Prediction of pharmacokinetic properties using experimental approaches during early drug discovery

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There has been a significant increase in the number of compounds synthesized in early drug-discovery programs with the advances in combinatorial chemistry and high-throughput biological screening efforts. Various *in silico*, *in vitro* and *in situ* approaches have been described in literature that achieve higher throughput pharmacokinetic screening. *In silico* methodologies have mainly attempted to quantify the prospects of oral absorption of compounds based upon their physico-chemical properties. There is a greater availability of *in vitro* and *in situ* approaches to screen compounds for intestinal permeability (as a surrogate for absorption) and metabolic stability (as a surrogate for clearance). More recent modifications of the *in vitro* and *in situ* approaches to assess the potential of absorption and metabolism have enabled a higher throughput and an ability to correlate better with *in vivo* pharmacokinetics of compounds.

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Abbreviations

AUC area under the curve
BCS Biopharmaceutics Classification Scheme
Caco-2 human colon adenocarcinoma
Cl clearance
CYP cytochrome P450
F oral bioavailability
Fa fraction absorbed into portal circulation
Fg fraction escaping gut clearance
Fh fraction escaping hepatic clearance
Fl fraction escaping lung clearance
GFR glomerular filtration rate
HTS high-throughput screening
IAM immobilized artificial membrane
J_0 flux
MDCK Madin–Darby canine kidney
MW molecular weight
NCE new chemical entity
P_0 permeability coefficient
PAMPA Parallel Artificial Membrane Permeation Assay
PK pharmacokinetics
PSA polar surface area
$T_{1/2}$ half-life

Introduction

Pharmacokinetics is the study of the time course of drug absorption, distribution, metabolism and excretion (ADME), and how these ADME processes are related to the intensity and time course of the pharmacological (therapeutic and toxic) effects of drugs [1]. The discipline of pharmacokinetics (PK) was intended, in part, to be a tool to optimize the design of biological experiments with drugs. Rapid advances in analytical chemistry allowed clinical applications of pharmacokinetics, which have resulted in significant improvements in drug therapy.

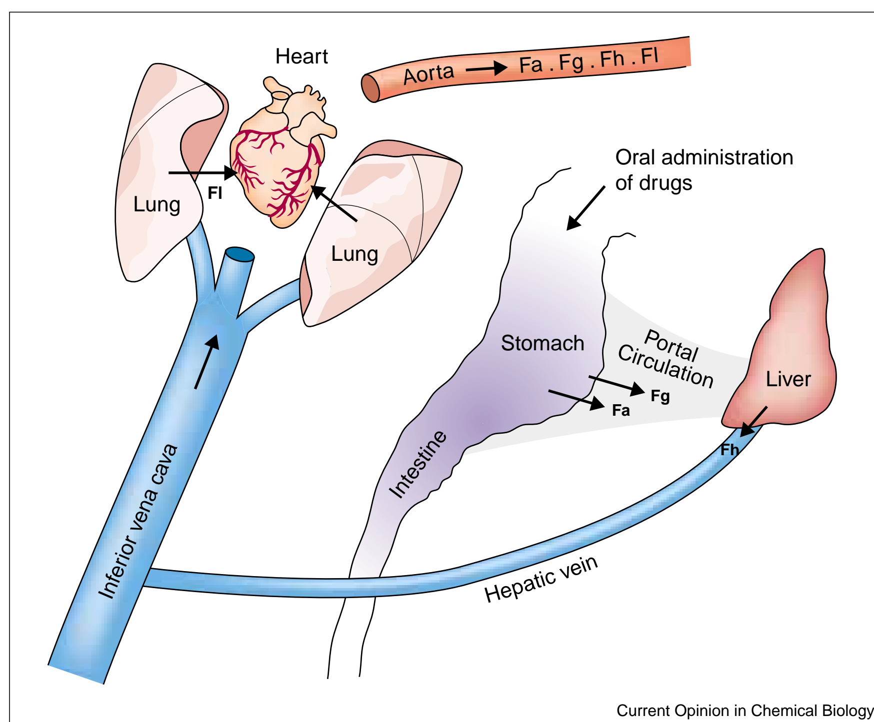
Orally delivered pharmacologically active compounds must have favorable absorption and clearance properties and satisfactory metabolic stability to provide adequate systemic exposure to elicit a pharmacodynamic response. If the compounds possess reasonable physico-chemical properties, have low to intermediate clearance and reasonable absorption, adequate oral bioavailability may be achieved. Because conducting an *in vivo* experiment is time-consuming and material-consuming, early discovery efforts have focussed on assessing *in vitro* or *in situ* absorption potential and *in vitro* metabolic stability [2,3••]. Such experiments are intended to screen compounds and identify candidates that are most likely to have adequate PK properties for further pharmacological evaluation.

Furthermore, in contemporary drug-discovery and development, novel molecular targets are being investigated to provide novel pharmacological approaches to therapeutics. This places an additional burden on the 'proof-of-concept' studies to support further clinical development of new chemical entities (NCEs). The three main reasons for clinical failure of an NCE are lack of efficacy, toxicity and unfavorable pharmacokinetic properties. All three are poorly understood and difficult to predict. Hence, drug-discovery research is under enormous pressure to increase the probability of ultimate success of the drug entering clinical trials [4].

Given these issues, there has been a concerted effort towards better prediction of pharmacokinetic properties and toxicity of NCEs in early discovery efforts. Through progress in linking '*in silico*' and higher throughput physico-chemical methods with *in vitro* approaches, progress is being made in predicting PK properties [5]. Such high(er)-throughput screening (HTS) efforts require rapid methods and the use of minimum amounts of compounds, so that new molecules can be synthesized based upon the predicted or experimental outcomes of PK and/or toxicity experiments in a timely fashion. In addition, oral administration is preferred for many classes of drugs because of the ease of administration and patient compliance. Predicting oral bioavailability of compounds during early drug-discovery efforts poses additional challenges to the pharmaceutical industry [6].

Several approaches have been described for predicting human PK properties from *in vivo* preclinical PK data [7,8]. The best technique to predict human PK is allometric scaling

Figure 1



Schematic representation of the process of drug absorption following oral administration of drugs. Drugs are absorbed into the portal circulation (Fa), with the fraction reaching the portal circulation being Fa . Fg, where Fg is the fraction not removed by the gut. The fraction of dose entering the hepatic vein is the fraction escaping liver clearance and can be estimated as Fa . Fg . Fh. The arterial bioavailability of the drug is the product of drugs escaping the intestinal, liver and lung clearance and can be estimated from Fa . Fg . Fh . Fl.

[3••,9–11]. Allometry is the study of relationships between body size and the structural and functional capacities of organs [12]. Because PK parameters are a function of the anatomical and physiological processes, they can be scaled allometrically across species [3••].

Because early drug-discovery efforts must also evaluate whether the NCE has the ability to be absorbed and become bioavailable, emphasis on predicting oral bioavailability rapidly and early is critical. Because reliable estimation of oral bioavailability requires *in vivo* experimentation, surrogate parameters for oral bioavailability are estimated from a battery of *in silico*, *in vitro* or *in situ* experiments. A retrospective study of marketed drugs indicated that physico-chemical properties including partition coefficient, molecular weight (MW), aqueous solubility and conformational flexibility, influence the oral bioavailability of drugs [13]. As such, oral bioavailability is influenced by several factors including solubility, permeability, intestinal and liver metabolism, rapid biliary and other efflux pump-mediated excretion and conditions in the gastrointestinal milieu [6,14]. Hence, a systematic approach is required in predicting oral bioavailability and other PK properties of NCEs in early discovery efforts.

The aim of this review is to summarize experimental approaches that can be utilized in a HTS mode during early drug discovery to facilitate the prediction of PK parameters. Although virtual and computational approaches [15] have also been proposed to predict PK parameters, this review will focus on the features and limitations of available experimental methods, which require (relatively) small quantities of test compounds, while improving the throughput of 'classical' pharmacokinetic evaluations.

Key pharmacokinetic parameters: theory and rationale

Bioavailability

The oral bioavailability (F) of drugs is defined as the fraction of the ingested dose that is available to the systemic circulation. Thus, both absorption and elimination processes determine the oral bioavailability of a given drug. A general representation of oral bioavailability is described in Figure 1 and can be estimated as:

$$F = Fa \,.\, Fg \,.\, Fh \,.\, Fl \qquad (1)$$

where F refers to systemic oral bioavailability, Fa is the fraction absorbed across the intestinal wall, and Fg . Fh . Fl is the product of the fractions escaping clearance by the gastrointestinal tract, liver and lung. The product of fraction available after gut and liver extraction (Fg . Fh) following oral administration primarily determines the oral clearance of the drug, although the contribution of lung clearance should also be considered [1].

Intestinal permeability as a predictor of the true 'fraction absorbed'

Gastrointestinal permeability is an estimate of the selective ability of intestinal epithelium to provide a barrier to the absorption of drugs. It is generally accepted that intestinal permeability and lipophilicity have a sigmoidal relationship. For compounds with no significant metabolism or

efflux via active transport, within a low MW range (~150 to 1200), drug 'absorbability' is governed by intestinal permeability via passive diffusion. The permeability coefficient (P_0) is calculated from the unbiased flux (J_0), concentration in the medium (C), and a conversion factor (cf) for tissue mass to surface area (cf = 24.0 cm^2/g) from the following equation [16]:

$$J_0 = P_0 \cdot C \cdot \langle cf \rangle \quad (2)$$

The theoretical relationship between the fraction of drug absorbed (Fa) and dimensionless effective steady-state permeability (P_{eff}) has been described by Amidon *et al.* [17].

$$Fa = 1 - \exp^{(-2.Peff)} \quad (3)$$

Assuming an aqueous drug diffusivity of 5×10^{-4} cm^2/min, and a rat intestinal radius of 0.2 cm, a theoretical estimate of rat gut permeability of 25×10^{-4} cm/min has been considered to be indicative of a well absorbed drug [18].

Clearance

The best estimate for drug elimination can be obtained by determining the 'total clearance' (Cl) of the drug. Organ clearance is defined as the volume of blood that must be cleared of drug in a unit of time in order to account for the rate of drug elimination. Thus, clearance is the ratio of elimination rate of the drug to the drug concentration in blood entering the organ. The total clearance is the sum of all individual organ clearances of the drug. However, it should be noted that not all organ clearances are additive. Because drugs are eliminated by various tissues, including gastrointestinal wall, liver, lungs and kidneys, the anatomical arrangement of organs is quite important. When the eliminating organs are arranged in parallel (such as the liver and the kidneys), the total clearance of the drug can be estimated by additive clearance from individual eliminating organs. When the eliminating organs are arranged in series (e.g. gastrointestinal tract and liver), a different approach using the products of organ clearance is required for the estimate of total clearance [19,20]. Because clearance plays an important role in both drug elimination and oral bioavailability, its prediction is of utmost importance in estimating PK properties of drugs [3••].

Most drugs are eliminated primarily by the liver and/or the kidney. Hence, prediction of hepatic and renal clearances are of prime importance in predicting PK properties of NCEs. It is well known that total systemic clearance (Cl_s) of a drug is estimated as the ratio of dose to area under the curve (AUC) following intravenous administration of the drug:

$$Cl_s = Dose_{(iv)} / AUC_{(iv)} \quad (4)$$

Where iv is intravenous. Following oral administration, Cl_{oral} is defined as:

$$Cl_{oral} = Dose_{(oral)} / AUC_{(oral)} \quad (5)$$

Combining the relationships for clearance and oral bioavailability, Cl_{oral} can also be estimated from:

$$Cl_{oral} = Cl_s / (Fa \cdot Fg \cdot Fh \cdot Fl) \quad (6)$$

where Fa and Fg. Fh. Fl are the fraction of drug absorbed into the portal vein, and fractions not subject to elimination by the gut, liver and lung, respectively. Assuming negligible gut and lung clearance, Equation 6 can be reduced to:

$$Cl_{oral} = Cl_s / (Fa \cdot Fh) \quad (7)$$

If drugs are cleared by both liver and kidneys,

$$Cl_s = Cl_h + Cl_r \quad (8)$$

where Cl_h is hepatic clearance and Cl_r is renal clearance. Thus,

$$Cl_{oral} = (Cl_h + Cl_r) / (Fa \cdot Fh) \quad (9)$$

Thus, it is evident from Equation 9, that if one were able to predict renal and hepatic clearance, as well as the fraction of drug absorbed into the portal circulation and fraction of drug escaping hepatic elimination, the estimates of oral clearance and oral bioavailability would be quite accurate.

Volume of distribution

The volume of distribution is a measure of the extent of drug distribution and is determined by the binding of the drug in plasma as well as tissues. Because it is assumed that the unbound drug can diffuse across membranes, it is implicit that the distribution to tissues is affected by plasma-protein binding. It is also important to understand that because of significant tissue binding for most drugs, the 'apparent' volume of distribution far exceeds the total body water (i.e. 58% of the adult human weight). As such, the volume of distribution is the proportionality constant relating the drug concentration in blood or plasma to the amount of drug in the body. Gibaldi and McNamara [21] have shown that:

$$V = V_b + V_t \cdot (f_b/f_t) \quad (10)$$

where V is the volume of distribution, V_b is the blood volume, V_t is the extravascular tissue space volume, f_b is the unbound fraction in blood and f_t is the unbound fraction in tissues.

Elimination half-life

The half-life ($T_{1/2}$) of any drug is related to its apparent volume of distribution (V) and its systemic clearance (Cl_s) as:

$$T_{1/2} = 0.693 \cdot (V/Cl_s) \quad (11)$$

Thus, the half-life of any drug is a function of blood and tissue binding of the drug as well as its total clearance and is a derived parameter from Cl_s and V. For drugs with high clearance, the half-life is relatively independent of changes in intrinsic clearance, whereas for drugs with low clearance, increases in intrinsic clearance result in decreased half-life.

Physico-chemical properties for oral absorption

The role of physico-chemical and physiological factors in drug absorption have been evaluated [6,14,22] and key determinants of oral bioavailability for NCEs with adequate metabolic stability are being identified. Because drug dissolution (*in vitro*) and gastrointestinal permeability are fundamental parameters controlling rate and extent of absorption of drugs, a Biopharmaceutics Classification Scheme (BCS) for correlating *in vitro* dissolution and *in vivo* bioavailability has been proposed [23].

Several approaches have focussed on predicting the 'requisite' physico-chemical properties for 'drug-like' absorption potential for NCEs. [15,24]. A retrospective analysis of the World Drug Index in terms of physico-chemical properties identified the 'rule of 5' [25]. Poor intestinal permeability of compounds is predicted for compounds with any two or more of the following: more than five hydrogen-bond donors (sum of OH and NH groups), more than 10 hydrogen-bond acceptors (sum of N and O), MW >500, and log P >5. Compounds that are substrates for biological transporters or gastrointestinal wall enzymes are exempted from this rule.

Recent studies have indicated that polar surface area (PSA) has a strong correlation with permeability. PSA is defined as the van der Waals surface that derives from N and O, and as such is related to the hydrogen-bonding capacity of the molecule. Studies combining MW and PSA have shown that orally bioavailable recently marketed drugs (from 1993–1995) mostly have a MW <500 and a PSA <120 $Å^2$ [26]. PSA has been shown to be useful in predicting oral absorption and intestinal permeability [2,27]. From data on 20 selected compounds, it has been shown that a PSA >140 $Å^2$ results in incomplete (<10%) oral absorption, whereas a PSA <60 $Å^2$ results in excellent (>90%) absorption [28]. However, the computation time for PSA is considerable and has rendered this approach unsuitable for high-throughput prediction of absorption potential [2]. Recently, a new, fast computational model, based on partitioned molecular surface areas, has been reported to predict intestinal permeability with an accuracy comparable to that obtained from time-consuming quantum mechanics calculations [29].

The lipophilicity (log P) of a drug is clearly related to its passive absorption, although the relationship of oral absorption and log P is complex and indirect. A retrospective analysis of 50 marketed drugs across six drug classes showed that drugs possessing log P values between 0 and 3 and molecular weight between 150 and 550 are most likely to demonstrate adequate oral bioavailability [13]. The major components of lipophilicity are considered to be hydrophobicity, molecular size and hydrogen bonding. In a 'deconstructed' analysis of lipophilicity, highly absorbed drugs (>80%) were found to have MW values less than 500 and clogP between 1 and 5 [30]. In addition to rendering calculations of clogP more predictive, newer approaches such as liposomal partitioning for calculating absorption potential have indicated sigmoidal correlation with intestinal permeability [31].

Aqueous solubility is, as a broad generality, inversely related to lipophilicity. Assuming good stability and lack of first-pass metabolism, the two key parameters controlling drug absorption are membrane permeability and dose-to-solubility ratio [17]. Recently, Horter and Dressman [32••] have shown that when aqueous solubility of drugs is less than 100 μg/ml, dissolution limits oral absorption of the drugs, especially when the volume of fluid required to dissolve the drug's dose exceeds one liter.

The BCS combines the solubility and permeability properties and identifies four classes of drugs: high solubility, high permeability; low solubility, high permeability; high solubility, low permeability; low solubility, low permeability [23]. Although the description of the mathematical approach to the BCS is beyond the scope of this review, it is important to note that the solubility component in this classification system is driven by the dissolution of the drug in the volume of the fluid available in gut lumen. The estimated fraction of the dose absorbed is driven by three dimensionless parameters described by dissolution number (D_n), dose number (D_o) and absorption number (A_n) [23]. It is also implicit, subject to this classification, that the drugs do not have significant 'first-pass' metabolism and their oral bioavailability is dissolution-limited. For instance, based upon a permeability approximately equal to that for glucose ($A_n = 10$), it is suggested that for a fraction absorbed of 50% for a given drug requiring doses of 5, 50 or 500 mg, a corresponding (gut) solubility of 0.5, 5.0 or 50.0 μg/ml will be required. Furthermore, if the permeability was reduced 10-fold ($A_n = 1$), the corresponding solubilities for the doses of 5, 50 or 500 mg would be 5.0, 50.0 or 500.0 μg/ml.

Based upon the BCS, one can expect good *in vitro* dissolution and *in vivo* bioavailability correlation for class II and IV drugs (dissolution is slower than gastric emptying time and thus rate-limiting). In the case of class II drugs, the choice of dissolution media to predict rate of solution of drugs is critical. Dressman and Reppas [33] have recently shown that the *in vivo* absorption of poorly soluble drugs such as danazol, ketoconazole, atovaquone and troglitazone could be predicted by using biorelevant dissolution media such as simulated gastric fluid and fluids modeling the fasted and fed states of the stomach and small intestine. Absorption for a class I drug is controlled by gastric emptying (rate-limiting step), and permeability controls the absorption of a class III drug [34].

Table 1

Comparison of apparent permeability coefficients (P_{app}) for highly absorbed model drugs from different laboratories.

Model drugs	$P_{app} \times 10^5$ cm/sec in Caco-2 monolayers*	Literature reported $P_{app} \times 10^5$ cm/sec in Caco-2 cells	Reported fraction absorbed in humans
Terbutaline	1.95	0.038‡	25–80¥, 60**
Testosterone	10.91	7.23§	100§
Enalapril	0.71	NR	60¶
Caffeine	2.51	5.05§	100§
Glucose	1.21	2.5#	100#
Metoprolol†	4.66	2.7‡	95#

*Obtained at Vertex Pharmaceuticals using 10 μM concentration in a two-hour experiment. †Metoprolol experiment conducted at 5 μM. ‡Artursson and Karlsson [38]. §Yee [39]. #Lennernas *et al.* [70]. ¶Leppert and Fix [35]. ¥Nyberg [40]. **Fagerholm *et al.* [51]. NR, not reported.

Methods of estimating intestinal permeability as a predictor of the fraction of the drug absorbed (Fa)

In vitro and *in situ* approaches have been used to estimate intestinal permeability of molecules to predict *in vivo* drug absorption. The three most widely used methods are rat *in situ* single-pass intestinal perfusion [17], rat everted intestinal rings [35], and the *in vitro* human colon adenocarcinoma (Caco-2) cell line [36,37]. These absorption assessment methods are primarily applicable to drugs that are absorbed via passive diffusion (i.e. permeability is a function of partition coefficient and pK_a of the molecule).

Absorption rate constants (expressed as apparent permeability coefficients) were determined for 20 drugs by Artursson and Karlsson [38] in Caco-2 monolayers. In that study, drugs that were completely absorbed in humans had apparent permeability coefficients $>1 \times 10^{-6}$ cm/sec, whereas drugs with human oral absorption between 1 and 100% had permeability coefficients between 0.1 and 1.0×10^{-6} cm/sec. Various investigators have indicated that Caco-2 monolayers are a good *in vitro* model for oral absorption. Furthermore, it has been suggested that apparent permeability coefficients of $<1 \times 10^{-6}$ cm/sec, $1–10 \times 10^{-6}$ cm/sec and $>10 \times 10^{-6}$ cm/sec can be classified as poorly (0–20%), moderately (20–70%) and well (70–100%) absorbed compounds [39]. Note that the permeability coefficient estimates between the latter proposal and those reported by Artusson and Karlsson [38] differ by a factor of about 10.

During validation of the use of Caco-2 monolayers, caution must be used while comparing permeability estimates among various laboratories. For instance, Caco-2 permeability estimates for highly absorbed (≥60%) model drugs in our laboratory are compared with reported literature estimates in Table 1. With the exception of terbutaline, it can be seen that literature P_{app} estimates are in good agreement (3–5-fold) with those obtained by us.

The absorption of terbutaline is complex and points to one of the limitations of the Caco-2 monolayers. The estimation of fraction absorbed for terbutaline is confounded by significant first-pass metabolism, particularly in the gut wall [40]. The complexity of absorption is, in part, also a result of different enantiomers of terbutaline governing the absorption and elimination processes. It has been reported that the (–)-enantiomer of terbutaline governs its absorption, whereas the (+)-enantiomer determines its elimination [41,42]. Thus, it is important to note that Caco-2 monolayers will over- or under-estimate the expected oral bioavailabilities for drugs undergoing presystemic elimination and/or having stereospecific absorption or elimination.

Limitations of Caco-2 monolayers to assess permeability include the following [2,18,37,39]:

1. 21-day culture times.

2. Homogenous cells that lack mucus.

3. Lack of standardization in cell culture and experimental procedures.

4. Transport properties, enzyme expression and transepithelial electrical resistance being representative of colon rather than small-intestinal cells.

5. Transformed nature of the human colon adenocarcinoma cells resulting in unpredictable differentiation markers.

Although Caco-2 cells are of human origin, the system is static and may have higher involvement of paracellular transport compared with human small intestine and usually provides low rates of transport [43]. To overcome the limitations of Caco-2 and to reduce tissue culture time, cost, and effort for permeability determination, the use of Madin–Darby canine kidney (MDCK) cells as a model for cellular barrier for assessing intestinal epithelial drug transport has been reported [44,45]. Like Caco-2 cells, MDCK cells differentiate into columnar epithelium and form tight junctions on semipermeable membranes. The permeability data from MDCK cells has been compared with that obtained from Caco-2 cells for 55 drugs [46••]. These data show that the permeability of passively absorbed compounds was similar to that obtained from Caco-2 cells. The major advantage of the use of MDCK cells is its ability to assess reliable permeability estimates after only three days of culture rather than the 21 days required by Caco-2 cells. Braun *et al.* [47] have suggested that the ease of handling of MDCK cells with shorter culture times (7–14 days) and their low expression of transporter proteins and metabolizing enzymes, make them perfect for evaluation of permeability of passively absorbed compounds.

The ability to assess the role of intestinal cytochrome P450 (CYP) 3A4 in first-pass metabolism *in vitro* has been difficult. Recently, human CYP3A4 has been introduced into Caco-2, MDCK and LLC-PK1 mammalian cells to assess permeability and intestinal metabolism [48]. Long-term stable expression of CYP3A4 was achieved in MDCK cells, but the highest level of CYP3A4 activity was observed in the LLC-PK1 cell line. More permeability and metabolism data from such cell lines will need to be correlated with human absorption to assess the true potential of these modifications.

The oral absorption potential of 12 drugs has been evaluated *in vitro* in everted intestinal rings [35]. These investigators have reported that the extent of drug accumulation in intestinal tissue over a short period (≤10 minutes) is a good predictor of human fraction absorbed rather than the rate of drug accumulation in the intestinal tissue. Under the conditions evaluated, it was found that the prediction of drug absorption was independent of gastrointestinal region, pH and co-solvent. An *in vitro* drug accumulation of at least 2500 pmoles/mg intestinal tissue was associated with good oral absorption (≥60%) in the study reported by Leppert and Fix [35]. Although this *in vitro* method is relatively quick and inexpensive, and is able to predict oral absorption potential of passively and actively absorbed drugs, critics of this approach take issue with the viability of the tissue, and limited uptake of the drug by tissue [18].

Rat single-pass intestinal perfusion is an *in situ* approach with intact anatomy and physiology. Thus, this approach is much more physiologically and pharmacologically relevant and input of drugs can be controlled in terms of concentration, pH and composition [49]. The assessment of permeability by this technique has been used to predict human oral absorption for both passive and carrier-mediated transport of compounds [17,50]. Its limitations include the technical difficulty, limited viability of tissue, and the need for larger amounts of compound for evaluation of drug absorption potential. From steady-state intestinal perfusion experiments in animals, dimensionless wall permeabilities have been estimated. Compounds with dimensionless intestinal wall permeability of 1.0 (equivalent to 25×10^{-4} cm/min) or higher have been shown to correspond to well absorbed drugs in humans, whereas drugs with dimensionless wall permeability of <0.1 have been poorly absorbed. [17,51].

Stewart *et al.* [18] have compared the estimated permeabilities of a set of drugs using the three approaches described above and have shown that regardless of the method of permeability estimation, intestinal permeabilities greater than approximately 20×10^{-4} cm/min were descriptive of well absorbed drugs. There appeared to be good correlation among the three methods for rank ordering the drug absorption potential of the compounds evaluated. However, they cautioned that reference compounds spanning a wide range of physico-chemical properties, with associated carrier-mediated absorption, may not be predictive of the oral drug absorption of all small molecules or peptidomimetic drugs by any one of these approaches.

Other approaches for rapid assessment of absorption potential include immobilized artificial membrane (IAM) chromatography [52] and the parallel artificial membrane permeation assay (PAMPA) [53]. The IAM chromatography offers the advantages of experimental simplicity and good correlations between IAM k′ values and Caco-2 permeabilities have been reported. PAMPA is based on a 96-well microtiter plate technology and allows reasonable throughput, although it lacks similarity to natural membranes in that it does not possess pores or active transport mechanisms. It enables fast determination of the trends in the ability of the compounds to permeate membranes by passive diffusion and is thus suited for the screening of large libraries. A caveat of the IAM and PAMPA approaches is to remember that they will underestimate the absorption of compounds subject to active or paracellular transport *in vivo* and overestimate the absorption of compounds subject to efflux pump transport [54].

Prediction of clearance (Cl_S)

Predicting renal clearance

The prediction of renal clearance for humans has been quite successful using interspecies allometric scaling approaches [55–57]. Although allometric scaling has successfully predicted renal clearance in humans, it requires experimentation in four to five species, thus limiting the practical value of such an approach [8]. A simpler approach for predicting human renal clearance is to use the ratio of glomerular filtration rate (GFR) between rats and humans [58]. With the latter approach, the ratios of renal clearance for various drugs in rats and humans were roughly similar to the ratio of GFR between these two species. Thus, knowledge of renal clearance in rats allows the approximate estimation of human renal clearance using the GFR ratios. Because of the reasonable accuracy of prediction of these two approaches, *in vivo* urinary excretion data in rats and other species has been used to estimate renal clearance of drugs eliminated by the kidneys.

Predicting hepatic clearance

Most drugs are eliminated by the liver either by metabolism or direct biliary excretion or a combination of both. Despite numerous attempts to predict human hepatic clearance from *in vivo* studies, interspecies scaling approaches for hepatic clearance have been less successful because of large inherent interspecies differences in the elimination processes [59]. Rane *et al.* [60] first predicted *in vivo* hepatic clearance in rats from *in vitro* data from liver microsomes, taking into consideration hepatic blood flow and unbound fraction of the drug. Based upon the good correlation of predicted and observed hepatic extraction ratios (by isolated rat livers), the potential for *in vitro–in vivo* hepatic clearance prediction was identified.

Table 2

Physiological and biochemical parameters important for scaling *in vitro* drug metabolism data*.

Parameter	Parameter value	Scaling factor for standard rat weight of 250 g
Liver weight	4.5 g/kg body weight	11 g
Liver blood flow	1.8 ml/min/g liver	20 ml/min
Hepatocyte number†	1.35×10^8 cells/g liver	1.5×10^9 cells
Microsomal protein yield†	45 mg protein/g liver	500 mg protein

*Houston [20]. †Represents literature average.

Models for prediction of hepatic clearance include the use of liver microsomes, isolated hepatocytes, 9000g supernatant (S9) fractions, recombinant (heterologously expressed) CYP isozymes, liver slices and *in situ* gastrointestinal/liver single-pass perfusion preparations. All these approaches are reasonably predictive of hepatic clearance when liver metabolism is the predominant contributor to clearance, although they each have some limitations. Heterologously expressed CYPs are not predictive of intrinsic clearance because of contributions of several CYPs in the metabolism of many drugs and because metabolic rates differ extensively with the expression system used [61••]. Liver slices are not useful for kinetic predictions due to a lack of uniform diffusion of compounds into all the cells within the slice because of the tissue thickness (~260 μm) [8].

Recent advances in understanding the role of efflux pumps such as MDR1 and MRP have brought into question the ability of some of the *in vitro* approaches to predict hepatic clearance mediated by a combination of liver metabolism and biliary excretion. Liu *et al.* [62] have recently reported that hepatocytes cultured in a collagen-sandwich configuration for up to five days establish intact canalicular networks, maintain MRP2, re-establish polarized excretion of organic anions and bile acids, and represent a potentially useful *in vitro* model for studying hepatobiliary elimination of compounds. However, to date there are no significant data on the predictive ability of this approach.

A simple four-stage strategy for the extrapolation of *in vitro* metabolic data to predict *in vivo* metabolic clearance was first proposed by Houston [20] using either initial rate of metabolite formation or the time profile for drug loss in liver microsomes and/or hepatocytes. Within a database of 25 drugs, using biological scaling factors for isolated hepatocytes and hepatic microsomes, it was shown that excellent prediction of *in vivo* metabolic clearance was possible over four orders of magnitude [20]. The *in vitro* methods using hepatic microsomes or hepatocytes are the most widely used approaches for estimating hepatic clearance using the physiological and biochemical parameters for scaling *in vitro* drug metabolism data (see Table 2) proposed by Houston [20]. Scaling factors for extrapolating both rat and human *in vitro* metabolism data to *in vivo* hepatic clearance have been estimated and are summarized in Table 3.

Houston [20] has shown that intrinsic clearance (Cl_{int}) is a pure measure of enzyme activity towards a drug and is independent of hepatic blood flow and protein binding. Thus,

$$Cl_{int} = V_{max}/K_m = \text{Rate of metabolism} / C_e \quad (12)$$

Where V_{max} is the maximum rate of metabolism, K_m is the Michaelis constant (substrate concentration for enzyme half saturation) for the drug–enzyme interaction and C_e is the concentration at the enzyme site and is equal to the free concentration of the drug in the liver. Houston [20] and Carlile *et al.* [63] have shown that the *in vitro* data (V_{max} and clearance terms) can be scaled to *in vivo* clearance terms using either the microsomal scaling factor (660 mg microsomal protein/250 g rat weight) or hepatocellularity (1.2×10^9 cells/250 g rat weight). Furthermore, assuming the dispersion model [64], it has been shown that hepatic clearance (Cl_h) can be expressed as a function of *in vivo* intrinsic clearance ($Cl_{int,\mathit{in\ vivo}}$), hepatic blood flow ($Q_h$), fraction unbound in plasma (f_u), blood-to-plasma concentration ratio (R_b) and the dispersion number (D_n) [59].

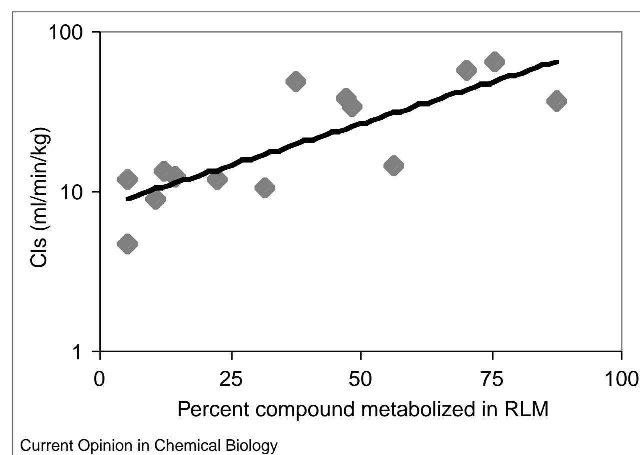
Although Houston [20] indicated that hepatic microsomes underpredicted the hepatic clearance for highly cleared drugs, recent work has shown that the underpredictions may be isoform or pathway-dependent phenomenon, rather than an upper limit of the metabolism rates in microsomes [63]. Other possible factors for perceived limitations of liver microsomes include different efficiencies of microsomal preparation among various laboratories, different CYP activities from disruptive microsomal preparation and possible product inhibition, resulting in slightly different microsomal scaling factors. Because hepatocellularity is not as variable, a smaller variability is associated with hepatocyte scaling factors, resulting in better predictions from *in vitro* hepatocytes to *in vivo* hepatic clearance [20,63].

The relationship between *in vitro* intrinsic metabolic clearance from hepatocytes and *in vivo* intrinsic metabolic clearance was studied for 29 marketed drugs in humans [59]. A good correlation was found between *in vitro* and *in vivo* results with most compounds being within three-to five-fold range of intrinsic to *in vivo* clearance values. However, there was a tendency for the *in vivo* metabolic clearance to be higher than the estimated *in vitro* intrinsic metabolic clearance. These differences were attributed to possible factors from *in vivo* contributions from other organs, incorrect assumption about rapid equilibration between the blood and hepatocytes, presence of active transport mechanisms and inter-individual differences [59].

The rapidity and convenience of evaluating the *in vitro* metabolism of test compounds by mouse, rat, dog, monkey or human microsomes has been adopted by our laboratory

over the years. Typically, we have been able to experimentally determine the depletion of the substrate following short incubations of the test material with liver microsomes from laboratory animal species (or humans). We have successfully evaluated the metabolic stability of compounds for numerous compounds in a HTS mode by this approach. For compounds where liver metabolism is the predominant (or sole) contributor to clearance, we have been able to predict the (*in vivo*) systemic clearance for this set of compounds with good accuracy. Figure 2 represents a typical prediction for one class of enzyme inhibitors during early discovery phase. An exponential (semilog) relationship between *in vitro* metabolism and systemic clearance in rats is evident for this set of compounds. Furthermore, once the percent depletion of the substrate exceeded 50% in one hour, the systemic clearance appeared to be in the 'highly cleared' range as described in the proposed classification in Table 4. It is important to note that the scope of prediction is limited to the testing conditions for the select classes of compounds studied.

Figure 2

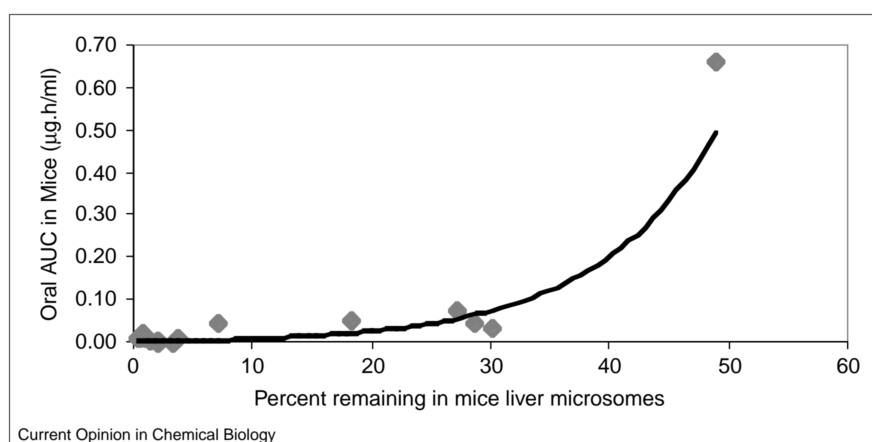


Predicted intravenous systemic clearance (Cl_s) for test compounds as a function of percent compound metabolized in an incubation of 20 μM of test compound for one hour in rat liver microsomes (RLM). Data represent mean of three animals (for *in vivo* clearance estimates) and duplicate incubations (for microsomes).

A similar relationship was obtained for another class of enzyme inhibitors using mouse liver microsomes to predict oral bioavailability in mice in our laboratory (Figure 3). Once again, an exponential relationship was evident between the percent of the compound remaining in incubates of mouse liver microsomes and AUC in mice receiving an oral dose of the test compound. In addition, there seemed to be a similar breakpoint in the percent not metabolized (~50%) for the compounds to start demonstrating reasonable oral exposure in mice.

Although many of the *in vitro* metabolism approaches have provided reasonable estimates of clearance, particularly within limited data sets, not all extrapolations are successful. A possible confounding factor in extrapolating *in vitro* metabolism data to predict *in vivo* hepatic clearance is the determination of fraction of the compound bound in plasma. A novel and convenient *in vitro* serum incubation method for predicting hepatic clearance has been proposed by [65]. This method uses the direct measurement of intrinsic hepatic clearance of a drug by using serum as the incubation medium for hepatocytes, which allows the prediction of hepatic availability (Fh) and hepatic clearance (Cl_h). Hepatocytes are preferred in this approach because they contain both phase I and II metabolizing enzymes and have physiological concentrations of cofactors. The major advantage of this method over the conventional hepatocyte preparation in Williams' E medium is the lack of necessity of estimating unbound fraction in plasma. The predicted hepatic clearance by this approach was in good agreement with systemic clearance for 16 marketed drugs, and the oral bioavailability for the marketed drugs was equal to or lower than the predicted Fh by this method. Thus, the serum incubation method for assessing hepatic availability and clearance may be a useful tool in early drug discovery.

Figure 3



Area under the plasma concentration-time curve following oral administration of test compounds in mice as a function of percent compound remaining (5 μM initial concentration) in mouse liver microsomes after 30 minutes of incubation. Data represent mean of three animals per time point for mouse AUC and triplicate incubations for microsomes.

Table 3

Biochemical parameters important for scaling *in vitro* rat and human drug metabolism data*.

In vitro parameter	Species Rat	Human
CYP450 content hepatocytes (nmol CYP450/10^6 cells)	0.27	0.14
Number hepatocytes/liver (cells/g liver)	125×10^6	120×10^6
CYP450 content in liver microsomes (nmol CYP450/mg microsomal protein)	0.66	0.32

*Data from Iwatsubo *et al.* [71].

Excellent reviews of the various approaches for estimating hepatic clearance by *in vitro* and allometric scaling approaches are available [3••,7]. Obach *et al.* [7] evaluated the prediction of hepatic clearance from *in vitro* microsomal incubation of parent drug, along with the determination of V_{max} and K_m for calculating intrinsic clearance, and allometric scaling methods with and without considering interspecies differences in plasma-protein binding. In the set of compounds evaluated by Obach *et al.* [7], the best approaches for predicting human hepatic clearance included the following:

1. Substrate depletion in human *in vitro* microsomal incubation systems without accounting for the unbound plasma fraction.

2. The substrate saturation experiments to estimate V_{max} and K_m without considering the unbound plasma fraction.

3. The allometric scaling method, which included the interspecies differences in plasma protein binding.

Lave *et al.* [3••] reported that allometric scaling combining *in vitro* and *in vivo* data from three animal species and *in vitro* data from human hepatocytes without considering protein binding was the best predictor of human hepatic clearance. Furthermore, these investigators reported that a less labor-intensive approach of *in vitro* and *in vivo* data from a single species and *in vitro* data from humans lead to acceptable prediction of human hepatic clearance.

An *in situ* approach to estimate the rate and extent of drug absorption and metabolism as well as to estimate the contribution of small intestine to first-pass effect following oral administration was proposed by Pang and co-workers [66,67]. This new *in situ* approach offers the unique advantage that the circulation and the morphology of the tissues remain intact. This model is more flexible in evaluating the combined (serial) intestinal and liver metabolism contribution when used in a single pass 'intra-arterial' perfusion mode; whereas the estimation of fraction bioavailable following 'oral' (intra-duodenal) administration in a recirculation mode is also possible. Data for model compounds (acetaminophen and enalapril) have illustrated the usefulness of this approach in predicting drug absorption, metabolism and excretion [66,67].

We have used the single pass *in situ* rat intestine and liver preparation to predict the systemic clearance of compounds in rats. Although this approach is labor-intensive, it allows the estimation of intestinal and hepatic availability (Fg . Fh) as well as the prediction of systemic clearance of compounds in early discovery efforts with minimal amount of compound (2–3 mg). Figure 4 illustrates the predictive ability of this approach for several compounds with a wide range of *in situ* intestinal and hepatic availability and intravenous clearance. Our approach of classifying low availability (Fg . Fh <0.3), moderate availability (0.3 <Fg . Fh <0.75), and high availability (Fg . Fh >0.75) is in agreement with the proposed classification for low and high extraction drugs by Houston [20] (see Table 4). Furthermore, as shown in Figure 4, it is quite evident that low intestinal and hepatic availability translated into high systemic clearance values (>50 ml/min/kg). Again, our classification system of low, moderate and high systemic clearance in rats, as shown in Table 4, is consistent with those proposed independently by Houston [20] and Shibata *et al.* [65].

Prediction of distribution parameter (apparent volume of distribution)

Allometric scaling approaches have been proposed for predicting the volume of distribution in humans. As seen from Equation 11, the estimation of volume of distribution requires knowledge of both blood-protein binding and tissue binding of compounds. The latter is methodologically quite difficult and few studies assess tissue binding of drugs. It has been reported by Fichtl *et al.* [68] that when propranolol volume of distribution was corrected for the unbound fraction, the unbound volume of distribution was virtually identical for all species. These investigators suggested estimating the unbound volume of distribution in laboratory animals and *in vitro* plasma-protein binding for humans, and predicting human volume of distribution of drugs. However, Boxenbaum [69] has shown that the unbound volumes of distribution of many benzodiazepines are significantly different between dog and man.

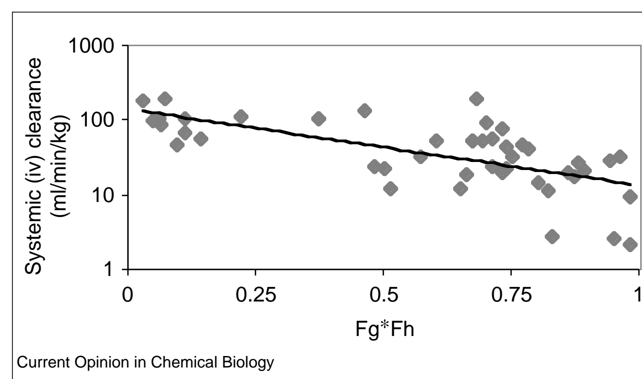
Obach *et al.* [7] have attempted to predict the human volume of distribution using four different methods. They found that allometric scaling across species, especially when excluding the interspecies protein-binding differences, was a poor predictor of human volume of distribution. When *in vitro* average fraction unbound in tissues of preclinical species was used with human plasma-protein binding data, best estimates of human volume of distribution were obtained. Furthermore *in vitro* plasma-protein binding data in dogs and humans along with intravenous PK in dogs was also a reasonable predictor of human volume of distribution in their analysis.

Prediction of human half-life ($T_{1/2}$)

Few reports are available on the prediction of drug half-life in humans. However, because half-life of drugs plays an

important role in dosing frequency, it would be good to have estimates of half-life in humans during the drug-discovery phase. Obach *et al.* [7] tried two approaches for predicting the human half-life for a set of proprietary compounds. One method relied on the direct correlations between animal and human half-life estimates, and the other method estimated individual volume of distribution and clearance, to yield $T_{1/2}$ values. They reported for the set of compounds evaluated that more accurate predictions of clearance resulted in more accurate predictions of half-lives for humans. Ironically, they found that *in vitro* clearance methods that excluded estimates of protein binding provided better half-life predictions than when including the protein binding differences among species. In addition, allometric clearance prediction methods, when combined with volume of distribution predictions, provided less accurate predictions than the *in vitro* methods of estimating clearance. Overall their study indicated that relatively simple animal–human correlation methods based on preclinical intravenous PK data provided reasonably accurate predictions of human drug half-lives and did not warrant the development of complex techniques of extrapolating *in vitro* metabolism data or PK data from multiple species.

Figure 4



Predicted intravenous systemic clearance (Cl_s) for test compounds as a function of the intestinal and hepatic availability (Fg . Fh) of the test compound from *in situ* rat intestine and liver perfusion experiments. Data represent the mean of three animals each for *in situ* and *in vivo* experiments.

Conclusions

It has been shown that different experimental approaches can be used to predict key human PK parameters of drugs. Allometric interspecies scaling, PK data from preclinical species and *in vitro* metabolism data can be used to predict oral clearance with varying degrees of accuracy and reliability. *In vitro* permeability measurements have provided a good estimate of the oral drug absorption potential for several drugs. However, all current approaches have limitations in their predictive abilities. Pending unforeseen advances, inter- and intra-subject (human) variability will continue to be a confounding factor in predicting oral bioavailability across the population.

For compounds having presystemic first-pass elimination, the estimates of oral absorption and thus oral bioavailability will be overestimated from *in vitro* permeability studies. Similarly, compounds with paracellular and carrier-mediated transport, or racemates with stereospecific absorption may be underestimated by currently used permeability assessment approaches. For compounds predominantly eliminated by the liver (via metabolism), *in vitro* assessment of metabolic stability in hepatic microsomes or isolated hepatocytes are good predictors of systemic clearance.

The use of *in vitro* approaches for predicting absorption and clearance have enabled rapid evaluation of the PK properties of compounds allowing more bioavailable compounds to move into development from discovery. It is important to decouple poor absorption due to poor permeability from poor absorption due to first-pass metabolism. In our laboratory, the use of *in vitro* metabolic stability and that of *in situ* rat intestine and liver perfusion approach have provided good tools to predict systemic clearance (particularly for peptidomimetics) during early drug-discovery phase.

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Table 4

Classification of poorly, moderately and highly available molecules: prediction from *in vitro*, *in situ* and *in vivo* rat data*.

Parameter	Low availability/ high clearance drugs	Moderate availability/ Moderate clearance	High availability/ Low clearance	Reference
Fg . Fh	≤0.3	0.3–0.75	≥0.75	Based on Vertex results from rat *in situ* perfusion
Liver extraction ratio	≥0.7	–	≤0.3	[20]
In vivo rat hepatic clearance	56 ml/min/kg	–	24 ml/min/kg	Estimated from [20]
In vivo rat hepatic clearance	>40 ml/min/kg	15–40 ml/min/kg	<15 ml/min/kg	[65]
Systemic (IV) clearance in rats	>50 ml/min/kg	20–50 ml/min/kg	<20 ml/min/kg	Predicted and observed data from Vertex *in vitro*, *in situ* and *in vivo* studies

*Literature estimates from Houston [20] and Shibata *et al.* [65].

the critical review of the manuscript by Ene Ette, Mark Murcko, Robert Silverman, Roger Tung and Yow-Ming Wang.

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