THE INFLUENCE OF NONSPECIFIC MICROSOMAL BINDING ON APPARENT INTRINSIC CLEARANCE, AND ITS PREDICTION FROM PHYSICOCHEMICAL PROPERTIES

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ABSTRACT:

The apparent intrinsic clearance of 13 drugs has been determined using rat liver microsomes at three different concentrations of microsomal protein. The kinetics was studied using the in vitro half-life method. The nonspecific binding of these drugs to the microsomes was also studied under the same conditions, except for cofactor removal, using equilibrium dialysis. The intrinsic clearances are shown to be dependent on the microsomal concentration, but are approximately constant when corrected for the extent of nonspecific binding to the microsomes. The large difference between observed intrinsic clearance and unbound intrinsic clearance that exists for some compounds, particularly lipophilic bases, is highlighted. A simple model has been developed for understanding the binding of compounds to microsomes and is demonstrated to accurately predict the extent of microsomal binding at one concentration of microsomes from measurement at another. The binding of a further 25 drugs to rat liver microsomes at a microsomal concentration of 1 mg/ml was also studied, along with measurements of lipophilicity using octanol-water partition coefficients. It is shown that the extent of microsomal binding is correlated with lipophilicity, but that basic compounds show a different behavior to acidic and neutral compounds. Microsomal binding is shown to be best predicted using a model where logP is used for basic compounds, and logD7.4 is used for acidic and neutral compounds. This model has been developed further so that the extent of binding to microsomes of any given concentration can be estimated purely from a knowledge of lipophilicity and ionization.

In recent years, kinetic measurements using liver microsomes have been increasingly used as a measure of the rate of metabolic drug oxidation in drug discovery. The results from these in vitro assays are then frequently processed, through a variety of methods, to produce predictions of the in vivo metabolic clearance of compounds in humans (Iwatsubu et al., 1997; Obach et al., 1997; Lave et al., 1999). Methods that are often used for scaling such in vitro data to in vivo predictions are the well stirred model and the parallel tube model, and these models contain plasma free fraction terms that account for the effect of plasma binding on the clearance of compounds (Pang and Rowland, 1977). However, it is frequently found that inclusion of the plasma binding correction can lead to underprediction of the in vivo clearance, particularly for nonacidic compounds, and better performance can often be obtained by ignoring the correction altogether (Obach, 1997, 1999; Obach et al., 1997). It has been recognized that nonspecific microsomal binding in the in vitro metabolic assays can significantly affect the observed kinetics of metabolism and hamper the accurate prediction of clearance, and there are now several examples where knowledge of the extent of microsomal binding can lead to a better understanding of the relationship between in vitro metabolism and in vivo pharmacokinetics (Lin et al., 1978, 1980; Bäärnhielm et al., 1986; St-Pierre and Pang, 1995; Obach, 1996, 1997, 1999; Carlile et al., 1999). It has been reinforced by several studies (Houston, 1994; Obach, 1996; Iwatsubu et al., 1997) that unbound intrinsic clearance (CL\textsubscript{int,U}) is for many compounds not the same as the experimentally observed intrinsic clearance (CL\textsubscript{int}) and that the two are related by eq. 1:

\[
CL\textsubscript{int,U} = \frac{V\text{\textsubscript{max}}}{K\text{\textsubscript{M}}} = \frac{CL\text{\textsubscript{int}}}{fu\text{\textsubscript{inc}}} 
\]

where \(V\text{\textsubscript{max}}\) is the maximum velocity, \(K\text{\textsubscript{M}}\) is the Michaelis constant based on free substrate concentrations, and \(fu\text{\textsubscript{inc}}\) is the free fraction of compound in the microsomal incubation. \(CL\text{\textsubscript{int}}\) is not a constant for a particular compound, but is a function of the microsome concentration, and is not an ideal parameter for use in predictions of in vivo clearance. However, for some compounds, the observed \(CL\text{\textsubscript{int}}\) does fortuitously lead to good predictions of in vivo clearance (Obach et al., 1997; Obach, 1999). When eq. 1 is substituted into the well stirred model, the predicted in vivo clearance is given by eq. 2 (Obach, 1996):

\[
CL = \frac{Q \cdot A \cdot B \cdot fu\text{\textsubscript{inc}} \cdot CL\text{\textsubscript{int}}}{fu\text{\textsubscript{inc}} + \left(\frac{Q \cdot A \cdot B \cdot fu\text{\textsubscript{inc}} \cdot CL\text{\textsubscript{int}}}{fu\text{\textsubscript{inc}}}\right)} 
\]

where \(A\) is a constant representing the milligrams of microsomes per gram of liver, \(B\) is a constant describing the grams of liver per kilogram of body weight, \(Q\) represents liver blood flow, \(fu\text{\textsubscript{inc}}\) is the free fraction of the compound in plasma, and \(fu\text{\textsubscript{inc}}\) is the free fraction of the compound in the microsomal incubation. From eq. 2, an interpretation of why \(CL\text{\textsubscript{int}}\) can provide good predictions of clearance can be given, because such good predictions could be the result of a compound that
has similar binding to plasma and to microsomes at the microsomal concentration used for the measurement of \( CL_{\text{int}} \), leading to the cancellation of the \( f_u/f_u^\text{inc} \) ratios in eq. 2. Using measured values of \( f_u \) and \( f_u^\text{inc} \), it has been shown that eq. 2 could be of more general utility in the prediction of clearance than either the well stirred model containing no free fraction corrections or only the \( f_u^\text{p} \) correction (Obach, 1999).

Knowledge of \( f_u^\text{inc} \) has also been shown to be important for the prediction of in vivo drug-drug interactions, where inhibition constants (\( K_i \)) have been converted to unbound \( K_i \) values followed by plasma binding corrections, leading to improved predictions of changes in area under the curve upon coadministration of drugs (Ishigam et al., 2001).

If eq. 2 is to be more widely used for clearance predictions and if unbound \( K_i \) values are going to be more commonly used then it would be advantageous to be able to predict the \( f_u^\text{inc} \) term from readily available physicochemical properties of compounds such as lipophilicity. Several studies have examined the extent of nonspecific microsomal binding of various compounds, and how this binding affects the observed kinetics of turnover of these compounds by the microsomes, and the quality of in vivo prediction (Obach, 1997, 1999; Carlile et al., 1999; Mclure et al., 2000; Venkatakrishnan et al., 2000; Kalvass et al., 2001). However, these studies have used a variety of microsomal protein concentrations, and the resulting data are consequently unsuitable for finding predictive quantitative relationships between the physicochemical properties of the compounds and the extent of microsomal binding. To find such relationships, data are required on a set of compounds with a variety of physicochemical properties, and where the binding is measured under identical conditions of microsomal protein concentration, which is the main purpose of this work.

**Materials and Methods**

**Materials.** Frozen hepatic microsomes from male Sprague-Dawley rats at a protein concentration of 20 mg/ml were obtained from In Vitro Technologies (Baltimore, MD). The same batch of microsomes was used for all of the studies and was stored at \(-80^\circ C\). 1-Octanol was obtained from Fisher Scientific (Loughborough, UK). \( \beta \)-NADPH, 2-ethoxybenzamide, albendazole, alprazolam, ami-iodarine, astemizole, bemetanide, carbamazepine, cinoxacin, clobenzapine, clozapine, colchicine, desipramine, glipizide, glyburide, ibuprofen, indapamide, indomethacin, ketoprofen, mebendazole, methocarbamol, meclozine, metyrapone, pimozide, piroxicam, prednisone, promethazine, quinidine, sulindac, tamoxifen, thioridazine, tolbutamide, tolmetin, triazolam, trimeprazine, trioxanol, verapamil, and warfarin were obtained from Sigma Chemical (Poole, Dorset, UK). Oxaprazin was obtained from Maybridge Chemicals (Trevillet, UK). Betaxolol, cerivastatin, chlorpromazine, diazepam, dichloralphenazone, diclofenac, diltiazem, diphenhydramine, imipramine, isradipine, losartan, phenoxymide, and sulfadoxine were obtained from the AstraZeneca compound collection.

**Instrumentation.** All sample handling was performed using a Genesis RSP 100 liquid handling robot (Tecan, Durham, NC) fitted with disposable tips and controlled by Gemini software. Centrifugations were carried out using an Allegro R6 (Beckman Coulter, Inc., Fullerton, CA). A Dianorm unit, and rotated in a water bath at 37°C for 18 h, for later use as control microsomes in sample preparation prior to HPLC/MS analysis. The dialysis cells were then emptied and the solutions were treated in the following way such that the final samples and standards for HPLC/MS analysis were all present in an identical matrix. To 380 \( \mu l \) of sample from the microsomal side of each dialysis cell was added 380 \( \mu l \) of phosphate buffer, pH 7.4, along with 10 \( \mu l \) of a DMSO solution of five compounds each at a concentration of 1 \( \mu M \) simultaneously in each dialysis cell. The mixtures of compounds were chosen such that all compound masses were at least 5 Da apart, allowing resolution of the compounds during MS quantification.

**Microsomal Incubations.** Five microliters of 100 \( \mu M \) compound solutions in DMSO was transferred to glass vials held in a thermostatically controlled metal incubation block heated at 37°C. To these same vials was also added 395 \( \mu l \) of microsomes of the appropriate concentration suspended in 0.1 M phosphate buffer, pH 7.4. The drug and microsome mixtures were then mixed thoroughly and left to equilibrate for approximately 3 min. After this equilibration period, the metabolic reactions were initiated by the addition of 100 \( \mu l \) of \( \beta \)-NADPH solution at a concentration of 50 mM in 0.1 M phosphate buffer, pH 7.4. At eight time points, covering a range of 3 min for the most rapid reactions, and up to 90 min for the slowest reactions, 50-\( \mu l \) aliquots of this mixture were then removed and quenched by addition to 300 \( \mu l \) of methanol that contained an appropriate internal quantification standard compound (indomethacin or 2-ethoxybenzamide) at a concentration of about 0.25 \( \mu M \). The plate of quenched samples was then centrifuged at 300g and 5°C for 20 min to sediment the precipitated proteins before quantitation using HPLC/MS.

The kinetic data were analyzed using a linear fit of the natural logarithm of the ratio of the compound peak area to the internal standard peak area against time. Using the assumption that the substrate concentration of 1 \( \mu M \) is well below the apparent \( K_m \), \( CL_{\text{int}} \) values were then calculated from the negative slope of the linear fit divided by the microsomal concentration (Obach et al., 1997).

**Measurement of Microsomal Binding.** The extent of binding of compounds to microsomes was determined using equilibrium dialysis of five compounds (each at a concentration of 1 \( \mu M \)) simultaneously in each dialysis cell. The mixtures of compounds were chosen such that all compound masses were at least 5 Da apart, allowing resolution of the compounds during MS quantification.

To one side of each of the dialysis cells was added 1 ml of microsomes of the appropriate concentration, suspended in 0.1 M phosphate buffer, pH 7.4, along with 10 \( \mu l \) of a DMSO solution of five compounds each at a concentration of 100 \( \mu M \). The other half of each dialysis cell was filled with 1 ml of 0.1 M phosphate buffer, pH 7.4. The cells were then sealed, clamped to the Dianorm unit, and rotated in a water bath at 37°C for 18 h. To a further volume of microsomes was added 1% (v/v) DMSO, and this solution was stored in a sealed tube at 37°C for 18 h, for later use as control microsomes in sample preparation prior to HPLC/MS analysis. The dialysis cells were then emptied and the solutions were treated in the following way such that the final samples and standards for HPLC/MS analysis were all present in an identical matrix. To 380 \( \mu l \) of sample from the microsomal side of each dialysis cell was added 380 \( \mu l \) of phosphate buffer, pH 7.4, and 1140 \( \mu l \) of methanol. To 380 \( \mu l \) of sample from the buffer side of each dialysis cell was added 380 \( \mu l \) of the control microsome solution and 1140 \( \mu l \) of methanol. A solution for dilution of HPLC standards was also prepared from control, buffer control microsome solution, and methanol in the same ratios as the other samples. All of these samples were then centrifuged at 300g for 10 min. Six standards of unknown absolute concentration, but known relative concentration (covering a 200-fold range of concentration) were then prepared from dilutions of the samples originating from the microsomal side of the dialysis cells using the dilution solution. All of these samples were then quantified using HPLC/MS, and the free fraction of each compound was determined from the ratio of the buffer to microsome concentrations, each interpolated from the six point calibration line. To validate this method of measuring binding in mixtures of five compounds simultaneously, measurements were carried out on 10 compounds singly, and also on the same 10 compounds as two mixtures of five compounds. The 10 measured free fractions from the singly measured group were compared with those from the mixtures group using a two-tailed paired \( t \) test and shown not to be significantly different (\( p = 0.31 \)).

**Measurement of \( \text{Log} D_{\text{int}} \).** Partitioning of compounds (40–400 \( \mu M \)) between 1-octanol and 0.02 M phosphate buffer, pH 7.4, at 20°C was determined using a standard shake flask method (Lee et al., 1971). Samples were analyzed by HPLC with MS quantitation of both layers of the partition mixture.

**Model for Microsomal Binding.** The most simple model for describing the binding of drugs to microsomes is to treat the system as a phase equilibrium, in the same way that drug binding to liposomes is normally described (Austin et al., 1995). The drug is assumed to be in equilibrium between an aqueous phase and a microsomal phase, and the equilibrium is described by a partition coefficient \( K_{\text{micro}} \) given by eq. 3:

\[
K_{\text{micro}} = \frac{\text{drug concentration in microsome phase}}{\text{drug concentration in aqueous phase}}
\]
Equation 4 can now be used to relate the value of $n_{\text{mics}}$ to the value of $C_V_{\text{mics}}$ at typical microsomal concentrations, eq. 6 can be simplified to give eq. 7:

$$K_{\text{mics}} = \frac{[\text{drug}]_{\text{mics}}}{[\text{drug}]_{\text{aq}}} = \frac{n_{\text{mics}}}{n_{\text{aq}}} \frac{V_{\text{aq}}}{V_{\text{mics}}}$$

where $[\text{drug}]_{\text{mics}}$ and $n_{\text{mics}}$ are the concentrations and amounts of drug in the microsomal phase, $[\text{drug}]_{\text{aq}}$ and $n_{\text{aq}}$ are the concentrations and amounts of drug in the aqueous phase, $V_{\text{aq}}$ is the volume of the aqueous phase and $V_{\text{mics}}$ is the volume of the microsomal phase. If we now set $r$ to be equal to the ratio of aqueous to microsomal phase volumes $V_{\text{aq}}/V_{\text{mics}}$, at a 1 mg/ml microsomal concentration then eq. 3 becomes the following:

$$K_{\text{mics}} = \frac{n_{\text{mics}}}{n_{\text{aq}}} C_{\text{mics}}$$

where $C_{\text{mics}}$ is the microsomal concentration in micrograms per milliliter. Equation 4 can now be used to relate the value of $n_{\text{mics}}/n_{\text{aq}}$ at one microsomal concentration ($C_1$) to the value of $n_{\text{mics}}/n_{\text{aq}}$ at another microsomal concentration ($C_2$) as shown by eq. 5:

$$\left(\frac{n_{\text{mics}}}{n_{\text{aq}}} \right)_{C_1} = \frac{C_2}{C_1} \left(\frac{n_{\text{mics}}}{n_{\text{aq}}} \right)_{C_2}$$

Equation 5 now needs to be expressed in terms of free fractions rather than ratios of amounts in each phase. The definition of free fraction is given by eq. 6:

$$f_u = \frac{[\text{drug}]_{\text{mics}}}{[\text{drug}]_{\total}} = \frac{n_{\text{aq}}}{n_{\text{aq}} + n_{\text{mics}}} \frac{V_{\text{aq}} + V_{\text{mics}}}{V_{\text{aq}}}$$

because $V_{\text{aq}} \gg V_{\text{mics}}$ at typical microsomal concentrations, eq. 6 can be simplified to give eq. 7:

$$f_u = \frac{n_{\text{aq}}}{n_{\text{aq}} + n_{\text{mics}}}$$

Equation 7 can now be rearranged to give eq. 8:

$$\frac{n_{\text{aq}}}{n_{\text{mics}}} f_u = \frac{1}{1 - f_u}$$

Equation 8 can now be substituted into eq. 5, followed by rearrangement, to give eq. 9, which is an expression for the free fraction at a second microsomal concentration ($f_u_2$) in terms of the observed free fraction at a first microsomal concentration ($f_u_1$) and the second and first microsomal concentrations ($C_2$ and $C_1$):

$$f_u_2 = \frac{1}{C_2} \left( \frac{1 - f_u_1}{f_u_1} \right) + 1$$

Results

A set of 13 compounds was selected that covers a range of lipophilicity (logD2,4 ranges from 0.3 to >5) and ionization class (five neutral, four acidic, and four basic). Acids are defined as compounds with a $pK_a$ for the formation of an anion of <7.4, and bases are defined as compounds with a $pK_a$ for the formation of a cation of >7.4. The kinetics of metabolism of these compounds was determined using the in vitro half-life approach (Obach et al., 1997) with rat liver microsomal protein concentrations of 0.25, 1, and 4 mg/ml.

The extent of binding of compounds, $f_u_{\text{mics}}$, was determined at these same microsomal protein concentrations using equilibrium dialysis. In the dialysis experiments, $\beta$-NADPH was omitted such that metabolism of the compounds did not occur. The results of these experiments are listed in Table 1.

Of the 13 compounds in Table 1 it was found that some of them had an approximately constant $C_{\text{int}}$ at the three different microsomal concentrations. An example of this type of behavior is given by bumetanide where $C_{\text{int}} = 157 \pm 6$ (microliters per minute per milligram of protein) at 0.25 mg/ml, 127 \pm 7 at 1 mg/ml, and 121 \pm 21 at 4 mg/ml. The other extreme of observed behavior is exemplified by compounds such as astemizole, where the $C_{\text{int}}$ values decrease with increasing microsomal concentration. These different types of behavior correspond to differing propensity for microsomal binding.

According to eq. 1 the correctly defined intrinsic clearance, which is equal to the observed intrinsic clearance divided by the free fraction of compound in the incubation medium, should be constant. The data in Table 1 shows that such values of $C_{\text{int},U}$ are much closer to being constant than the values of $C_{\text{int}}$. Figure 1A shows a plot of $C_{\text{int}}$ at 1 mg/ml microsomal protein against $C_{\text{int},U}$ at 0.25 mg/ml, where the error bars indicate 95% confidence intervals calculated from the four replicate data points. If $C_{\text{int},U}$ were constant then the points would lie along the indicated line of unity, but clearly several of the compounds deviate away from the line of unity, often very strongly. In contrast, Fig. 1B is a plot of $C_{\text{int},U}$ at 1 mg/ml microsomal protein against $C_{\text{int},U}$ at 0.25 mg/ml, where the error bars indicate 95% confidence intervals propagated from the 95% confidence intervals calculated from the four replicate $C_{\text{int},U}$ values and the four replicate $f_u_{\text{mics}}$ values. Most of the compounds lie on the indicated line of unity within experimental error, whereas the remaining compounds only show small deviations.

The microsomal binding data in Table 1 can be used to develop a better understanding of how the extent of microsomal binding de-
pends on microsomal protein concentration. A simple model for microsomal binding has been developed that treats the system as a microsome/buffer phase equilibrium (under Materials and Methods, eqs. 3–9). From this model of binding, it is possible to estimate the extent of microsomal binding at one microsomal concentration from the observed extent of binding at a different microsomal concentration using eq. 9. From the data in Table 1, eq. 9 was used to estimate \( f_{u \text{inc}} \) at one microsomal concentration from that observed at a different concentration, and Fig. 2 shows a plot of the resulting predictions. The data points scatter fairly evenly about the indicated line of unity, and the average deviation in the predicted \( f_{u \text{inc}} \) values is only 15%. The simple phase equilibrium model therefore seems to be a good model for microsome binding.

The binding data at 1 mg/ml microsomal protein concentration in Table 1 was augmented with binding measurements on a further set of 25 compounds, and with logD\(_{7.4}\) measurements on the full set of 38 compounds. Microsomal binding data for the full set of compounds is shown in Table 2 along with \( \log P \), logD\(_{7.4}\), and \( \log K_a \) data. Table 2 also contains microsome binding data from Obach (1997, 1999). Most of the additional data from Obach is from human liver microsomes, whereas the current study has used rat liver microsomes. However, it has been shown that the extent of microsome binding shows very little difference between human, rat, dog, and monkey (Obach, 1997). The Obach data were determined using a variety of microsomal protein concentrations between 0.3 and 10 mg/ml, and any data not determined at 1 mg/ml have been converted to an estimated extent of binding at 1 mg/ml using eq. 9. The combination of our data with Obach’s produces a microsomal binding data set on 56 compounds of diverse structure where all of the data are either measured at 1 mg/ml microsomal protein or have been converted to 1 mg/ml using a method that has been shown to work reliably.

The extent of nonspecific binding to microsomes is expected to be dominated by lipophilicity. Figure 3 shows a plot of log((1 – \( f_{u \text{inc}} \))/\( f_{u \text{inc}} \)) against logD\(_{7.4}\). The microsomal binding data are plotted in this way because (1 – \( f_{u \text{inc}} \))/\( f_{u \text{inc}} \) is similar to an equilibrium constant (eq. 8), which is the appropriate property to use when searching for linear free energy relationships. Figure 3 shows a strong relationship between microsomal binding and logD\(_{7.4}\). The extent of microsomal binding generally increases with increasing lipophilicity, but basic compounds clearly show enhanced binding over neutral or acidic compounds of similar lipophilicity. This is expected because it has been shown that basic compounds exhibit enhanced affinity for phospholipid membranes, compared with octanol, as demonstrated by liposome binding studies (Austin et al., 1995; Krämer et al., 1998). To account for the pattern shown in Fig. 3, a modified lipophilicity descriptor has been introduced that accounts for the enhanced microsomal binding of basic compounds. This descriptor will be called logP/D and corresponds to logP for basic compounds and logD\(_{7.4}\) for acidic and neutral compounds (note, logD\(_{7.4}\) = logP for neutral compounds). Figure 4 shows a plot of log((1 – \( f_{u \text{inc}} \))/\( f_{u \text{inc}} \)) against logP/D, where only those compounds with \( f_{u \text{inc}} \) < 0.9 have been included. This is because it is very difficult to discriminate between compounds with free fractions between 0.9 and 1.0 using equilibrium dialysis, and it is not important to model this range of binding because all of these compounds are close to 100% free. Using the logP/D descriptor, Fig. 4 shows a much better correlation than that in Fig. 3. The logP/D descriptor has collapsed the separate acid/base/neutral behavior shown in Fig. 3 into a single trend of high statistical significance. The equation of the least-squares regression line in Fig. 4 is given by eq. 10:

\[
\log((1 - f_{\text{inc}})/f_{\text{inc}}) = 0.53\log P/D - 1.42
\]

\( n = 37, r^2 = 0.82, F = 160.0, p = 1.3 \times 10^{-14} \)
Table 2

<table>
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<tr>
<th>Compound</th>
<th>Charge Type</th>
<th>logD_{2,4}</th>
<th>pK_{a}</th>
<th>logP</th>
<th>f_{u,uc}</th>
<th>log(1 – f_{u,uc})</th>
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<tbody>
<tr>
<td>2-Ethoxybenzamide</td>
<td>Neutral</td>
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<td>1.34</td>
<td>0.98</td>
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<td>Alprazolam</td>
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<td>1.84</td>
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<td>Amodarone</td>
<td>Base</td>
<td>&gt;5</td>
<td>8.7</td>
<td>&gt;6.35</td>
<td>0.002</td>
<td>2.70</td>
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<td>Atrimoxilone</td>
<td>Base</td>
<td>4.14</td>
<td>8.35</td>
<td>5.14</td>
<td>0.012</td>
<td>1.92</td>
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<td>Acid</td>
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<td>4.5</td>
<td>3.21</td>
<td>0.92</td>
<td>-1.06</td>
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<td>0.92</td>
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<tr>
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<td>9.38</td>
<td>5.25</td>
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<td></td>
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<td>5.9</td>
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<td>0.96</td>
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<td>4.29</td>
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<td>-0.66</td>
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<td>0.29</td>
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<td>4.6</td>
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<td>0.92</td>
<td>-1.06</td>
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<td>4.1</td>
<td>4.13</td>
<td>0.90</td>
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<tr>
<td>Methobendazole</td>
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<td></td>
<td>4.96</td>
<td>0.081</td>
<td>1.05</td>
</tr>
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<td>8.60</td>
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<td>Acid</td>
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<td>4.40</td>
<td>3.98</td>
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<td>Acid</td>
<td>1.00</td>
<td>5.27</td>
<td>3.13</td>
<td>&gt;0.97</td>
<td>-1.51</td>
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<tr>
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<td>Neutral</td>
<td>2.07</td>
<td>7.96</td>
<td>2.07</td>
<td>&gt;0.97</td>
<td>-1.51</td>
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<tr>
<td>Hexobarbital</td>
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<td>8.37</td>
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<td>-1.33</td>
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</table>

Discussion

When one wishes to scale in vitro metabolic kinetic data to in vivo clearances, the issue of nonspecific microsomal binding needs to be addressed in addition to the plasma or blood binding that is more frequently incorporated into the scaling method. It has been demonstrated that eq. 2 could be of more general utility in the prediction of clearance than either the well stirred model containing no free fraction corrections, or only the fu_{u,uc} correction (Obach, 1999). Cancellation of the fu_{u,uc} / fu_{u,uc} ratios cannot be relied upon, particularly because fu_{u,uc} can depend strongly on the microsomal protein concentration that one chooses to use as shown in Table 1 and by other studies (Obach, 1996, 1997; Mclure et al., 2000; Venkatakrishnan et al., 2000). If eq. 2 is to

**Note:** The table includes data on various compounds with their respective charge types, logD_{2,4}, pK_{a}, logP, fu_{u,uc}, and log(1 – fu_{u,uc}). The data represent physicochemical and microsomal binding data on compounds studied using 1 mg/ml rat liver microsomes.
stable with CLint,
microsomal protein amiodarone seems to be metabolically rather
alternative method for the estimation of func.

Clearance assays is also a strong driving force for the generation of an
capacity and highly automated efficiency of microsomal intrinsic
equilibrium dialysis experiments have not nearly reached the high
water partition coefficients would be very useful. The fact that
mal binding using commonly measured properties such as octanol-
calculated from chemical structure, but the ability to estimate microso-
capacity and on chemical structure is re-
understanding of how nonspecific microsomal binding depends on
importance of eq. 1 using a wider set of compounds.

bolic screening in drug discovery research, to further highlight the
importance and validity of eq. 1 have previously been demonstrated using full determinations of Vmax
and apparent Km (Obach, 1997; Venkatakrishnan et al., 2000; Kalvass et al., 2001) on limited numbers of compounds. This study has used the
in vitro t1/2 method, the most frequently used method for metabolic screening in drug discovery research, to further highlight the
importance of eq. 1 using a wider set of compounds.

In addition to the well established dependence of fu inc on micro-
somal protein concentration, it is also possible that fu inc depends on the compound concentration, and also that the binding is saturable. It has been shown that the microsomal binding of phenytoin and tolbu-
tamide (Carlile et al., 1999), amitriptyline (Venkatakrishnan et al.,
2000), and imipramine and propranolol (Obach, 1997) is independent of compound concentration over the range of concentration studied
(up to 1000 µM). Evidence for concentration dependence has been shown for nortriptyline (Mclure et al., 2000) and for warfarin (Obach,
1997). However, the observed concentration dependence is weak in both cases. It therefore seems that nonspecific microsomal binding is
normally independent of compound concentration and that saturation of the binding does not occur at the low micromolar concentration
levels used in modern metabolic assays, which use sensitive MS
detection. If strong concentration dependence were frequently ob-
served then the commonly used description of the process as nonspe-
cific microsomal binding would clearly be inappropriate. The type of
model involving defined binding sites (Romer and Bickel, 1979) that has been suggested as appropriate for modeling microsomal binding of
drugs (Mclure et al., 2000; Kalvass et al., 2001) seems to be
unnecessarily complicated. Binding that is independent of compound concentration is indicative of a phase equilibrium-type process, where clearly defined individual binding sites do not exist, much like organ-
ic/aqueous partitioning systems. The success of this model of micro-
somal binding (eq. 9) is shown by the quality of the predictions in Fig.
2. It therefore seems that eq. 9 represents a suitable general model for the description of microsomal binding, particularly when the drug
concentration is low, and should be of general use for converting microsomal binding data between different microsomal protein con-
centrations.

Equation 9 has been used to augment our own microsomal binding
data set of 38 compounds all measured at 1 mg/ml microsomal protein with literature data on 18 further compounds, leading to a fairly large
data set suitable for finding a structure-binding relationship. The enhanced binding shown by basic compounds in Fig. 3 led us to search for a more suitable lipophilicity-related descriptor. The enhanced phospholipid binding of compounds containing protonated
basic groups is thought to be due to a favorable electrostatic interac-
tion between the protonated base and phosphate groups on the phos-
pholipid (Austin et al., 1995; Krämer et al., 1998). A result of this is
that phospholipid membrane affinity at pH 7.4 for basic compounds is
much closer to the octanol-water logP of the molecule than to logD7.4,
whereas for acidic molecules (where only the neutral form of the
molecule has significant membrane affinity) the membrane affinity at
pH 7.4 is much closer to the octanol-water logD7.4 than logP (Austin et al.,
1995). Therefore, because the affinity of compounds for microsomes is likely to be dominated by the affinity for the phospholipid
membrane content of the microsomes, this should be best modeled by
octanol-water logP for bases and by logD7.4 for acidic and neutral
(note, logD7.4 = logP for neutral compounds) compounds. This de-
scriptor will be called logP/D and does indeed lead to a much better
correlation with extent of microsomal binding (Fig. 4; eq. 10).
The correlation shown in Fig. 4 seems to be of higher quality where 
logP/D > 2, and this may due the equilibrium dialysis method not
being able to accurately distinguish between the low levels of binding
(50–100% free) of hydrophobic compounds. For predicting the binding
of more lipophilic molecules it might be better to only fit the data in
Fig. 4 where logP/D > 2, and simply state that for less lipophilic
molecules fu inc > 0.5. This approach should lead to better predictions
for more lipophilic molecules where fu inc can be very small (hence
corrections to CL int would be large). Accurate prediction of microsomal
binding of hydrophobic molecules is not so important because the
resulting corrections to CL int would be less than a factor of 2 (of the
56 compounds in Table 2, 28 have logP/D < 2 and none of these has
fu inc < 0.5). The correlation in Fig. 4 also seems to be better for basic
compounds than for neutral or acidic compounds. Again this is likely
to be due to the fact that most of the basic compounds exhibit higher
binding than the neutral or acidic compounds (a large proportion of
the bases have logP/D > 2), which is more easily discriminated by the
equilibrium dialysis method. Equation 10 shows that microsomal
binding is only significant for rather lipophilic neutral and acidic
compounds (logD 2.4 > 3), but will be significant for many more basic
compounds. For example, a basic compound with logD 2.4 of only 1
and pK a of 10 will have logP = 3.6 and from eq 10 will have fu inc of
about 0.2.

The fact that the literature microsomal binding data and the data
from the current study both fit well in the correlation in Fig. 4 adds
validation to two important facts about microsomal binding that are
emerging. First, it is consistent with the observation that microsome
binding is independent of species (Obach, 1997); and second, it gives
further evidence of the general utility of eq. 9 for converting data
between different microsomal concentrations. Equation 10 can only
predict the extent of binding to microsomes with a concentration of 1
mg/ml. However, the good correlations shown in Figs. 2 and 4 suggest
that eqs. 9 and 10 can be combined to give a reliable prediction of fu inc
at any microsomal concentration from lipophilicity using eq. 11

\[
fu_{inc} = \frac{1}{C \cdot 10^{1.1 \log P - 1.41} + 1} \quad (11)
\]

where C is the microsomal protein concentration in milligrams per
milliliter, logP/D is the logP of the molecule if it is a base (basic pK a
> 7.4), and logP/D is the logD 2.4 of the molecule if it is neutral or an
acid (acidic pK a < 7.4). Along with a knowledge of lipophilicity and
ionization, eq. 11 should prove to be useful for gaining better insight
into observed kinetics of microsomal metabolism, for converting
CL int data to CL int,U for further testing and validating models for
clearance prediction such as eq. 2, and for conversion of K i to
unbound K i as part of the process of predicting in vivo drug-drug
interactions.

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