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Research Paper

Predictions of the *In Vivo* Clearance of Drugs from Rate of Loss Using Human Liver Microsomes for Phase I and Phase II Biotransformations

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Purpose. The utility of *in vitro* metabolism to accurately predict the clearance of hepatically metabolized drugs was evaluated. Three major goals were: (1) to optimize substrate concentration for the accurate prediction of clearance by comparing to K_m value, (2) to prove that clearance of drugs by both oxidation and glucuronidation may be predicted by this method, and (3) to determine the effects of nonspecific microsomal binding and plasma protein binding.
Methods. The apparent K_m values for five compounds along with scaled intrinsic clearances and predicted hepatic clearances for eight compounds were determined using a substrate loss method. Nonspecific binding to both plasma and microsomal matrices were also examined in the clearance calculations.
Results. The K_m values were well within the 2-fold variability expected for between laboratory comparisons. Using both phase I and/or phase II glucuronidation incubation conditions, the predictions of *in vivo* clearance using the substrate loss method were shown to correlate with published human clearance values. Of particular interest, for highly bound drugs (>95% plasma protein bound), the addition of a plasma protein binding term increased the accuracy of the prediction of *in vivo* clearance.
Conclusions. The substrate loss method may be used to accurately predict hepatic clearance of drugs.

KEY WORDS: clearance prediction; intrinsic clearance; microsomes.

INTRODUCTION

Measurement of the loss of substrate from an *in vitro* incubation has been shown to be a convenient way to allow for the prediction of drug properties as simple as the relative *in vitro* metabolic stability of a new chemical entity (NCE) to the prediction of the *in vivo* clearance of the NCE (1). The utility of measuring substrate loss is primarily attributable to its ease of determination, because only the parent compound needs to be analyzed. Thus the measurement of substrate loss can be performed earlier in the drug development process because only the loss of parent is followed, rather than the formation of a metabolite or metabolites (for the traditional approach). The metabolic scheme of the compound need not be known to perform the substrate loss method and thus this method can be applied during the early stages of drug development when the metabolic scheme is often not known. However, this method may not be as useful with low intrinsic clearance compounds where the accurate measurement of small changes in substrate may be difficult.

Physiologically based scaling is a method where *in vitro* parameters are scaled to a whole species using various physiological parameters for that species. The values for many of the physiological parameters and processes of the human body required for the scaling of *in vitro* metabolic clearance data to *in vivo* clearance are known, such as content of microsomal protein in a typical liver, along with average liver weight and liver blood flow. Such exercises may be performed by scaling up the *in vitro* measurement of loss of substrate via the various physiological parameters to whole body clearance. This method of predicting human clearance was investigated recently by several laboratories (2–5). Although the substrate loss method was shown to be effective in the prediction of clearance of drugs oxidized by microsomal drug metabolizing enzymes, its usefulness for drugs cleared by glucuronidation was not extensively studied. Recently, however, scaling of substrates cleared by glucuronidation was explored using product formation methodology (6,7). Thus, the usefulness of physiologically based scaling of substrate loss *in vitro* for the prediction of human clearance requires further evaluation.

The inclusion of several additional *in vitro* parameters may be necessary before an accurate prediction of *in vivo* clearance can be obtained. For example, the nonspecific binding of the compound to the microsomal milieu in the incubation mixture may lead to an underestimation of the clearance as a result of the alteration in the availability of the substrate to the enzymes (8–10). The concentration of the

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; K_m value, Michaelis constant value; LC/MS/MS, liquid chromatography tandem mass spectrometry.

substrate used must also be low enough to ensure that the concentration is within the linear range of the depletion curve. This is of some concern because saturation of the enzyme as substrate concentration exceeds the K_m value for the enzyme activity may lead to a substantial underestimation of the clearance of a substrate circulating at concentrations below the K_m value. The optimal substrate concentration used in the experiment should be high enough to allow for bioanalytical measurement of rapid loss of substrate, yet low enough to prevent reaching nonclinically relevant saturation of metabolic enzymes. For the substrate loss method, few studies have examined the role of substrate concentration in the prediction of *in vivo* clearance.

Another factor that needs to be examined when predicting *in vivo* clearance is the potential effect of plasma protein binding of the NCE. For example, a compound that is a high extraction compound *in vitro*, with extensive protein binding, may behave as a low extraction compound *in vivo*. *In vivo* evidence has demonstrated that protein binding may have an effect on the clearance of some drugs. Midazolam, a benzodiazepine anxiolytic drug, has been shown to have a greater clearance in elderly *vs.* healthy younger subjects (11). This unexpected result was postulated to be attributable to the increased free fraction of drug in elderly subjects compared with control subjects. Thus, the increased free fraction of midazolam, or lower protein binding value, increased clearance of the drug. On the other hand, the clearance of propranolol, a β-receptor antagonist, was shown to be unaffected by differences in protein binding and thus the amount of free drug (12). The effect of plasma protein binding on the clearance of drugs has been extensively discussed in the literature (13).

In the current study, several different experimental conditions were investigated in search of a paradigm for the prediction of *in vivo* clearances from *in vitro* microsomal studies. These conditions include the use of cofactors and incubation conditions to allow for oxidative and glucuronidation pathways to be active individually or simultaneously with two compounds to initially explore this metabolic scheme. Furthermore, the effects of substrate concentration, nonspecific microsomal binding, and plasma protein binding on the prediction of *in vivo* clearance were also explored—yielding a paradigm that differs from that previously reported, for the use of these different factors that results in the optimum prediction of human clearance.

MATERIALS AND METHODS

Chemicals

Imipramine, desipramine, midazolam, diclofenac, ketoprofen, dextromethorphan, buprenorphine, propranolol, verapamil, clomipramine, metoprolol, alprazolam, niflumic acid, uridine 5′-diphosphoglucuronic acid (UDPGA), alamethicin, saccharolactone, and β-nicotinamide adenine dinucleotide 2′-phosphate reduced (β-NADPH) were obtained from Sigma (St. Louis MO, USA).

Human Tissue

Incubations were performed using human liver microsomes pooled from six different livers [human liver G (HLG), human liver R (HLR), human liver T (HLT) IU 6/6/96, IU 12/12/96, and Strom 6/26/97] obtained under approved protocols and prepared according to the method described by van der Hoeven and Coon (14). Different pretreatment and buffer conditions, as indicated below, were used for oxidation and glucuronidation metabolic incubations.

Instrumentation

All samples were analyzed using a HTC PAL injector (CTC Analytics) and a Shimadzu LC AD90 high-performance liquid chromatography (HPLC) system.

For propranolol, imipramine, diclofenac, desipramine, buprenorphine, verapamil, midazolam, dextromethorphan, clomipramine, metoprolol, alprazolam, and niflumic acid, liquid chromatography tandem mass spectrometry (LC/MS/MS) was performed on a Micromass Quattro LC mass spectrometer operating in electrospray positive ionization mode using the MassLynx v 4.0 software package. Mobile phase used for these samples was 0.1% formic acid in water with 5% acetonitrile and 0.1% formic acid in water with 95% acetonitrile. A Metachem Monochrom® octadesylsilyl, 50 × 30 mm column was used for chromatographic separation of analytes.

Analysis of ketoprofen, and niflumic acid (internal standard) was performed on an AB Sciex Instruments API 4000 mass spectrometer operating in electrospray negative ionization mode using Analyst v 1.2 software. Mobile phase used for these samples was 5 mM ammonium acetate in water with 5% acetonitrile and 5 mM ammonium acetate in water with 95% acetonitrile. A Phenomenex LUNA phenyl–hexyl, 100 × 2 mm column was used for chromatographic separation of analytes. Further analytical parameters are found in Table I.

Substrate concentrations were quantitated by comparing peak area ratios (analyte/internal standard) to the peak area ratios generated with a standard curve. Standard curve correlation coefficients (r^2 values) were greater than 0.99.

Table I. Mass Spectrometry Conditions for Analyses of Substrates

Compound	Electospray ionization	Transition	Retention time (min)
Imipramine	Positive	281.2 > 86.2	2.0
Clomipramine[a]	Positive	315.2 > 86.2	2.0
Verapamil	Positive	455.2 > 165.1	4.1
Dextromethorphan	Positive	272.2 > 272.2	3.8
Desipramine	Positive	267.2 > 72.3	1.9
Clomipramine[a]	Positive	315.2 > 86.2	2.0
Midazolam	Positive	326.0 > 291.1	2.3
Alprazolam[a]	Positive	309.0 > 205.0	2.0
Propranolol	Positive	260.1 > 116.2	3.2
Metoprolol[a]	Positive	268.2 > 116.2	4.1
Buprenorphine	Positive	468.0 > 468.0	3.2
Dextromethorphan[a]	Positive	272.2 > 272.2	4.1
Diclofenac	Positive	295.9 > 213.9	2.2
Mefenamic acid[a]	Positive	242.1 > 224.1	2.3
Ketoprofen	Negative	253.0 > 208.9	3.2
Niflumic acid[a]	Negative	280.9 > 176.8	3.2

[a] Internal standard (verapamil and dextromethorphan used as internal standard for the other substrate).

Incubation Conditions

Incubation Milieu

Oxidation Incubations. Human liver microsomes were added to the incubation buffer (100 mM sodium phosphate, pH = 7.4) at a final concentration of 0.5 or 1 mg/mL^{-1}. Substrates examined for oxidative metabolism were propranolol, dextromethorphan, desipramine, imipramine, midazolam, diclofenac, and verapamil at the concentrations indicated below.

Glucuronidation Incubations. Human liver microsomes were added to the incubation buffer (100 mM potassium phosphate, 1 mM $MgCl_2$, and 5 mM saccharolactone, pH = 7.1) at a concentration of 0.5 or 1 mg mL^{-1}. Microsomes were activated by the addition of alamethicin at a final concentration of 50 μg mg^{-1} of microsomal protein and incubating for 30 min on ice (15). The substrates selected were buprenorphine and ketoprofen, which were incubated using these conditions. To explore the combined oxidation and glucuronidation of buprenorphine, both UDPGA and NADPH were utilized to initiate the reactions with microsomes pretreated with alamethicin.

Substrate Concentrations. Substrate concentrations used for the determination of K_m values for diclofenac were 1, 3, 5, 10, 30, and 50 μM; for buprenorphine 2, 6, 20, 60, 120, and 180 μM; for dextromethorphan 0.1, 0.3, 0.5, 1, 3, 5, 10, 30, 50, and 100 μM; for imipramine 0.1, 0.3, 0.5, 1, 3, 5, 10, 30, 50, and 100 μM; and for midazolam 1, 3, 5, 10, 30, 50, and 100 μM. All other substrates were incubated at a single 0.1 μM concentration.

Incubation Procedures. The mixtures were preincubated at 37°C for 3 min at which point cofactor, NADPH (final concentration: 1 mM) and/or UDPGA (final concentration: 5 mM) were added to initiate the reaction (final volume: 650 μL). Incubations were quenched by taking a 100-μL aliquot and adding it to 25 μL of 25% formic acid at 0, 5, 10, 20, and 30 min. The percent substrate remaining was calculated by dividing the amount of substrate at each of the 5-, 10-, 20-, and 30-min time points by the amount of substrate determined at time 0 and multiplying by 100%. The percent substrate remaining was then used to estimate the rate constant of substrate loss, k_{loss}, using the following equation using the WinNonLin software package:

$$\%\ \text{substrate remaining} = 100 \times e^{-k_{loss} \times time} \tag{1}$$

The K_m value and the rate constant of substrate loss at a very low substrate concentration, $k_{loss\ ([S] \approx 0)}$, were determined by fitting the modified Michaelis–Menten equation to the data:

$$k_{loss} = k_{loss(S \approx 0)} \times \left(1 - \frac{[S]}{[S] + K_m}\right) \tag{2}$$

Microsomal and Plasma Protein Binding. Microsomal and plasma protein binding were performed at a 1 μM concentration using a 96-well equilibrium dialysis method similar to that of Banker *et al.* (16). The concentration of substrate on each side of the membrane was determined and fraction unbound in plasma ($f_{u\ \text{plasma}}$) and fraction unbound in microsomes ($f_{u\ mics}$) were calculated using the following equation:

$$f_{u(\text{plasma or microsomes})} = \frac{\text{concentration of drug in buffer}}{\text{concentration of drug in plasma or microsomes}} \tag{3}$$

Calculation of Predicted Human Clearance

Scaled intrinsic clearance (Cl^s_{int}) was calculated according to the following formulas:

$$\text{Scaled intrinsic clearance} = Cl^s_{int} = k_{loss[S] \approx 0} \times \frac{\text{ml incubation}}{\text{mg microsomes}} \times \frac{\text{mg microsomes}}{\text{gm liver}} \times \frac{\text{gm liver}}{\text{kg bw}} \tag{4}$$

where *bw* is body weight of a typical human (70 kg) (17) and microsomal content is 45 mg microsomal protein g liver^{-1} (18).

Assuming a well-stirred model, total and free hepatic clearances (Cl_H) were determined as follows:

(a) without protein binding

$$Cl_H = \frac{Q \times Cl^s_{int}}{Q + Cl^s_{int}} \tag{5}$$

where Q is liver blood flow (21 mL min^{-1} kg^{-1}) (17);

(b) with plasma protein binding

$$Cl_H \text{ with plasma protein binding} = \frac{Q \times f_{u(\text{plasma})} \times Cl^s_{int}}{Q + f_{u(\text{plasma})} \times Cl^s_{int}} \tag{6}$$

(c) with plasma protein and microsomal binding

$$Cl_H \text{ with plasma and microsomal binding} = \frac{Q \times f_{u(\text{plasma})} \times \frac{Cl^s_{int}}{f_{u(mic)}}}{Q + f_{u(\text{plasma})} \times \frac{Cl^s_{int}}{f_{u(mic)}}} \tag{7}$$

Determination of Prediction Error

Prediction error was determined by the following equation:

$$\text{Prediction Error} = \left(\frac{Cl_{predicted} - Cl_{observed}}{Cl_{observed}}\right) \times 100 \tag{8}$$

Observed clearances were obtained from Goodman and Gilman (19) and for buprenorphine from Mendelson *et al.* (20).

RESULTS

Initially, the kinetics of the oxidation of diclofenac, imipramine, dextromethorphan, and midazolam, and the glucuronidation or oxidation of buprenorphine were examined by the substrate loss method. Figure 1 shows the concentration *vs.* rate constant (k_{loss}) curves for the determination of K_m values using the substrate loss method for four oxidation, one glucuronidation, and one glucuronidation plus oxidation pathways. The k_{loss} value for each was obtained by fitting Eq. (1) to the % substrate remaining *vs.* time data. This k_{loss} was determined for several of the substrates at numerous concentrations (6–10) to determine K_m values for total turnover of the substrate. The modified Michaelis–Menten equation [Eq. (2)] was fit to the k_{loss} data and apparent K_m values were obtained. These K_m values represent the biotransformation to one or more metabolites for each substrate.

Table II compares the K_m values of major metabolites for each substrate from the literature determined using the classical metabolite formation method and the substrate loss method as described above. The apparent K_m values obtained for midazolam, diclofenac, dextromethorphan, imipramine, verapamil, and buprenorphine with phase II and combination of both phase I and phase II metabolism using the substrate loss method were found to be very similar to the corresponding mean K_m values obtained from literature (21–26). The fold differences observed between the K_m values obtained by the substrate loss and the metabolite formation method were: diclofenac 4′-hydroxylation, 0.9-fold; imipramine demethylation, 0.6-fold; imipramine hydroxylation, 1.6-fold; dextromethorphan *O*-demethylation, 1.5-fold; midazolam 1′-hydroxylation, 0.6-fold; and buprenorphine glucuronidation, 1.6-fold. The K_m value obtained for buprenorphine *O*-demethylation plus glucuronidation was found to be similar (Table II) to that of *O*-demethylation alone

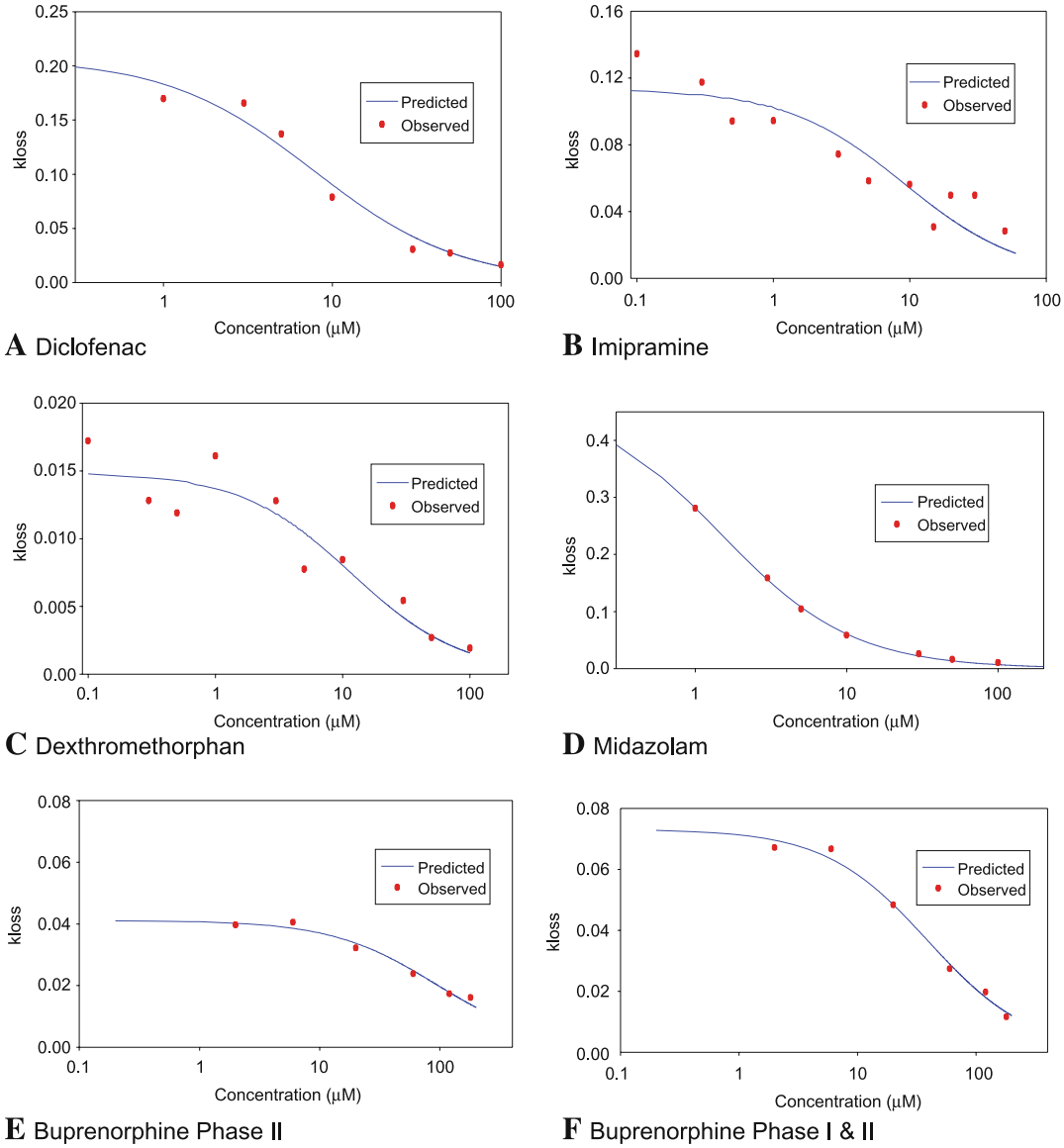

Fig. 1. Substrate depletion curves for determination of K_m values.

Table II. Apparent K_m Values (±SE) Determined using a Substrate Loss Method and Published Apparent K_m Values Determined Using Product Formation

Compound	Predicted apparent K_m (μM)	Published apparent K_m (μM)
Diclofenac (CYP2C9)	7.8 ± 2	9
Imipramine	9.4 ± 0.3	15 and 6[a]
Dextromethorphan (CYP2D6 and CYP3A4)	12 ± 4.3	8
Midazolam (CYP3A4)	2.4 ± 0.4	4
Buprenorphine (UGT1A1)	92 ± 12	57
Buprenorphine (UGT1A1 and CYP3A4)	39 ± 4.4	39[b]

[a] Demethylation and 2-hydroxylation, respectively.
[b] *O*-Demethylation.

(39 μM). All these comparisons were well within the expected 3-fold variation for between-laboratory comparisons (27).

When a full kinetic evaluation was performed, the parameter determined by the substrate loss method that was utilized to calculate the *in vivo* intrinsic clearance was not the K_m value but the $k_{loss\,([S]\approx 0)}$ value [Eq. (4)]. However, examination of the plots of k_{loss} *vs.* substrate concentration (Fig. 1) for K_m determinations clearly demonstrates that as the concentration of substrate decreases, k_{loss} essentially plateaus at a value approximating the $k_{loss\,([S]\approx 0)}$ value. Table III shows the predicted clearances of eight probe substrates determined using the substrate loss method. For diclofenac, imipramine, midazolam, and buprenorphine, the k_{loss} value used to calculate scaled intrinsic clearance was the $k_{loss\,([S]\approx 0)}$ value obtained during the determination of the K_m value. To demonstrate the observation that k_{loss} of a low substrate concentration approximates $k_{loss\,([S]\approx 0)}$, the scaled intrinsic clearance for imipramine was determined to be 9.9 mL min^{-1} kg^{-1} with all of the data *vs.* 12.1 mL min^{-1} kg^{-1} when determined at the single 0.1 μM concentration (data not shown). The various clearance values were extremely similar whether using all of the data from the kinetic determination or a single low substrate concentration. Thus, the scaled intrinsic clearances (Cl^s_{int}) of propranolol, desipramine, verapamil, and ketoprofen (Table III) were predicted using a k_{loss} value determined at a single low substrate concentration of 0.1 μM.

Comparison of the predicted total hepatic clearance, Cl_H, obtained using the well-stirred model [Eq. (5)], with published total clearance values of predominantly hepatically cleared drugs showed that the predicted value determined without the addition of binding parameters for several of the test compounds were well within a 2-fold error (Tables III and IV). Specifically, the Cl_H values (Table III) for propranolol, desipramine, imipramine, buprenorphine, and verapamil all agree very well with the $Cl_{observed}$ literature data when using this simple model, yielding prediction errors of less than 47% (Table IV). A dramatic overprediction, however, was observed for ketoprofen, midazolam, and diclofenac (Table III). This overprediction ranged from a 212% prediction error for midazolam to 633% for ketoprofen (Table IV).

The additions of plasma protein and microsomal binding terms to the well-stirred model were explored to determine their effect on the reliability of the predictions. Plasma protein and microsomal binding were determined for each of these substrates using an equilibrium dialysis method. The fraction unbound values were calculated using Eq. (3), and the fractions bound are shown in Table IV. Correction for the volume shift observed in the equilibrium dialysis of plasma was performed using the Boudinot formula (28). Prediction of free hepatic clearance with the addition of a plasma protein binding term, $Cl_{H+\text{plasma protein binding}}$, was determined using Eq. (6). The addition of the plasma protein term showed a marked improvement in the accuracy of prediction for the three compounds, ketoprofen, midazolam, and diclofenac, whose clearances were overpredicted using the well-stirred model without binding correction. The overprediction of midazolam was reduced from 212% without the binding term to 65% with the plasma protein binding term included, whereas for ketoprofen the binding correction resulted in a change from a 633% overprediction to a 75% underprediction and for diclofenac 379% overprediction to 7% underprediction (Table IV). For the probes that were predicted well without the binding term, the addition of this value led to a less accurate prediction for four of the five compounds (Table IV), although the difference was not as large as that for the original three overpredicted compounds. It is interesting to note that those compounds for which the

Table III. Clearance Calculations (mL min^{-1} kg^{-1}) using the Substrate Loss Method

Compound	$Cl_{int}{}^s$	Cl_H	$Cl_{H\,+\,\text{plasma protein binding}}$	$Cl_{H\,+\,\text{plasma and microsome binding}}$	$Cl_{observed}$[a] (19,20)
Propranolol[b]	35.8	13.2	5.9	10.7	16 ± 5
Desipramine[b]	7	5.3	1.2	4.7	10 ± 2
Imipramine[c]	18.7	9.9	2.5	5.5	15 ± 4
Buprenorphine[c] (glucuronidation and oxidation)	86.1	16.9	8	13.1	15 ± 5
Verapamil[b]	657.6	20.4	15.5	18.2	15 ± 4
Ketoprofen[b] (glucuronidation)	15.1	8.8	0.3	0.3	1.2 ± 0.3
Midazolam[c]	1132	20.6	10.9	12.8	6.6 ± 1.8
Diclofenac[c]	483	20.1	3.9	11.2	4.2 ± 0.9

[a] ±SD.
[b] Values determined using k_{loss} obtained with a single low (0.1 μM) substrate concentration.
[c] Values determined using $k_{loss\,[S]\approx 0}$.

Table IV. Binding Values and Prediction Error [Eq. (8)] using the Substrate Loss Method with and without Various Correction Terms (Negative Values Indicate Underprediction, Positive Values Overprediction)

Compound	Cl_H	$Cl_{H\ +\ plasma\ protein\ binding}$	$Cl_{H\ +\ plasma\ and\ microsomal\ binding}$	Plasma protein binding (% bound)	Microsomal binding (% bound)
Prediction error (%)					
Propranolol	−18	−63	−33	77	62
Desipramine	−47	−88	−53	82	79
Imipramine	−34	−83	−63	85	62
Buprenorphine [a]	13	−47	−13	85	63
Verapamil	36	3	21	91	57
Ketoprofen [b]	633	−75	−75	98	0
Midazolam	212	65	94	98	31
Diclofenac	379	−7	167	99	80

All clearance values in mL min^{-1} kg^{-1}.
[a] Glucuronidation and oxidation.
[b] Glucuronidation.

inclusion of plasma protein binding improved the prediction were those with >95% binding (Table IV). However, all Cl_H with plasma protein binding term predictions were well within a 2-fold prediction error (Table IV).

Nonspecific binding to microsomes and plasma protein were also considered in the calculation of free hepatic clearance ($Cl_{H\ +\ plasma\ and\ mic\ binding}$) using Eq. (7). For propranolol, desipramine, imipramine, and buprenorphine, the obtained results were marginally less accurate than when no binding terms were added (Table IV). The addition of plasma protein and microsomal binding terms to the well-stirred model showed an improvement for the prediction of verapamil from the situation where no binding terms were included, but less improvement compared to clearance predicted using plasma protein binding alone. However, all values were well within a 2-fold prediction error (Table IV).

DISCUSSION

The purpose of the present study was to evaluate *in vitro* metabolism methods to accurately predict the clearance of hepatically metabolized drugs and determine optimal conditions for the prediction of clearance of an NCE using a substrate loss method. Several parameters were examined to determine the optimal paradigm for clearance prediction including substrate concentrations, inclusion of cofactors for phase I and phase II reactions, and UGT activators in the *in vitro* incubations for two compounds. Furthermore, in clearance calculations the inclusion of microsomal and/or plasma protein binding terms were evaluated. The purpose was to define a paradigm that reliably, defined as less than 2-fold prediction error, predicted the clearance of a test set of eight drugs metabolized by phase I and/or phase II enzymes.

K_m values were determined for a variety of predominantly hepatically cleared substrates for which *in vitro* kinetic information was available in this laboratory and from the literature. The chosen compounds were: diclofenac, a CYP2C9 probe substrate; midazolam, a CYP3A probe substrate; buprenorphine, a UGT1A1 probe substrate and also a substrate of CYP3A; dextromethorphan, a CYP2D6 substrate; along with imipramine and verapamil, both metabolized by a several P450 forms. K_m values obtained for each of the probe substrates determined using the substrate loss method showed a good agreement with K_m values obtained from the literature, which used product formation for the major metabolic pathway. K_m values, along with k_{loss} determined in the method, are a conglomeration of values from all of the operative pathways. The alteration of a particular pathway through allelic variation in the enzyme or addition of another drug can alter the K_m and k_{loss} values to the value of the unaltered pathway when using this method.

The K_m values obtained for these substrates fell well within 3-fold of the reported K_m values. The 3-fold difference was considered acceptable for between-laboratory comparisons in a recently published "consensus" paper (27). Thus, the substrate loss method yielded reliable estimates of K_m values for these substrates without the necessity of syntheses of metabolites or development of multiple assays for the determination of the formation of metabolites of the substrate. Obach and Reed-Hagen (29) recently reported determining K_m values for a set of P450 probe substrates by the substrate loss method using individually expressed cytochromes P450. However, a thorough review of the literature indicated that the K_m values reported here seem to be the first to use human liver microsomes and the substrate loss method. In addition, K_m values have not been determined for glucuronidation reactions in human liver microsomes using a substrate loss approach. This work attempts to address both aspects for *in vitro* to *in vivo* prediction of drug clearance.

The plots of k_{loss} *vs.* substrate concentration (Fig. 1) demonstrated that at low substrate concentrations the rate constant of substrate loss significantly levels off to become effectively a constant. Therefore, a full kinetic treatment of a substrate (that is, multiple substrate concentrations) should not be necessary to obtain a reliable prediction of the *in vivo* clearance; instead, a single low concentration should suffice. As most drugs circulate at concentrations that are much lower than the K_m values of the enzymes metabolizing them, a single substrate concentration reflecting the low concentration will yield an estimate of k_{loss} that will result in a good prediction of *in vivo* clearance. Thus the substrates verapamil, desipramine, propranolol, and ketoprofen were incubated at a single 0.1 μM concentration. This concentration was

chosen because it was at the plateau region of all six biotransformations studied in detail (Fig. 1), representing a plasma concentration of about 30 ng mL^{-1} for a typical small molecule drug; it is also most often the concentration that allowed for measurement of 90% substrate loss before reaching the lower limit of quantitation of the bioanalytical methods. These observations allow for the rapid determination of k_{loss} for the prediction of clearance for an NCE.

The predictions of clearance were then performed using either the $k_{loss\,(s \approx 0)}$ with those substrates for which a K_m value was obtained or the k_{loss} obtained with the single low substrate concentration. Clearance values were well predicted for those substrates with low plasma protein binding even when no protein binding terms were included in the calculation (Table IV). Specifically, these protein binding terms were not applied to the predictions for imipramine, desipramine, propranolol, and buprenorphine, all compounds with protein binding of less than 90%, and showed good agreement (<47% prediction error) between predicted and observed clearances (less than 2-fold prediction error).

On the other hand, those compounds with plasma protein binding exceeding 95% showed good correlation between predicted and observed clearance values when the plasma protein binding terms were included (Table IV). That is, diclofenac, midazolam, and ketoprofen all showed much better correlation (less than 95% prediction error) when the plasma protein binding was included in the calculation of predicted *in vivo* clearance. In the *in vitro* situation with a relatively large amount of the substrate available for metabolism along with a high *in vitro* microsomal extraction ratio, a dramatic overprediction of clearance was observed. The high plasma protein binding of these compounds results in a smaller amount of free drug available for the metabolism of drug *in vivo*, and thus lower clearance. For example, two of these highly plasma protein bound drugs, midazolam and diclofenac, exhibited high scaled total intrinsic clearances (1,132 and 483 mL min^{-1} kg^{-1}, respectively), with corresponding high predicted total hepatic clearances. Yet, because of low free fractions (0.02 and 0.01), low *in vivo* clearances are observed, 6.6 and 4.2 mL min^{-1} kg^{-1}, respectively, which were substantially better predicted when the plasma protein binding terms were included.

The inclusion of nonspecific microsomal binding is often discussed as a potential correction factor for the prediction of clearance. However, the conclusion drawn from the data reported here that microsomal binding does not dramatically affect clearance prediction is not surprising. As observed in k_{loss} *vs.* substrate concentration plots, as substrate concentrations become sufficiently low, k_{loss} values actually plateau and thus change very little as substrate concentration decreases. Concentrations used for the prediction of clearances in this test set of compounds are all expected to be in this plateau region of the kinetic relationship. As shown with Eq. (7), microsomal binding correction is a correction factor for the determination of scaled intrinsic clearance (Cl^{s}_{int}). The only value in the determination of Cl^{s}_{int} that is obtained in the substrate loss experiment is the value for k_{loss}. Because, as discussed above, the value for k_{loss} does not substantially change at very low concentrations, the correction for microsomal binding would yield very little adjustment as it essentially further lowers the substrate concentration and thus does not significantly alter the value. The same situation cannot be expected for plasma protein binding, where drug is bound in the central compartment preventing it from entering the hepatocyte where it can be metabolized and thus cleared.

A previous report by Obach postulated that protein binding considerations were not necessary for the accurate prediction of *in vivo* clearances of basic drugs, but were necessary for the accurate prediction with acidic drugs (5). Although protein binding considerations seem to be necessary for the nonsteroidal anti-inflammatory (NSAID) class of acidic drugs, results in the current study indicate that this seems to be attributable to the high level of plasma protein binding observed with these drugs and not to their acidic nature. This class of drugs is known to be very highly plasma protein bound with many reported fractions bound in excess of 99.5%. Two of the three acidic drugs in the Obach report (5) with low plasma protein binding, hexobarbital and methohexital, showed good prediction without correction for protein binding, consistent with the results in the current work. Furthermore, the basic drugs examined in the Obach report that had fairly high plasma protein binding showed better prediction when the plasma protein binding term was included in the calculation. For example, midazolam with reported 95% plasma protein bound was more accurately predicted when the plasma protein binding term was included. This, too, is consistent with the current report, where neutral and basic drugs with fairly high plasma protein binding, midazolam with 98% bound and verapamil with 92% bound, also show a better correlation between predicted and observed *in vivo* clearances when the plasma protein binding terms are included in clearance prediction. Those drugs that were not highly plasma protein bound, imipramine (85% bound), desipramine (82% bound), propranolol (77% bound), and buprenorphine (85% bound), are basic drugs that showed a good prediction without addition of binding factors. Thus, the results of both reports are consistent with the concept that it is not whether or not a drug is acidic or basic that is important, but instead whether or not the drug is highly plasma protein bound, that determines when protein binding should be included in the prediction of clearance.

The prediction of clearances for substrates with a substantial contribution of clearance due to phase II metabolism has not been studied extensively (6,7). In this study, two substrates, buprenorphine and ketoprofen, showed substantial substrate loss with the inclusion of alamethicin and UDPGA during incubation. Specifically, buprenorphine is known to be extensively conjugated by UGT1A1, and ketoprofen is metabolically cleared primarily by UGT2B7. Each of these compounds was metabolized in the activated human liver microsomes at a fairly extensive rate, as indicated by their intrinsic clearances (buprenorphine 7 mL min^{-1} mL^{-1} and ketoprofen 15 mL min^{-1} mg^{-1}). Furthermore, as shown in Table IV, the substrate depletion method adequately predicted *in vivo* clearances of these glucuronidated drugs when plasma protein binding for the highly protein bound ketoprofen is taken into account. Thus, the substrate depletion method seems to accurately predict the clearances of drugs metabolized by glucuronidation ± oxidation pathways.

In summary, this report has shown that *in vivo* clearances of several substrates with reported *in vivo* clearances ranging from 1.5 to 16 mL min^{-1} mg^{-1} can be accurately predicted using the substrate loss method with incubations using human liver microsomes. The use of the pore-forming peptide alamethicin and the cofactor UDPGA also allows for the prediction of clearance of substrates metabolized through glucuronidation. Although this has been performed with a limited number of substrates, it has been shown to be a valid method and a more vigorous validation is now warranted. Furthermore, it is possible to utilize a single substrate concentration to estimate k_{loss} if the incubations are carried out at a concentration well below the K_m value for the biotransformation. Accurate prediction also requires the appropriate use of plasma protein binding terms in calculating a predicted clearance. Correction for binding is not necessary for those drugs that are less than 90% bound, whereas for those compounds that are highly plasma protein bound (>95% bound), plasma protein binding terms dramatically improve the prediction of clearance. The inclusion of plasma protein binding terms allowed for the correlation of *in vitro* scaled clearances with *in vivo* scaled clearances for these highly bound drugs with high *in vitro* microsomal extraction ratios. The use of the microsomal binding term in the clearance calculation does not seem to substantially improve the prediction of *in vivo* clearance. In conclusion, the paradigm that is proposed for the prediction of clearance from substrate loss experiments is that for compounds with high plasma protein binding (>95%), correction for this binding term should be included in the calculation, whereas in compounds with lower plasma protein binding the term may be safely ignored independent of whether the drug is metabolized by oxidation or glucuronidation, or is acidic or basic.

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