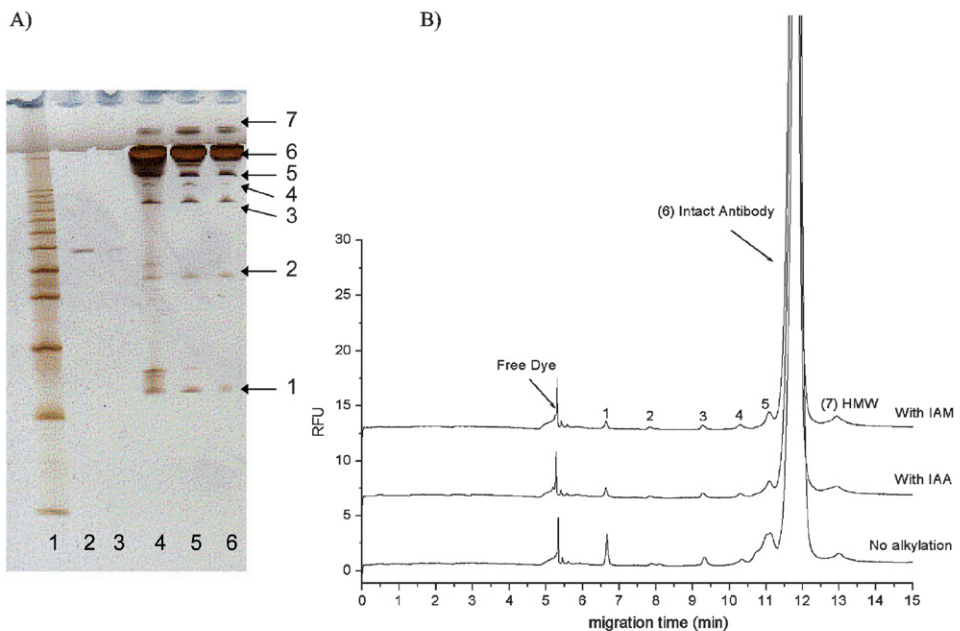

PLATE 4 **Effect of sample preparation on the fragmentation of an rMAb observed in (A) SDS-PAGE and (B) CE-SDS with LIF detection. SDS-PAGE lanes: (Lane 1) molecular weight standards; bovine serum albumin at (Lane 2) 8 ng and (Lane 3) 2 ng; (Lane 4) rMAb control; after alkylation with (Lane 5) iodoacetic acid and (Lane 6) iodoacetamide.**

16

CE AS AN ORTHOGONAL TECHNIQUE TO CHROMATOGRAPHY

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ABSTRACT

Recently, the use of dissimilar or orthogonal chromatographic systems, i.e., systems showing considerable selectivity differences, to develop a method for drug impurity profiling is gaining importance. The screening of a new drug on a limited number of these systems not only enhances the chance that all impurities are revealed, but also allows choosing an appropriate system for further method optimization. However, this innovating approach does not always give a satisfactory separation for all impurity mixtures. In this chapter, it is shown that capillary electrophoresis (CE) can be considered as a valuable orthogonal technique to high-performance liquid chromatography (HPLC). First of all CE is based on a totally different separation mechanism and as a consequence provides selectivity differences toward HPLC. Second, CE has the capacity to determine polar compounds, which co-elute with the injection peak in a conventional reversed-phase HPLC method. Finally, CE has also shown a better efficiency in separating isomers compared to chromatographic analyses.

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I. INTRODUCTION

The term chromatography covers several separation techniques where the separation is most frequently based on differences in partition of the solutes between a stationary and a mobile phase. For instance, a substance preferentially dissolved in the mobile phase will move faster through the chromatographic system (CS) than one with a higher partition in the stationary phase. As a consequence, the times needed to elute the compounds are different and separation is achieved. The compounds can then be identified based on their retention time, i.e., the time elapsed between the moment of injection and the moment when the maximum concentration of the component elutes.[1,2] Among these techniques, liquid chromatography, in particular high-performance liquid chromatography (HPLC), has emerged as the preferred technique for the separation and quantitative analysis of a wide range of pharmaceutical samples because of its ease, flexibility, and good efficiency.[3]

Most HPLC applications are performed with non-polar columns, thus in the reversed-phase mode (RPLC), since it allows simple and versatile conditions. Another advantage is that in general the applied mobile phase is an aqueous buffer. Moreover in RPLC chemical equilibria such as ion suppression, ion-pair formation, metal complexation, and micelle formation can easily be exploited to optimize separation selectivity. This explains the large number of commercially available non-polar HPLC columns.[2,4–6]

Octadecylsilyl (ODS) or C_{18} bonded phases, dominating the majority of the RPLC applications, are still being improved and new variations are developed. Also, new bonded moieties are constantly introduced to provide new interaction possibilities with the solute and to shield solutes from reactive silanols, which may cause peak tailing. For instance, long-chain alkyl phases as C_{30} are recently commercialized.[6] Another popular category of RP silica-based columns still explored are the polar-embedded phases, which allow analysis in low organic, highly aqueous environments.[5,6] Analysis in broader pH ranges than applicable on the classical silica-based columns can be performed with newer materials as hybrid, polymeric, and zirconia phases.[6–8] The latter phase is also well-known for its high-temperature stability.[7,8] This large pool of commercialized columns provides different selectivities, meaning that differences in the retention of a set of solutes will be caused by differences in solute–stationary phase interactions. As an advantageous consequence, a broader range of components can be separated.[5,6]

CSs, defined by a given stationary and mobile phase combination, showing large differences in selectivity are called orthogonal or dissimilar systems. They are very useful to screen unknown mixtures as a first step in the development of a separation method. Since on orthogonal systems different separations can be obtained for a given mixture, this enhances the possibility that all compounds are revealed. Moreover, this screening approach allows choosing a suitable CS for further method development.

An important application of dissimilar systems, studied by Van Gyseghem et al.,[9–11] is the evaluation of an impurity profile for a new drug, as is required by the International Conference on Harmonization.[12] This is a real analytical challenge because the impurities are unknown both in structure and in number. Moreover, they often have molecular structures similar to that of the main compound. Such mixtures are first screened on a series of dissimilar CSs coupled with a mass spectrometer. Second, the number of compounds is determined from the chromatograms by means of peak-tracking algorithms and a suited system is selected for further method development. In the subsequent optimization procedure, the mobile phase buffer pH, the organic modifier composition, the gradient time, and the column temperature are considered.

Pellett et al.[13] presented a procedure to develop orthogonal methods for nine routine RPLC methods from six pharmaceutical laboratories. During this study, several drug products

and their impurities were separated with two orthogonal RPLC systems. Another application exploiting orthogonality is the study performed by Silva et al.[14] in which amino acids were characterized with respect to their lipophilic behavior by means of several dissimilar RPLC systems differing in stationary phase and buffer pH.

Analytical separations obtained with capillary electrophoresis (CE) are based on the differences in electrophoretic mobilities of ions in (buffered) electrolyte media inside small capillaries.[15] Compared to column liquid chromatography, CE has several advantages: it reduces method development time, operating costs, and solvent consumption, and provides higher efficiencies.[16–18] Since CE is based on a totally different separation mechanism, it shows also large selectivity differences compared to chromatography. As a consequence, it can be said that CE is a technique orthogonal to chromatography, analogous to the dissimilarity, or orthogonality concept within chromatography.

Pluym et al.[19] compared the use of CE to that of HPLC in chemical and pharmaceutical quality control. They stated that CE could be considered as a complementary technique to HPLC because of its large separation capacity, its simplicity, and its economical benefits. Jimidar et al.[17] decided that CE offers high separation efficiency and can be applied as an adjunct in HPLC method validation. Mol et al.[20] evaluated the use of micellar electrokinetic chromatography (MEKC) coupled with electrospray ionization mass spectrometry (ESI−MS) in impurity profiling of drugs, which resulted in efficient separations.

The previous examples confirm that it can be very useful to include CE methods in an orthogonal screening set for unknown mixtures, e.g., in impurity profiling.[18]

II. ORTHOGONAL CHROMATOGRAPHIC SYSTEMS

A. Orthogonality

In the strict mathematical sense, two parameters are orthogonal when the Pearson's correlation coefficient between both is zero.[21] Practically, it means that they are uncorrelated. Considering comprehensive two-dimensional chromatography, two systems are called orthogonal when the constituent dimensions operate independently and the synentropy across the dimensions is zero.[21] However, in one-dimensional chromatography, as considered above, often a less strict definition is applied for orthogonal systems, "systems that differ significantly in selectivity."[22] As a consequence, some analysts rather prefer the term dissimilar to orthogonal in such situations.

The term orthogonality or dissimilarity of systems or techniques is broad and can be interpreted in several ways. First, one can compare systems within one mode of the same separation technique, for instance RPLC. Van Gyseghem et al.[9,23] defined a set of dissimilar silica-based RPLC systems and an orthogonal set containing more diverse RP stationary phases. These sets can be used as generic screening sets in the first step of impurity profiling. A second option is to assess orthogonality within different modes of the same separation technique, e.g., NPLC (normal phase LC) and RPLC. Fields et al.[24] compared the selectivities of a zirconia−polybutadiene column under high-temperature liquid chromatography (HTLC) and RPLC conditions to that of a conventional C_{18}−silica HPLC system. A broader option to consider orthogonality is between different separation techniques, for instance HPLC and CE. Steuer et al.[25] compared HPLC, supercritical fluid chromatography (SFC), and capillary zone electrophoresis (CZE) primarily to evaluate the orthogonality of the provided information and secondly to determine their ability to separate several groups of substances. In Figure 1, it can be seen that different selectivities occurred for a protein digest applying either HPLC or CE. It can also be noticed that the CE method is less time-consuming and produces smaller

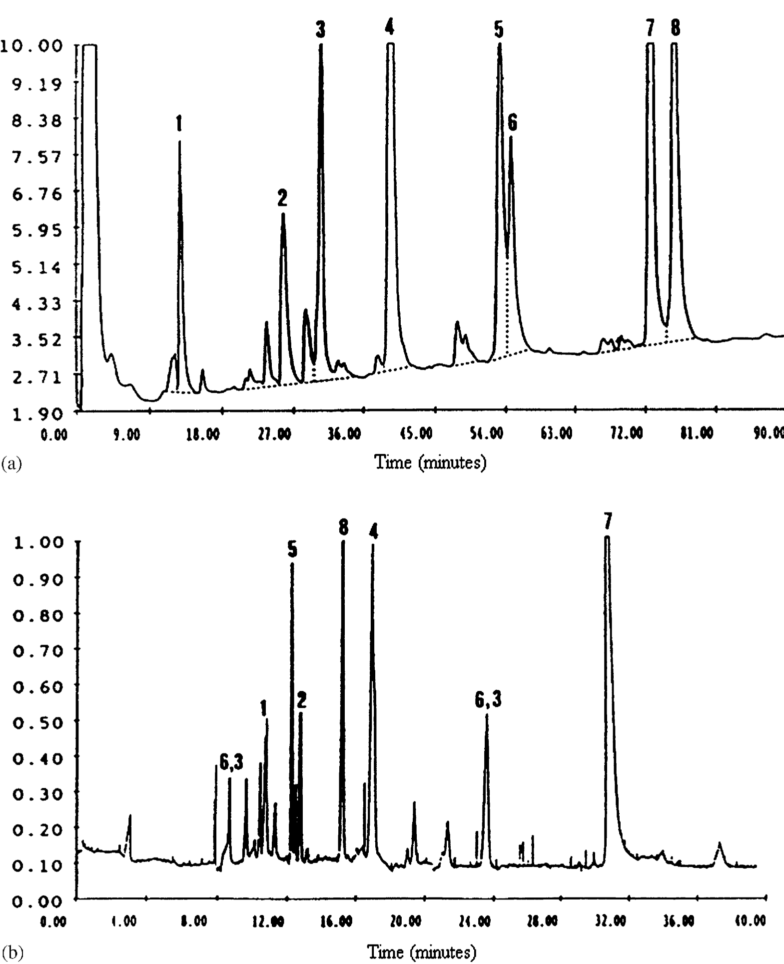

FIGURE 1 Comparison of the separation of a protein digest by (a) HPLC and (b) CZE, reprinted with permission from reference 25.

peaks. Finally, orthogonality can also be studied in two dimensions (comprehensive chromatography), as already indicated earlier, e.g., by performing a GC × GC separation.[26]

In regular one-dimensional chromatography, the stationary phase has the largest influence on the selectivity and is thus most responsible for selectivity differences between systems. For example, at low pH a zirconium-based stationary phase can show both anion and ligand exchange properties beside the classical RP properties, and as a consequence, it is found dissimilar toward silica-based phases.[9,23] However, also within the silica-based phases, even of one type (e.g., C_{18}), large selectivity differences can occur. This is a benefit in our context, but is undesired for compendial analyses.[27,28]

Secondly, the pH can have an effect on the selectivity. In pharmaceutical analysis, mainly compounds with either basic or acidic properties have to be analyzed. Since the pH influences the dissociation and charge of these substances, it will affect their retention behavior. Changing the organic modifier does not lead to major selectivity differences, but it can enhance the differences induced by pH and/or stationary phase changes.[9] Also, the column

temperature can affect selectivity in RP separations, particularly when separating solutes with different functionalities.[29] Unfortunately, the direction and extent of this effect are not easily predicted and therefore varying temperature is not generally used for adjusting selectivity nor to select dissimilar systems.[29]

In summary, it can be stated that the stationary phase and the mobile phase (buffer) pH are the most important factors determining the generic selectivity of a CS. The organic modifier composition and the column temperature can influence the selectivity locally, i.e., when separating a specific mixture of rather similar compounds, e.g., a drug impurity profile.

B. Evaluation of Orthogonality Between (Chromatographic) Systems

Van Gyseghem et al.[9,23,30] assessed the (dis)similarity between two CSs by calculating the Pearson's correlation coefficient (r) between their retention data. A generic set of 68 pharmaceutical compounds differing in molecular weight, structure, log P, pK_a, and pharmacological and chemical classes were injected on the studied systems to determine their retention factors (k), i.e., the difference between the retention time and the dead time divided by the dead time. Then r was calculated between both series of k. The more dissimilar the CSs are, the lower the correlation coefficient. This is caused by differences in selectivity between both systems, i.e., the retention of substances is uncorrelated, and when plotted, an unstructured cloud of points is seen (Figure 2a). A high r value indicates a direct (linear) relationship between the considered responses (Figure 2b), which implies that the solutes have a similar elution behavior resulting in correlated retention factors. However, a low correlation coefficient does not always imply generic selectivity differences. For instance, in Figure 2c the retention factors between most compounds are highly correlated, while outlying values for only few compounds cause a low r value. Therefore, it might be recommended to evaluate the dissimilarity between systems with a low r value visually.

A similar approach was performed by Neue et al.[31] to determine selectivity differences in RP separations, focusing on the influence of the stationary phase.

The above approach could also be applied to evaluate the orthogonality between CE systems and CSs using migration and retention parameters, respectively.

C. Selection of Orthogonal Chromatographic Systems

The first step in a method development strategy, prior to the screening of unknown mixtures, is the determination of a generic set of dissimilar (chromatographic) systems. These can be selected out of a pool of CSs with very diverse stationary phases[23] or out of a limited set with only one particular type of columns, for instance silica-based RP columns.[9] One can also broaden the pool to systems from different techniques (including CE) depending on the needs and possibilities of the analyst. The number of systems included in the resulting set should be high enough to provide generic selectivity differences, but also as low as possible to limit the number of experiments. The composition and size of the set is often guided by practical considerations, e.g., the number of instruments, detectors, and column switchers available.

Many chemometric techniques have already been tested to create a simple and fast selection procedure.[9,23,30,32–34] The preferred approach of Van Gyseghem et al. is building a dendrogram with the weighted pair group method using arithmetic averages (WPGMA) combined with an r-color map.[9,23,30] The WPGMA, a hierarchical clustering technique, sequentially merges the most similar objects, i.e., the CSs with the highest

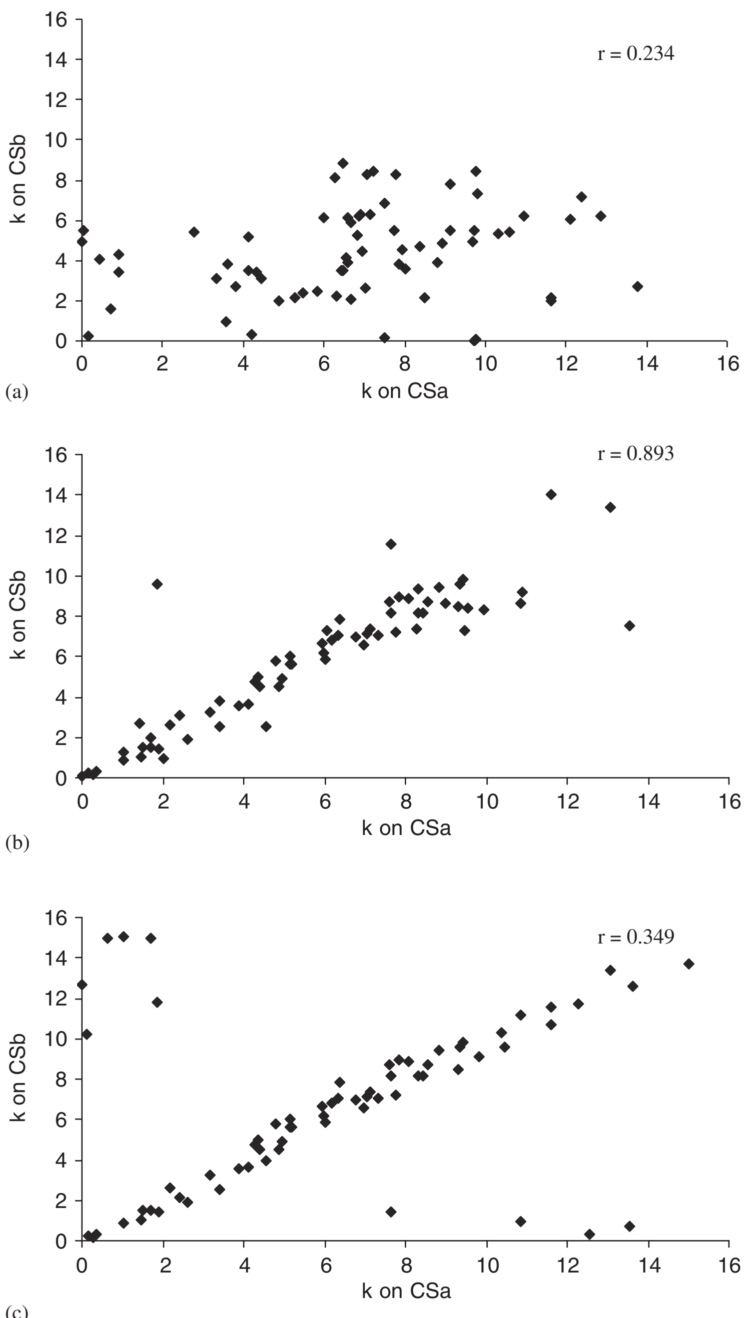

FIGURE 2 Retention factors on two chromatographic systems: (a) dissimilar CS, (b) similar CS, and (c) similar CS with some atypical correlation results. r = Pearson's correlation coefficient.

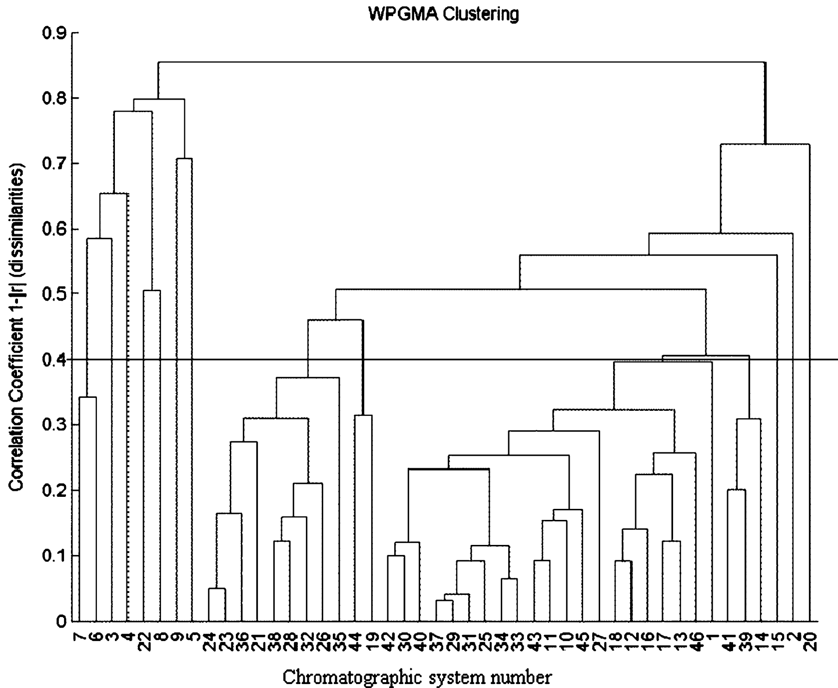

FIGURE 3 Dendrogram for 46 chromatographic systems resulting from the WPGMA technique applied on the retention factors of 68 substances using $1-|r|$ as dissimilarity criterion, reprinted with permission from reference 23.

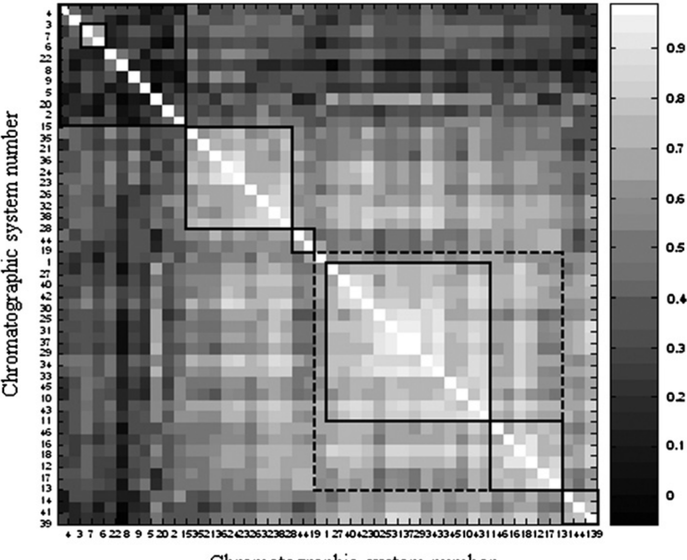

FIGURE 4 Color map of correlation coefficients between the retention factors of 68 substances on 46 systems, with the systems ranked according to decreasing dissimilarities ($1-|r|$) in the WPGMA dendrogram, reprinted with permission from reference 23.

Pearson's correlation coefficients (r) between the retention factors obtained on the considered systems. The results of this clustering can be visualized in a dendrogram (Figure 3), where the height of the connections between the branches in the tree is a measure of (dis)similarity. The higher two systems are connected, the higher their dissimilarity value, $1-|r|$, and thus they are the more dissimilar. An arbitrary limit of dissimilarity beneath which the systems are considered similar was defined as 0.4.[30] However, the values of interest will also depend on the diversity of the set of systems considered (e.g., diverse stationary phases versus silica-based RP phases). Additionally, the correlation coefficients between the retention factors on the systems can be visualized in a color map (Figure 4), where the colors scale from dark blue for low correlation values to brown for high r values. When the systems are ranked on decreasing dissimilarity, based on the WPGMA dendrogram, a blue colored group of systems can be distinguished in the left-upper corner of the map. This group represents the most dissimilar systems since the systems have low r values with most other systems of the test set. Several groups of similar systems (colored red) are also revealed.[30]

When a combination of CE and HPLC systems would be considered, the most dissimilar from the global set could be selected according to the above approach. For the CE methods, a response should be selected and applied with values in the same order of magnitude as the retention factors of the CSs, e.g., the migration times. Another possibility would be to use the so-called normalized migration indexes (see further Section III.C) for both the CE and the HPLC measurements.[35]

Other agglomerative clustering techniques such as single linkage,[36] complete linkage,[36] unweighted pair group method using arithmetic averages (UPGMA)[36] were evaluated by Van Gyseghem et al.,[23] once applying $1-|r|$ as dissimilarity criterion and once using the Euclidean distance. However, the WPGMA approach was preferred.

In the same study, a uniform mapping algorithm, the Kennard and Stone algorithm,[37,38] was tested to select the orthogonal systems. Put et al.[33] applied auto-associative multivariate regression trees (AAMRT)[39] to select orthogonal systems. Forlay-Frick et al.[32] used the generalized pairwise correlation method (GPCM)[40] combined with parametric and non-parametric statistical tests for the same purpose. These different methods seem to perform rather similarly. Dumarey et al.[34] proposed a systematic comparison approach to evaluate the orthogonality of the systems selected by the different selection methods. The Kennard and Stone algorithm seems to select the most dissimilar systems first. In the same paper, Dumarey et al. evaluated the use of the orthogonal projection approach (OPA) to select orthogonal systems. However, this method does not seem to perform as well as the others.

D. Developing the Separation for an Unknown Mixture

An unknown mixture can be screened on a set of orthogonal systems as a first step in the method development procedure. The chromatographic and/or electrophoretic system, on which the best separation was achieved, can then be retained for further method optimization. Sequentially, the pH[41,42] and the organic modifier composition of the mobile phase[29,41] can be adjusted to improve the separation on the CS. If necessary, also the temperature can be modified, while for gradient methods the gradient slope can be considered.[29] For CE methods, the optimization steps will be different from RP chromatography methods. Other factors will be optimized depending on the type of CE method, e.g., CZE and MEKC. However, for the development of CE methods, we would like to refer to Chapter 4 of this book.

III. CE AS AN ORTHOGONAL TECHNIQUE

A. Selectivity Challenges in Applications of RPLC

Although RPLC is a very powerful technique that solves the majority of separation problems, a few critical zones in the chromatogram can be indicated (Figure 5).[17]

First of all, two compounds can co-elute and as a consequence only one peak is observed (regions 1 and 4 in Figure 5). This problem can often be detected by MS. However, two structural or stereoisomers cannot be distinguished since they have identical molecular weights. This occurs frequently during drug impurity profiling because main compound and degradation products have similar structures. For example, in Figure 5, there is a possible co-elution of the main compound with an impurity in region 4, while in region 1 two impurities might be represented by one peak. A separation of all compounds can then mostly be achieved by adjusting the selectivity of the CS by choosing an appropriate mobile phase.[17] Usually the stationary phase will not be changed because this might cause major selectivity differences, which are not necessarily desired.

To identify all compounds of the drug-impurities mixture, the use of an orthogonal set of systems can be beneficial. The expected selectivity differences between these systems combined with MS detection and the use of peak tracking and matching algorithms should enhance the chance that all different compounds occurring in the mixture can be identified.

A second problem possibly arising is that some non-polar compounds elute late or even not from the column (region 2 or beyond in Figure 5). Gradient elution might provide a possibility to solve this problem.

The last critical region in the chromatogram (region 3 in Figure 5) is around the injection peak. A polar compound might not be retained by the stationary phase, and as a consequence, elutes at the time of the injection peak.[17] For this type of problem, a CE method can be an alternative. For example, Herrero et al.[43] succeeded in distinguishing several polar compounds, which were not retained by RPLC, by the use of a CE method.

B. The Surplus of CE Methods

Where RPLC often fails in separating polar compounds from the injection peak, ordinary CE mostly succeeds efficiently. As a consequence, it can be very useful to add at least one CE method to an orthogonal chromatographic set.[17,43]

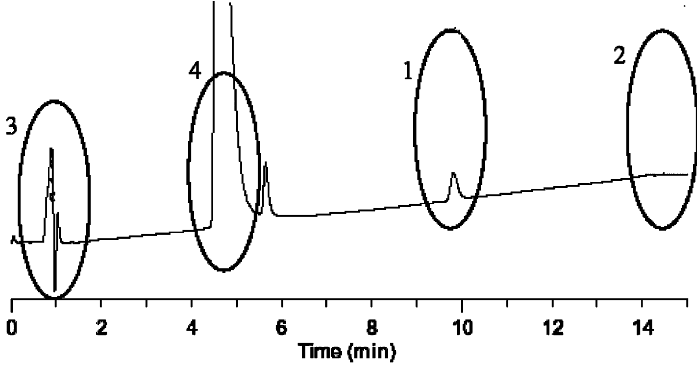

FIGURE 5 Critical regions in an HPLC chromatogram of a drug impurity profile with respect to potential selectivity challenges: (1) co-elution of impurities, (2) highly retained (non-polar) compounds, (3) non-retained (polar) compounds, and (4) co-elution with the main compound.

A second motivation to include CE methods is the excellent performance of chiral CE, which is often the first choice technique to separate stereoisomers.[44] Such method can be used complementarily to avoid potential co-elution of isomers or related products, e.g., degradation products, with similar chromatographic properties. Practically, one can fractionally collect the peak volume, lyophilize it, and dissolve the resulting mass in an appropriate solvent. The pre-concentrated sample can then easily be analyzed with a selective and efficient CE method. Another option is to develop an on-line coupling between HPLC and CE to facilitate the analysis.[17]

Finally, one should also keep in mind that CE is mainly based on electromigration, a totally different separation mechanism than conventional partition chromatography. This implies potentially large selectivity differences between both techniques, which can be very advantageous to screen unknown mixtures.[17,19] Within CE systems also, orthogonality can be obtained by adding electrolyte additives as ion-pair reagents, cyclodextrins, polymer additives, complexing agents, organic acids, metal ions, or a combination of these possibilities. Moreover, in the nineties, an even broader range of selectivities was introduced by the development of some new CE-based methods such as MEKC, microemulsion electrokinetic chromatography, and non-aqueous CE.[44] In MEKC, neutral solutes will partition with the micelles in a chromatographic way and selectivity differences can then be caused by varying the micelle-creating surfactants.[44] In non-aqueous CE, the electrolytes are prepared in 100% organic solvents. The latter affects the solvation of the ions and also alters the pK_a values of the solutes. As a consequence, selectivity can be adjusted by varying the organic solvent.[44,45]

C. Parameters for Orthogonality Evaluation Between HPLC and CE Systems

Vassort et al.[35] introduced a normalized migration index (NMI) to allow the direct comparison of HPLC and CE-based methods. For HPLC, this index was calculated with the following equation:

$$\mathrm{NMI}_{\mathrm{HPLC}} = \frac{t_{\mathrm{R}} - t_0}{t_{\mathrm{last}} - t_0} \quad (1)$$

where t_R is the retention time of the analyte, t_0 the dead time, and t_{last} the retention time of the last eluting compound. The NMI for CE methods was calculated with the following equation:

$$\mathrm{NMI}_{\mathrm{CE}} = \frac{\mu_{\mathrm{e}} - \mu_{\mathrm{fastest}}}{\mu_{\mathrm{last}} - \mu_{\mathrm{fastest}}} \quad (2)$$

where μ_e is the electrophoretic mobility of the analyte, $\mu_{fastest}$ of the fastest migrating component, and μ_{last} of the last migrating compound. The correlation between these indexes can then easily be calculated to assess orthogonality between two systems.

D. Applications

Jimidar et al.[17] proposed a set of highly selective CE methods applied as orthogonal methods to assess peak purity in HPLC (see also Section III.B). This set is able to analyze both charged and neutral compounds. High selectivity is not only obtained by the influence of the pH, but also by using specific additives such as surfactants (SDS), cyclodextrins, and organic solvents. The analyses are performed in the context of drug impurity profiling.

Saavedra et al.[46] used CE as orthogonal technique to HPLC to confirm the identification of a degradation product in alprazolam tablets during their stability assay.

Marsh et al.[47] tested selectivity differences within the CE technique by studying separations of nicotine and nicotine-related alkaloids by various capillary electrophoretic modes.

Vassort et al.[35] developed three orthogonal capillary zone electrophoresis electrospray ionization mass spectrometry (CZE−ESI−MS) methods for the analysis of six drug candidates and their respective process-related impurities. Not only the orthogonality between the CE techniques was evaluated, but also the selectivity differences between the three CE methods and four established HPLC−MS methods were investigated. It was found that HPLC versus CZE systems provided improved dissimilarity compared to CZE versus CZE or HPLC versus HPLC. Vassort et al.[35] did not limit the orthogonality assessment to one[35] parameter, correlation, but peak spreading angles, theoretical peak capacities, and practical peak capacities were also investigated.

Pluym et al.[19] used a combination of CZE and HPLC methods to develop the impurity profile of domperidone. Each individual method was unable to resolve all impurities.

Mol et al.[20] applied MEKC coupled with ESI−MS successfully for the analysis and characterization of impurities in drug substances.

IV. CONCLUSIONS

The orthogonality between CSs has already been studied extensively and has proven its importance in many applications. In this chapter, it was shown that CE can add important value as orthogonal technique to chromatography, for instance in drug impurity profiling. First of all CE is based on a totally different separation mechanism than partition chromatography and thus shows selectivity differences toward conventional HPLC. This implies that CE should be able to provide additional information about a sample. Moreover, CE proved its importance in the critical zones of a chromatogram, i.e., where the co-elution of two structural or stereoisomers or of a compound with the injection peak occurs. As a consequence, it can be very useful to include a CE method as orthogonal technique in a set of dissimilar CSs used to screen unknown mixtures. Primarily, this approach enables to determine the number of compounds in a mixture, and secondly, a suited system can be chosen for further method development.

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17 CAPILLARY ELECTROCHROMATOGRAPHY OF PHARMACEUTICALS

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ABSTRACT
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ABSTRACT

Capillary electrochromatography (CEC) is a miniaturized separation technique that combines aspects of both interactive chromatography and capillary electrophoresis. In this chapter, the theory of CEC and the factors affecting separation such as the stationary phase and mobile phase parameters have been discussed. The chapter focuses on the types and preparation of columns for CEC and describes the progress made in the development of open-tubular, particle-packed, and monolithic columns. The detection techniques in CEC such as the traditional UV detection and improvements made in coupling with more sensitive detectors such as mass spectrometry are also described. The chapter provides a summary of some applications of CEC in the analysis of pharmaceuticals and biotechnology products.

I. INTRODUCTION

Capillary electrochromatography (CEC) is a hybrid microseparation technique that combines the high selectivity of High performance liquid chromatography (HPLC) and the high separation efficiency of CE. The flow of mobile phase is driven through the column by an electric field, a phenomenon known as electroosmosis (electrostatic flow, EOF). The foundation of CEC dates back to 1974 when Pretorius et al. realized the advantage of the flat flow profile generated by EOF in both thin layer and column chromatography.[1]

An advantage of CEC is that the pressure drop across the column is very low so that small particles and longer columns can be used. Also, the electroosmotic flow results in a plug flow profile as opposed to a parabolic or laminar flow derived from a pressure-driven flow (Figure 1). The combination of these advantages leads to highly efficient columns that can be applied to separate components in a mixture.

Plug flow in CEC is due to the fact that the driving force of the EOF (i.e., charge on the capillary wall) is uniformly distributed along the capillary, which means that no pressure drop is encountered and the flow velocity is uniform across the capillary. HPLC has a pressure-induced flow, generating a parabolic flow that leads to band broadening and, therefore, lower efficiencies compared to CE and CEC. Moreover, HPLC requires large quantities of packing materials, solvents, and analytes. Even though there are attempts to reduce the amount of materials and solvents with micro-HPLC, technical problems due to low dead-volume fittings are encountered. In CEC smaller column packing materials can be used while in HPLC the size of the particles is limited by the generation of back pressure. Since the EOF in CEC does not generate a pressure drop across the column, the use of micro particles is possible. A comparison of the operational parameters in HPLC, CE, and CEC are outlined in Table 1.

A major advantage of CEC over CE is the ability to separate neutral and charged analytes in one sample mixture due to the combination of both electrophoretic and chromatographic

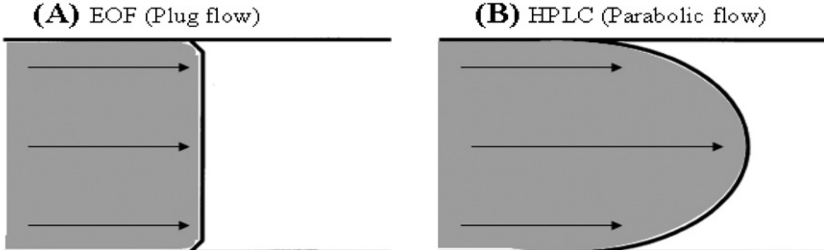

FIGURE 1 The flow profiles showing the plug or flat flow in CE (A) and the laminar or parabolic flow in HPLC (B).

TABLE 1 Comparison of the Operational Parameters in HPLC, CE, and CEC

	HPLC	CE	CEC
Column format	Capillary to preparative scale	Capillary	Capillary
Particle size	Usually 3–10 μm	Open tubular (OT)	OT/particle packed (Typically 3 μm/ porous rod (monolith)
Flow	Parabolic	Laminar	Laminar
Application mode	Pressure	Voltage	Voltage and/or pressure
Amount of materials and solvents	Large	Small	Small
Efficiency	Low/high[a]	High	Very high
Analysis time	Long	Short	Short

[a]Capillary formats produce high efficiencies.

processes. Neutral compounds can, however, be separated in CE by micellar electrokinetic chromatography (MEKC) by the addition of surfactants such as sodium laurylsulphate (SDS) to the running buffer phase. However, most of these additives with the exception of perfluorinated ones do not allow MS detection due to their nonvolatile nature.[2]

Despite the advantages of CEC over CE and HPLC, particle-packed columns are plagued with problems such as the difficulty in the preparation of frits to retain the stationary phase and bubble formation that results in current leakage and EOF breakdown.[3] These problems set the pace for the development of column technology to overcome the problems associated with particle-packed columns and to improve on the speed of separation of analytes in mixtures. The fabrication of a continuous porous rod (monoliths), not requiring any frits and ensuring a constant and uniform current flow to give a stable EOF has so far proved a potential development for microseparations.[4]

A. CEC Instrumentation

A typical CEC equipment includes a high voltage power supply, solvent and sample vials at the inlet and a vial to collect waste at the outlet of the capillary column, a column that simultaneously generates an EOF and separates the analytes, and a detector that monitors the component peaks as they elute from the column (Figure 2). In addition to the basic building blocks it also includes a module that enables pressurization of the vials at both the inlet and outlet ends of the CEC-packed capillary column to about 1.2 MPa. This prevents the formation of bubbles that can lead to a noisy baseline and drying out of the capillary. Typically, equal pressures of an inert gas, such as nitrogen, is applied to both vials in CEC mode to avoid the flow that would otherwise occur resulting from the pressure difference. Hydraulic pressure applied only at the inlet end of the capillary column is occasionally used in pressure-assisted electrochromatography (pCEC)[5,6] which also reduces the tendency for bubble formation.[55] However, with monolithic columns, where bubble formation is not a problem, pressure is usually only applied at one end.

It is, however, important to note that because of the rapid development of the techniques in the last decade, most and potential CEC applications initially utilized currently available CE instruments with packed columns where the maximum external pressure that can be

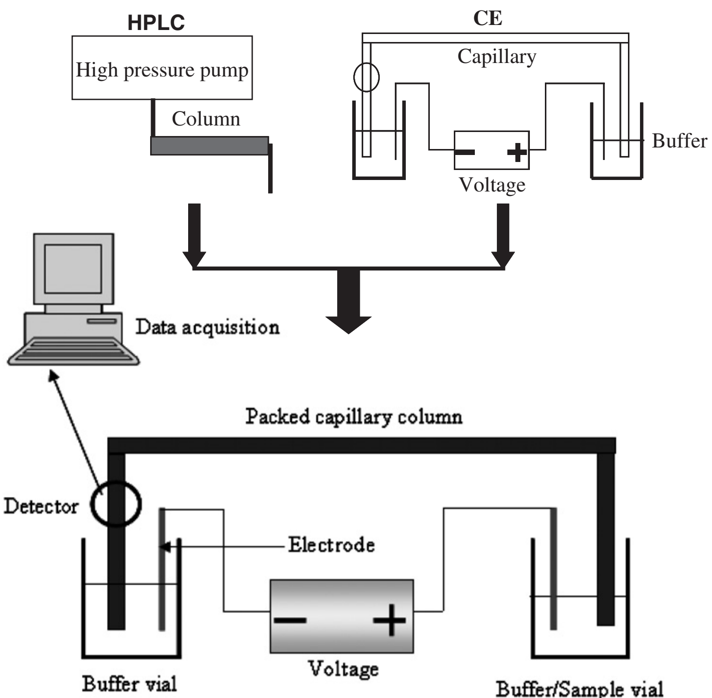

FIGURE 2 A simplified schematic diagram of a CEC instrument.

applied is not more than 12 bars. Instrument manufacturers quickly developed or modified existing instruments. A summary of CE instruments that are adaptable to CEC application and of dedicated commercially available CEC instruments is presented in Tables 2 and 3, respectively. Some home-built configurations have also been used successfully in CEC. For a detailed discussion on CEC instrument hardware configurations, refer to Rapp and Tallarek.[7]

B. Retention Mechanisms in CEC

Since CEC is a hybrid technique, the differential migration of analytes through the stationary phase bed will generally involve lipophilic, electrostatic, and electrophoretic processes between the analytes and the stationary and mobile phases.[8,9] Consequently, the applied voltage and electrical field strength and the mobile phase properties such as pH, buffer concentration, ionic strength, temperature, and the organic content will all affect the separation. For a detailed discussion, also refer to reference 10.

II. FACTORS AFFECTING THE ELECTROPHORETIC AND CHROMATOGRAPHIC PARAMETERS IN CEC

Similar to HPLC and CE there are a number of important variables that affect the separation process in CEC. These include the electric field strength, mobile phase parameters, and stationary phase surface chemistry that are described as follows.

TABLE 2 Commercial CE Instruments Adapted for CEC Applications

Specifications	Agilent 1100 series	Waters CapLC™	LC Packings UltiMate™	Eldex MicroPro™
Flow rates	0.01–2500 L min^{-1} in 0.01 μL min^{-1} increments	0.25–40 μL min^{-1}, increments of 0.01 μL min^{-1}	0.05–1000 μL	0.01–10,000 μL min^{-1} in increments of 0.01 μL min^{-1}
Gradient mixing	(binary) Pre-split high pressure mixing	(binary or ternary) Splitless high-pressure mixing	Low pressure (quaternary) mixing	(up to quaternary) Splitless high-pressure mixing
Pressure range	up to 400 bar	up to 5000 psi (≈345 bar)	up to 400 bar	up to 10,000 psi (≈700 bar)
Column oven	Yes	Yes	Yes	not integrated
Detection systems	UV-Vis, MWD detector, and PDA, MS systems from Agilent and other manufacturers	PDA and MS systems	Fast scanning UV-Vis, several MS systems	A variety of detectors from different manufacturers

Source: Reproduced with permission from reference 7.

TABLE 3 Commercial μHPLC/CEC/pCEC Instruments

Specifications	Unimicro Tri-Sep™ 2000GV	Micro-Tech Ultra-Plus II™	ProLab Evolution 200
Flow rates	1 μL min^{-1}–10 mL min^{-1}	5–300 μL min^{-1} without split, split down to 10 nL min^{-1}	Isocratic: 0.02–200 μL min^{-1} Gradient: 1–200 μL min^{-1}
Gradient mixing	Binary back-pressure regulated pre-split mixing	Binary two-stage dynamic high pressure pre-split mixing	High pressure passive mixing inside a mixing tee
Pressure range	up to 1000 psi (∼70 bar)	up to 10,000 psi (∼700 bar)	up to 400 bar
Column oven		Cooling for pCEC	Dry, Peltier-thermostated forced air
Detection systems	UV-Vis, variable or fixed wavelength	Electrochemical or UV/Vis (variable wavelength)	Variety of detectors from different manufacturers

Source: Reproduced with permission from reference 7.

A. Applied Voltage and Field Strength

Separation by electrophoresis is based on differences in solute velocity in an electric field. The velocity of an ion is given by

$$v = \mu_e E \tag{1}$$

where v is the ion migration (electrophoretic) velocity ($m\,s^{-1}$), μ is the electrophoretic mobility ($m^2\,V^{-1}\,s^{-1}$), and E is the applied electric field (or electric field strength) ($V\,m^{-1}$).

$$E = \frac{V}{L} \tag{2}$$

where V is the applied voltage and L is the length of the capillary.

Equations (1) and (2) establish the relationship between the applied voltage and field strength. Generally, the electroosmotic\ velocity is directly proportional to the field strength; hence a plot of μ_{eof} versus E is linear over a wide range of voltages. In practice, varying the field strength is a useful means by which the flow velocity can be manipulated in CEC separations. It is often useful to operate at high field strengths to achieve shorter analysis times. However, practical limitations are imposed by Joule heating effects and by power supplies that usually do not exceed 30 kV. The effect of the voltage on the linear velocity of analytes has been reported (Figure 3).[11]

For a given E, values of μ_{eof} for particles with different diameters are similar (Figure 3). While small differences are observed at higher electric field strengths, it is likely that any variations are a consequence of ohmic heating and resultant changes in viscosity, since dissipation of heat depends on packing efficiency, which depends on particle diameter. Since μ_{eof} depends on E, an increase in column length must be accompanied by an increase in voltage. Most CEC experiments so far have been carried out with applied voltages up to 30 kV, but equipment allowing voltages up to 90 kV has also been reported.[12]

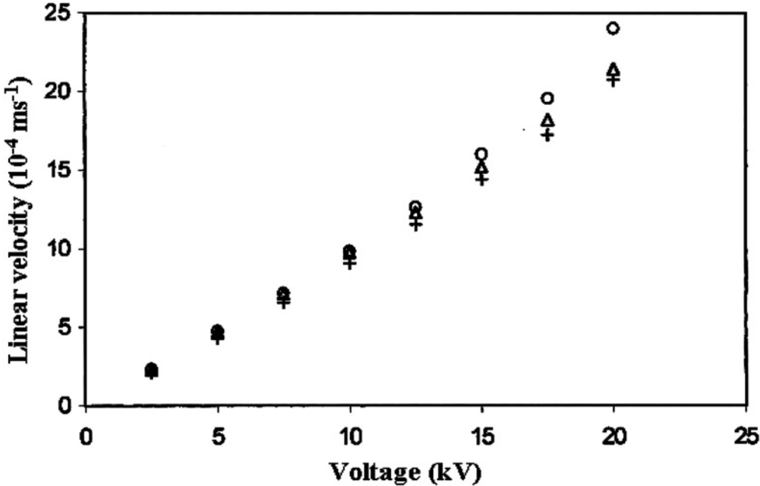

FIGURE 3 Effect of voltage in CEC using columns with different particle size: 3 (Δ), 5 (O), and 10 (+) µm ODS1 material (8 nm pore size). (Reproduced with permission from reference 11.)

B. The Mobile Phase in CEC

Most CEC separations are predominantly reverse phased with mobile phases that are mixtures of aqueous buffers and organic solvents. The mobile phase plays the dual role of carrying and interacting with the analytes as well as conducting electricity. As the EOF is created by ionized functionalities and the extent of their effect on the flow rate depends on the pH of the mobile phase, the mobile phase must be buffered to a desired pH value in order to achieve the optimum flow velocity. Critical among the mobile phase parameters that have to be controlled are the percentage of organic solvent, the concentration (ionic strength) of the buffer solution, the pH of the buffer solution, and the temperature of the mobile phase.

1. Organic Solvent

The effect of the organic content of the mobile phase on CEC separation is very similar to that of HPLC. The type and proportion of organic solvent in the mobile phase is predicted to influence the EOF mobility through the ratio of permittivity to viscosity, ε_r/η, in Equation (3). For the separation of a series of neutral compounds, an increase in the percentage organic reduces the retention time (Figure 4).[13] This has been demonstrated in CEC experiments (Figure 4), and indicates that well-established theories used in HPLC method development should be equally applicable to the separations in CEC.[14] However, increased organic content also decreases the selectivity and affects the EOF.

2. Buffer Concentration

The electroosmotic velocity (μ_{eof}) in CEC can be defined from the von Smoluchowski equation:

$$\mu_{eof} = \frac{\varepsilon_0 \varepsilon_r \xi E}{\eta} \tag{3}$$

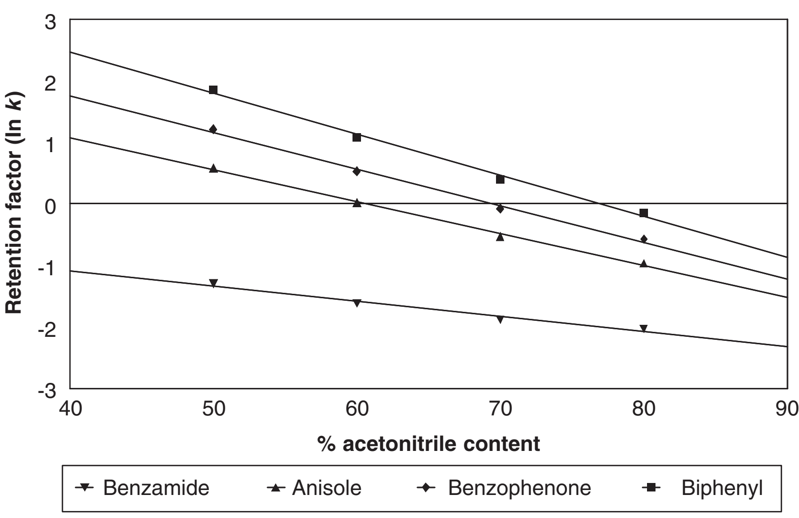

FIGURE 4 The effect of organic modifier content on retention factor (ln *k*) in CEC. (Reproduced with permission from reference 13.)

where ε_r is the dielectric constant of the medium, ε_0 is the permitivity of the vacuum, ξ is the zeta potential of the capillary inner wall (the voltage drop between the wall and the surface of shear), E is the electric field strength defined as V/L where V is the voltage and L is the total length of the capillary column, and η is the viscosity of the bulk solution.

The zeta potential at the surface of the shear is defined as

$$\xi = \frac{\sigma\delta}{\varepsilon_0\varepsilon_r} \tag{4}$$

where σ is the charge density at the surface of the shear and δ is the thickness of the double layer.

$$\delta = \left[\frac{\varepsilon_0\varepsilon_r RT}{2F^2 c}\right]^{0.5} \tag{5}$$

where R is the gas constant, T is the temperature, F is the Faraday constant, and c is the concentration of the electrolyte.

Combining Equations (3), (4), and (5) gives

$$\mu_{\infty f} = \frac{\sigma[\varepsilon_0\varepsilon_r RT/2F^2 c]^{0.5}}{\eta E} \tag{6}$$

The electroosmotic velocity decreases with the square root of the salt concentration of the buffer. At high ionic strengths, compression of the double layer results in a decrease in the magnitude of the EOF. However, an increase in concentration of the electrolyte also increases the conductivity of the mobile phase producing a rapid increase in the current. High currents generate more Joule heat, thereby increasing the temperature within the column. Although very low buffer concentrations should afford high electroosmotic flow and prevent Joule heating, their buffering capacity may be quickly depleted. Therefore buffer solutions with convenient concentrations in the range of 5–50 mM are suggested to achieve good CEC separations. The zeta potential is therefore governed by the thickness of the electrical double layer (δ) and the surface charge density (σ).[15] The electrical double layer (δ) is inversely proportional to the square root of the concentration of the electrolytes in the mobile phase. The charge density (σ) depends on the number and the degree of ionization of the free silanol groups and the effective surface area of the stationary phase. The number of silanol groups and other chargeable ligands, which obviously influences the EOF, varies, depending on the type and the nature of the micro particles used. For their stationary phases, manufacturers utilize various types of silica, with differing properties that differ in characteristics, such as purity, surface area, and pore size. In addition, different chemical processes are used to bond; for example, the alkyl groups to the silica and possibly end-cap and/or base-deactivate.[15,16] There are a considerable number of stationary phase capillary columns available for CEC ranging from silica to polymer-based with the possibility of modification of the surface chemistry.

3. pH of the Buffer

The pH of the buffer has complex effects on separation of analytes since it affects the ionization of the chargeable groups at the surface of the stationary phase. This is particularly important for stationary phases in which the weakly acidic silanol groups are the only driving

force for the EOF. As the buffer pH increases, there is increased dissociation of (Si–OH) to ($Si{-}O^{-}$) on the inner surface of the capillary and since the zeta potential is proportional to the surface charge, an increase in pH increases the ionization of the silanol group producing a greater zeta potential and therefore an increase in the EOF. Hence in general, the separation of neutral compounds is considerably accelerated in a buffer with higher pH values (pH > 8) compared to an acidic pH (2–3) at which the acidic silanol groups are less ionized producing a lower EOF. This is, however, not the case for capillary columns with strong ion-exchange functionalities. For example, pH changes in the range of 2–10 do not significantly affect the overall ionic mobility in the mobile phase with monoliths with strongly acidic sulfonic acid functionalities since the sulfonic acid group remains dissociated in this range with the current remaining almost constant. This has been demonstrated by Smith and Evans.[17] in the investigation of the effect of pH on EOF, dimethyloctadecylchlorosilane (ODS) (Spherisorb ODS-1), and Spherisorb mixed-mode packed columns (3 μm).[17] The mixed-mode contains bonded C_6 and sulfonic acid groups. While the ODS column showed an increase in the EOF with the increases in pH, the EOF in the C_6/strong cationic exchangers (SCX) mixed-mode phase is much less dependent on pH because of the presence of $-SO_3H$ groups that are strong acids and therefore always ionized. The pH of the mobile phase also affects the ionization of acidic and basic analytes and hence their electromigration. Since this migration can be opposite to that of the electroosmotic flow, it may both improve and impair the separation. This effect is particularly important in the separation of proteins and peptides that contain a number of ionizable functionalities. Ericson and Hjerten[18] used the derivatization of monolithic columns to control the extent of EOF and electromigration.

4. Effect of Temperature

Temperature is an important variable in all modes of chromatography since it affects the mobile phase viscosity, as well as solute partitioning, solute diffusivity, the degree of ionization of buffers, and the buffer pH.[19] Increased temperature (T) reduces the mobile phase viscosity as described by the exponential relationship:

$$\eta \propto \exp\left(\frac{\text{constant}}{RT}\right) \tag{7}$$

where R is the gas constant.

An increase in temperature decreases the viscosity and hence increases the EOF. Thus, for a given voltage, more rapid analysis is possible. Temperature also affects the solute partitioning between the mobile and stationary phases and therefore the chromatographic retention.[20] The distribution of the solute between the mobile and stationary phases is a function of its solubility in the liquid phase and adsorption on the solid stationary phase. This is characterized by the distribution ratio K defined as the ratio of the concentration of the solute in the stationary phase to its concentration in the mobile phase. Retention factors are influenced by increasing column temperature because of the increased partition into the mobile phase according to the Van't Hoff equation:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{8}$$

where ΔH is the enthalpy associated with the transfer of the solute to the stationary phase and ΔS is the corresponding change in entropy.

Van't Hoff plots of ln$K$ versus reciprocal absolute temperature are linear. Since the column temperature controls both the overall flow rate and the retention of the individual

compounds, a programmed temperature gradient can be used to shorten the CEC run times and optimize selectivity. Interestingly, it is recognized that the retention mechanism and thermodynamic constants of analytes in CEC are different from those obtained in HPLC.[21]

C. Stationary Phase Surface Chemistry

One of the important operational variables in CEC is the analyte–sorbent interaction. In reversed-phase separations (typical in CEC) the hydrophobicity of the stationary phase determines the selectivity of the separation, and retention can be controlled by adjusting the surface chemistry of the packing, composition of the mobile phase, and temperature. In contrast to HPLC, the CEC column plays a dual role in providing a flow driving force and separation unit at the same time; hence electrophoretic and chromatographic processes are operational. The stationary phase chemistry is dealt with in detail in Section III on column technology.

D. Efficiency and Band Broadening in CEC

Electrochromatography involves the use of a stationary phase; hence the concepts of efficiency and band broadening are similar to those that occur in conventional liquid chromatography. The efficiency, expressed in number of theoretical plates N can be obtained directly from an electropherogram using the following equation:

$$N = 5.54\left(\frac{t}{w_{1/2}}\right)^2 \tag{9}$$

where t is the migration time and $w_{1/2}$ is the peak width at half height.

This can be related to the height equivalent to a theoretical plate (HETP), H, by

$$H = \left(\frac{L}{N}\right) \tag{10}$$

In HPLC retention factor (k) is defined as

$$k = \frac{t_r - t_o}{t_o} \tag{11}$$

where t_r is the elution time of a retained solute and t_o is the elution time of an unretained solute.

However, in CEC the velocity factor (k_e) is given by

$$k_e = \frac{t_{eo} - t_m}{t_m} \tag{12}$$

where t_{eo} is the migration time of an EOF marker and t_m is the migration time of the analyte.

With CEC, the retention factor is a hybrid of the k (HPLC) and k_e (CE) terms.[22] Similar to HPLC the efficiency in CEC is expressed as theoretical plate (number of plates per meter). One of the main advantages of CEC, compared to HPLC, is that its efficiency is much greater.[23]

In liquid chromatography (LC) the plate height H is related to the various band broadening terms as described by the van Deemter equation:[24]

$$H = \frac{B}{u} + A + C_s u \tag{13}$$

where H is the plate height, u the linear flow, B the longitudinal diffusion term, A the eddy diffusion term, and C_s the mass transfer term. Hence, the lower the value of H, the greater the efficiency. A plot of the linear velocity against the plate height (H) gives a minimum value of H at which efficiency is optimum.

The van Deemter plots for CEC and HPLC differ, as the minimum of the CEC plot is smaller and flatter.[25] These smaller plate heights are generally attributed to the plug flow profile of CEC (Figure 1). Since, in CEC, the flow variation between channels is smaller, the eddy diffusion is substantially lower. In addition, the particles in the bed do not hinder the flow, as they do in HPLC, but propel the liquid through the column, as the EOF originates at the particle surface.[16] Since there is no pressure drop over the column in CEC, it is possible to use much smaller particles and longer columns than in HPLC. The efficiency can therefore be even greater, as both A terms and C terms in the van Deemter equation decrease with the smaller particle diameters.[26] The A term in Equation (13) relates to the diffusion arising from different flow paths that solute molecules can take through the packed bed. Due to the laminar flow in HPLC in contrast to plug flow in CEC solute molecules between streams move at different velocities. However, in CEC the contribution of eddy diffusion to band broadening is significantly lower because the velocities between the channels are identical. It is clear from the van Deemter equation (Equation (13)) that a reduction in the particle diameter will lead to more densely packed and uniform columns and in turn a smaller contribution from the A term. The C-term reflects band broadening due to slow equilibration of the solutes (resistance to mass transfer) between the mobile and stationary phases and is increased as the mobile phase velocity increases because less time is available for equilibration. The contribution to band broadening from the C-term also can be reduced by the use of small diameter packing materials. The effect of particle size on efficiency, when comparing pressure-driven and electro-driven flow has been investigated by Knox and Grant,[26] indicating that smaller particle sizes produce higher efficiencies.

The effect of particle size has also been investigated by Channer et al.[27] where lower retention times and efficiencies were obtained with larger particle size (Figure 5).

Grant[28] calculated that if the particle diameter was reduced to 0.5 μm then the contribution to the plate height from the A-term (eddy diffusion) would be $\sim$ 0.5 μm and the C-term 0.025 μm; thus the major contribution to the plate height (2 μm) would be from axial molecular diffusion, i.e., the B-term in the van Deemter equation. If the particle size is reduced significantly, for example to <1 μm then the A and C terms will be reduced to such a level that the dominant contribution to band broadening will be the B-term, i.e., only axial molecular diffusion is a contributing factor to band broadening and now H is given by the plate height equation used in CE:[28]

$$H = \frac{2D_m}{\mu} \tag{14}$$

where μ is the linear velocity and D_m is the diffusion coefficient of the solute.

The B-term in the equation is the contribution to the plate height resulting from longitudinal diffusion (molecular diffusion in the axial direction) and arises from the tendency of the solute band to diffuse away from the band center as it moves down a column. It is proportional to the time that the sample spends in the column and also to its diffusion

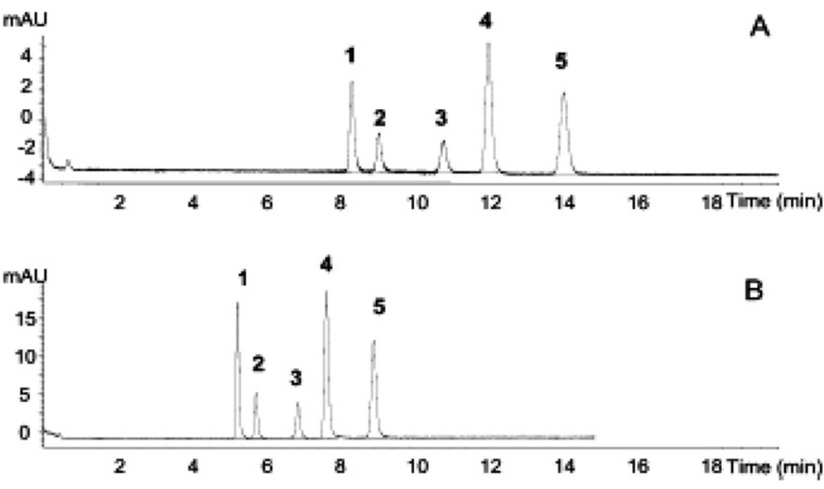

FIGURE 5 **Separation of the neutral analytes: thiourea (1), benzamide (2), anisole (3), benzophenone (4), and biphenyl (5) on Hypersil CEC Basic C18 of differing particle size. A = 5 µm and B = 12 µm. CEC conditions: 8:2 v/v Acetonitrile: 50 mM MES (pH 6.1); 20 kV; thermostatted temperature 20°C; detection = signal 254, 8 and reference 450, 80 nm; injection, 0.5 MPa/6 s. (Reproduced with permission from reference 27.)**

coefficient in the mobile phase. The longer a solute spends in the column the greater the extent of diffusion and therefore the *B*-term only becomes significant at low flow rates.

III. COLUMN TECHNOLOGY IN CEC

Column technology is an active area of exploration in CEC for efficient and fast separations. The issues dealt in this section include capillary materials and column types used in CEC.

A. Column Tubing (Capillary Technology)

Generally CEC is performed in fused silica capillary tubing that is capable of withstanding high temperatures. Fused silica tubing also has a high electrical resistance, high-pressure resistance, good UV transparency, high thermal conductivity, good chemical inertness, good flexibility, and high mechanical strength. The outer wall of the fused silica tubing is normally coated with a thin layer of polyimide coating to offer it mechanical stability as well as protecting the fused silica from scratches and invasion by oxygen and water vapor. The superior properties of fused silica tubing make it the preferred choice for CEC. Despite its significant properties, fused silica tubing is also associated with several problems. When UV absorption or another optical detection method is used, a detection window must be fabricated in the polyimide-coated fused silica tubing by simply burning off or dissolving away a small section of the coating, making the window section of the capillary very fragile. When the column is immersed in a mobile phase containing a high percentage of organic solvent for a period of time, polyimide swelling gradually occurs and the polyimide coating easily peels off into the buffer vial. Fused silica is also known to gradually dissolve in the

mobile phase if the mobile phase pH is greater than 10 or lower than 2. Moreover, some analytes can be adsorbed onto the polyimide coating during sample injection, resulting in sample cross-contamination.

Teflon coating is UV transparent and does not absorb the majority of compounds. Teflon-coated fused silica tubing can be directly used for UV detection without the fabrication of a detection window. Teflon-coated fused silica tubing also exhibits no sample cross-contamination. However, Teflon has high oxygen and water vapor permeability that causes the capillary to age and lose its flexibility. Poly (etheretherketone) (PEEK) capillary tubing exhibits excellent chemical resistance, good biocompatibility, excellent stability (over a pH range of 0–14), high mechanical strength, and flexibility. Recently, Fujimoto et al.[29] successfully used PEEK capillary tubing to fabricate an open-tubular column for CEC. However, PEEK tubing is not UV transparent. Coupling of a detection window to the PEEK capillary column is necessary for UV detection, which can cause loss of column efficiency due to extra-column band dispersion. The commercially available minimum inner diameter for PEEK tubing is 65 μm that is too large for open-tubular CEC columns. Moreover, PEEK capillary tubing is not suitable for packed columns because it is hard to fabricate on-column end-frits in the tubing and it is extremely difficult to chemically modify the inner wall.

IV. TYPES OF COLUMNS USED IN CEC

CEC is generally performed in one of three types of columns, namely open-tubular (coated columns), packed columns, or continuous-bed (monolithic) columns.[30]

A. Open-Tubular Columns

An open-tubular column is a capillary bonded with a wall-supported stationary phase that can be a coated polymer, bonded molecular monolayer, or a synthesized porous layer network.[31,32] The inner diameters of open-tubular CEC columns should be less than 25 μm that is less than the inner diameters of packed columns. The surface area of fused silica tubing is much less than that of porous packing materials. As a result, the phase ratio and, hence, the sample capacity for open-tubular columns are much less than those for packed columns. The small sample capacity makes it difficult to detect trace analytes.

One way to increase the phase ratio of open-tubular columns is to use a polymeric stationary phase instead of a bonded molecular monolayer (Figure 6).

Such columns can be used for the CEC separation of small neutral compounds. The problem with this type of open-tubular column, however, is the low efficiency obtained due to the small diffusion coefficients of the analytes in the polymeric stationary phase, and the heterogeneous film structure caused by Rayleigh instability.

Another way to increase sample capacity is to increase the surface area for conventional chemically bonded phases. Two methods have been reported for increasing surface area: (a) laying down a thin layer of porous material on the surface and (b) etching the surface. The precursors and catalyst dictate the characteristics of the final sol-gel. Manipulation of the components and procedures in the sol-gel process can control the phase ratio and the retention properties of the sol-gel-derived phase.

Compared to packed columns, open-tubular columns have no bubble formation problems because end-frits are not needed, small internal diameter columns are used, and the stationary phase is homogeneous. The column length can also be easily shortened. Excellent mass sensitivity can be achieved by using capillaries with smaller inner diameters. The EOF in an open-tubular column is higher than that in a packed column because a greater

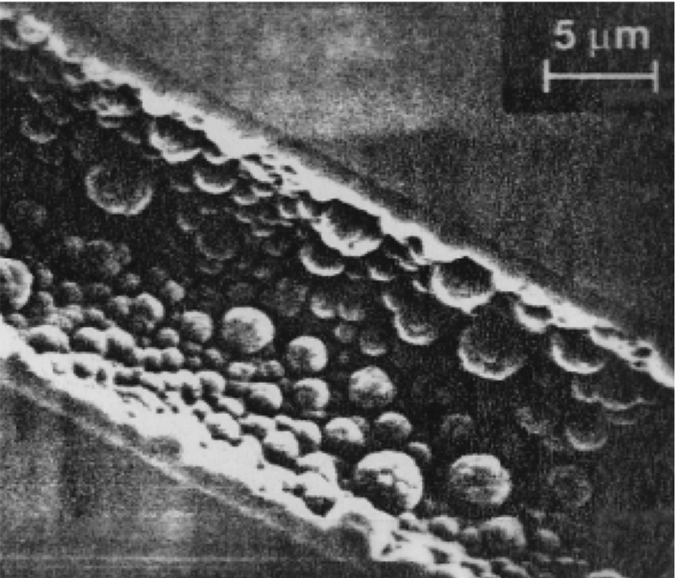

FIGURE 6 **Scanning electron micrograph of a polymeric porous layer in capillary column. (Reproduced with permission from reference 33.)**

voltage can be applied due to fast heat dissipation from the narrow column. However, open-tubular columns suffer some serious disadvantages compared to packed columns. On-column UV detection is difficult with open-tubular columns because the optical path length (the column inner diameter) is short and difficult to align. Although many efforts have been paid to increase the surface area and, hence, phase ratio of open-tubular columns, the retention factors for small molecules in open-tubular columns still are much lower than those in packed columns. A further increase of the surface area is necessary to improve sample capacities. To increase the surface area, the laying down of multiple porous layers deserves some investigation. Deeply etching capillaries with thick walls and narrow inner diameters followed by coating with a porous sol-gel may also significantly increase the phase ratio.

B. Packed Columns

Capillaries packed with a stationary phase are the most widely used columns in CEC.[33–35] Suitable phase particles are packed into the capillary and frits are employed at the ends to retain the bed. The retaining frits are mainly produced by hydrothermal or sol-gel fritting technologies.[27] A comparative study of the utility of sol-gel and hydrothermal fritting technologies demonstrated that the tetraethoxysilane (TEOS) sol-gel procedure (Figure 7) was a fast and reliable procedure, producing packed columns with comparable efficiencies (170,000–190,000 m^{-1}) to those obtained using the hydrothermal fritting technology.[15]

The CEC phases must be capable of carrying a charge to generate an EOF and appropriate moieties to facilitate the chromatographic processes. Silica-based reversed-phase packing materials have been most widely used in CEC. The use of polymeric and mixed-mode bonded particles has also been reported.[36] For the silica-based phases, the carbon chains bonded on the silica surface provide the retention and selectivity for analytes, and the residual silanol groups on the surface of the silica are ionizable and generate the EOF.

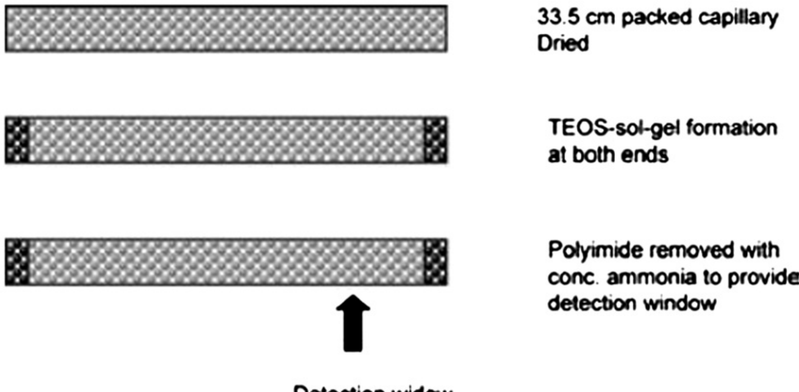

FIGURE 7 **CEC-packed capillary with TEOS-sol-gel frits. (Reproduced with permission from reference 27.)**

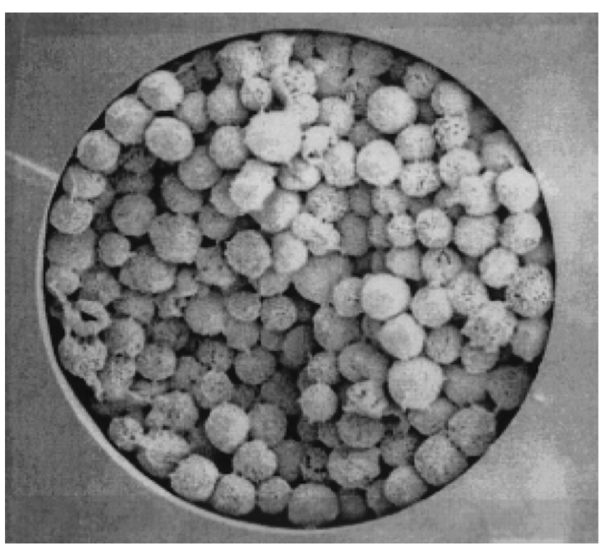

FIGURE 8 **Scanning electron micrograph of sol-gel bonded particles.[37]**

The EOF generated with such packing materials is strongly dependent on the pH of the mobile phase due to the pH-dependent dissociation of the silanol groups. At acidic pH values, where the EOF values are very low, the analysis of neutral and negatively charged analytes becomes impractical. To provide suitable analysis times, the pH range for CEC using silica-based reversed-phase packing materials is usually between 5 and 10. Moreover, the silica-based packing materials can break down gradually at pH values less than 2 and greater than 10.

Packed capillary columns (Figure 8) have a greater sample capacity than open-tubular columns because of the increased surface area and, hence, greater phase ratio. Greater sample capacities result in increased sensitivity and selectivity. More than 95% of the CEC columns

reported in the literature are packed columns. There are however technical problems with packed CEC columns that include the difficulty in their preparation (selection of packing materials, column packing, and the preparation of end-frits), lack of reproducibility, their tendency to act as a catalyst for bubbles, their unpredictable influence on the electroosmotic flow and band-spreading.[36,37]

C. Continuous-Bed or Monolithic Columns

A continuous-bed or monolith is a capillary containing a wall-supported porous continuous bed that is formed in situ. These columns have been developed for CEC use in recent years.[38–40] The surface chemistry can be functionalized to convert it into a phase with the desired chromatographic properties.[41] Monolithic columns are stable and have shown great potential for CEC due to the absence of a requirement for retaining frits, thereby eliminating the drawbacks in OT-CEC and packed columns.

The preparation of monoliths dates back to the 1970s and was advanced by Ross and Jefferson[42] and Hileman et al.[43] who prepared monolithic open-pore polyurethane foam for both high-performance liquid and gas chromatography. However, their use was short lived due to their excessive swelling and softening in some solvents. Satisfactory continuous bed media were developed by Hjertén et al.[44] in 1989 and were successfully used for chromatographic separations. In the early 1990s, Svec and Fréchet[45] introduced the rigid macroporous polymer monoliths, which had numerous applications and gradually generated wider research into both polymer and silica-based monoliths.[46–48] Monolith formats have found applications in solid-phase extraction,[49] sample enrichmeny,[49,50] analysis of pharmaceuticals,[51,52] environmental chemicals and biomolecules.[53,54] Monoliths or continuous beds can be classified into two general categories, i.e., silica-based and organic polymer-based monolithic columns.

1. Silica-Based Monoliths

Silica-based monolithic columns (Figure 9) are generally prepared using sol-gel technology. This involves the preparation of a sol solution and the gelation of the sol to form a network in a continuous liquid phase within the capillary. The precursors for the synthesis of these monoliths are normally metal alkoxides that react readily with water. The most widely used are alkoxysilanes such as tetramethoxysilane (TMOS) and TEOS.

The sol-gel process for the preparation of silica-based monoliths involves heat treatment at lower temperatures for gelation and aging and the formation of mesopores by heating at high temperatures.

Even though the technology for developing continuous silica supports dates back to 1970, useful silica monoliths for chromatographic applications only began to appear in 1996 when Fields[46] developed a silica-based reverse-phase column for HPLC in a fused-silica column using a potassium silicate solution with 10% w/v ODS in dry toluene. Although the method can produce a continuous silica xerogel with mean pore diameter of approximately 2 μm, the morphology of the material was heterogeneous. Minakuchi et al.[47] developed the sol-gel method in preparing a porous silica rod by hydrolytic polycondensation of alkoxysilanes accompanied by phase separation in the presence of water-soluble organic polymers. The process of gelation, aging and drying was involved in the preparation procedure The monolithic silica thus formed can be surface modified with octadecyldimethyl-*N*,*N*-diethylaminosilane to form a reversed-phase chromatographic surface. The formation of sol-gel columns with embedded particles such as octadecylsilane (ODS) particles within the capillary has been reported by Tang et al.[56] Significant advantages observed over the

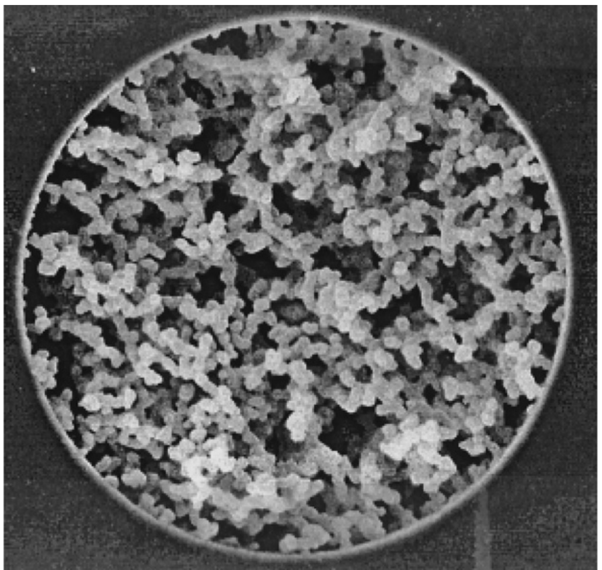

FIGURE 9 Scanning electron micrograph of monolithic silica-based capillary column. (Reproduced with permission from reference 55.)

conventional particle-packed columns included the production of a lower current without the formation of bubbles, a higher EOF velocity due to silanol groups in the matrix, and an increased efficiency (*N*). Since the year 2000, there has been a rapid development of the methodology in constructing sol-gel-based columns. In a typical sol-gel procedure TMOS was added to a solution of polyethylene oxide (PEO) in water with a suitable catalyst, such as acetic acid. The mixture was stirred at 0°C for 30 min. The resultant homogeneous solution was poured into a cylindrical polycarbonate mould and allowed to react at 40°C. Gelation occurred within 2 h and the gelled sample was subsequently aged at the same temperature for 24 h. The wet silica rod produced was washed with distilled water and then immersed in an aqueous solution of ammonium hydroxide in order to tailor the mesopore structure. Evaporation drying and heat treatment were successively performed, which led to the decomposition of organic constituents and stabilization of the surface of the hydrophilic silica gel. Since the gel shrunk during the aging and drying process, the resultant silica gel had to be encased in heat-shrinking polytetrafluoroethylene (PTFE) tubing and compressed with external pressure to ensure that there was absolutely no space between the silica rod and tube.[57] Ishizuka and co-workers in 2002 perfected silica rods by studying the mechanism of pore formation and its relevance to the separation of proteins and peptides.[58] The process of macropore (> 50 nm) and mesopore (between 2 and 50 nm) formation could be controlled with a temperature-monitored post-gelation treatment. Derivatization of the silica-based monolith has been achieved with compounds such as octadecyldimethyl-*N*,*N*-diethylamino-silane and *N*-octadecyldimethyl [3(trimethoxysilyl)propyl] ammonium chloride (C_{18}-TMS) and TMOS to produce positively charged surfaces.[59] The chemical structure of C_{18}-TMS is unique and specifically suitable for the preparation of CEC monolithic phases. The structural design of this precursor contains three important features: (i) The octadecyl moiety capable of providing chromatographic interactions with the analytes. (ii) Three methoxy groups attached to the silicon atom that can undergo hydrolysis, followed by condensation. This facilitates the in situ creation of a chemically bonded monolithic matrix throughout the entire solution-filled

inner-capillary volume. (iii) The positively charged quaternary ammonium moiety that can provide a positive surface charge within the matrix to support the essential EOF in CEC. Separation efficiencies of up to 1.75×10^5 plates m^{-1} were achieved on a 50 cm × 50 μm ID column using polycyclic aromatic hydrocarbons and aromatic aldehydes and ketones as test solutes. Again SCXs have been incorporated to produce mixed-mode columns. Surface modification has been achieved by octadecylsilyation to C_{18} phase by an on-column reaction for evaluation of chromatographic performance.[58]

2. Polymer-Based Monolith

Porous organic polymers (Figure 10) are potential electrochromatographic stationary phases for the analysis of pharmaceuticals. The polymer network is generally formed inside the capillary by a stepwise chain polymerization reaction. Polymerization reaction mixtures usually consist of a combination of monomers and cross-linker, initiator, and a porogenic mixture of solvents.

A variety of monomers can be employed to manufacture the final monolith. A combination of charged and hydrophilic monomers generates an EOF, while a combination of uncharged and hydrophobic monomers allow reversed-phase interactions. The cross-linker concentration can be adjusted to vary the degree of cross-linking that influences the overall porosity. An initiator is required to instigate the stepwise chain reaction, and is often 2,2′-azo-bis-isobutyronitrile (AIBN) that can be initiated by UV light or thermal treatment. The polymer monolith precipitates and becomes insoluble in the reaction medium as a result of both the cross-linking and choice of porogen (a poor solvent for the polymer), which is commonly a mixture of alcohols.

In general the preparation of a polymer monolithic rod is performed as a multi-step procedure (Figure 11). Generally, the stages involved are pre-treatment and preparation of the monolithic matrix by polymerization and derivatization or functionalization. Pre-treatment of the bare capillary is sometimes needed in order to obtain good physical stability. Most columns are therefore polymerized in silanized columns. The capillary column is first washed with a strong alkaline solution such as 1.0 M sodium hydroxide so that the siloxane groups at

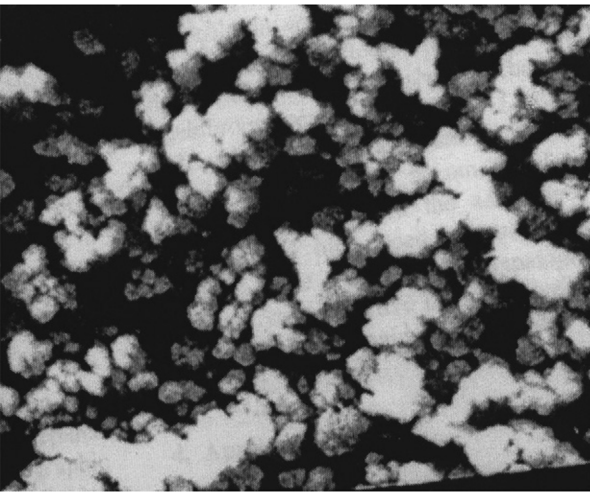

FIGURE 10 Scanning electron micrograph of the inner part of a methacrylate-based monolith. (Reproduced with permission from reference 59.)

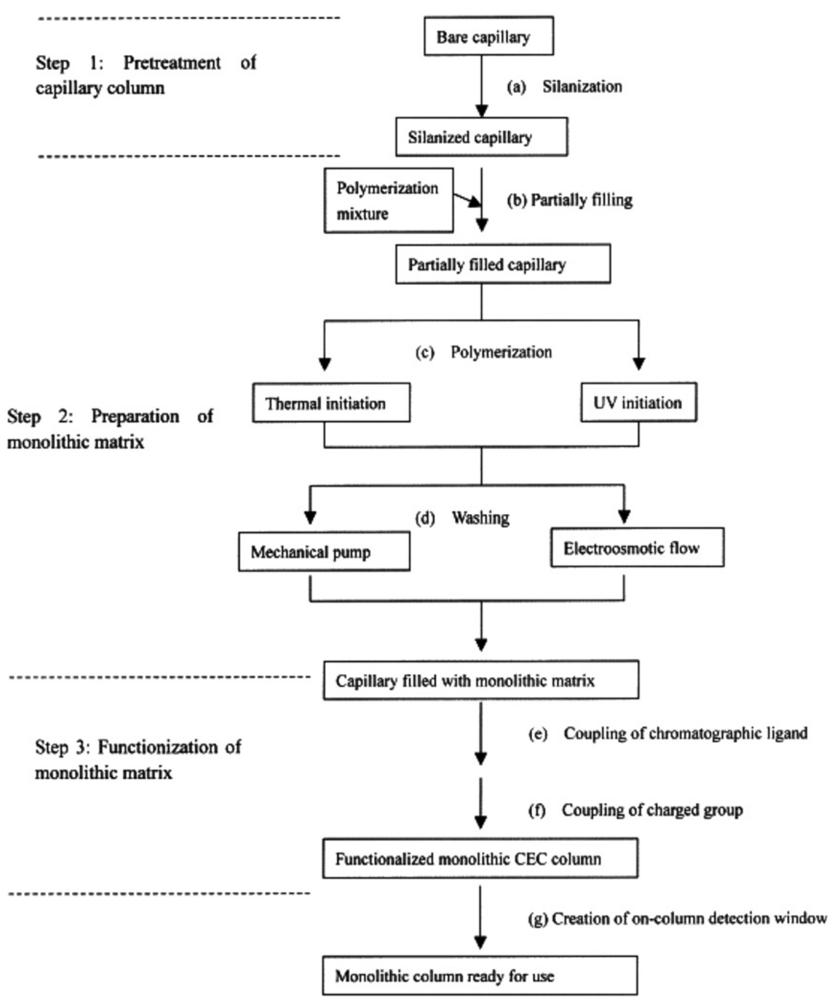

FIGURE 11 Schematic representations for the preparation of monolithic CEC columns. (Reproduced with permission from reference 59.)

the inner surface of raw fused-silica capillaries are hydrolyzed. This increases the density of the silanol groups that serve as anchors for the subsequent silanization. Then the capillary column is filled with a solution of a bifunctional reagent, typically γ-methacryloyloxypropyl-trimethoxysilane (γ-MAPS) also called 3-trimethoxysilylpropyl methacrylate (TMSPM) in acetone, and allowed to react for a period of time. In the derivatization reaction the bifunctional coupling agent, γ-MAPS reacts with the silanol groups on the silica surface through the trimethoxysilyl moiety. The other functionality, the methacrylate group, is the anchor for the monolith to be synthesized in a radical polymerization reaction. In this case, Si–O–Si–C bonds are formed between the capillary wall and the reactive methacryloyl groups that are available for subsequent attachment of the monolith to the wall during the polymerization reaction.[60,61]

After pre-treatment, the monolithic mixture consisting of the monomers, cross-linker, initiator, and the porogenic solvents is filled into the capillary and then sealed at both ends.

The polymerization is then initiated thermally (55–80°C) or by UV light. The seals are then removed and the monolithic capillary is then washed by flushing solvent through the column to remove the porogens and other soluble compounds that remain in the monolithic rod after polymerization. After the monolith support is prepared, the column can be subsequently derivatized depending on the chromatographic property required. For example, functional groups such as hydrophobic groups used in reversed-phase chromatography can be coupled to the matrix. In addition, charged groups can also be coupled to the matrix in order to generate EOF in CEC.

Three main types of polymer-based monoliths are polymethacrylate-based monoliths where methacrylate forms the major component of the monomers for polymerization, polyacrylamide-based monoliths where cross-linked polyacrylamide is synthesized directly within the capillary, and polystyrene-based monoliths that are usually prepared from styrene and 4-(chloromethyl) styrene as monomers and divinylbenzene (DVB) as the cross-linker.

A new type of CEC column has been prepared with a charged polymer layer on the inner wall of the capillary and a neutral monolith as the bulk stationary phase (Figure 12). After silanization of the capillary wall, polyethyleneimine was covalently bound to the wall, to provide charged moieties.

Afterwards, a bulk monolith was prepared in situ by copolymerization of vinylbenzyl chloride and ethylene glycol dimethacrylate. The benzyl chloride functionalities were then hydrolyzed to benzyl alcohol groups and the monolith used in separating a peptide test mixture.

A summary of some of the typical phases used in CEC is presented in Table 4.

V. ANALYTE DETECTION IN CEC

The sensitivity of devices such as UV detectors depends on the optical path length in accordance with the Beer–Lambert's law. In CEC, there is a requirement for small volume and

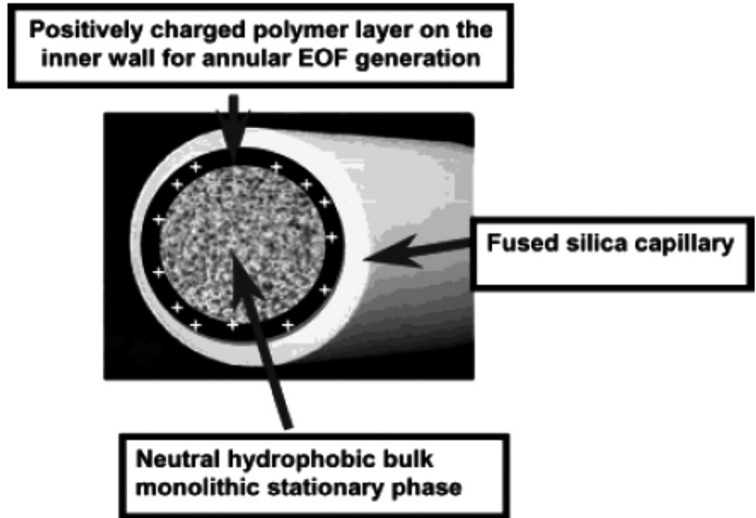

FIGURE 12 Wrapped structure of a monolithic column with annular EOF generation. (Reproduced with permission from reference 62.)

TABLE 4 Stationary Phases Employed in Different CEC Modes

	Dynamic	**Static**
Open-tubular column	Polymeric surfactants	Neutral hydrophilic layer, e.g., polyacrylamide derivatized
	Charged polymers	Epoxide-based, diol, polyethylene glycol.
	Neutral polymers, e.g., polysaccharides, polyvinyl alcohol, polyethylene oxide	
Packed columns	Porous and non-porous silica	
	Lipophilic modified silica gels, e.g., C18	
	Mixed mode phases, e.g., C8/SCX	
	Silica-based	**Polymeric**
Monoliths	Silica and lipophilic modified silica	Polyacrylamide, polystyrene, and polymethacrylate-based phases
	Mixed-mode (alkyl/charged moiety e.g. sulfonic acid, quaternary ammonium compounds)	Affinity
		Chiral
	Ion exchange	Molecular imprinted polymers
	Chiral	

sensitive devices as a result of the short optical path length and low peak volumes.[63] UV monitors are the most common detectors used in CEC analysis and the detection is carried out *on-column* (i.e., through the unfilled section of a CEC capillary). However, the short optical path lengths (50–100 μm) and the fact that the incident light impacts on a curved surface rather than a flat surface results in losses in sensitivity.[63] UV detection in CEC can be carried out through the stationary phase (*in-column* detection) after removal of the polyimide coating on capillaries or by using columns made from transparent materials. Although the background noise is usually increased with *in-column* detection, the high concentration of analyte zones in the stationary phase results in signal enhancement compared to that obtained with the eluting zones in *on-column* detection.

Fluorescence or laser-induced fluorescence (LIF) detectors can be used in CEC to obtain higher sensitivities compared with UV detection. However, these detection systems are only limited to analytes that are intrinsically fluorescent or can be derivatized to fluorescent analogues.

The coupling of a mass spectrometer to CE and CEC provides a powerful system for the analysis of pharmaceuticals and complex biological mixtures. This can replace or complement other conventional detection methods such as UV, electrochemical, or LIF that provide less structural information. The use of mass spectrometer as a detector enhances the usefulness of the CE and CEC and allows an efficient separation and identification of complex mixtures, obtaining structure and/or molecular mass information.[64,65] The choice of mass analyzers used in CE/CEC–MS depends on factors such as sensitivity, mass resolution, requirement for structural elucidation, and the type of application (Table 5). The analyzers that have been used in CEC analysis include time-of-flight (TOF), quadrupole (Q), ion-trap (IT), fourier

TABLE 5 General Specifications and Features for Mass Analyzers Used in CE/CEC-MS[66]

Criteria	Q	IT	DFMS	TOF	FTICR
Mass range	2–1000 Da	10–1000 Da	2–4000 Da	5–1500 Da	10–3000 Da
Mass accuracy	100 pm	100 pm	$<$ 5 ppm	5–10 ppm	1–2 ppm
Speed	Low	Medium	Low	Very high	High
Monitoring mode	Full scan, SIM	Full scan, SIM, product ion scan	Full scan, SIM	Full range spectrum	Full scan, SIM, product ion scan
Sensitivity	Medium	Medium	High	High	High
Mass resolution	Unit mass resolution	Unit mass resolution	$>$ 10,000	1000–10,000	$>$ 10,000
MS/MS	None (except triple quadrupole MS)	MS^n	None	None	MS^n
Performance/cost	Low	Low	Very high	High	High

Key: Q = Quadrupole; IT = ion trap; DFMS = Double focusing magnetic sector; TOF = Time of flight; FTICR = Fourier transform ion cyclotron resonance.

transform-ion cyclotron resonance (FTICR), and double focusing magnetic sector (DFMS) instruments.[66]

Due to their comparatively low costs and easy operation, quadrupole instruments are the most common instruments used for hyphenation in CEC analyses. However, these instruments only operate at low mass resolution. Sensitivity can be enhanced by operating in selected ion monitoring mode instead of full scanning acquisitions. Unfortunately, this leads to the loss of structural information. The expansion of biological applications has been largely accommodated by the TOF, quadrupole mass filter, and ion-trap instruments. The major advantage of TOF is its potential for speed, resolution, and good mass accuracy.

VI. APPLICATIONS OF CAPILLARY ELECTROCHROMATOGRAPHY

The highly polar nature of pharmaceuticals containing amine functional groups makes the use of chromatography quite complex. Ion pairing reagents and stringent column regeneration are often necessary to reduce non-specific ionic interactions that occur with reversed-phase chromatography. Both CE and CEC are appropriate for the analysis of basic compounds because their properties can be exploited to enhance resolution. The most common and simplified format of operation involves the use of a specific acidic pH such that ionization of the silanol groups is suppressed whilst the analyte's amine functional groups are maximally ionized. The incorporation of a stationary phase in the bare fused silica capillary in CEC offers it orthogonal selectivity with respect to CE.

One of the challenges of drug discovery is in developing analytical methods for the pharmacokinetic profiling of new drug candidates and hence, important to this process is the development of rapid, generic methods that allow the screening of large numbers of compounds isolated from complex sample matrices. While CE has been used to quantify drugs in biological fluids and matrices, the application of CEC would require extensive sample cleanup to prevent poisoning of the phase by macromolecules such as proteins. The use of clean samples will make CEC a more versatile analytical screening tool.

The use of μLC and voltage-assisted μLC in CEC have been applied successfully to the separation of neutral and basic compounds. Figures 13 and 14 illustrate the potential of the technique in the analyses of complex mixtures.[67]

CEC has a variety of applications in the analysis of pharmaceuticals, biochemicals, food, and industrial and environmental substances. However, for the purpose of this chapter we shall limit ourselves to pharmaceutical applications.

A. Pharmaceutical Applications of CEC

Many research groups have studied the potential applicability of CEC for the separation of different types of pharmaceutical compounds. However, an important issue to be addressed before CEC will be accepted as a method for routine analysis is the repeatability of the experiment to provide quantitative results.

CEC has found wide pharmaceutical applications including impurity profiling and analyses of pharmaceuticals in biofluids, in proteomics and genomics, and in the food industry (Table 6).

For most of the applications outlined in Table 6, high precisions have been obtained. For example, for the determination of barbiturates in human serum, the intra-day precision for spiked serum samples was better than 2%, and the inter-day precision better than 5%.[76] Figure 15 shows the chromatograms of a blank and a spiked serum sample.

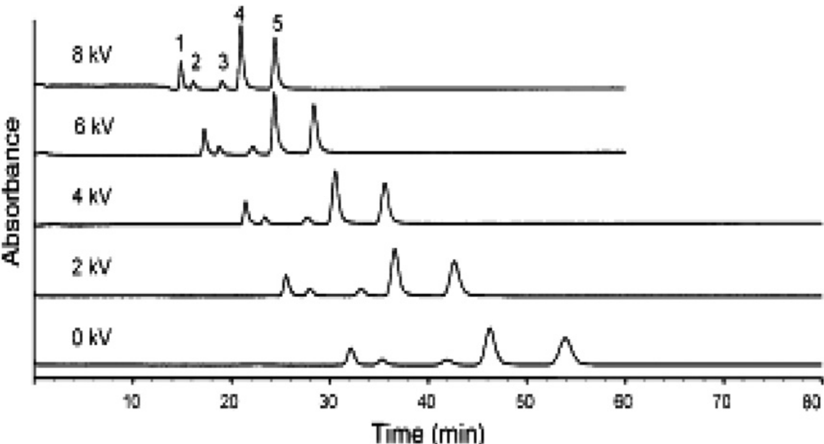

FIGURE 13 Separation of the neutral analytes: thiourea (1), benzamide (2), anisole (3), benzophenone (4), and biphenyl (5) using a 240 × 0.1 mm capillary packed with 3 μm Hypersil CEC Basic C18 in micro-LC and voltage-assisted micro-LC mode; 1.0 MPa of pressure applied to the inlet vial; applied voltage as stated in figure; mobile phase ACN/50 mM Tris buffer pH 7.8, 8:2 (v/v); cartridge temperature, 20°C; Detection, 254 nm; Injection hydrodynamic 0.8 MPa/15 s. (Reproduced with permission from reference 67.)

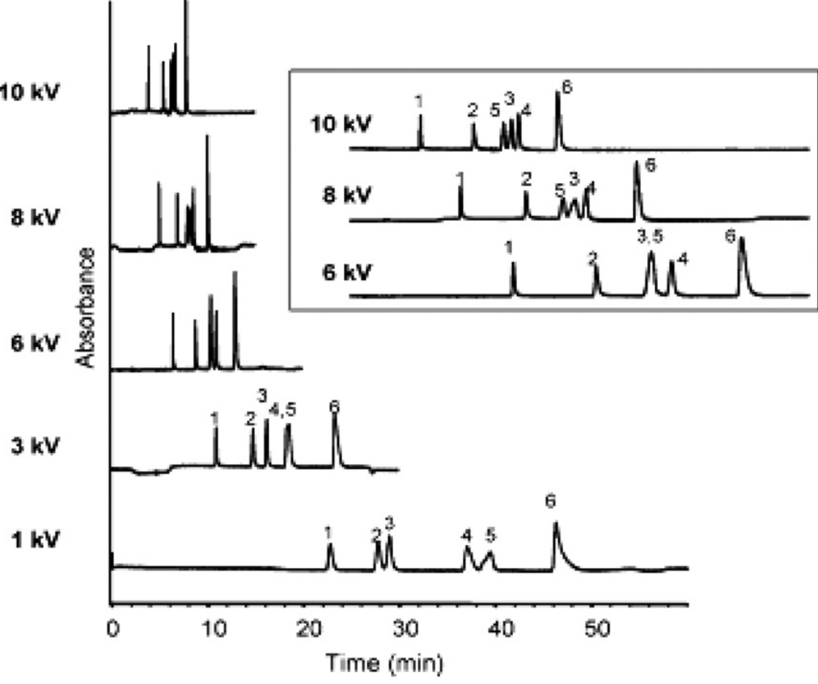

FIGURE 14 Separation of six basic analytes: benzylamine (1), procainamide (2), AR-D080301 (3), AR-R12924 (4), diphenhydramine (5), and nortriptyline (6) by micro LC and voltage-assisted micro-LC using a 240 × 0.1 mm capillary packed with 3 μm Hypersil CEC Basic C18 material. Injection, 0.8 MPa/15 s. Conditions: Applied pressure to inlet vial, 1.0 MPa; voltages as shown in the figure applied across the capillary (340 mm), mobile phase: ACN:H_2O:KH_2PO_4 buffer (50 mM pH 2.3) 4:4:2 (v/v/v); 20°C; 210 nm. (Reproduced with permission from reference 67.)

TABLE 6 Selected Applications of CEC

Applications	Details	Technique	Reference
Drugs and impurities	Separation of amphenicol and macrolide antibiotics	X-Terra RP18 column with anionic surfactant	68
	Analysis of non-steroidal anti-inflammatory drugs (ibuprofen, ketoprofen, naproxen, fenoprofen, flurbiproen, and suprofen)	Histidine-coated capillary columns	69
	Impurity profiling of ketorolac, a chiral non-steroidal antiinflammatory drug	C_{18} packed columns	70
	Impurity profiling of a non-steroidal analgesic drug	A commercial packed column (3 μm particles, C18)	71
Chiral separations	Non-acidic pharmaceuticals	Non-aqueous CEC using teicoplanin chiral stationary phase	72
	Warfarin, praziquantel	Affinity CEC using a strong anionic exchange phase	73
Bioanalysis	Determination of non-steroidal antiinflammatory drugs (ibuprofen and related compounds) in urine.	Fused-silica capillary of which the wall was modified with histidine	74
	CEC separation of barbiturates	5 μm Particle packed columns	75
	Determination of barbiturates in human serum	Capillary columns packed with polysulfoethyl A (prepared by chemical bonding of a polypeptide, poly (2-sulfoethyl aspartamide), to silica particles)	76
	Determination of oxazepam in a urine sample	Capillary column packed with 3 μm C18 particles	77
Amino acids	Separation of amino acids	Proline-coated capillary columns	78
	Histamine and its methylated metabolite in human urine samples	Monolithic ODS column	79
	Determination of amino acids in the analysis of dialyzed rat cerebrospinal fluid	Methacryloxypropyltrimethoxysilane (MPTMS) polymerized monolith	80,81
	Chiral CEC separations of amino acids	Cross-linked 2-hydroxyethyl methacrylate (HEMA) polymer monolith incorporated with	82

TABLE 6 (Cont.)

Applications	Details	Technique	Reference
		a chiral monomer derived from cinchona alkaloid	
	Enantiomeric separation of amino acids	Silica particles derivatized with a chiral selector ((teicoplanin aglycone (TAG)) and polymerized with a methacrylamide	83
	Enantioseparation of dansyl amino acids, free amino acids, hydroxy acids, and dipeptides	Monolithic sol-gel column modified with L-hydroxyproline	84
	Determination of L and D forms of *N*-nitro-arginine (NNA)	75 μm ID capillary packed with C18 particles	85
Peptides	Separations of peptides, including highly basic peptides	Photografted columns	86
	Separation of a peptide mixture	Monolithic column with a zwitterionic stationary phase	87
	Peptide mapping (the separation of the tryptic digest) of β-lactoglobulin and human growth hormone (hGH).	Silica particle packed columns bearing an embedded cationic quaternary amine and a C_{23} chain	88
	Separation of a peptide mixture	Butyl methacrylate columns bearing sulfonic acid functionalities	89
	Separation of a tryptic digest of cytochrome *c*	ODS particle packed columns	90
	Analysis of neuropeptides	Open tubular CEC-MS (OT-CEC-MS) bearing positively charged alkylamino silyl monomers	91
	Analysis of cytochrome *c*	OT-CEC modified with different surface moieties (butylphenyl, cholesteryl, and biphenyl compounds)	92–95
	Peptide analysis	Glycidyl methacrylate monolithic	96
	Peptide analysis	Coated open tubular CEC	97
	Analysis of tryptic digest of BSA	CEC-MS using methacrylate monolith, modified with *N*-ethylbutylamine	98
	Analysis of charged peptides	CEC-MS using methacrylate monolith, modified with *N*-ethylbutylamine (investigation of quality of spectra using volatile and non-volatile buffers)	98

	Separation of water-soluble and membrane proteins	Cationic stearyl-acrylate monoliths	99
	Affinity-based CEC separations for the analysis of mannose-binding proteins	Mannan, the polysaccharide portion of the mannoprotein from yeast, was immobilized on a positively charged methacrylate-ester-based monolith to the epoxy groups	100
	Separation of proteins	Butylacrylate monolithic column	101
Nucleosides and nucleotides	Separation of nucleosides	Methacrylate monolithic column	102
	Separation of nucleic acid bases and their nucleosides	Silica monolithic columns with a cyano phase	103
	Separation of nucleosides and nucleic acid bases	Mixed-mode CEC stationary phase modified with a sulfonated naphthyl compound	104
	Separation of nucleotide bases	A molecular imprinted polymer coating comprising 9-ethyladenine for the OT-CEC	105
Food and natural products	Separations of plant sterols and related sterol esters	Silica particle packed capillaries	106
	Determination of preservatives (sorbic and benzoic acid, parabens) in food products	Methacrylate-ester-based monolithic column	107
	Determination of phenylurea herbicide residues in vegetables and vegetable processed food	Capillary column packed with 5 μm C18 stationary phase particles	108
	Separation of flavonoids from extracts of *Adinandra nitida* leaves	Methacrylate monolithic columns	109
	Separation of the basic alkaloids extracted from *Coptis chinensis Franch* and *Rhizoma corydails*	Silica monolith	110
	Separation of triterpenoids from a crude extract of *Ganoderma lucidum*	A hybrid organic-inorganic monolith	111
	Separation of triterpenes	3 μm ODS particle packed CEC columns	112

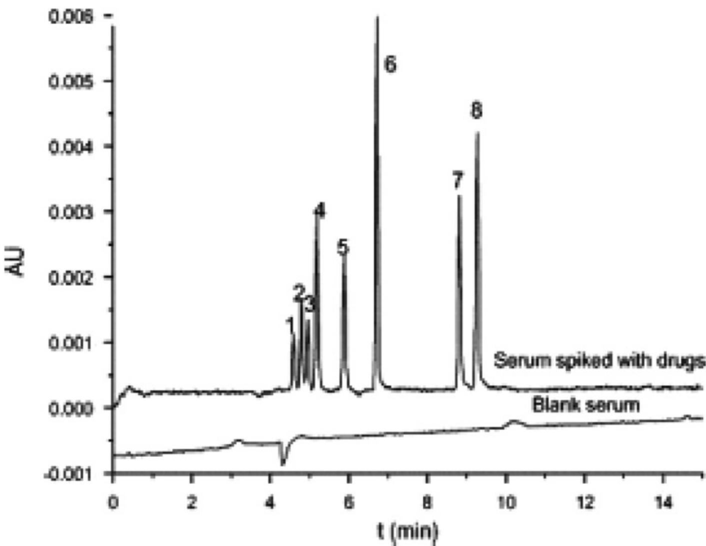

FIGURE 15 **Electrochromatograms obtained for the separation of basic drugs spiked in a human serum compared with a blank in a hydrophobic interaction CEC. Column: 5 μm 300 Å polysulfoethyl A particles, 20 cm packed length, 50 μm ID; mobile phase: ACN/TEAP buffer (80:20); applied voltage, 10 kV; detection at 214 nm. Drugs: (1) amobarbital; (2) phenobarbital; (3) barbital; (4) caffeine; (5) sulfanilamide; (6) theophylline; (7) 2,4-dimethylquinoline; (8) propranolol. (Reproduced with permission from reference 76.)**

The order of elution of peptides (charged compounds) is governed by a combination of electrophoresis and partitioning, with hydrophobic as well as electrostatic contributions.[89] In this study it was demonstrated that sulfonic acid functionalities in the methacrylate monolith provide high stability and maintain a constant EOF over a wide range of pH (2–12). It was also demonstrated that a better separation of a mixture of therapeutic peptides was obtained at high pH values (Figure 16) due to the suppression of electrostatic attraction.

In a related study this group also demonstrated the use of non-volatile solvents in CEC–MS without compromising the quality of spectra that has also been demonstrated[113] using polymer-based monolithic column prepared by in situ copolymerization of butyl methacrylate with sulfonic acid functionalities.

Ammonium formate and phosphate buffers CEC–ESI–MS for the analysis of leucine enkephalin and substance P, which are respectively singly and triply charged peptides. The good mass spectra obtained for the peptides in both the volatile and non-volatile buffers (Figures 17 and 18) indicate the non-crystallization of the non-volatile buffer which is further diluted by the sheath liquid.

The sensitivities obtained with the volatile buffer (ammonium formate) and non-volatile buffer (sodium borate) in CEC–ESI–MS were assessed by comparing the spectra obtained in the two modes using leucine enkephalin and substance P that carry charges of +1 and +3, respectively.

Figure 17 shows the mass spectra obtained for leucine enkephalin (charged state +1 at pH 2.8 and −0.5 at pH 9.5) and Figure 18 for substance P (charged state +3 at pH 2.8, and +1.5 at pH 9.5). The analytes undergo ionization at the interface to yield the positively charged ions as a result of the addition of the formic acid sheath liquid to the alkaline buffer.

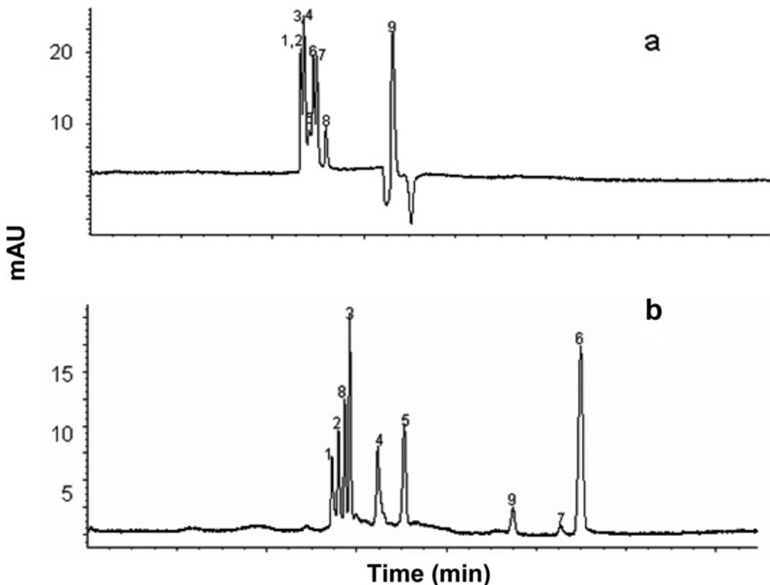

FIGURE 16 **Separation of a nine-peptide mix under acidic conditions (a) and basic conditions (b). Peak assignment [bradykinin (1), vasopressin (2), luteinizing hormone releasing hormone (3), substance P (4), bradykinin fragment 1–5 (5), leucine enkephalin (6), methionine enkephalin (7), bombesin (8), and oxytocin (9)]. Conditions: 100 μm ID, 375 μm OD, total length 33.5 cm, effective length 25.0 cm; 10 kV; 200°C; 5 kV/2 s, detection at 206 nm; 6 bar pressure in both vials [ACN/H2O/50 mM ammonium formate buffer pH 2.8 for (a) and 50 mM sodium borate buffer pH 9.5 for (b) (70:10:20 by volume for each mobile phase).[89]**

This observation widens the potential applications of this monolithic chemistry in that the high selectivities obtained with non-volatile buffers do not have to be sacrificed to obtain good ESI spectra.

The application of monoliths for the analysis of proteins by CEC has been demonstrated by several workers. In these investigations the various mechanisms of interactions such as affinity, ion-exchange, and hydrophobic interactions have been demonstated.[99–105] Again it has been demonstrated that CEC produces shorter separation times and that a smaller amount of sample is required compared to conventional HPLC.

VII. TWO-DIMENSIONAL SEPARATIONS

Two-dimensional (2D) separation systems that involve the coupling of two separating techniques are of interest because of their increased peak capacity compared to one-dimensional separations. Several column-based two-dimensional separation schemes have been developed in order to help reduce the analysis time and labor involved with separate one-dimensional modes.[114] For these systems, several interfaces have been designed to inject the effluent from the first-dimension column into a second dimension including automated switching valves,[115] parallel columns in the second dimension,[116] flow gating,[117,118] and optical gating.[119] Two complementary techniques that can be coupled together relatively easily are microcolumn reversed-phase LC and capillary electrophoresis (CE).[118]

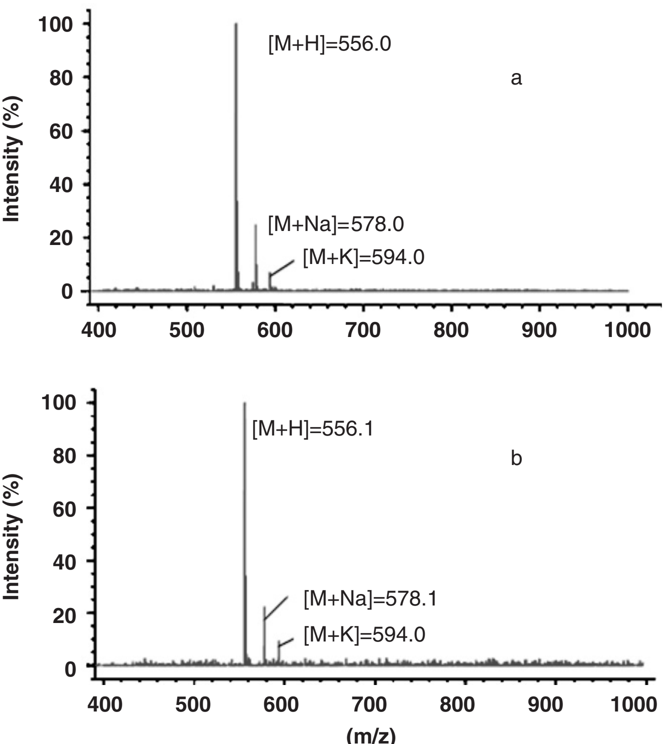

FIGURE 17 Mass spectrum of leucine enkephalin in CEC–MS. conditions: BMA monolith 100 μm ID, 375 μm sheath buffer 0.25% formic acid in methanol (30:70 by volume), sheath buffer flow rate (4.0 μL min^{-1}), nebulizing gas pressure (7.0 psi), drying gas flow rate (7.0 L min^{-1}), drying gas temperature (250°C), fragmentor voltage (70 V), electrospray voltage (3500 V) (a) ACN/H2O/ 50 mM ammonium formate buffer pH 2.8 (70:10:20 by volume) and (b) ACN/H_2O/50 mM sodium borate buffer pH 9.5 (70:10:20 by volume).[113]

Microfabricated fluidic devices (microchips) are potentially useful for multidimensional separations because high-efficiency separations can be achieved and small sample volumes can be manipulated with minimal dead volumes between interconnecting channels. Electrokinetically driven separation techniques demonstrated on microchips include CE,[120–122] MEKC,[123,124] electrochromatography,[125,126] and gel electrophoresis.[127,128] Recently, a microfluidic device for 2D separations of peptide mixtures using MEKC in the first dimension and CE in the second was reported.[129] Also, a two-dimensional separation system on a microfabricated device has been demonstrated using open-channel electrochromatography as the first dimension and CE as the second for the analysis of fluorescently labeled products from tryptic digests.[130]

Jia et al. (2005) developed a two-dimensional (2-D) separation system of coupling chromatography to electrophoresis for profiling *Escherichia coli* metabolites. Capillary LC with a monolithic silica-octadecyl silica column (500 × 0.2 mm ID) was used as the first dimension, from which the effluent fractions were further analyzed by CE acting as the second dimension.[131] Multi-dimensional separations have found wide applications in biomedical and pharmaceutical analysis.[132]

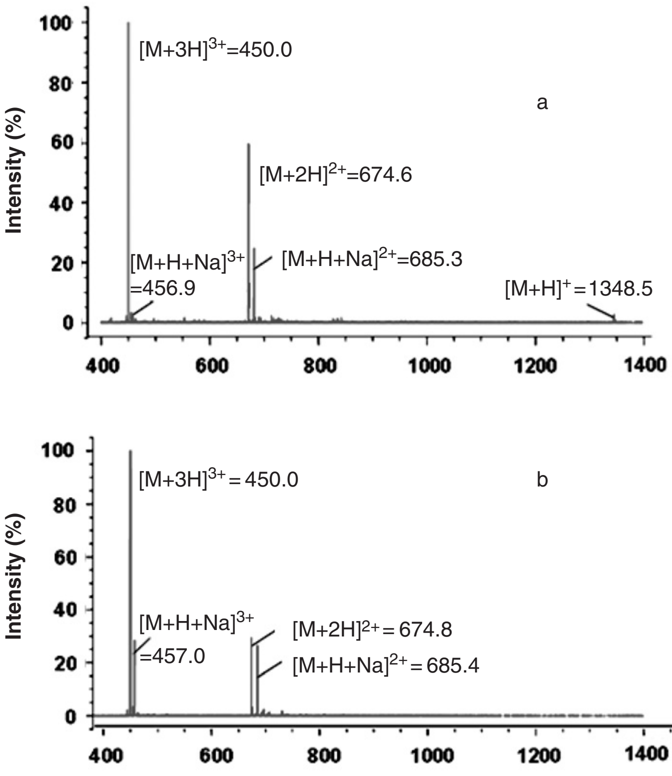

FIGURE 18 **Mass spectrum of substance P in CEC–MS. Conditions: BMA monolith 100 μm ID, 375 μm OD, effective length 50.0 cm, voltage 20 kV; 20°C; injection: 5 kV/2 s, sheath buffer 0.25% formic acid in methanol (30:70 by volume), sheath buffer flow rate (4.0 μL min–1), nebulizing gas pressure (7.0 psig), drying gas flow rate (7.0 L min^{-1}), drying gas temperature (250°C), fragmentor voltage (70 V), electrospray voltage (3500 V) (a) ACN/H2O/50 mM ammonium formate buffer pH 2.8 (70:10:20 by volume) and borate buffer (b) ACN/H_2O/50 mM sodium borate buffer pH 9.5 (70:10:20 by volume).[113]**

VIII. VALIDATING CEC METHODS FOR PHARMACEUTICAL ANALYSIS

CEC is still evolving and has not yet been adopted by the regulatory authorities in pharmacopoeial monographs. However, its use in analysis is subject to the same rigorous validation criteria as HPLC and CE methods. There is the requirement for *specificity, linearity, accuracy, precision* (repeatability, intermediate precision, and reproducibility), *robustness*, and *limits of detection and quantification*. System suitability tests, such as resolution and peak symmetry that provide an indication of peak efficiency in CE and HPLC, can also be applied to CEC analysis. Fabre and Altria[133] described the similarities in validation procedures in CE and HPLC with regards to the above criteria. These procedures are generally applicable to CEC that is effectively a hybrid of CE and HPLC. Some important considerations in the use of CEC are variations in capillaries from different lots and suppliers; electrolysis of buffers leading to changes in EOF and compound ionization with a resulting loss of repeatability of

selectivity; and robustness testing. A more comprehensive description of validation criteria for electrophoretic methods is provided by Fabre and Altria.[133]

IX. CEC—PROSPECTS AND POTENTIALS

The emerging of CEC and the increased scientific work on the preparation of different phases, characterization, and applications of the CEC columns have given much credence to their future potentials in microseparations. The fabrication and availability of different phases for analysis with both particle-packed and monolithic columns give the technique a great future. This is because a variety of mechanisms can be exploited in the analysis and separation of compounds that could otherwise be difficult to analyze with HPLC or CE alone. The ease of coupling CEC to sensitive detectors such as mass spectrometers for enhanced sensitivity, structural elucidation, and characterization bestows the technique with great versatility.

However, CEC can be seen as a complementary and not direct replacement to HPLC or CE. Despite the increased research into the fabrication and use of different types of phases in CEC, numerous factors are holding back the technique from steady development and routine use. Instrumentation is mainly restricted to the use of available CE instruments and a limited number of CEC instruments. This prevents the exploitation of operating factors such as pressure that influences separation in CEC. Problems with column preparation and the inter-batch and intra-batch reproducibility still present drawbacks in the technology. However, the gradual development from particle-packed columns to monolithic columns where frits are not needed has paved the way for increased analysis in CEC. Still faced with the problems of cumbersome preparation procedures and reproducibility, the use of two-dimensional separation techniques and gradual development of the chip technology has provided exciting separation breakthroughs.

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18

COUPLING CE AND MICROCHIP-BASED DEVICES WITH MASS SPECTROMETRY

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ABSTRACT

Recent developments in capillary electrophoresis coupled to mass spectrometry (CE–MS) are reviewed from over 400 references covering the significant challenges, issues, and results obtained with CE–MS analyses.

A particular attention is paid to instrumental aspects for successful hyphenation of CE with MS, regarding interfaces and ionization sources. A special section is dedicated to quantitative results, and various methodologies to achieve sensitive and repeatable analysis are considered.

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Dedicated applications of capillary zone electrophoresis (CZE) coupled to MS are discussed, particularly in the field of drug analysis. Development of other capillary-based electrodriven separation techniques such as non-aqueous capillary electrophoresis (NACE), micellar electrokinetic chromatography (MEKC), and capillary electrochromatography (CEC) hyphenated with MS are also treated. The successful coupling of these electromigration schemes with MS detection provides an efficient and sensitive analytical tool for the separation, quantitation, and identification of numerous pharmaceutical, biological, therapeutic, and environmental compounds.

Chip-based microdevices are finally discussed, regarding fabrication methods, designs, MS interfacing, and applications. Current capabilities and limitations for future use are emphasized considering improvements in methodology and instrumentation.

I. INTRODUCTION

Capillary electrophoresis (CE) is currently a powerful separation technique and has found numerous applications in various analytical fields. Several advantages such as a high-efficiency rapid method development, simple instrumentation, and low sample consumption are the main reasons for this success. UV-Vis spectrophotometry is probably the most widely used detection technique with CE because of the simplicity of the on-line configuration. However, its sensitivity, directly related to the optical pathlength afforded by the internal diameter of capillaries, which is in the μm range, is low and remains the major bottleneck of this technique. UV detection thus requires relatively high analyte concentrations, and is often unsuitable for numerous applications such as the bioanalytical field or impurities' peak profiling. Therefore, other detectors are required for the analysis of complex mixtures and many detection techniques have been already hyphenated with CE such as laser-induced fluorescence (LIF) and electrochemical (EC) detections. Because pharmaceutical and chemical structures do not always possess a strong chromophore or fluorophore, a derivatization procedure is often mandatory for a sensitive spectroscopic detection while EC is limited to electroactive substances. In this context, the on-line combination of high-efficiency CE separations and mass spectrometry (MS) is an attractive perspective and presents some major benefits. Among them, it enhances sensitivity and enables determination of co-migrating compounds with different mass-to-charge ratios (m/z). MS provides a higher potential for an unambiguous identification and confirmation of components in complex mixtures, and potentially gives some information concerning the structure of the separated compounds. Therefore, due to its high sensitivity and specificity, MS is a detector of choice for CE, and CE−MS coupling provides a powerful combination for performing rapid, efficient, and sensitive analysis.

The present chapter will review instrumental aspects for successful coupling of CE with MS, regarding interfaces, ionization sources, and analyzers. Practical considerations concerning different CE modes such as CZE, NACE, MEKC, and CEC coupled with MS will also be discussed and illustrated with a focus on recent pharmaceutical applications. Additionally, quantitative CE−MS will be presented and various methodologies used to achieve sensitive and repeatable analysis will be discussed. Finally, the final section of this chapter will give an overview on new devices (i.e., microchips), hyphenated to MS, in terms of fabrication methods, microchip designs, MS interfacing, and applications.

II. CE−MS COUPLING AND INSTRUMENTATION

Since the introduction in the mid-1980s, different MS systems and ionization interfaces have been described.[1−8] In the last few years, CE−MS techniques were successfully applied in a variety of fields with commercially available setups. Today, manufacturers propose user-ready CE−MS, and the technique can be routinely employed in numerous dedicated fields of

analytical chemistry. Technological developments in instrumentation continue to occur, and improvements in homebuilt instruments are regularly reported, which offer advantages in terms of reduced cost, capillary dimension, applied voltage, and interface modifications.

Several approaches have been investigated for CE−MS coupling. Most of the developed interfaces were initially used for LC hyphenation and adapted to the constraints of CE analysis. In establishing on-line CE−MS interfacing, several difficulties have to be considered. (i) The electrical connection at the interface side of the separation capillary must be achieved with the cathode end of the capillary directly connected to the MS interface. (ii) The typical flow rate in CE capillary, resulting from the electroosmotic flow (EOF), does not exceed 100 nL/min and is generally not compatible to conventional LC−MS interfaces. Therefore, either a make-up liquid or a miniaturized electrospray system should be implemented. (iii) The presence of nonvolatile constituents included into the background electrolyte (BGE), such as selectivity modifier additives, may be detrimental to the MS performance, owing to ion source or analyzer contamination.

A. Interfaces

As indicated above, modified LC−MS interfaces are generally applied in CE−MS.[9−17] In this section, attention is focused on the coupling devices that ensure an adequate operation of CE−MS interfacing. Two configurations are conventionally distinguished, with the addition of a make-up liquid or without additional liquid, in a miniaturized interface. A growing interest in the development and use of new sheathless interfaces occurs according to its improved sensitivity over the sheath-flow approach. However, due to its instrumental simplicity, versatility, and robustness, the coaxial sheath-flow interface still represents the most common approach when hyphenating CE with MS. With these interfaces, the electrical connection and flow rate compatibility issues are resolved, but a special care must be taken to avoid any loss in CE efficiency due to external peak broadening.

1. Sheath-Flow Interfaces

In addition to the electrical connection and flow rate issues, the analytes must be released from solvent molecules and brought into the gas phase. Liquid flow rates in conventional CE suggest that evaporation is easy but droplets can be formed at the tip of the CE capillary, which causes an unstable flow to the mass analyzer.[18] To overcome this problem, make-up flows are often used. Although the sheath liquid system is relatively easy to implement and use, it is rather demanding in terms of optimizing the operational parameters to obtain a stable and repeatable spray (e.g., capillary-tip position, sheath liquid flow rate, and composition). Two types of sheath-flow interfaces can be distinguished: the coaxial sheath liquid interface and the liquid-junction interface. The distinction between both interfaces is based on the integration of the make-up liquid: In the coaxial configuration, liquid addition takes place proximal to the MS orifice while the liquid-junction geometry provides the make-up liquid distal to the sprayer tip.

(a) Coaxial Sheath-Flow Interface:

With the coaxial interface, initially reported by Smith et al.,[19−21] the problem of irregular droplets formation is circumvented by using a sheath flow in the μL/min range surrounding the CE capillary and mixing with the CE buffer at the tip of the separation capillary. It provides electrical contact at the outlet end of the separation capillary, appropriate flow, and solvent conditions for ionization and evaporation, independent on the nature of the CE buffer solution. It is the most commonly used interface and can be easily implemented. Furthermore,

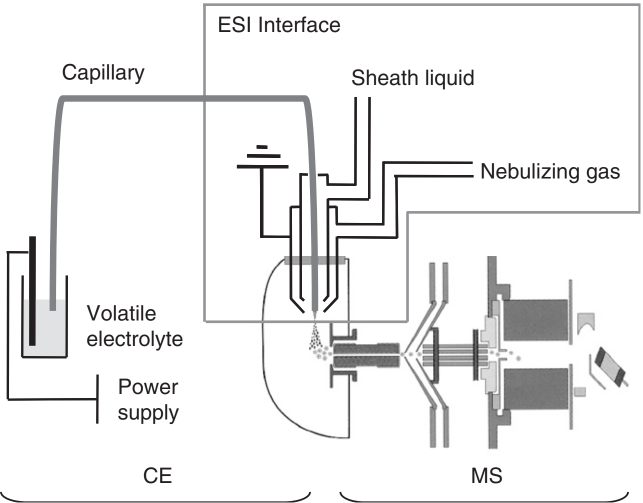

FIGURE 1 **Experimental setup for CE−ESI/MS with a coaxial sheath-flow interface.**

high buffer-salt concentrations, which have unintended negative effects on the efficiency of the spray, are diluted with the sheath liquid, and spray stability is usually improved. Dilution of the CE effluent by the sheath liquid flow rate does not significantly affect the sensitivity of the detection since the sheath liquid is also evaporated during the spray process. A number of papers have described sheath liquid interface optimization and practical aspects in setting up a coaxial CE−MS interface.[21−26] A typical coaxial sheath-flow interface is depicted in Figure 1.

(b) Liquid-Junction Interface:

This type of interface was first developed by Henion and co-workers[27,28] and further modified by different authors.[21,29−31] The electrical connection is established at the junction between the CE capillary and a second one leading to the mass spectrometer. In an open connection, the junction of both capillaries is usually placed under a liquid reservoir together with the electrode. This liquid serves as make-up flow and carries the analytes toward the detector both with the siphoning action of the field generated by the source potential and with the aspirating effect of the nebulizing gas. Because the liquid-junction interface is partially disconnected from the emitter (both physically and electrically), the latter can be easily replaced and problems related to the emitter are isolated from those linked to the separation capillary. However, the gap between both capillaries is a critical element and can introduce significant external peak broadening. Therefore, an extreme care must be taken for proper alignment.

2. Sheathless Interfaces

Several reports concerning the development of stable and rugged sheathless interfaces were proposed.[32,33] The first sheathless interface was developed by Olivares et al.,[19] and two types of sheathless interfaces are currently distinguished. The first one consists of a nanospray needle, which is inserted with a connection unit to the CE capillary. This setup allows changing the spray needle alone independently on the capillary exchange.[26,34−40] The second approach involves the use of the end of capillary tip as an emitter with the help of a capillary-outlet conductive coating[41−53] or by inserting a conductive wire into the capillary outlet.[54−56] This type of device is usually homebuilt, and different procedures for modifying the capillary tip and applying the conductive layer are reported.[57−64]

The main advantage of sheathless interfaces is their high sensitivity compared to sheath-flow interfaces. First, a larger portion of produced ions reaches the MS because the nanospray tip is commonly positioned more closely to the MS orifice than other interfaces; second, the smaller droplets obtained due to the reduced flow rate improve analyte ionization; finally, no sample dilution by the additional sheath flow occurs.[65] Furthermore, compatibility with the low flow rates present in CE is improved and no additional chemical substances are introduced at the ionization stage. Therefore, possible interferences from the sheath solvents are eliminated. On the other hand, sheathless interfaces appear less stable due to deposits or flaking of the coating that cause a short lifetime. In addition, simultaneous adequate electrophoretic and ionization currents are not straightforward due to low liquid flows eluting from the CE capillary.

B. Ionization Sources

Analytes must be liberated from their associated solvent molecules as well as be ionized to allow mass separation. Several ionization methods enable ion production from the condensed phase and have been used for the coupling of CE to MS. Among them, atmospheric pressure ionization (API) methods, matrix-assisted laser desorption/ionization (MALDI), and inductively coupled plasma (ICP) ionization are mainly used. API techniques are undoubtedly the most widespread ionization sources and cover different analyte polarity ranges.

1. Atmospheric Pressure Ionization Sources

(a) Electrospray Ionization:

Electrospray Ionization (ESI) is the predominant ionization method for on-line CE−MS as it is well suited for the analysis of ionizable or polar compounds ideally separated by capillary zone electrophoresis (CZE). ESI is a soft ionization method that produces, in the gaseous phase, ions from charged evaporating liquid droplets in a high electrical potential toward the MS.[20,66−68] A countercurrent flow of heated gas is often used to speed up desolvation, and nebulization is assisted by a nebulizing gas surrounding the CE capillary. Advantages of ESI are its simplicity, high ionization efficiency in terms of ions released from charged droplets, and ability to produce multi-charged ions.[69] The main limitation of CE−ESI/MS concerns the limited compatibility of electrophoretic buffers commonly used in CE−UV, such as phosphate or borate. Therefore, volatile electrolytes, such as formate, acetate, carbonate, and ammonium, are often recommended. Furthermore, BGE ionic strength must be low since the ionization process in ESI is impaired by highly conductive solutions. Selectivity modifiers such as micelles, microemulsions, ion-pairing agents, and chiral selectors are not suitable or can even be detrimental for on-line CE−ESI/MS since they may contaminate the MS ionization source and cause significant ion suppression.[70,71] Different strategies have been reported to address these difficulties, such as the partial-filling technique (PFT) (see Section III.A.2) or capillary electrochromatography (CEC) (see Section III.D). To decrease the negative effect of BGE composition, other ionization sources such as atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) could be envisioned since both techniques vaporize the sample and the CE electrolyte prior to the ionization process.

(b) Atmospheric Pressure Chemical Ionization:

The basic principle of APCI consists in nebulizing the effluent that is transferred through a heated vaporizer allowing complete evaporation of the solvent. A "corona discharge" electrode is placed in the source next to the vaporizer and produces electrons initiating the

chemical ionization process with ionization of the gas formed from the evaporated effluent.[72,73] Some attempts were reported for CE−APCI/MS hyphenation,[74−77] but led to very poor sensitivities because most of APCI interfaces were initially designed for LC−MS. Since APCI is a mass-flow-dependent source, sensitivity is improved with LC flows but impaired with low flow rates conventionally encountered in CE.

(c) Atmospheric Pressure Photoionization:

APPI was introduced by Robb et al. in 2000[78] as a complementary technique to ESI and APCI for broadening the range of ionizable analytes by API techniques.[79,80] This ionization source is mostly used in the analysis of non-polar compounds.[78,81−87] APPI source is very similar to APCI, i.e., the sample is vaporized in a heated nebulizer before ionization and non-volatile salts can be easily removed during this step. However, the ionization is afforded by a discharge lamp instead of the corona needle used in APCI. The absorption by the analyte of a photon, generally emitted from a krypton lamp, can occur if the lamp energy is higher than the analytes ionization potential (IP). To significantly improve the ionization process, a doping agent is often added[86,87] to participate in the ionization process via a charge or proton-transfer mechanism to the compound of interest.[88] For several reasons, implementing APPI instead of APCI can lead to an improvement in sensitivity (height-to-noise ratio, *H*/*N*). First, common solvents such as water, acetonitrile, or methanol are not affected by discharge lamp as their IP are above 10 eV,[89,90] thus resulting in a low background noise. Second, APPI sources appear to be less sensitive to ion suppression (compared to APCI and ESI).[91−94] Finally, APPI achieves significantly better sensitivity than APCI over a wider range of flow rates, particularly at the very low flow rates generated by CE separations. Therefore, it can also be considered in the sheathless configuration.[90,95] Currently, only few publications are dedicated to CE−APPI/MS[96−100] with experiments achieved on a conventional CE−MS system equipped with the sheath-flow configuration. To adapt the APPI interface, a spacer is positioned between the nebulizer and the vaporizer, and the dopant is usually added within the sheath-liquid interface. A wider choice of CE electrolytes (buffer and additives) can be used for the analysis of both polar and non-polar compounds without background noise and source contamination. As indicated by Mol et al., sodium dodecyl sulfate (SDS) also has no negative effect on photoionization efficiency; therefore, APPI provides a good solution for MEKC−MS[96] and for MEEKC−MS.[100] As presented in Figure 2, analysis of five steroids was performed by MEEKC−APPI/MS without extensive background noise or contamination of the ionization source.

2. Matrix-Assisted Laser Desorption/Ionization Interfaces

In MALDI, the energy of a laser is used to vaporize the CE effluent and ionize the analyte molecules. A light-absorbing compound is needed as a matrix for energy absorption, and the solution used as the matrix can act as both CE electrolyte and laser energy absorber at the interface. The off-line hyphenation of CE with MALDI has been recently reviewed.[101−105] It requires either direct sample deposition onto a MALDI target or CE fraction collection for subsequent MALDI/MS analysis.[106−109] However, the complexity and the high cost of the equipment has prevented its widespread use, even though some successful applications are reported,[110−113] particularly for the characterization of large molecules with molecular masses up to several hundred kilodaltons (kDa).[114]

3. Inductively Coupled Plasma Ionization Interfaces

ICP interfaces are used to completely fragment the analyte of interest for elementary composition determination. The principal field of application for CE−ICP/MS is the analysis of metals for the selective detection of specific elements where low detection limits are

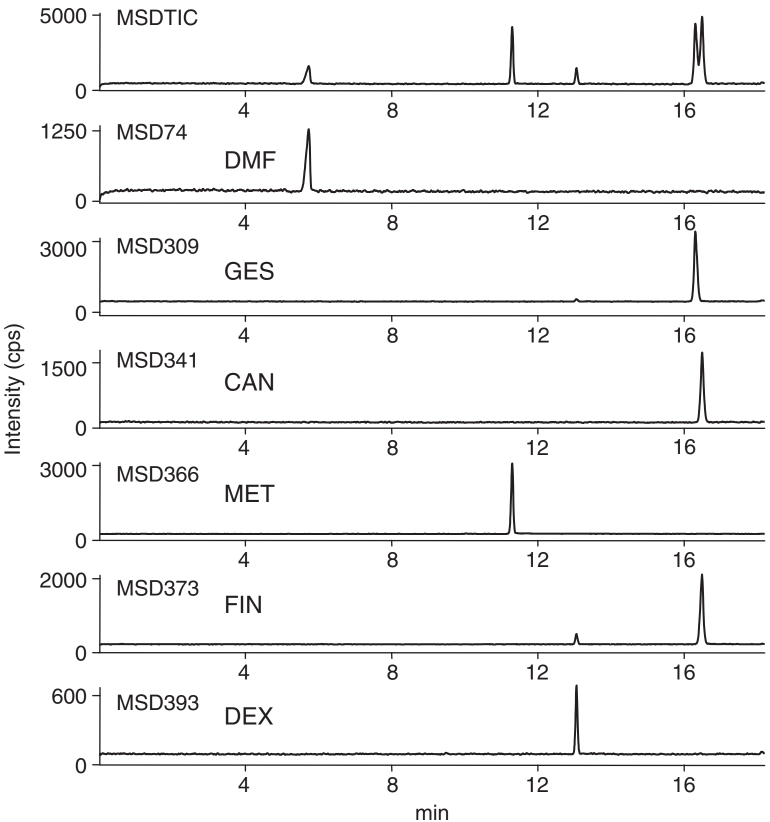

FIGURE 2 MEEKC–APPI/MS analysis of five steroids. Total ion current (TIC) and extracted ion currents (XIC) of gestrinone (GES), canrenone (CAN), metolazone (MET), finasteride (FIN), and dexamethasone (DEX) and an EOF marker (*N,N*-dimethylformamide, DMF).

required.[115–121] Applications of the technique in the field of trace-element speciation analysis are currently increasing with the strong interest in analysis of inorganic (metal ions determination), organometallic (mercury speciation), and bio-inorganic (selenium and arsenic speciation) compounds.

C. Analyzers

All mass analyzers can be potentially hyphenated to CE. However, the very high efficiencies achieved in CE lead to very short peak widths. Only few seconds are available to record the analyte signal; therefore, this short analysis time window should be compatible with the MS sampling rates. For example, full-scan acquisition over a large mass range with a single quadrupole can be problematic regarding both peak definition and sensitivity due to the relatively slow cycle time afforded by this analyzer (>1 s). Although most research on CE–MS was performed using single quadrupole instruments,[122–125] combinations of CE with ion trap,[126–128] triple-quadrupole,[46,129] time-of-flight (TOF),[130–133] and Fourier transform-ion cyclotron resonance (FT-ICR)[134–138] mass spectrometers were also described. This section gives a short overview of the most common MS instruments hyphenated with CE. For additional information, the reader is referred to dedicated literature on MS.[139–141]

Quadrupole mass spectrometers has been used mainly in CE−MS because they can be obtained at relatively low cost, possess small dimensions, and are easy to operate. As previously mentioned, the scanning process is relatively slow and allows operation of only a small fraction of the available ions, which is not suitable with very narrow CE peaks. The use of selected ion monitoring (SIM) mode greatly improves sensitivity and duty cycle, but it is not always appropriate for the detection of complex mixtures. This kind of analyzer is currently used as a low-resolution instrument and for quantitative determination.

Ion-trap mass spectrometers, also referred to as "quistors" (quadrupole ion storage traps), are three-dimensional analogues of the above-mentioned quadrupole systems. Ions of different masses are stored in the trap and released one at a time by scanning the applied voltages. Ion traps can accumulate ions of pre-selected m/z values, with a resulting gain in sensitivity compared to single quadrupole systems. They also present faster scanning rates, to be able to record a large number of spectra per second. Therefore, ion traps make efficient use of incoming ions and offer the possibility of MS^n experiments, providing additional information by multiple-stage fragmentation of the analytes.

The most notable advance has been the recent commercialization of TOF analyzers as detectors for CE. Basic principle of a TOF mass spectrometer involves measuring the flight time of an ion through the mass spectrometer, yielding its m/z value. Fast acquisition rates that provide extremely short times to generate a mass spectrum are achieved by a TOF instrument allowing complete characterization of the CE peaks with the additional advantages of high mass resolution, high mass accuracy (<10 ppm), extended mass range, and high sensitivity (sub-femtomole detection limits). Thereby, new applications in the proteomics and metabolomics are accessible with CE−TOF/MS.[142,143]

In terms of overall sensitivity (one molecule detection), mass resolution (in excess of 10^5), mass accuracy (<2 ppm), scan speed, and MS^n capabilities, FT-ICR analyzers remain a very promising technique for the on-line coupling with CE. However, its technical demands, in terms of vacuum technology, and its high price currently limits its use mostly to fundamental studies.

III. APPLICATIONS

On-line coupling of CE with MS has generated a wealth of data in bioanalytical ("life science"),[23,142−151] pharmaceutical,[152−156] forensic,[157] and environmental[158−160] domains, and was the subject of a number of comprehensive reviews.[14,15,161] The reader is thus referred to these sources for a systematic coverage of the field and a more extensive discussion of CE−MS applications than can be given in this context. On the basis of this consideration, all applications of electromigration techniques coupled with MS are not intended to be covered. The present section is directed toward recent examples on the on-line coupling of CZE and related electrophoresis-based techniques with MS in the field of pharmaceutical research. Indeed, CE is mainly used in pharmaceutical industries for the determination of major drugs and their by-products in pharmaceutical preparations (quality assurance purposes) as well as active components and their metabolites in biological fluids.[162,163] Therefore, CE−MS emerges as a suitable technique that fulfills key requirements such as sensitivity, selectivity, and peak assignment certainty.[164]

A. CZE−MS

CZE is the most common separation technique in CE since high speed and high-resolution separations of low-molecular-weight acidic and basic compounds can be achieved

with the appropriate choice of BGE (see Chapter 2). Applications of CZE−MS for the analysis of a broad range of pharmaceutical compounds have been extensively demonstrated,[165] and the present section gives a general overview of both achiral and chiral recent applications.

1. Drug Analysis by CZE−MS

(a) Drug Discovery:

Physicochemical and pharmacokinetic profiling of new chemical entities allows the rapid identification and elimination of compounds with unsuitable properties for further drug development.[166] For instance, the aqueous dissociation constant (pK_a) is an important parameter as the ionization state of a drug affects its absorption, distribution, metabolism, and excretion (ADME). CZE is an alternative method for pK_a measurement to commonly used potentiometric titration or UV spectral shift methods, and the use of MS detectors extends the application range to non-UV-absorbing and poorly soluble analytes. Moreover, the additional selectivity offered by MS allows a greater pooling of compounds per analysis, which provides an increased throughput particularly important in the drug discovery stage. For instance, Wan et al. developed a method for the simultaneous pK_a measurement of more than 50 compounds in less than 150 min with CZE hyphenated with an ion-trap mass analyzer.[167]

(b) Drug Impurity Profiling:

Impurity profiling of pharmaceutical products is another issue in the pharmaceutical field. The speed and reliability of analytical data regarding impurities, which can be both synthetic and degradative products, have a direct impact on the eventual success or failure of a promising drug. In this context, CZE can advantageously be used as an orthogonal technique to high-performance liquid chromatography (HPLC) since separation principles are different.[168] For instance, CZE−MS equipped with an ESI source and an ion trap was implemented by Visky et al. in the method development approach to support impurity profiling of galantamine formulations under harsh conditions. Two degradation products were detected at concentrations lower than 0.05% and further identified by MS/MS[169] as shown in Figure 3.

(c) Drug Metabolism Study:

Biotransformation of a therapeutic agent to metabolites possessing different biological and structural properties can modify both the extent and the duration of parent drug's activity. Therefore, the subsequent isolation and structural characterization of the resulting metabolites are important to understand the pharmacological effects of therapeutic agents. The high efficiency of CE, combined with the versatility of ionization techniques to analyze metabolites of varying polarity and the power of MS/MS to provide reliable structural information, evolves as an efficient tool in drug metabolism studies as well as a rapid and sensitive screening strategy for drug candidate selection.[170] Using CZE coupled to ion-trap ESI−MS, the analysis of diphenhydramine metabolites in human urines was performed by Baldacci et al., and CE−MS^n appeared suitable for identifying urinary drug metabolites for which no standards were available.[171]

(d) Illicit Drugs of Abuse:

Routine analysis of common designer drugs in human biological fluids (e.g., plasma, urine) is a major concern in doping control, surveillance of drug substitution, clinical toxicology, as well as forensic science.[172] Method sensitivity is often an issue since many drugs

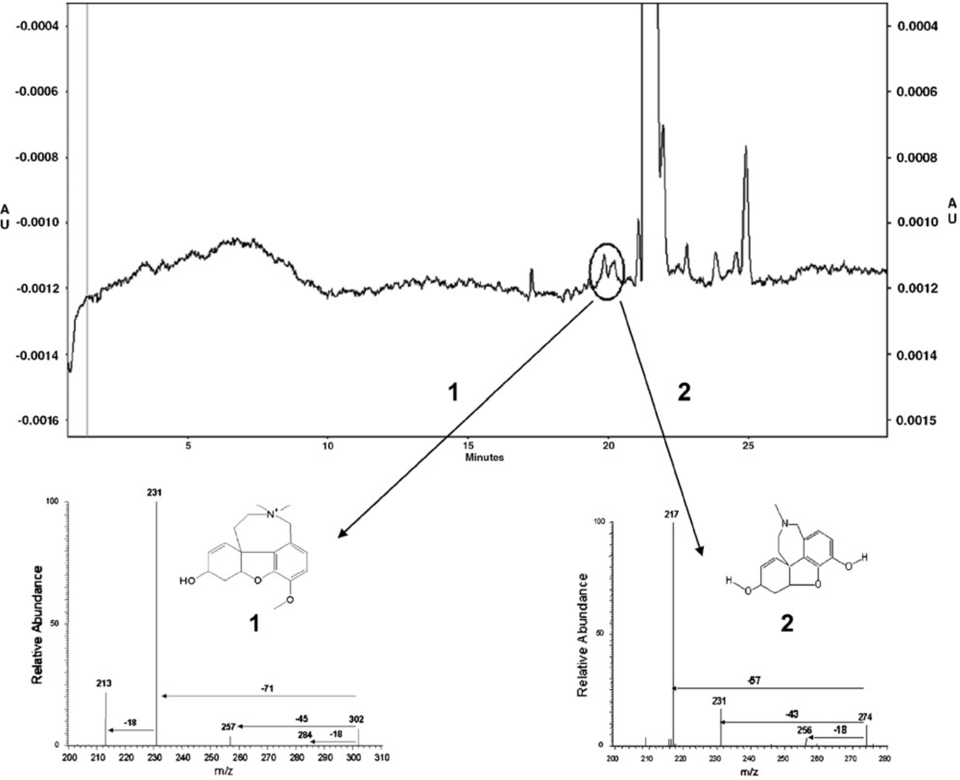

FIGURE 3 CE−ESI/MS analysis of a stressed galantamine sample (18 months, 25°C, 60% relative humidity) with structures and MS/MS spectra of both identified degradation products (peaks 1 and 2, *m/z* 302 and 274, respectively). Adapted from reference 169 with permission from Wiley-VCH Verlag GmbH.

possess a high volume of distribution, resulting in low concentration levels. CZE−MS can be a useful tool for the determination of such substances in body fluids, especially when low sample amounts are available.[173] Schappler et al. analyzed amphetamine analogues most consumed illegally worldwide and pharmaceutical compounds such as tramadol and methadone by CZE−ESI/MS with a single quadrupole.[174] Detection limits of 200 pg/mL in plasma were reached with an appropriate sample preparation (liquid−liquid extraction, LLE) followed by an electrokinetic injection.

2. Chiral Drug Analysis by CZE−MS

An extremely important aspect in pharmaceutical research is the determination of drug optical purity.[155,175] The most frequently applied technique for chiral separations in CZE remains the so-called dynamic mode where resolution of enantiomers is carried out by adding a chiral selector directly into the BGE for in situ formation of diastereomeric derivatives. Various additives, such as cyclodextrins (CD), chiral crown ethers, proteins, antibiotics, bile salts, chiral micelles, and ergot alkaloids, are reported as chiral selectors in the literature,[176−178] but CDs are by far the selectors most widely used in chiral CE.

(a) Direct Coupling:

First experiments in chiral CZE−MS with CD were achieved by Sheppard et al.[179] in 1995, who demonstrated selectivity and sensitivity advantages of MS for the separation of terbutaline and ephedrine enantiomers. An important gain in sensitivity (1000-fold) was obtained by MS compared to UV detection. Both the free drug enantiomers and the enantiomer−CD inclusion complexes were detected by MS, demonstrating, for the first time, the interference problem given by the presence of chiral selectors in the ionization chamber. Other authors such as Otsuka et al.[180] recommended to work with non-aqueous conditions to reduce the noise afforded by the presence of heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) as chiral selector in the BGE. The negatively charged highly sulfated γ-CD (HS-γ-CD) was used by Iwata et al.[181] for the simultaneous chiral separation of amphetamine-type stimulants. By using a relatively high concentration of HS-γ-CD, amphetamines migrated as negatively charged complexes and were detected at the anode (reversed polarity mode). The complexes were dissociated at the ESI interface set in cationic ionization mode, and only amphetamines were detected by MS. Unfortunately, the important electrophoretic current due to the presence of the negatively chiral selector at high concentration led the authors to work in aqueous media without buffering constituents.

(b) Partial-Filling Technique:

Because interfacing chiral CE with ESI/MS was severely troubled by the presence of CD, the PFT was investigated. The PFT, first introduced by Valtcheva et al.[182] and further modified by Tanaka et al.,[183] involves the filling of a discrete portion of the CE capillary with BGE containing a chiral selector (i.e., partial filling). This method is particularly adapted in chiral CE−MS and presents major advantages over the traditional approach.[184,185] Neutral CDs were initially used, but charged chiral selectors are currently preferentially employed.

(c) PFT with Neutral Chiral Selectors:

When using PFT with a neutral selector, it is quite difficult to avoid any entrance of the chiral selector into the ionization source, particularly at a high pH, where EOF is important. The use of BGE at low pH and/or coated capillary to minimize EOF is therefore mandatory. However, the coaxial sheath gas, which generally assists the ionization process, leads to an aspirating phenomenon of the chiral selector in the MS direction. Javerfalk et al.[186] were the first to apply PFT with a neutral methyl-β-CD for the separation of racemic bupivacaine and ropivacaine with a polyacrylamide-coated capillary and an acidic pH buffer (pH 3). Cherkaoui et al. employed another neutral CD (HP-β-CD) with a PVA-coated capillary for the analysis of amphetamines and their derivatives.[187] To prevent a detrimental aspiration effect, analyses were carried out without nebulization pressure. Numerous other studies presented excellent results such as the enantioselective separation of adrenoreceptor antagonist drugs using tandem mass spectrometry (MS/MS);[188] the separation of clenbuterol enantiomers after solid-phase extraction (SPE) of plasma samples;[189] or the use of CD dual system for the simultaneous chiral determination of amphetamine, methamphetamine, dimethamphetamine, and *p*-hydroxymethamphetamine in urine.[190]

(d) PFT with Charged Chiral Selectors:

The use of a charged chiral selector is probably the best solution to improve the classical PFT when CE is hyphenated with MS. Better solubility, additional electrostatic interactions, and improvement of the stereoselective separation power afforded by the self-mobility of the chiral additives into the BGE are among the numerous advantages of these charged selectors. When electromigration of the chiral species and the analytes are opposite (PFT-countercurrent approach), the mobility difference between free and complexed analytes is increased, leading to a higher resolution than with a neutral chiral selector.[191−194] In optimized countercurrent

conditions, analytes reach the detector while the charged chiral selector migrates toward the opposite side of the MS. Hence, the stability of the ionization is improved by minimal contamination of the source and detector during the electrophoretic run. Cationic and anionic chiral selectors were employed for the stereoselective CZE−MS analysis of acidic and basic analytes, respectively.

Vancomycin was one of the most employed chiral selectors for separating acidic compounds. Introduced by Armstrong et al. for the separation of a wide range of compounds,[195−197] vancomycin is a glycopeptide antibiotic that contains numerous stereogenic centers and a high number of functional groups. Chiral anionic arylpropionic acids such as ibuprofen, etodolac, and their metabolites were analyzed by Fanali et al.,[198] and advantages of MS were shown by unambiguous compound identification for several unresolved peaks in biological matrices. Vancomycin and other charged antibiotics were also used by Tanaka et al. for the separation of isocitric acid lactone, ibuprofen, ketoprofen, warfarin, and camphor sulfonic acid.[199]

Regarding basic compounds, which represent more than 85% of ionizable drugs in the pharmaceutical domain, numerous CZE−MS applications with negatively charged CD were developed. For instance, sulfobutylether-β-CD (SBE-β-CD) was used for the first time by Schulte et al. for the separation of pharmaceutical cationic analytes (etilefrine, mianserine, dimethindene, and chloropheniramine).[200] The potential of the PFT-countercurrent in CZE−MS has been further demonstrated for the stereoselective analysis of other chiral drugs such as bupivacaine, mepivacaine, prilocaine and ketamine,[201] fluoxetine, and methadone.[202] The latter was also used as a model compound for a fundamental study on the PFT-countercurrent approach based on a chemometric approach.[203] Analysis of complex samples such as biological matrices or plant material was also achieved, for atropine[165] for instance. A drug metabolism study was performed as well, and a simultaneous enantioseparation of tramadol and its five phase I metabolites was accomplished by CZE−ESI/MS, using negatively charged CD combined with the PFT-countercurrent methodology.[204] The same strategy was also applied to the chiral separation of amphetamine derivatives at very low levels (sub-nanogram per milliliter) using an electrokinetic injection.[174] As depicted in Figure 4, concentration of 1 ppb of each analyte, corresponding to an enantiomeric concentration of 0.5 ppb, was detected.

It is noteworthy that the use of PFT-countercurrent requires the presence of these negatively charged chiral selectors at relatively low concentration inducing a low conductivity in the BGE. As a result, the generated current generally did not exceed the instrument limitation even when high voltage was applied, which led to high efficiencies.[205]

B. NACE−MS

The recent introduction of non-aqueous media extends the applicability of CE. Different selectivity, enhanced efficiency, reduced analysis time, lower Joule heating, and better solubility or stability of some compounds in organic solvent than in water are the main reasons for the success of non-aqueous capillary electrophoresis (NACE).[206−208] Several solvent properties must be considered in selecting the appropriate separation medium (see Chapter 2): dielectric constant, viscosity, dissociation constant, polarity, autoprotolysis constant, electrical conductivity, volatility, and solvation ability.[209−212] Commonly used solvents in NACE separations include acetonitrile (ACN); short-chain alcohols such as methanol (MeOH), ethanol (EtOH), isopropanol (*i*-PrOH); amides [formamide (FA), *N*-methylformamide (NMF), *N*,*N*-dimethylformamide (DMF), *N*,*N*-dimethylacetamide (DMA)]; and dimethylsulfoxide (DMSO). Since NACE−UV may present a lack of sensitivity due to the strong UV absorbance of some solvents at low wavelengths (e.g., formamides),[213,214] the on-line coupling of NACE

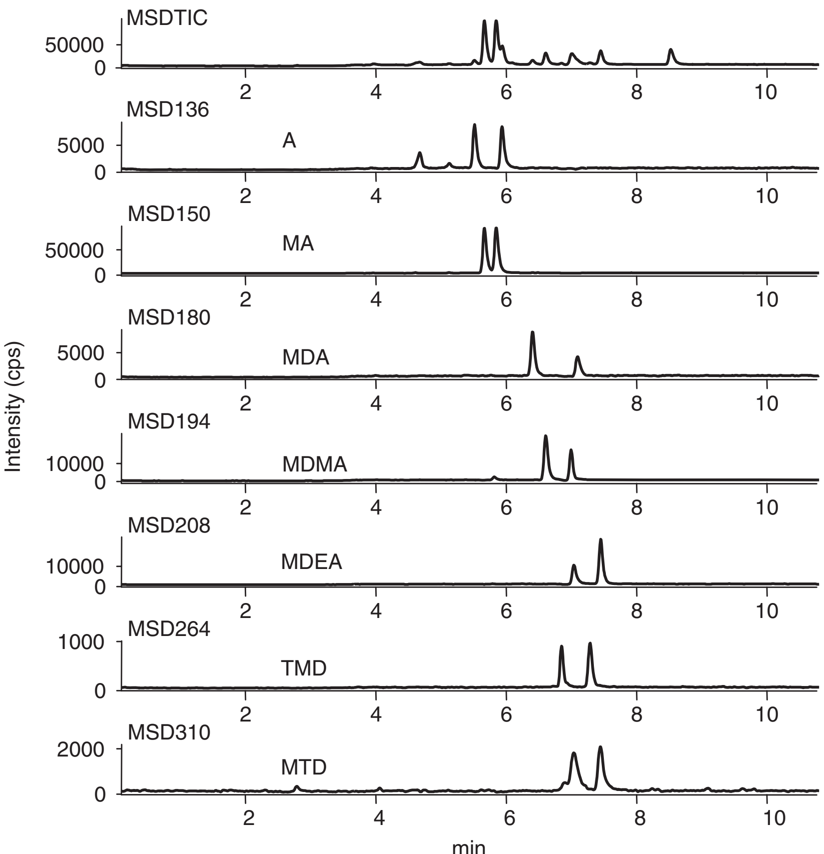

FIGURE 4 Chiral CE−ESI/MS analysis of five amphetamine derivatives and two pharmaceutical compounds. Total ion current (TIC) and extracted ion currents (XIC) of amphetamine (A), methamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), and methylenedioxyethylamphetamine (MDEA), and tramadol (TMD) and methadone (MTD) in plasma after LLE with electrokinetic injection.

with MS is particularly interesting. In addition, low evaporation temperature and surface tension of organic solvents are additional features that are favorable for sensitivity and spray stability.[215] Finally, the generated electric currents are lower in the presence of organic mixtures than in water, allowing stable CE−MS conditions, as illustrated for the analysis of six amphetamines in ACN/MeOH with formate buffer such as the BGE by NACE−ESI/MS.[216]

Several reviews were published on NACE—MS,[217−219] and numerous fundamental studies were performed to assess the influence of the solvent on CE and MS performance, either with a sheath liquid[220] or a sheathless nanospray[29,221] configuration. BGE with volatile electrolytes in MeOH, ACN, DMSO, FA, NMF, and DMF were evaluated for the separation of basic analytes with NACE−ESI/MS vs. CE−ESI/MS.[222] Organic solvents improved the separation selectivity and sensitivity due to a better ionization from facilitated evaporation of the solvent. NACE−MS is also readily applied to impurity profiling. For example, Cherkaoui and Veuthey described a NACE−ESI/MS method for the simultaneous analysis of fluoxetine, its *meta*-isomer, and other related compounds.[223] Under selected NACE conditions, baseline separation of the investigated compounds was possible in contrast to

aqueous CE where co-migration of the closely related compounds was observed and attributed to similar electrophoretic behavior causing insufficient resolution. Finally, the use of NACE−MS has proven to be valuable in pharmacological and clinical studies,[224,225] as well as in investigations of drugs and metabolites.[12,226−230]

C. MEKC−MS

Besides CZE and NACE, micellar electrokinetic chromatography (MEKC) is also widely used, and ionic micelles are used as a pseudo-stationary phase. MEKC can therefore separate both ionic and neutral species (see Chapter 2). Hyphenating MEKC with ESI/MS is problematic due to the non-volatility of micelles, which contaminate the ionization source and the MS detector, resulting in increased baseline noise and reduced sensitivity. However, MEKC−ESI/MS was applied by Mol et al. for identifying drug impurities in galantamine samples.[231,232] Despite the presence of non-volatile SDS, all impurities were detected with submicrogram per milliliter sensitivity and could be further characterized by MS/MS.

To overcome the limitations of MEKC−MS, several methods have been implemented: (i) the use of volatile surfactants,[233] (ii) the use of low-molecular-weight (unpolymerized) surfactants either at low concentrations[70] or with the previously described partial-filling approach (see Section III.A.2),[234−240] (iii) the use of high-molecular-weight surfactant, and (iv) the use of APCI or APPI sources instead of ESI.[241] The possibility of using high-molecular-weight surfactants is an attractive approach because these polymerized micelles are difficult to ionize due to the covalent bond formed between the surfactant monomers. Moreover, they exhibit a low surface activity resulting in a stable spray and a signal increase in the MS.[242] Because APCI and APPI processes are less affected by non-volatile salts, they also provide several possibilities for MEKC−MS to reduce background noise and source contamination. Takada et al. reported an MEKC−APCI/MS method for xanthine derivatives using SDS directly introduced into the ionization source of the MS detector without a severe decrease in sensitivity.[243] Mol et al. demonstrated that SDS had also little negative effect on photoionization efficiency and MEKC−APPI/MS was successfully applied for both polar and non-polar compounds.[96] The analysis of enantiomers by chiral MEKC−MS is also readily performed, and Shamsi was the first to report the use of chiral surfactants.[244] However, most chiral surfactants substantially contribute to chemical noise and suppress analyte ionization because of micelle dissociation during ionization process into surfactant monomers with high surface activity.

D. CEC−MS

CEC (see Chapter 17) is an analytical approach that combines the advantages of both electrophoresis and partitioning separation processes, i.e., the high efficiency of CE with the high loading capability of particle-based columns.[245] In CEC, capillary columns are usually classified into three main formats: (i) packed-CEC, where a fused-silica capillary is filled with a typical HPLC packing material; (ii) open-tubular CEC (OT-CEC), where the retentive stationary phase is present only on the walls of the capillary;[246,247] and (iii) monolithic-CEC, where a monolithic stationary phase is prepared by in situ polymerization within the capillary. The main drawback of packed-CEC is the production of frits and the need for junctions between transfer segments and the filled capillary to prevent packed bed movements under EOF. The pressurized inlet and outlet reservoirs are also used in most CEC systems to suppress bubble formation associated with frits.[248] Continuous bed-type columns such as OT-CEC and monolithic-CEC columns have thus emerged as alternatives to packed-CEC to circumvent

these problems and obtain a completely frit-less packed column system that eliminates band broadening, peak distortion, or bubble formation.[249]

The three types of support are used in CEC−MS coupling, and hyphenation appears effortless since additional selectivities (e.g., hydrophobic or chiral interactions) are given by the stationary phase. Therefore, CEC can overcome some drawbacks of CE−MS coupling and prevent source contamination by non-volatile additives such as CD or SDS. The first interface ever used for the hyphenation of CEC with MS was the continuous flow-fast atom bombardment (CF-FAB) interface.[250,251] Because of technical difficulties to maintain a stable electrical current, this type of interface was replaced by the more convenient API interfaces. Few reports exist on the hyphenation of CEC with MS, using APCI[252] or APPI,[99] and main developments involve ESI coupling. Considerations encountered in interfacing CEC−MS with API sources are analogous to those met in CE−MS, i.e., electrical contact and flow rate issues.[253] Similar to CE−MS coupling (See Section II.A), both can be overcome with an appropriate interface such as the sheath-flow or the sheathless configurations.[254] Whereas OT-CEC and monolithic-CEC do not require a special setup, care must be paid when coupling packed-CEC with MS. As mentioned earlier, the use of pressurized inlet and outlet reservoirs is recommended to suppress bubble formation and such pressurized system are currently not compatible with API source. Therefore, packed columns are directly connected to the interface, and three different configurations of column outlet were reported. The first configuration uses columns with fritted termination[255−258] and consists in using only the packed portion of the capillary column, i.e., without open connecting tube. Therefore, the column is terminated after the outlet retaining frit and the CEC effluent is directly sprayed into the atmospheric area of the ion source. This configuration is generally used with a sheath-flow interface. The next possibility is using columns with tapered ends as flow restrictors where the packed capillary column can be either externally[259] or internally[133] tapered. Both configurations are preferentially used in a sheathless arrangement, and CEC columns with an integrated, conductive nanospray tip have been recently commercialized. The third employs columns with a connecting tubing and uses a piece of fused-silica capillary as the connecting device, generally in a coaxial sheath-flow or liquid-junction configuration.[260,261] In this arrangement, the CEC column is electrically grounded after the terminating frit and the connecting tube is coupled to the packed-CEC column via a zero-dead-volume union. This configuration presents the advantage that backpressure due to the flow through the open tube is enough to overcome the frits issue. Furthermore, tip dimensions are usually decreased, resulting in a very stable spray. Applications of CEC, including the determination of pharmaceutical compounds and impurities, as well as chiral separations, were extensively reported.[262,263] The next section gives a brief outline of some relevant applications.

1. Drug Analysis by CEC−MS

CEC−MS was applied for the analysis of various pharmaceuticals using either packed-CEC,[264−269] OT-CEC,[270,271] or monolithic-CEC columns.

(a) Packed-CEC−MS:

Lord et al. analyzed a mixture of steroids by CEC−ESI/MS and interfaced externally tapered CEC columns in both sheathless and sheath-flow arrangement. Sensitivity was found 20-fold higher in the sheathless configuration.[259] The same conclusion was drawn by Warriner et al., who evaluated CEC-nanospray/MS vs. CEC-microspray/MS with an ion trap using five corticosteroids.[272] Cahours et al. used CEC−ESI/MS for a drug metabolism study and obtained a simultaneous baseline separation of flunitrazepam and its major metabolites. For CEC−ESI/MS coupling, the commercially available packed-CEC column was connected

without any dispersive effect using a Teflon connection.[273] CEC−ESI/MS was also implemented in high-throughput drug discovery by Paterson et al. who reported the separation of 13 structurally related compounds from a parent drug candidate.[274] Finally, electroosmotically driven solvent gradients were used for the separation of 12 amino acids by reversed-phase CEC coupled with TOF/MS. Capillary columns with an internal taper at the column outlet were used in the sheath-flow configuration.[133]

(b) OT-CEC−MS:

Zhu et al. coupled OT-CEC to ESI/MS for the analysis of β-blockers and benzodiazepines. The authors described the use of a polymeric surfactant as a stationary-phase coating that enabled minimal surfactant introduction in the MS compared to MEKC−ESI/MS, thus avoiding interferences from non-volatile micelles in ESI/MS.[275]

(c) Monolithic-CEC−MS:

Kato et al. evaluated three different silica monolithic columns by changing the poly(ethylene glycol) (PEG) contents for the simultaneous analysis of cationic, neutral, and anionic compounds using CEC−ESI/MS with a sheath-liquid configuration.[276] Que and co-workers also developed novel types of polar monolithic-CEC columns for the analysis of complex mixtures of saccharides with CEC−ion-trap MS[277] and CEC−FT-ICR MS,[278] enabling low-femtomole sensitivities with mass accuracy <4 ppm in the m/z range of 200−2000. This column technology provided a nearly universal system that could separate a wide range of carbohydrates, including monosaccharides and oligosaccharides, saccharide alditols, and anomers.

2. Chiral Drug Analysis by CEC−MS

As discussed in previous sections, adding a chiral selector to CZE (see Section III.A.2) or MEKC (see Section III.C) buffers, either directly or indirectly using PFT, is possible for analysis of CE−ESI/MS enantiomers. However, the use of such chiral selectors or additives can produce a significant enhancement of background noise. An alternative is to attach or bond the chiral selector as a chiral stationary phase (CSP) either to a packed-CEC or monolithic-CEC column,[279,280] or to an OT-CEC column.[281−284]

(a) Chiral Packed-CEC−MS:

Brush-type, proteins, CDs, natural molecular imprint-based polymers (MIP), and macrocyclic antibiotics[285] have been immobilized as chiral selectors on packed-CEC columns. Zheng and Shamsi demonstrated the possibility of using chiral CEC−ESI/MS with a commercially packed column for the determination of warfarin enantiomers in human plasma using coumachlor as an internal standard (IS).[286] Robustness of this chiral CEC capillary was recently improved by a novel procedure[287] and applied for the simultaneous enantioseparation of height β-blockers with multimodal CSP using different combinations of vancomycin and teicoplanin, as presented in Figure 5.[288]

(b) Chiral OT-CEC−MS:

Although the majority of chiral CEC−MS applications still involve packed columns, few reports on chiral OT-CEC−MS are found in recent literature. The feasibility of coupling OT-CEC (using a short Chirasil-Dex-coated capillary column) to MS and MS/MS for trace analysis of hexobarbital enantiomers in biological fluids was reported by Schurig and Mayer.[289] More recently, Kamande et al. investigated polyelectrolyte multilayer (PEM) coating as a new medium for the separation of chiral analytes, and PEM-coated capillaries were successfully coupled to ESI/MS for the stereoselective analysis of five β-blockers.[290]

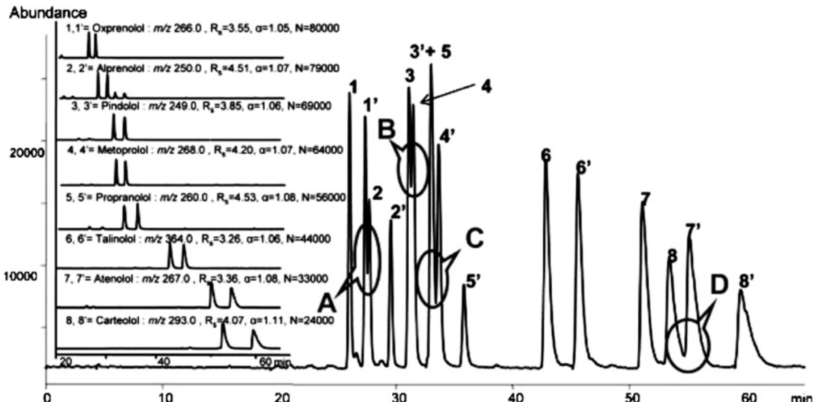

FIGURE 5 **Chiral CEC−ESI/MS analysis of eight β-blockers: 1,1′ = oxprenolol, 2,2′ = alprenolol, 3,3′ = pindolol, 4,4′ = metoprolol, 5,5′ = propranolol, 6,6′ = talinolol, 7,7′ = atenolol, 8,8′ = carteolol. Reprinted from reference 288 with permission from Wiley-VCH Verlag GmbH.**

IV. QUANTITATIVE ASPECTS

Quantitative CE−MS studies were scarcely reported.[126,173,291−293] This subject is however of prime importance, particularly for the pharmaceutical industry where the reliability of analytical data is essential. For this reason, method development is generally followed by an evaluation of quantitative performance using an appropriate validation procedure performed in agreement with criteria established by the International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) and the Food and Drug Administraction (FDA) guidelines, or Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) commissions.[294−298]

A major difficulty in terms of quantitative CE−MS studies can be linked to the sheath-flow interface geometry. Instrumental measurements of ionization currents were described by Bruins[66] and depend on different parameters, including the positioning of the capillary inside the nebulizer. An incorrect or unrepeatable positioning of the capillary inside the interface leads to an unstable spray, which in turn yields inaccurate ionization performance and low sensitivity. Therefore, tests must be performed prior to starting a sequence of analyses to adjust the capillary position and ensure that these electric currents remain stable. Geiser et al. suggested a daily procedure based on constant ionization currents to achieve good sensitivity and enhanced system stability. With this applied procedure, precision and trueness were considerably improved.[299] A second possible source of error is generally attributed to the electrophoretic process itself and to the impossibility to obtain uniform EOF, particularly in presence of capillary laminar flow due to both the nebulizing gas and the sheath liquid. These effects also appear to be detrimental for repeatable sample injections. Finally, it is difficult to correctly thermostate the final part of the capillary in CE−MS configuration, and this can lead to shifts in migration times and/or peak broadening.

To eliminate problems associated with quantitation in CE−MS and reduce the impact of system variability on method accuracy, it is mandatory to use an IS. The variability obtained in CE−MS, typically around 20%, can be lowered significantly when the IS is taken into account. Using a simple structurally unrelated IS, the precision of a CE−MS method can be improved by a factor of 2[300] and increased up to a factor 4 with a closely migrating

compound.[216] However, even with closely migrating IS, analyte and IS still reach the ionization source at different times and short-term variations of the ionization process may be of concern, particularly in ESI. To achieve repeatable results, ionization conditions must be constant, without ion suppression or enhancement, namely matrix effects. Matrix effects have been described by several authors, particularly for LC−ESI/MS analysis of biological samples.[301−304] Evaluation of the matrix effect usually consists of a post-column infusion of the analyte mixture while different matrices are injected onto the chromatographic system, leading to the visualization of ionization suppression or enhancement. Adapted from the configuration proposed by Bonfiglio et al.,[305] a post-capillary infusion system was used with the sheath-liquid interface as an analyte-delivering device.[174] A drug mixture solution was continuously infused with the sheath liquid through the nebulizer and effects associated to the migration of interfering compounds were assessed by measuring MS responses with the injection of blank or spiked matrices.

Ionization changes can be efficiently corrected with the use of an isotopically labeled IS, which possesses identical ionization response and fragmentation pattern.[306,307] Therefore, deuterated IS can be used to correct both the overall method variability (e.g., sample preparation, injection, electrophoretic process, etc.) as well as matrix effects since the amount of suppression from interferents is expected to be similar. However, the total concentration of analyte and IS should be below the saturation of the ionization process.[308] Guidelines to obtain a reproducible CE−MS method were published by Ohnesorge et al.[309] and took into account the use of an isotopically labeled IS.

Because most of the uncertainty and potential non-linearity in MS do not refer to the analyzer but rather to the ionization process, all types of analyzers can be used for quantitation. Sensitivity, scan speed, mass resolution, and cost are the key points to be compromised. In the case of target analysis, the sensitivity, and thus accuracy, is best for quadrupole instruments. Generally, the time resolution should be adapted to the small peak widths of CE; therefore, the SIM mode is preferred, which also improves sensitivity. Multitarget analysis and screening can be better performed with ion trap or TOF mass spectrometers.[181] On the other hand, the use of MS/MS can greatly enhance selectivity using the selected reaction-monitoring (SRM) mode that ensures more accurate measurements and lower limits of quantitation (LOQ) by reducing the chemical noise.

Finally, when comparing precision, number of samples, analyte concentration, and ionization yield should be considered.[310,311] A small amount of data can lead to unrepresentative values. Furthermore, when the concentration is close to the LOQ, the variability increases and analytes with higher ionization efficiencies yield present lower relative standard deviations (RSD) values because of *H*/*N* differences.

V. NEW DEVICES

Chip-based microdevices represent a new emerging and particularly advanced technology from the early work of Manz and co-workers in 1990.[312] The technology relies on the integration of different steps of the analytical process into a miniaturized flow system. Potential microchip's major advantages are: high speed for maximum "time-to-result decrease," reduced sample volume and reagent consumption, integration of operational elements, disposability, portability, and high-throughput capabilities via parallel processing or automation.[313] They are engineered for sample preparation such as SPE;[314] solid-phase microextraction (SPME);[315,316] LLE; protein digestion;[317−320] sample desalting;[321−323] or derivatization, preconcentration,[324,325] and for different separation strategies, namely CE,[326−329] CEC,[330,331] and nano-HPLC.[332] However, the latter technique is less popular since electrokinetically driven flows are more easily integrated to microstructures. It is simpler

to establish a voltage drop across microchannels than pressure drops that would require miniaturized valves and pumps. Furthermore, microchips often include integrated detection methods such as optical and EC detectors,[333] and can also be hyphenated to MS. Although there are many efforts invested in developing miniaturized and multiplexed MS,[334–338] the main drawback of this coupling presently is that MS cannot be miniaturized and integrated to the microchip. Early microfluidic systems hyphenated with MS were mainly used as sample delivery devices for infusion experiments, with the potential to rapidly load a large number of different samples. Recently, new developments enable the combination of MS with complex microdevices that integrate the whole analytical process. The present section is restricted to the description of microfluidic separation devices hyphenated with MS in which the flow is electrokinetically driven. It will focus on fabrication methods, microchip designs, MS interfacing, and applications.

A. Fabrication Methods

CE chips are mainly obtained using various glass substrates, from inexpensive soda-lime glass to high-quality quartz.[339,340] Various polymer materials are also used.[341,342] The choice of a particular material depends on its surface properties, ease of fabrication, which can be quite different according to the material origin, disposability, and price. Microfabrication processes were recently reviewed and the reader is thus referred to dedicated literature for additional useful information on microfluidic device fabrication.[343,344]

1. Silica-Based Microfabricated Devices

Glass substrates are most commonly used because of their good optical properties, well-understood surface characteristics, and well-developed microfabrication methods adapted from the microelectronics industry. Structures on glass substrates are usually generated using standard photolithographic technologies. The glass substrate is coated with a sacrificial and a photoresist layer, which is exposed to UV light in a region defined by a photomask presenting the appropriate pattern design. The mask pattern is then chemically etched with hydrofluoric acid. Following microchannel etching, the photoresist and sacrificing mask layers are removed. Finally, the substrate is bonded to another piece of substrate to form a finished microchip. Thermal diffusion[345] is the most often used method for glass bonding as well as chemical-activated bonding[346] and adhesive annealing.[347]

2. Polymer-Based Microfabricated Devices

Optical properties of the material are less critical for microchips hyphenated with MS than for devices with on-chip optical detection where low background absorption or fluorescence is mandatory. Thus, completely opaque polymers like glassy carbon[348] or polyimide[349] can be used as microfabrication substrates. Furthermore, polymer microchips are of great interest because their potentially low manufacturing costs may allow them to be disposable. Methods used for the fabrication of plastic chips include laser ablation and molding methods.

The photoablation process consists of the absorption of a short-wavelength laser pulse to break covalent bonds in polymer molecules and eject decomposed polymer fragments.[350,351] Channels of various geometries and dimensions can be obtained using an appropriate mask. Many commercially available polymers can be photoablated, including polycarbonate, poly(methyl methacrylate) (PMMA), polystyrene, nitrocellulose, poly(ethylene terphtalate) (PET), and poly(tetrafluoroethylene) (Teflon).[352]

The formation of microchannels using molding methods involves the fabrication of a molder, followed by the transfer of the channel pattern from the molder to the polymeric substrate, and finally microchannels' enclosure. In contrast to chemical etching (i.e., isotropic etching), microchannels obtained with molding methods possess vertical walls. Depending on the channel dimensions and precision requirements, the molder can be produced with various techniques. For instance, photolithography of an X-ray resist was performed for very small channels ($<100\,\mu m$), followed by electroplating to form the suitable molder.[353] Replication of the molder to produce microchips can be accomplished by injection molding, embossing, or casting. In the injection molding process,[354] the polymer is melted and injected against the molder in a molding chamber. In the embossing process,[355] the polymer substrate and the embossing tool are heated separately under vacuum to a temperature just above the glass transition temperature of the polymer material, and then they are brought into contact. The casting process[356] involves polymer material that is poured onto the top of the molder and hardened at atmospheric pressure and temperature. Finally, the grooves are sealed with thermal lamination[357] to form microchannels. Thus, plastic chips possess two different surface types, three walls of the polymer substrate, and one wall of the laminated film. Special care must be given to well match the materials to avoid disrupted plug flows. An alternative consists in annealing the molded plate directly to another plate with oxygen plasma.[358,359] With this technique, channels with four equivalent walls are formed and separation is less impaired.

B. Designs

A typical CE microchip is around $10\,cm^2$ in size and a few millimeters thick. Dimensions of the microchannels are $1-5\,cm$ in length, $5-50\,\mu m$ in depth, and $20-100\,\mu m$ in width, although channels with depths of less than $5\,\mu m$ were reported. Thus, the small cross section of the channels allows Joule heat dissipation, thus high electric fields can be applied on microchips to achieve sub-second separations. The change of the classic CE format with relatively long effective separation length to shorter microfabricated channels on microchips enables rapid separations without significant peak broadening. Because efficiency is independent of the migration length, shorter separation paths can be used without loss in resolution. However, short separation channels on microchips make the sample introduction more critical. Apart from usual stacking procedures, separation channel's length can be increased by introducing serpentine- or spiral-shaped channels without any increase of zone dispersion from turns.[360,361]

The design of microchips for CE has undergone significant development from single-channel structures to increasingly complex models, and current designs allow on-chip reactions and separation in multiple channels. Arrays of separation channels were also designed on microchips to simultaneously analyze a large number of samples, but they are usually constrained by the detection method. A basic microchip includes two crossed channels and four reservoirs for sample, waste, and cathode and anode electrolytes. As mentioned above, the controlled injection of nanoliter or picoliter sample volumes in the separation channel is required to minimize dispersion effects. Electrokinetic injections are the most widely used sampling techniques since no additional elements such as valves or pumps need to be integrated onto the microstructure. Many injection schemes have been developed and the integrated injectors are usually either cross-channel injectors, formed by orthogonally intersecting the sample-to-waste channels, or double-T injectors, where the two arms of the sample-to-waste channels are offset to form a larger injector region. To date, cross-intersection is mostly employed, and with this design, several injection techniques such as floating,[362,363] pinched,[364–371] dynamic,[372,373] and gated[374–378] injections can be implemented depending on electric field strength distributions.

Implementation of microanalytical devices presents some issues mostly related to the scale of the volumes. In fact, successive reduction in the sample volume may compromise analysis either because the measurement limit of the analytical method is exceeded or because the sample is no longer representative of the bulk specimen. Another drawback for microchip devices is microvolume evaporation of both sample and reagent from the microchip, compromising quantitative determination or inducing unwanted hydrodynamic flows. This problem has been addressed by designing pipetting systems that automatically replace fluid lost by evaporation or by enclosing the chip in a controlled environment.[379]

C. MS Interfacing

Over the past decade, significant progress in the field of microfluidics, instrumentation miniaturization, and the integration of the whole analytical process has evolved, but microchip−ESI/MS interfacing has also received a great attention since it combines small sample volume's handling, fast separation with sensitive, nearly universal detection, and possible structure elucidation of separated compounds. Furthermore, despite the mismatch in physical dimensions, MS is a promising alternative to optical and EC detections due to the perfect and natural compatibility between flow rates required for ESI/MS and those generated by CE chips. An overview on the combination of microsystems and MS was given in general reviews,[380−382] and three approaches are currently distinguished to interface microfluidic devices to MS: (i) spraying directly from the chip, (ii) spraying from a capillary sprayer attached to the chip, or (iii) using a nanospray emitter integrated into the microchip. Several designs are illustrated schematically in Figure 6.

1. Spraying Directly from the Chip

The first on-chip separations prior to MS were reported in 1999 by several groups and consisted in spraying the fluid out of the microchip directly from the microchannel.[324,383] The main focus was to create stable ESI conditions and reduce background noise. Designs were quite attractive because they did not require any complex setup, since the outlet was simply formed by dicing the chips. On the other hand, the flat-face from which the sample was sprayed led to the formation of large droplets at the surface of the chip, which caused excessive band broadening and sample dilution. Attempts were made at minimizing droplet size by coating the outlet orifice with a hydrophobic agent or by pneumatically assisting the droplet formation.[384] However, these approaches did not provide efficiencies as high as obtained with a transfer capillary or a nanospray emitter. Another methodology was implemented by Girault and co-workers,[349,385] who developed a polyimide microfluidic system by plasma etching with a precise nanospray exit nozzle designed in a tip shape. The typical isotropic etching of the plasma as well as the thickness of the tip edge (only 20 μm) contributed to the onset of a spray in contrast to thicker devices in which droplets are formed at the outlet. Furthermore, the thin outlet walls made of hydrophobic polymer avoided the wetting of the spray edge, resulting in the formation of an efficient and stable nanospray.

2. Spraying from a Capillary Sprayer Attached to the Chip

Although previous studies showed that direct ionization from the outlet separation channel at the chip surface with Taylor cone formation could be possible, this is probably not optimal because the ESI cone volumes are larger than peak volumes. With short

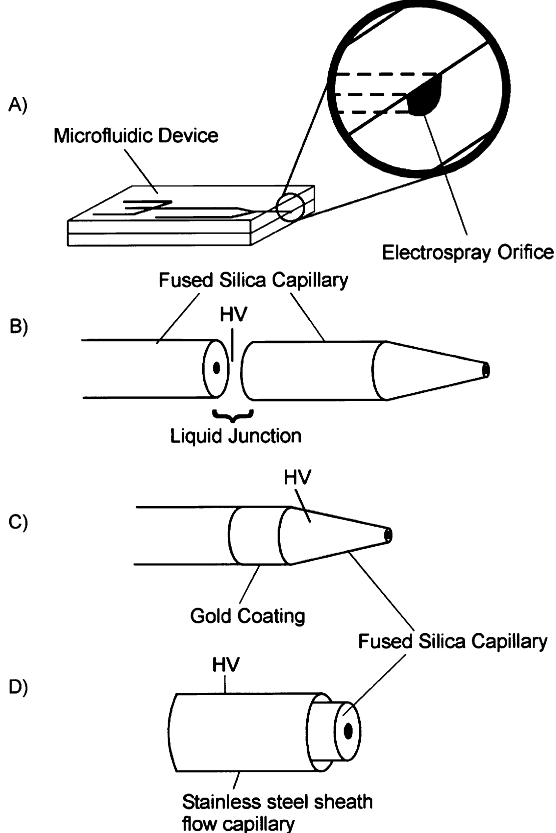

FIGURE 6 Schematic representation of different interfaces for chip CE–ESI/MS: (A) spray directly from the chip, (B) liquid-junction capillary interface, (C) gold-coated capillary interface, and (D) coaxial sheath-flow configuration. Reprinted from reference 410 with permission from Elsevier Science B.V.

transfer capillaries attached to the microdevice, as first introduced by Figeys et al.,[386] an external electrospray is implemented. Special care must be paid when using transfer capillaries for joining microfluidic devices. Precise, low dead-volume alignment of the chip separation channel and transfer capillary is mandatory for maintaining efficiency, especially because injected sample volumes on the microchips range from 0.1 to 0.5 nL compared with 2–5 nL in conventional CE. Thus, Bings et al. developed a method to obtain low dead-volume connectors, as small as 0.7 nL.[387] When the spray is generated from a transfer capillary, either a disposable nanospray emitter[388,389] or a fused-silica capillary transfer line can be inserted in the microchip. For the former, the ESI nozzle is usually made with a sharp, tapered capillary end from which the liquid sprays to produce small, well-defined droplets. An advantage of this setup is that the nanospray tip can be removed and replaced without changing the microchip, as clogging is a significant problem when using capillaries with tapered ends. For the latter, interfacing options are similar to those used in conventional CE–MS coupling and include liquid junction[324,390–392] and coaxial sheath-flow configurations.

3. Spraying from an Integrated Nanospray Emitter

Microchips' fabrication with integrated tips can result in improved spray repeatability and efficiency since alignment and dead volume are not a critical issue anymore. However, production of fine and robust nanospray emitters as an integral part of a microdevice is not trivial, and highly specialized microfabrication procedures are required. Microfluidic devices with integrated ESI tips have been produced for infusion experiments,[393−397] but to date, no microchips with such a design was fabricated for CE separation prior to MS detection.

D. Applications

The most prominent field of applications for microchip−MS concerns identification and analysis of large molecules in the field of proteomics[392,398−402] according to the reduced separation time compared to conventional approaches such as gel-based methods for protein analysis.[403] High-throughput analyses, with lower contamination and disposability, are other features of microfabricated devices that allow the fast screening of proteomic samples in the clinical field. Applications also include the analysis of low-molecular-weight compounds such as peptides[404] or pharmaceutical[405] samples.

Henion and co-workers investigated the use of microfabricated devices coupled with MS for the determination of several drugs. In a first study,[406] they evaluated the potential of a polymer-based microchip CE−MS system for on-chip separation and quantitative detection of polar small molecules. A silicon master was microfabricated using photolithographic and dry etching processes; microchannels were embossed in the plastic and thermally enclosed with the same polymer. The microchip was coupled to a microsprayer via a liquid junction formed between the chip and sprayer, and a triple-quadrupole mass spectrometer was operated in SIM and SRM modes to produce CE−MS results. No surface treatment of the polymer was necessary to obtain a sufficient and repeatable EOF. A baseline separation of carnitine, acylcarnitine, and butylcarnitine was obtained in less than 10 s for injection quantities of 0.2 nmol of each compound. This group assessed the applicability of another glass device for quantitative microchip CE−MS analysis of the above-mentioned drugs in human plasma[407] and urine[408] samples. LOQ as low as 5 μg/mL were obtained with good performance in terms of trueness and precision, which demonstrated the feasibility for on-chip CE separation and ESI/MS detection in bioanalytical applications. Tachibana et al. described a robust and simple interface for microchip CE−MS using a spray nozzle connected to the exit of the separation channel of the microchip.[409] The analysis of several basic drugs such as pindolol, nicardipine, sulpiride, and trimipramine was successfully performed in less than 20 s using the optimized system that consisted of a spray nozzle with a small bore size and a separation buffer with high viscosity. This system was also applied to the separation of peptides and trypsin-digested proteins with the adsorption of peptides to the quartz microchip alleviated by adding acetonitrile to the separation buffer.

VI. SUMMARY AND CONCLUSIONS

The use of different electromigration-based separation techniques hyphenated with MS has become a standard technique in modern pharmaceutical analysis. With the possibility of several commercially available instruments, including various interfaces, the coupling between CE and MS can now be easily achieved. Therefore, numerous issues can be resolved according to the wide choice of operation techniques afforded in CE and the various ionization modes and/or analyzers. CE−MS has emerged as a good alternative for trace

analysis (i.e., degradation substances, impurities) or for compounds without strong chromophores such as sugar, peptides, or amino acids. Furthermore, MS detection in the SIM mode enhances selectivity and sensitivity, and expands the potential for quantitation using CE. Despite the fact that CE−MS is widely used in qualitative analyses, few quantitative applications have been published for biological matrices and pharmaceutical formulation analyses.

Much progress has been made in coupling microfabricated devices with MS, and this field continues to move forward with the commercialization of new microfluidic systems. The latter allow the handling of very small samples with short analysis times and at reduced analysis costs. Furthermore, microfabrication enables large-scale integration of the entire analytical process, multiplexing, and therefore high-throughput analysis. Thereby, this emerging technology may become a dominant force in the very near future, as it perfectly couples the concept of limited sample amounts with the requirements for high separation efficiency and detection sensitivity. An important requirement for high-throughput qualitative and quantitative determination of small molecules in pharmaceutical drug discovery also demands highly selective screening methods for further testing. To achieve this goal, new developments such as multiplexed chip−MS are promising.

ABBREVIATIONS

ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
BGE	Background electrolyte
CD	Cyclodextrin
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CF-FAB	Continuous flow-fast atom bombardment
CSP	Chiral stationary phase
CZE	Capillary zone electrophoresis
DMA	*N*,*N*-dimethylacetamide
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
EC	Electrochemical
EOF	Electroosmotic flow
ESI	Electrospray ionization
EtOH	Ethanol
FA	Formamide
FDA	Food and durg administraction
FT-ICR	Fourier transform-ion cyclotron resonance
H/*N*	Height-to-noise ratio
HPLC	High-performance liquid chromatography
HP-β-CD	Hydroxypropyl-β-cyclodextrin
HS-γ-CD	Highly sulfated γ-cyclodextrin
ICH	International conference on Harmonisation
ICP	Inductively coupled plasma ionization
IP	Ionization potential
i-PrOH	Isopropanol
IS	Internal standard

LC	Liquid chromatography
LIF	Laser-induced fluorescence
LLE	Liquid–liquid extraction
LOQ	Limit of quantitation
m/*z*	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MIP	Molecular imprint-based polymer
MS	Mass spectrometry
NACE	Non-aqueous capillary electrophoresis
NMF	*N*-methylformamide
OT-CEC	Open-tubular capillary electrochromatography
PEG	Polyethylene glycol
PEM	Polyelectrolyte multilayer
PET	Polyethylene terphtalate
PFT	Partial-filling technique
PMMA	Polymethylmethacrylate
RSD	Relative standard deviation
SBE-β-CD	Sulfobutylether-β-cyclodextrin
SDS	Sodium dodecyl sulfate
SFSTP	Société française des sciences et techniques pharmaceutiques
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring
TIC	Total ion current
TOF	Time-of-flight
TM-β-CD	Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin
XIC	Extracted ion current

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