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THE BINDING OF DRUGS TO HEPATOCYTES AND ITS RELATIONSHIP TO PHYSICOCHEMICAL PROPERTIES

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ABSTRACT:

The binding of 17 drugs to rat hepatocytes has been determined using equilibrium dialysis in combination with metabolic inhibitors and a kinetic model for the binding and dialysis processes. Metabolic inhibitors were used to retard the main routes of metabolism such that the half-life for turnover of the drugs was comparable to or greater than the time scale of the equilibrium dialysis process. Further experiments were carried out to determine the kinetics of diffusion of the compounds across the dialysis membrane and the observed extent of binding to hepatocytes. Knowledge of the rate of metabolism of the drugs in the presence of the inhibitors, the kinetics of the dialysis process, and the observed extent of binding was then used with a kinetic model of the system to give true free fractions of the drugs in live hepatocytes. Further studies show that, for this set of compounds, there is no significant difference in the extent of binding to live or dead hepatocytes. The extent of hepatocyte binding is correlated with lipophilicity, and the best model for binding uses log *P* for basic compounds and log $D_{7.4}$ for acidic and neutral compounds. Hepatocyte binding is also demonstrated to be highly correlated with microsome binding.

The kinetic data from microsome or hepatocyte intrinsic clearance assays are often used for the prediction of in vivo metabolic clearance through the use of in vitro-in vivo scaling using methods such as the well stirred model and the parallel tube model (Pang and Rowland, 1977). It has been recognized that nonspecific binding in the in vitro metabolic assay medium can significantly affect the observed kinetics of metabolism and hamper the accurate prediction of clearance. Nonspecific binding has been fairly well studied using microsomes, and there are several examples where knowledge of the extent of microsomal binding leads 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).

Unbound intrinsic clearance ($CL_{int,U}$) is for many compounds not the same as the experimentally observed intrinsic clearance (CL_{int}), and the two are related by eq. 1:

$$CL_{int,U} = \frac{V_{max}}{K_M} = \frac{CL_{int}}{fu_{inc}} \quad (1)$$

where V_{max} is the maximum velocity, K_M is the Michaelis constant based on free substrate concentrations, and fu_{inc} is the free fraction of compound in the in vitro incubation. Studies of microsomal binding have shown that the difference between CL_{int} and $CL_{int,U}$ can be large for some compounds (Obach, 1999; Austin et al., 2002). When eq. 1 is substituted into the well stirred model, the predicted in vivo clearance is given by eq. 2 (Obach, 1996):

$$CL = \frac{\frac{Q \cdot A \cdot B \cdot fu_p \cdot CL_{int}}{fu_{inc}}}{\left(Q + \frac{A \cdot B \cdot fu_p \cdot CL_{int}}{fu_{inc}}\right)} \quad (2)$$

where A describes either the milligrams of microsomes per gram of liver or number of hepatocytes per gram of liver, B describes the grams of liver per kilogram of body weight, Q is the liver blood flow, fu_p is the free fraction in plasma, and fu_{inc} is the free fraction in the microsome or hepatocyte incubation. Using measured values of fu_p and fu_{inc}, it has been shown that eq. 2 could be of more general utility in the prediction of clearance from human liver microsomes than either the well stirred model containing no free fraction corrections or only the fu_p correction (Obach, 1999). However, recent studies have suggested that the use of measured values of fu_p and fu_{inc} in eq. 2 may still lead to poor predictions of human clearance for some compounds from microsomes and hepatocytes (Naritomi et al., 2001, 2003). Equation 2 could be more widely tested and validated if it were possible to predict the fu_{inc} term with reasonable accuracy from readily available physicochemical properties of molecules. Our previous study found that the extent of microsomal binding of a diverse set of drugs can be fairly well predicted from a knowledge of lipophilicity and ionization (Austin et al., 2002). However, less is known about the dependence of hepatocyte binding on physicochemical properties. Studies on three lipophilic pesticides have shown that the binding to rat hepatocytes is a passive process with a well defined equilibrium position that is reached rapidly (Ichinose and Kurihara, 1985). The extent of hepatocyte binding was found to increase with increasing lipophilicity, although the extent of binding solely to hepatocytes was unclear due to the presence of BSA in the buffer. The binding of parathion to rat hepatocytes has also been shown to be

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; MS, mass spectrometry; BSA, bovine serum albumin.

rapid and reversible, and to reach a defined equilibrium position (Nakatsugawa et al., 1980). Most other investigations relating to hepatocyte binding have focused on kinetic studies of uptake and metabolism of compounds by hepatocytes in which active uptake processes were expected to be present; for example, with drugs known to have extensive biliary clearance. The kinetics of uptake of benzylpenicillin and cefpiramide by rat hepatocytes has been found to occur through both a saturable transport mechanism and a passive process, whereas that of cefazolin takes place purely through a passive process (Tsuji et al., 1986). Other compounds with both active and passive hepatocyte uptake mechanisms include glycyrrhizin (Ishida et al., 1993), iodipamide (Joppen et al., 1985), tyramine (Zhong et al., 1993), pravastatin (Yamazaki et al., 1993), and bumetanide (Petzinger et al., 1989). The purpose of the current study is to determine the extent of hepatocyte binding for a set of drugs that exhibit a range of physicochemical properties, and to determine how the binding to hepatocytes is related to the physicochemical properties of the molecules.

Materials and Methods

Chemicals. 1-Octanol, formic acid, and ammonium acetate were obtained from Fisher Scientific (Loughborough, UK). Dulbecco's modified Eagle's medium, sodium bicarbonate, HEPES, albendazole, 1-aminobenzotriazole, astemizole, bumetanide, carbamazepine, clozapine, 2-ethoxybenzamide, glyburide, indapamide, metoprolol, metyrapone, propranolol, salicylamide, trioxsalen, and verapamil were obtained from Sigma Chemical (Poole, Dorset, UK). Oxaprozin was obtained from Maybridge Chemicals (Trevillet, UK). Betaxolol, cerivastatin, diazepam, isradipin, omeprazole, quinotolast, and troglitazone were obtained from the AstraZeneca compound collection.

Instrumentation. All sample handling was performed using a Tecan Genesis RSP 100 liquid handling robot (Tecan, Durham, NC) fitted with disposable tips and controlled by Gemini software (Tecan, Durham, NC). Centrifugations were carried out using a Beckman Coulter (Fullerton, CA) Allegra R6 centrifuge. A Dianorm system with cells of 1-ml volume was used for equilibrium dialysis experiments, along with Diachema cellulose membranes with molecular weight cut-off of 5000 (Dianorm, Munich, Germany). All HPLC analyses were carried out using a Waters 2700 autosampler (Waters, Milford, MA), a Waters 2690 separations module, a Waters 996 diode array detector, and a Waters Micromass ZMD mass spectrometer using a selected ion recording quantitation method. Waters Symmetry C8 5 μm $\times$ 3.9 mm $\times$ 20 mm columns were used along with a gradient of 1% actonitrile/99% 0.05% aqueous ammonium acetate to 99% actonitrile/1% 0.05% aqueous ammonium acetate at a flow rate of 2 ml/min over 3.5 min. For analysis of propranolol, metoprolol, and betaxolol, the 0.05% ammonium acetate was replaced with 0.1% formic acid.

Isolation of Rat Hepatocytes. Rat hepatocytes were isolated from male Sprague-Dawley rats (250–300 g) using a procedure based on the method of Seglen (1976). Briefly, following anesthesia, the liver was perfused via the portal vein, first with Hepatocyte Liver Perfusion Medium (Invitrogen, Paisley, UK) and then with a collagenase containing Hepatocyte Liver Digest Medium (Invitrogen). After release from the liver, the hepatocytes were suspended in hepatocyte medium (Dulbecco's modified Eagle's medium plus 25 mM sodium bicarbonate and 10 mM HEPES), filtered, centrifuged, and resuspended in hepatocyte buffer. Viability was determined by tryptan blue exclusion. In some experiments, 0.2% (w/v) BSA was also added to the hepatocyte medium.

Hepatocyte Incubations. To freshly prepared rat hepatocytes was added a DMSO solution of 1-aminobenzotriazole (400 mM, 2.5 μl per ml of hepatocytes), followed by incubation in a thermostated water bath at 37°C for 60 min to allow P450 inactivation to occur. To this solution was then added a DMSO solution of salicylamide (300 mM, 5 μl per ml of hepatocytes). After 5 min, the metabolic reactions were initiated by adding this solution to magnetically stirred glass vials (495 μl in each vial) containing a DMSO solution of drug (5 μl, 300 μM) held in a thermostatically controlled metal incubation block heated at 37°C. At eight time points, covering a range of 120 min, 50-μl aliquots of this mixture were then removed and quenched by addition to 300 μl of methanol, which contained an appropriate internal quantification standard compound (trioxsalen or carbamazepine) at a concentration of 1 μM. The plate of quenched samples was then centrifuged at 300*g* 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. The kinetics of each compound were measured in duplicate. Using the assumption that the substrate concentration of 3 μM is well below the apparent K_m, CL_{int} values were then calculated from the negative slope of the linear fit divided by the microsomal concentration (Obach et al., 1997). The validity of this assumption does not affect the use of the kinetic data for the calculation of corrected free fractions because the observed pseudo first order rate constants, rather than derived CL_{int} values, were used for the later modeling. For reactions not containing inhibitors, an identical method was used except that DMSO was added to the hepatocytes rather than DMSO solutions of inhibitors.

Dialysis Kinetics. Solutions of drug in buffer were prepared from hepatocyte medium (7 ml) and a DMSO solution of drug (70 μl, 2 mM). This solution was added (1 ml) to one half of five dialysis cells, with the other half being filled with hepatocyte medium (1 ml). The cells were then sealed, clamped to the Dianorm unit, and rotated in a water bath at 37°C for 2 h. At 10 time points, 250-μl aliquots were removed from both sides of the dialysis cells and analyzed by HPLC with UV quantification. Each of the five cells was sampled at two time points. Since the system is not under sink conditions, the kinetic model for analyzing the data needs to take into account the effect of back flux from the receiver compartment. Application of Fick's law to this system gives eq. 3 (Palm et al.,1999):

$$-\frac{dC_D}{dt} = \frac{P_e A}{V_D} C_D - \frac{P_e A}{V_D} C_A \quad (3)$$

where C_D and C_A are the concentrations of drug in the donor and acceptor compartments, respectively, P_e is the permeability of the drug, A is the surface area of the dialysis membrane, and V_D is the volume of solution in the donor cell. In this system, the following mass-balance relationship (eq. 4) and equilibrium relationship (eq. 5) apply:

$$C_D^0 = C_D + C_A \quad (4)$$

$$C_D^e = \frac{C_D^0}{2} \quad (5)$$

where C_D^0 and C_D^e are the initial and equilibrium concentrations of drug in the donor compartment, respectively. Equations 4 and 5 can be substituted into eq. 3 followed by integration to give eq. 6:

$$\ln\left(\frac{C_D - C_D^e}{C_D^0 - C_D^e}\right) = -\frac{2P_e A}{V_D} t \quad (6)$$

The values of P_e were then determined from the slope of a plot of $\ln(C_D - C_D^e)$ against t.

Measurement of Hepatocyte Binding. To freshly prepared rat hepatocytes was added a DMSO solution of 1-aminobenzotriazole (400 mM, 2.5 μl per ml of hepatocytes), followed by incubation in a water bath at 37°C for 60 min. A DMSO solution of salicylamide (300 mM, 5 μl per ml of hepatocytes) was then added, followed by a further 5-min incubation. This hepatocyte solution was then added (1 ml) to one side of each dialysis cell along with a DMSO solution of drug (10 μl, 300 μM), and hepatocyte medium (1 ml) was added to the other side of each cell. The cells were then sealed, clamped to the Dianorm unit, and rotated in a water bath at 37°C for 3 h. To another volume of hepatocytes was added 1.75% (v/v) DMSO, and this solution was stored in a sealed tube at 37°C for 4 h, for later use as control hepatocytes in sample preparation before 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 μl of sample from the hepatocyte side of each dialysis cell was added 380 μl of pH 7.4 hepatocyte buffer and 1140 μl of methanol. To 380 μl of sample from the buffer side of each dialysis cell was added 380 μl of the control hepatocyte solution and 1140 μl of methanol. A solution for dilution of HPLC standards was also prepared from buffer, control hepatocyte solution, and methanol in the same ratios as the other samples. All of these samples were

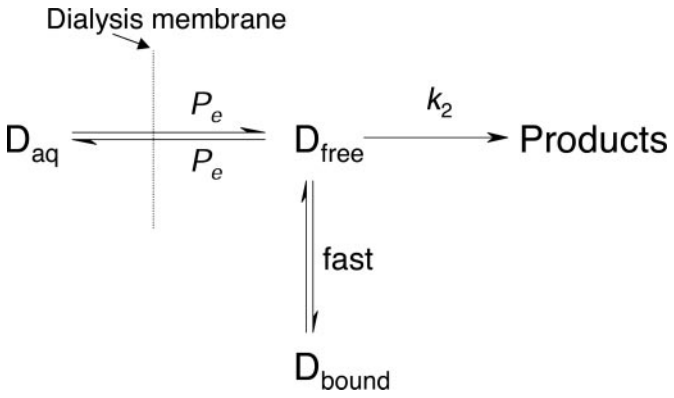

FIG. 1. Kinetic scheme for the binding of compounds to hepatocytes with simultaneous metabolism by the hepatocytes and transport across a dialysis membrane.

then centrifuged at 300*g* for 10 min. Five standards of unknown absolute concentration, but known relative concentration (covering a 200-fold range of concentrations) were then prepared from dilutions of the samples originating from the hepatocyte 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 hepatocyte concentrations, each interpolated from the five-point calibration line.

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

Kinetic Model for the Hepatocyte Metabolism and Equilibrium Dialysis Processes. The kinetic scheme is shown in Fig. 1. Drug bound to hepatocytes is denoted by D_{bound}, which is in equilibrium with free drug, D_{free}. The free drug is metabolized by the hepatocyte enzymes in a pseudo first order process (we assume here that $[D_{free}] < K_m$), with observed rate constant k_2. The free drug can also cross the dialysis membrane with permeability P_e to give drug in aqueous solution on the other side of the dialysis membrane, D_{aq}. The metabolism process controlled by k_2 and the dialysis process controlled by P_e both have half-lives on the order of tens of minutes, and it is reasonable to assume that the kinetics of the nonspecific binding process are fast in comparison. The differential equation describing the change in $[D_{aq}]$ is given by eq. 7:

$$-\frac{d[D_{aq}]}{dt} = k_1[D_{aq}] - k_1[D_{free}] \quad (7)$$

where $k_1 = P_eA/V$, in which A is the surface area of the dialysis membrane and V is the volume of half the dialysis cell. The differential equation describing the change in total concentration of drug in the hepatocyte containing half the dialysis cell, $[D_t]$ is given by eq. 8:

$$-\frac{d[D_t]}{dt} = -k_1[D_{aq}] + k_1 fu[D_t] + k_2[D_t] \quad (8)$$

where *fu* is the free fraction of the drug in that medium. By assuming that the kinetics of the hepatocyte binding process is fast compared with the other process, $[D_{free}]$ has been replaced with $fu[D_t]$. The differential eqs. 7 and 8 are analogous to the equations that arise from the two-compartment open model with elimination from the central compartment that is widely used in pharmacokinetics (Wagner, 1993), and the solutions consequently have a very similar form. The solutions arising from the boundary conditions of $[D_{aq}] = 0$ and $[D_t] = D_{t,0}$ when $t = 0$ are given by eqs. 9 to 12:

$$[D_{aq}] = \frac{k_1 fu[D_{t,0}][\exp(-\alpha t) - \exp(-\beta t)]}{\beta - \alpha} \quad (9)$$

$$[D_t] = \frac{[D_{t,0}][(k_1 - \alpha)\exp(-\alpha t) - (k_1 - \beta)\exp(-\beta t)]}{\beta - \alpha} \quad (10)$$

TABLE 1

Intrinsic clearance data for metabolism of compounds by rat hepatocytes

Data are the mean of two experiments. Average coefficient of variation of duplicate data is 25%.

Compound	CL_{int}/μl/min/10^6 Cells		
	Data with 0.2% BSA Present	Data with 0.2% BSA and Inhibitors Present	Data without BSA but with Inhibitors Present
2-Ethoxybenzamide	19.4	<1	1.7
Albendazole	2.5	0.8	1.2
Astemizole	8.3	2.5	1.1
Betaxolol	6.8	0.6	0.9
Bumetanide	2.1	<1	1.3
Cerivastatin	<1	<1	<1
Clozapine	22	2.3	2.7
Diazepam	3.2	<1	<1
Glyburide	<1	<1	3.2
Indapamide	1.7	0.4	0.8
Isradipine	7.5	1.1	1.1
Metoprolol	6.2	<1	<1
Metyrapone	5.5	4.5	5.5
Omeprazole	7.4	<1	<1
Oxaprozin	3.1	<1	1.1
Propranolol	32	0.9	2.6
Verapamil	20	4.2	2.8

$$\alpha = \frac{-k_1(1 + fu) - k_2 \pm \sqrt{(k_1(1 + fu) + k_2)^2 - 4k_1k_2}}{2} \quad (11)$$

$$\beta = \frac{k_1k_2}{\alpha} \quad (12)$$

The observed *fu* at a given time is given by the ratio of free to total concentration at that time as shown by eq. 13:

$$fu_{obs} = \frac{[D_{aq}]}{[D_t]} \quad (13)$$

Substitution of eq. 9 and eq. 10 into eq. 13, followed by rearrangement, finally gives an expression for *fu* in terms of the measured quantities fu_{obs}, k_1, and k_2:

$$fu = \frac{fu_{obs}[(k_1 - \alpha)\exp(-\alpha t) - (k_1 - \beta)\exp(-\beta t)]}{k_1[\exp(-\alpha t) - \exp(-\beta t)]} \quad (14)$$

Results

A set of 17 drugs was selected that covers a range of lipophilicities (log $D_{7.4}$ ranges from −0.2 to 4.1) and ionization classes (seven neutral, four acidic, six 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 intrinsic clearance of these compounds in rat hepatocytes was determined using the in vitro half-life approach (Obach et al., 1997), with rat hepatocyte concentrations of 10^6 cells/ml. The kinetics were measured under three conditions: in a medium containing 0.2% BSA and no metabolic inhibitors, in the same medium but with metabolic inhibitors included, and in a medium without BSA but with metabolic inhibitors present. The inhibitors used were 1-aminobenzotriazole and salicylamide, and were used to increase the half-life of the reactions such that they became similar to or longer than the half-life for transfer of the compounds across a dialysis membrane in later binding experiments. The kinetic data are shown in Table 1 and indicate that the inhibitors have a significant effect in reducing the rate of metabolism of all the compounds except for cerivastatin, where no turnover can be found under any of the conditions, and metyrapone, where the CL_{int} is constant at about 5 μl/min/10^6 cells. The highest measured CL_{int} in the presence of metabolic inhibitors was 5.5 μl/min/10^6 cells, which corresponds to a half-life of 2 h.

TABLE 2

Dialysis kinetic data and physicochemical data

Compound	Charge Type	10^5 P_e/cm s^{-1}	Log $D_{7.4}$	pK_a	log P
2-Ethoxybenzamide[a]	Neutral	19	1.34		1.34
Albendazole[a]	Neutral	2.5	3.29		3.29
Astemizole	Base	1.8	4.14	8.35[b]	5.14[c]
Betaxolol[a]	Base	7.4	0.42	9.38	2.40
Bumetanide[a]	Acid	7.9	0.31	4.5	3.21
Cerivastatin[a]	Acid	8.9	1.44	4.3	4.54
Clozapine[a]	Base	24.8	2.9	8.0	3.60
Diazepam[a]	Neutral	15.9	2.25		2.25
Glyburide[a]	Acid	5.1	2.19	5.3	4.29
Indapamide[a]	Neutral	11.4	1.76		1.76
Isradipine[a]	Neutral	14.1	3.75		3.75
Metoprolol	Base	8.3	−0.21	9.6[d]	1.99[c]
Metyrapone[a]	Neutral	9.6	1.37		1.37
Omeprazole	Neutral	14.2	2.08		2.08
Oxaprozin[a]	Acid	10	1.61	4.2	4.81
Propranolol[a]	Base	21.6	1.05	9.45	3.10
Verapamil[a]	Base	6.7	2.57	8.92	4.10

[a] Log $D_{7.4}$, log P, and pK_a data from Austin et al. (2002).
[b] Data from Dollery (1999).
[c] Calculated using $\log P = \log D_{7.4} + \log(1 + 10^{pK_a - 7.4})$ (Taylor, 1990). Log P = log $D_{7.4}$ for compounds designated as being neutral.
[d] Data from Medchem Database (Daylight Chemical Information Systems, Inc., Mission Viejo, CA).

The extent of binding of these compounds to live hepatocytes can be measured using equilibrium dialysis, but the observed results would be incorrect if the rate of transfer across the dialysis membrane were comparable to or slower than the rate of metabolism. Experiments were first carried out to characterize the kinetics of the dialysis process. Buffer was added to both halves of dialysis cells, and drug was added to one half of each cell, followed by removal of aliquots from each half of the cells at various time points and analysis by HPLC with UV quantification. Permeability coefficients (P_e) were then derived from these data using eq. 6, and are shown in Table 2. The values of P_e range from 1.8×10^{-5} cm s^{-1} for astemizole to 2.5×10^{-4} cm s^{-1} for clozapine, corresponding to a range in half-life of 65 to 5 min. Table 2 also lists lipophilicity and ionization data for the set of compounds.

An equilibrium dialysis method was used to determine the extent of binding of the 17 drugs to rat hepatocytes. The binding was measured under three conditions: in the presence of 0.2% BSA and metabolic inhibitors, no BSA but with metabolic inhibitors, and no BSA and no inhibitors but with hepatocytes that had been left to die for 24 h. The observed free fractions are listed in Table 3 for each of the three experimental conditions. In the two sets of experiments using live hepatocytes, it is possible, depending on the relative values of the rate constants for metabolism and crossing of the dialysis membrane, for the observed free fraction to be significantly different from the true free fraction. Since these rate constants are known, and a kinetic model has been derived that describes the complete system, the observed free fractions have been corrected to free fractions using eq. 14, and these data are listed in Table 3. Figure 2A shows a plot of *fu* from hepatocytes containing 0.2% BSA against *fu* from hepatocytes without BSA. Most of the compounds, and in particular, the acids, have data points that lie below the indicated line of unity, showing that binding to BSA is occurring in addition to hepatocyte binding. Figure 2B shows a plot of *fu* from dead hepatocytes against *fu* from live hepatocytes in the absence of BSA. The data are highly correlated, and a two-tailed paired *t* test indicates that the extent of binding to live and dead hepatocytes is not significantly different ($p = 0.42$).

The extent of nonspecific binding to hepatocytes is likely to be dominated by lipophilicity. Figure 3A shows a plot of log((1 − *fu*)/*fu*) against log $D_{7.4}$, along with the line of best fit from linear regression. The hepatocyte binding data are plotted using this transformation, since it is similar to a logarithm of an equilibrium constant, which is the appropriate property to use in linear free energy relationships (Austin et al., 2002). The equation of the regression line in Fig. 3A is given by eq. 15, along with the corresponding statistical parameters:

$$\log((1 - fu)/fu) = 0.38 \log D_{7.4} - 1.10$$

$$n = 17,\ r^2 = 0.55,\ \text{S.D.} = 0.42,\ F = 18.35,\ p = 6.5 \times 10^{-4} \qquad (15)$$

Figure 3A shows enhanced binding of some of the basic compounds compared with neutral or acidic compounds of similar lipophilicity. This finding is similar to that of previous microsome binding studies in which a combined lipophilicity descriptor (log *D/P* = log *P* for basic compounds and log $D_{7.4}$ for acidic and neutral compounds) was used to give a better correlation with the binding data (Austin et al., 2002). Figure 3B shows a plot of log((1 − *fu*)/*fu*) against the combined lipophilicity descriptor, log *D/P*, along with the line of best fit from linear regression. The linear regression parameters are shown in eq. 16:

$$\log((1 - fu)/fu) = 0.40 \log D/P - 1.38$$

$$n = 17,\ r^2 = 0.65,\ \text{S.D.} = 0.37,\ F = 27.86,\ p = 9.3 \times 10^{-5} \qquad (16)$$

TABLE 3

Hepatocyte binding data (mean fu ± S.D. from three replicate measurements)

Compound	Hepatocytes Containing 0.2% BSA		Hepatocytes without BSA		Dead Hepatocytes without BSA ($fu_{observed}$)
	$fu_{observed}$	$fu_{corrected}$	$fu_{observed}$	$fu_{corrected}$	
2-Ethoxybenzamide	0.96 ± 0.02	0.95 ± 0.02	0.93 ± 0.01	0.90 ± 0.01	0.98 ± 0.01
Albendazole	0.52 ± 0.07	0.60 ± 0.07	0.54 ± 0.01	0.66 ± 0.01	0.50 ± 0.06
Astemizole	0.029 ± 0.0072	0.038 ± 0.0085	0.038 ± 0.016	0.056 ± 0.023	0.023 ± 0.010
Betaxolol	0.71 ± 0.06	0.70 ± 0.06	0.72 ± 0.07	0.70 ± 0.07	0.78 ± 0.03
Bumetanide	0.29 ± 0.02	0.28 ± 0.02	0.95 ± 0.06	0.91 ± 0.06	0.86 ± 0.06
Cerivastatin	0.41 ± 0.1	0.40 ± 0.1	0.69 ± 0.01	0.67 ± 0.01	0.71 ± 0.02
Clozapine	0.24 ± 0.04	0.23 ± 0.04	0.51 ± 0.04	0.50 ± 0.04	0.22 ± 0.06
Diazepam	0.66 ± 0.09	0.65 ± 0.09	0.82 ± 0.03	0.81 ± 0.03	0.87 ± 0.01
Glyburide	0.040 ± 0.004	0.041 ± 0.004	0.67 ± 0.01	0.60 ± 0.01	0.46 ± 0.02
Indapamide	0.75 ± 0.04	0.75 ± 0.04	0.83 ± 0.01	0.82 ± 0.01	0.87 ± 0.01
Isradipin	0.26 ± 0.04	0.25 ± 0.03	0.28 ± 0.02	0.28 ± 0.02	0.26 ± 0.03
Metoprolol	0.84 ± 0.08	0.82 ± 0.08	0.97 ± 0.03	0.95 ± 0.03	0.96 ± 0.02
Metyrapone	0.72 ± 0.07	0.65 ± 0.07	0.88 ± 0.04	0.77 ± 0.04	0.95 ± 0.02
Omeprazole	0.81 ± 0.02	0.80 ± 0.02	0.94 ± 0.02	0.93 ± 0.02	0.86 ± 0.03
Oxaprozin	0.30 ± 0.10	0.29 ± 0.10	0.85 ± 0.02	0.83 ± 0.02	0.66 ± 0.05
Propranolol	0.68 ± 0.05	0.67 ± 0.05	0.54 ± 0.08	0.53 ± 0.08	0.50 ± 0.01
Verapamil	0.49 ± 0.1	0.42 ± 0.09	0.69 ± 0.06	0.63 ± 0.05	0.69 ± 0.11

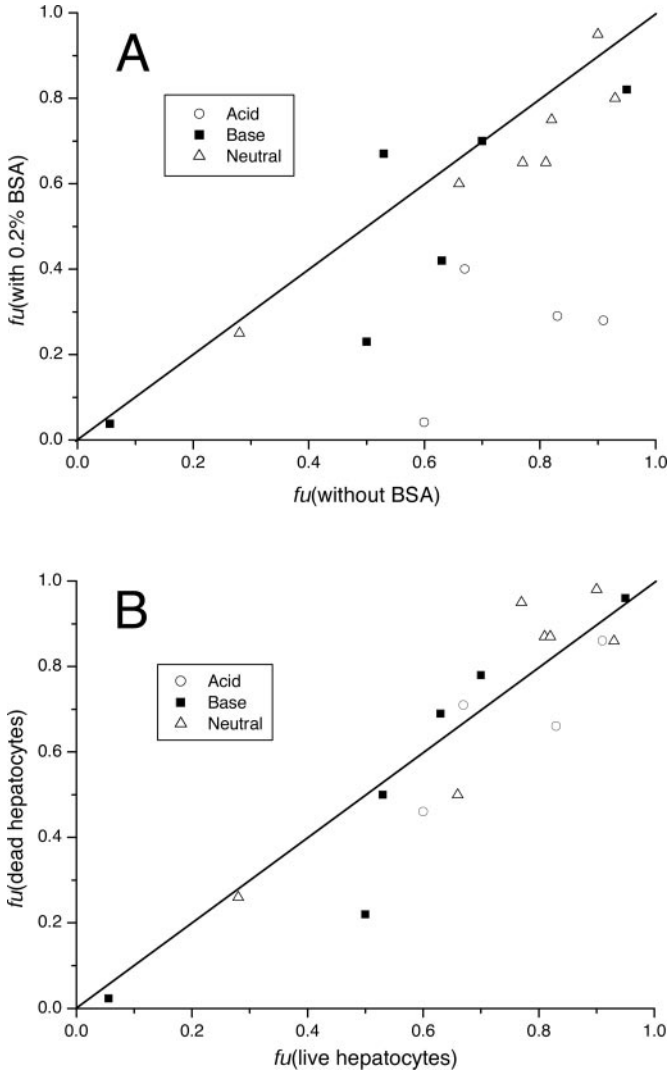

FIG. 2. Plot of *fu* from hepatocytes containing 0.2% BSA against *fu* from hepatocytes without BSA (A), and plot of *fu* from dead hepatocytes against *fu* from live hepatocytes (B).

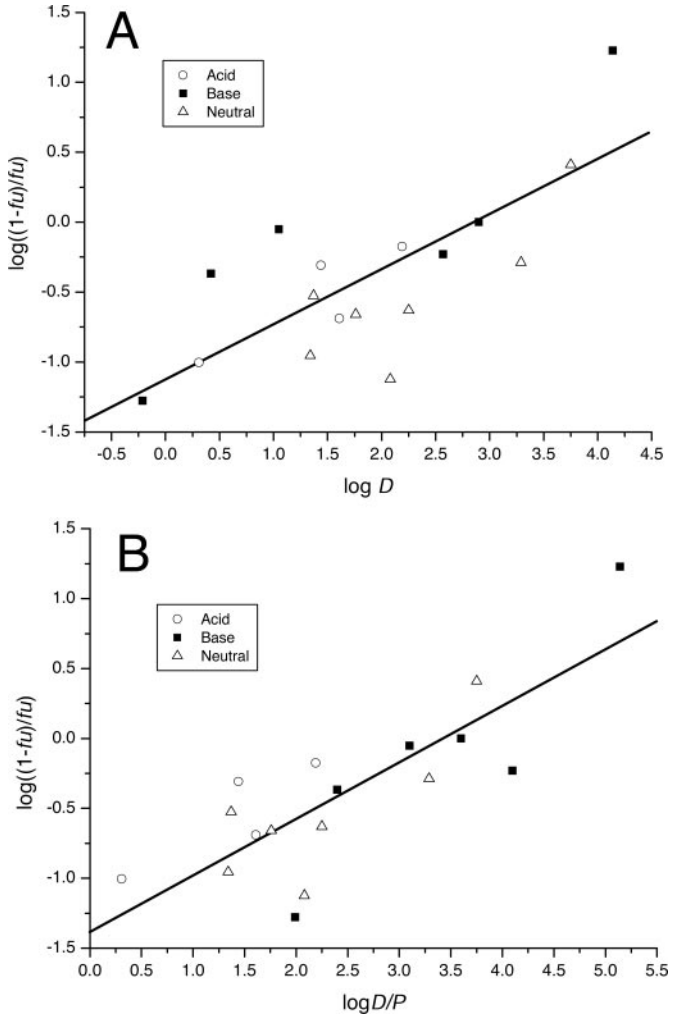

FIG. 3. Plot of $\log((1 - fu)/fu)$ in the absence of BSA against $\log D$ (A) and against $\log P/D$ (B).

Comparison of eq. 13 and eq. 14 shows that the log *D/P* does give a significant improvement in the quality of the linear regression model.

Discussion

The observed kinetics of in vitro metabolism are dependent on the extent of nonspecific binding to the incubation medium. This has frequently been demonstrated with microsomes as the incubation medium, but the same principles apply to hepatocytes, liver slices, or other incubation media (Obach, 1997, 1999; Carlile et al., 1999; Mclure et al., 2000; Venkatakrishnan et al., 2000; Austin et al., 2002). Since all of these incubation media contain hydrophobic compartments such as phospholipid bilayers in cell walls, the extent of binding is expected to increase with increasing lipophilicity of the drug. The quantitative dependence of nonspecific binding on drug lipophilicity has been demonstrated with rat liver microsomes (Austin et al., 2002), and we wished to investigate whether such a trend also occurs with rat hepatocytes. It is not uncommon for proteins such as BSA to be added to hepatocyte incubations (Ichinose and Kurihara, 1985; Lave et al., 1997; Shibata et al., 2000) in quantities that could lead to further binding of drugs in addition to hepatocyte binding. Consequently, we have carried out experiments with and without added 0.2% BSA to see how significant any additional binding is for our chosen set of drugs.

Most reports of microsomal binding determinations have used the equilibrium dialysis method, and the complication of simultaneous metabolism of the compounds is easily removed through omission of the NADPH cofactor. This is not possible with hepatocytes, and consequently, most hepatocyte binding studies have used rapid centrifugation methods to separate drug in solution from that bound to or contained within the hepatocytes (Joppen et al., 1985; Zhong et al., 1993; Naritomi et al., 2003). The centrifugation methods are not suitable for measuring free fractions in this study, since the presence in many of the samples of added BSA will cause the resulting supernatants to contain both free drug and that bound to BSA. Equilibrium dialysis does allow the measurement of free drug, but the rather slow equilibration time can be larger than the half-life for metabolism of the compounds by the hepatocytes. This outcome will cause the observed free drug concentration in the buffer-containing half of the dialysis cell to be different from the actual free concentration in the hepatocyte-containing cell. Consequently, it is important to reduce the rate of metabolism such that, ideally, it is slow compared with the dialysis process. The correction to the observed free fraction given by eq. 14 should then only be quite small, leading to a more reliable estimate of the true free fraction.

Reduction of the rates of metabolism was achieved using 1-amino-

benzotriazole as a cytochrome P450 suicide inhibitor (Huijzer et al., 1989) and salicylamide as a competitive inhibitor of uridine diphosphate glucuronyl transferase (Koike et al., 1981). These inhibitors are known not to be toxic to rat hepatocytes at the concentrations used in this study (Shiba and Shimanato, 1999; Nakagawa and Tayama, 2000). Incubations of the 17 drugs with hepatocytes, in the presence of 0.2% BSA, showed that 2-ethoxybenzamide, clozapine, propranolol, and verapamil were all fairly rapidly metabolized, with half-lives of 30 min or less (Table 1). The other drugs were less rapidly metabolized, but for many of them, the rate of metabolism was still too rapid for the purpose of binding experiments using equilibrium dialysis followed by correction using eq. 14. Further incubations with the metabolic inhibitors added led to a half-life of greater than 2 h for all 17 drugs, both with and without added BSA (Table 1). The inhibitors had no effect on the metabolism of metyrapone, which has a CL_{int} of about 5 μl/min/10^6 cells under all three of the conditions. The lack of effect of the inhibitors on metyrapone is likely to be due to the fact that this compound contains a ketone group, which is metabolized by a reductase rather than by cytochrome P450 or uridine diphosphate glucuronyl transferase (Imamura et al., 1997). However, despite the absence of inhibition, the half-life for metabolism of metyrapone of 2 h is sufficiently long for use in the later dialysis experiments. The lack of effect of the inhibitors on the rate of metyrapone metabolism further confirms that the inhibitors do not significantly affect the hepatocyte viability. No turnover of glyburide was detected in BSA medium either in the presence or absence of the inhibitors. However, when the BSA was absent but inhibitors present, a small amount of turnover was detected, leading to a CL_{int} of about 3.2 μl/min/10^6 cells. This result is consistent with a large amount of binding to BSA, giving a larger inhibiting effect on the observed rate of metabolism than the actual metabolic inhibitors, and this is confirmed by the later binding data showing that glyburide has $fu = 0.041$ in the presence of BSA and $fu = 0.60$ in the absence of BSA (Table 3). The hepatocyte binding data in Table 3 show that the corrections to the observed free fractions using eq. 14 are small in most cases. The largest correction is for astemizole, because it has the lowest permeability coefficient of all the drugs. If metabolic inhibitors had not been used, then the corrections would have been much larger, leading to less reliable estimates of the extent of hepatocyte binding. A potential drawback of the use of inhibitors is the possibility of disruption of the binding of the drugs to hepatocytes through displacement by the inhibitors. However, our previous studies of microsomal binding and analysis of literature microsomal binding data show that microsomal binding can be described by a simple nonsaturable mechanism that behaves as a phase equilibrium (lipid phase and aqueous phase), and that specific and saturable binding sites do not lead to a significant component of the observed binding (Austin et al., 2002). If the mechanism of hepatocyte binding for this set of drugs is similar to that for microsomal binding, and is dominated by nonspecific partitioning into the hydrophobic compartment of the hepatocytes (principally the cell wall), then significant displacement of the drugs by the inhibitors is unlikely. The correlation between hepatocyte binding and microsomal binding for these drugs (Fig. 4) is consistent with a similar mechanism of binding.

For many of the drugs, $fu_{corrected}$ is lower in the presence of 0.2% BSA. This is particularly marked for bumetanide (*fu* without BSA = 0.91, *fu* with BSA = 0.28), glyburide (*fu* without BSA = 0.60, *fu* with BSA = 0.041), and oxaprozin (*fu* without BSA = 0.83, *fu* with BSA = 0.29). These three drugs are all acidic (Table 2), and their high affinity for albumin is typical for this class of compound. These results clearly show that adding albumin to hepatocyte incubations will shift observed values of CL_{int} downwards and away from $CL_{int,U}$.

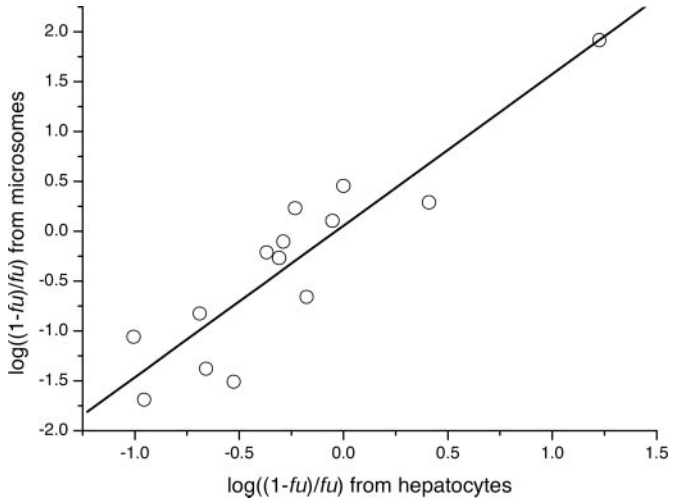

FIG. 4. Plot of microsome binding from Austin et al. (2002) against hepatocyte binding.

The shift in 0.2% BSA can be greater than 10-fold, and would be greater still in the serum incubation method (Shibata et al., 2000), where the concentration of albumin is about 4%. The challenge in such situations would then be to accurately quantify the resultant lower $CL_{int.}$ It is possible for displacement of drug from BSA by the inhibitors to occur in the experiments using added BSA. However, the main purpose of the BSA-containing experiments was to demonstrate that the added protein can significantly decrease *fu* for some drugs, and this is very clearly the case for bumetanide, glyburide, and oxaprozin, even in the presence of potential displacement.

The extent of binding of the drugs to live hepatocytes (with inhibitors) is highly correlated with the extent of binding to dead hepatocytes (without inhibitors), as shown in Fig. 2B. A two-tailed paired *t* test indicates that, taking the whole set of compounds into consideration, the extent of binding in live and dead hepatocytes is not significantly different at the 95% confidence level ($p = 0.42$). However, two of the individual drugs, astemizole and clozapine, have free fractions that are more than 2-fold smaller in dead hepatocytes than in live hepatocytes. Conversion of the standard deviations in Table 3 to 95% confidence intervals shows that this difference is not significant for astemizole but is significant for clozapine. The observed higher free fraction of clozapine in live hepatocytes could be an example of some displacement of the drug from binding sites by the two metabolic inhibitors, which were absent from the experiment using dead hepatocytes.

The correlation between the extent of hepatocyte binding and log *D/P* shown in Fig. 3 and described by eq. 16 indicates that reasonable estimates of hepatocyte binding may be obtainable from lipophilicity and ionization data. A good test of the predictivity of eq. 16 is the prediction of some recently published rat hepatocyte binding data acquired using a centrifugation technique (Naritomi et al., 2003). The physicochemical properties of the compounds along with the observed *fu* and predicted *fu* from eq. 16 are given in Table 4. For all five of these compounds, the predicted *fu* lies within a factor of 2 of the observed data, indicating a useful level of predictivity from eq. 16.

Fourteen of the compounds from this hepatocyte binding study were also the subject of previous microsomal binding determination (Austin et al., 2002). Figure 4 shows a plot of microsomal binding against hepatocyte binding (in the absence of BSA) and clearly demonstrates that these two types of binding are highly correlated. The equation of the line of best fit in Fig. 4 is given by eq. 17:

TABLE 4

Prediction of rat hepatocyte binding data from Naritomi et al. (2003) using eq. 14

Compound	Charge Type	Log *P*	Log $D_{7.4}$	$fu_{observed}$	$fu_{predicted}$ [a]
				cells/ml	
Diltiazem	Base	2.80[b]		0.53 (0.5×10^6)	0.78
Quinotolast	Acid		0.67	0.94 (0.5×10^6)	0.96
Troglitazone	Acid		4.60	0.22 (0.5×10^6)	0.41
Zidovudine	Neutral	0.05[b]		1.00 (2×10^6)	0.92
Acetaminophen	Neutral	0.51[b]		0.93 (1×10^6)	0.94

[a] Where the cell density from Naritomi et al. (2003) differs from 1×10^6 cells/ml, eq. 14 was used to predict *fu* at 1×10^6 cells/ml, followed by a correction to the appropriate cell density using eq. 9 from Austin et al. (2002).

[b] Data from Medchem Database (Daylight Chemical Information Systems, Inc., Mission Viejo, CA).

$$\log((1 - fu)/fu)_{\text{microsomes}} = 1.52 \log((1 - fu)/fu)_{\text{hepatocytes}} + 0.06$$

$$n = 14,\ r^2 = 0.83,\ \text{S.D.} = 0.41,\ F = 57.35,\ p = 6.6 \times 10^{-6} \qquad (17)$$

In addition to being highly correlated, the extents of binding to microsomes at a concentration of 1 mg/ml microsomal protein and to hepatocytes at a concentration of 1×10^6 cells/ml are of the same order of magnitude. However, at significantly different microsomal or hepatocyte concentrations, this will clearly not be the case. Since the microsomal binding and hepatocyte binding are highly correlated, they both have a similar dependence on lipophilicity, and this is manifested in the similarity of eq. 16 and the corresponding equation for microsomal binding ($\log((1 - fu)/fu) = 0.53 \log D/P - 1.42$) (Austin et al., 2002).

The experimental method and kinetic model used in this work allow determination of the free fraction of drug in a hepatocyte suspension (free concentration in solution bathing the hepatocytes/total concentration in hepatocyte suspension), but give no direct information (and require no information) about whether the observed "binding" is predominantly to the cell wall or is due to intracellular accumulation resulting from active uptake, or to a combination of both mechanisms. However, the similarity of binding to live and dead hepatocytes and the correlations of hepatocyte binding with microsomal binding and with lipophilicity indicate that active uptake into hepatocytes is likely to be negligible for this set of compounds, and that the mechanism of binding is predominantly nonspecific binding to the hydrophobic compartments of the hepatocytes such as the cell wall. We are currently investigating the use of the microsome and hepatocyte binding information for improving the quality of in vitro-in vivo scaling.

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