The Use of *in Vitro* Metabolic Stability for Rapid Selection of Compounds in Early Discovery Based on Their Expected Hepatic Extraction Ratios

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Purpose. The *in vivo* hepatic extraction ratio of cynomolgus monkeys was correlated with the corresponding *in vitro* extraction ratios that were determined in monkey microsomal incubations.

Method. For compounds that are eliminated mainly through liver phase I metabolism, the extraction ratio calculated from liver microsomal stability studies should correlate with their *in vivo* hepatic extraction ratios and also with their oral bioavailability in monkey. We used both well-stirred and parallel tube models of intrinsic clearance for the correlation. We also calculated extraction ratios for compounds within a given therapeutic area from fraction absorbed values that were estimated from the Caco-2 absorption model.

Result. The present data show that *in vitro* extraction ratios in monkey microsomes are predictive of the *in vivo* hepatic extraction ratios in monkeys. All compounds with high extraction ratio (>70%) *in vivo* were successfully classified as high-extraction-ratio compounds based on the *in vitro* monkey microsomal stability data. From the results of this study, it appears that the parallel tube model provided a slightly better classification than the well-stirred model.

Conclusions. The present method appears to be a valuable tool to rapidly screen and prioritize compounds with respect to liver first-pass metabolism in monkeys at an early phase of drug discovery.

KEY WORDS: *in vitro-in vivo* correlation; hepatic extraction ratio; monkey; liver microsomes; rapid screening; Caco-2.

INTRODUCTION

Traditionally in drug discovery, scientists rely heavily on animal pharmacokinetic data in selecting the lead candidates. This involves various in-life studies that are expensive and time consuming. These studies also require the maintenance of large colonies of the different animal species used. As a result of high-throughput combinatorial chemistry and parallel synthesis methods, an increasingly larger number of potential drug candidates require drug metabolism screening. Accordingly, there is a need to rapidly evaluate large numbers of compounds to eliminate highly metabolically unstable compounds and to prioritize the selected compounds for further pharmacokinetic investigation in animal models.

Recently, several laboratories (1–10) have reported alternate approaches such as the use of *in vitro* metabolism studies to predict *in vivo* hepatic clearance and ultimately to predict human pharmacokinetics. For example, Lavé *et al.* (7) demonstrated a correlation between intrinsic clearance in human hepatocytes and hepatic extraction ratio in humans and used the correlation to classify compounds as having low, intermediate, or high hepatic extraction ratios in humans. Rane *et al.* (6) had also successfully predicted hepatic extraction ratios using rat microsomes. Obach *et al.* (1) used hepatic microsomal intrinsic clearance to predict human *in vivo* clearance, and Houston and Carlile (8) compared the use of microsomes, hepatocytes, and liver slices for prediction of hepatic clearance in rats. They showed that both microsomes and hepatocytes might be useful for obtaining the rank order of clearances. Schneider *et al.* (2) used multivariate statistical models and artificial neural networks to predict the hepatic drug clearance in humans from *in vitro* hepatocyte data. Zuegge *et al.* (9) compared different mathematical models—allometric scaling, physiologically based direct scaling, empirical *in vitro–in vivo* correlation, and supervised artificial neural networks—for the prediction of hepatic metabolic clearance. Naritomi *et al.* (10) used *in vivo* animal (rat and dog) experiments and *in vitro* microsomal data (rat, dog, and human) to predict human hepatic clearance. Lavé *et al.* (11) also integrated *in vitro* data with allometric scaling to predict human clearance. These approaches can give us an idea about the pharmacokinetic behavior in both humans and animals early on, so there is less likelihood for failure when the compound gets into development.

In this study, we have assumed that compounds were eliminated mainly through phase I metabolism in liver; thus, the extraction ratios obtained from *in vitro* monkey liver microsomal stability studies should correlate with the respective *in vivo* hepatic extraction ratios and oral bioavailabilities in monkeys. The extraction ratios predicted from *in vitro* monkey microsomal incubation using both well-stirred and parallel tube models of intrinsic clearance were used for the correlation with *in vivo* data. The Caco-2 model was used to estimate fraction absorbed when *in vivo* absorption data were not available. The present method seems to be valuable in rapidly screening compounds with respect to liver first-pass metabolism in monkeys at an early phase of drug discovery and to prioritize compounds for further pharmacokinetic investigation in the monkey model.

MATERIALS AND METHODS

Materials

The 29 compounds selected were early-discovery compounds from the Schering-Plough Research Institute (Kenilworth, NJ). The molecular weights of the compounds ranged from 350 to 1,023 (structures of these investigational compounds cannot be disclosed at this time). HPLC-grade acetonitrile, methanol, and reagent grade DMSO, phosphoric acid, acetic acid, and potassium phosphate monobasic were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium acetate, NADPH, glucose-6-phosphate dehydrogenase, $MgCl_2$, and glucose-6-phosphate were from Sigma (St. Louis, MO). The water used for preparing aqueous solutions was obtained from an EASYpureUV compact ultrapure water system (Barnstead, Dubuque, IA).

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In Vivo Pharmacokinetic Data

In vivo pharmacokinetic and metabolic data in cynomolgus monkeys were generated in house following both intravenous (IV) and oral (PO) administration at a dose range of 1 to 10 mg/kg. Bioavailability (F) was calculated from the ratio of AUC_{po}/AUC_{iv}. Fraction absorbed (f_a) was determined with radiolabeled studies from the ratio of $AUC_{po,\ total\ radioactivity}/AUC_{iv,\ total\ radioactivity}$. The AUC values used in this calculation were dose normalized whenever oral and IV doses were different.

In Vitro Microsomal Incubation

Monkey liver microsomes were obtained from In Vitro Technologies. Triplicate monkey liver microsomal incubations were carried out at a final test concentration of 0.8 to 1 μM ($<< K_m$) and a CYP450 concentration of 0.05 to 1.75 μM (adjusted according to the observed metabolic rate of the compounds) in a 0.1 M KH_2PO_4 buffer at pH 7.4 at 37°C. A cofactor solution (1 ml) that contained 0.1 M phosphate buffer (pH 7.4), 1.2 mM NADPH, 1 U glucose-6-phosphate dehydrogenase, 9.2 mM $MgCl_2$, Glucose-6-phosphate (5 mM) was added to each tube following 3 min of preincubation. At 0, 5, 10, 20, 30, 40, 60, and 120 min, a 200-μl aliquot was removed and added to a glass culture tube containing 200 μl of acetonitrile. The tubes were centrifuged at 4,000 rpm for 20 min, and the supernatant was transferred into a 96-well plate. The plate was stored at −20°C until analyzed. An in-house compound, SCH 226395, was used as a positive control in all experiments.

HPLC/APCI/MS

The HPLC system consisted of a Perkin-Elmer 200 series pump and injector. The column used was Waters Symmetry C18 3.9 × 50 mm. The mobile phase was a gradient with (A) 80:20 water/methanol with 0.01 M ammonium acetate and (B) 100% methanol with 0.01 M ammonium acetate. The flow rate was 0.8 ml/min. A linear gradient of 40% to 90% B over 1 min, followed by 90% B for 4 min and back to 40% B over 1 min was used. The samples were analyzed in the positive-ion mode using the heated nebulizer interface of a PE/Sciex 150 (Perkin-Elmer, Norwalk, CT) single quadrupole mass spectrometer with nebulizer probe temperature at 475°C. To achieve optimum signal-to-noise ratio, the parent ions were selectively monitored.

Caco-2 Transport Studies

The Caco-2 monolayers were maintained as described earlier by Krishna *et al.* (12). The transephithelial electrical resistance (TEER) of Caco-2 monolayers was measured by using an EVOM epithelial voltohm meter (World Precision Instruments, Sarasota, FL). Caco-2 monolayers exhibiting TEER values greater than 160 $\Omega.cm^2$ were used within 30 days postseeding. The monolayers were preincubated at 37°C for 30 min in a CO_2 incubator with prewarmed transport media (TM). The TM consisted of Hanks Balanced Salt Solution with calcium and magnesium (HBSS, Biowhittaker), 10 mM HEPES buffer (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid, Biowhittaker), 25 mM D-glucose, and the pH was adjusted to 7.4. At the end of preincubation period, the TM was completely removed, and the appropriate labeled and/or unlabeled compound solution (10 μM) in the TM was added to the apical side. The compounds were solubilized in DMSO and reconstituted with TM to achieve a final concentration of 5% DMSO. The basolateral TM consisted of 4% bovine serum albumin (BSA, Sigma). The concentration of DMSO used in this study was previously shown not to affect Caco-2 monolayer integrity in the presence of 4% BSA in the basolateral side (12). The Caco-2 monolayer integrity was assessed by inverted light microscopy, measurement of TEER at the beginning and end of the experiment, and the use of radioactive mannitol, a poorly permeable compound. A completely absorbed compound in humans, propranolol, was used as a high-permeability reference (13). The data from Caco-2 monolayers with TEER values exhibiting a drop of no more than 20% of the initial value were used to calculate the apparent permeability value (P_{app}). The samples were taken from both apical and basolateral sides. The basolateral sample volume removed was replenished with fresh TM. The samples were analyzed by LC/MS/MS and/or scintillation counting using a Packard Tricarb 2250 CA scintillation counter. The apparent permeability was calculated as $P_{app} = (dM/dt)/(A \cdot C)$, where P_{app} is apparent permeability (in cm/s), dM/dt is the cumulative amount per unit time (μmol/s) in the basolateral side, A is the surface area (4.71 cm^2), and C is the initial concentration (μmol/ml) on the apical side. The transport index (TI) was calculated by dividing the permeability of the compound with that of propranolol, a highly permeable compound that is completely absorbed in humans (13).

Calculation of *in Vitro* Intrinsic Clearance

The *in vitro* measurements of the rate of clearance were based on substrate disappearance over the 2-h incubation time. The results were converted to percentage drug remaining, using the t = 0 values as 100%. The slope of the linear regression from log percentage remaining vs. incubation time relationships (−k) was used in the conversion to *in vitro* $t_{½}$ values from the equation $t_{½} = -0.693/k$. The intrinsic clearance was calculated using the integrated Michaelis-Menten equation:

$$CL'_{\text{int}} \cdot f_{ui} = \frac{0.693}{t_{1/2}} \cdot \text{liver weight (g/kg)} \cdot \text{P450 (nmol/g liver)} \cdot \frac{1}{\text{P450 (}\mu\text{M)}} \quad (1)$$

where f_{ui} is the unbound fraction in the microsomal incubation (assumed to be 1.0). A value of 18 nmol of P450/g liver and 32.5 g of liver weight per kilogram body weight were used.

Prediction of Hepatic Clearance from Intrinsic Clearance

The hepatic clearance was predicted from intrinsic clearance using the equations for both the well-stirred and parallel-tube models.

Well-stirred model:

$$CL_{hep} = \frac{Q \cdot f_{ub} \cdot CL'_{\text{int}}}{Q + f_{ub} \cdot CL'_{\text{int}}} \quad (2)$$

Parallel-tube model:

$$CL_{hep} = Q(1 - e^{-\frac{f_{ub} \cdot CL_{int}}{Q}}) \quad (3)$$

where f_{ub} is the fraction unbound in blood, which was assumed to be 1, and Q is the liver blood flow (44 ml/min/kg for monkey).

Prediction of Extraction Ratio from *in Vitro* Metabolism Data

$$ER = \frac{CL_{hep}}{Q} \quad (4)$$

Calculation of Extraction Ratio from *in Vivo* Pharmacokinetic and Caco-2 Transport Data

The *in vivo* extraction ratio is calculated from the following equation:

$$E_h = \frac{f_a - F}{f_a} \quad (5)$$

where f_a is the absorption and F is bioavailability.

For compounds for which *in vivo* absorption data were not available, the fraction absorbed, f_a, was estimated from the %TI in the Caco-2 absorption model. For selected compounds, the f_a estimates from both *in vivo* and *in vitro* Caco-2 absorption models were available and therefore were used. The %TI of compounds was calculated as %TI = 100 × ($P_{app,compound}/P_{app,propranolol}$). The f_a were estimated to be low (mean 15% from the %TI range of 0–30), medium (mean 50% from the %TI range of 30–70), and high (mean 85% from the %TI range of 70–100). The f_a was assumed to be 85% for compounds with permeability values that were higher than that of propranolol because propranolol is completely absorbed.

RESULTS

The fraction absorbed and bioavailability values for 20 compounds from nine therapeutic areas were determined from ^{3}H-labeled monkey IV and PO studies. Therefore, *in vivo* extraction ratios were calculated from Eq. (5). The *in vitro* measurement of the rate of metabolism was based on substrate disappearance. The CL_{int} was calculated using equation (1), and the CL_{hep} then calculated from Eqs. (2) and (3).

The relationships between the *in vivo* and *in vitro* extraction ratios in monkey according to the well-stirred and parallel-tube models are summarized in Fig. 1, and the corresponding data are reported in Table I. A reasonable correlation was found between the observed and the predicted extraction ratios. The R^2 values based on the linear regressions for the well-stirred and parallel-tube model were 0.72 and 0.79, respectively. The compounds were then classified into low- ($E_h < 0.3$), intermediate- ($0.3 < E_h < 0.7$) and high ($E_h > 0.7$)-extraction ratios categories. Based on these criteria, the present data show that *in vitro* intrinsic clearance values from monkey microsomes are predictive of the *in vivo* hepatic extraction ratios in monkeys. All 10 compounds with high extraction ratio (>70%) *in vivo* were successfully classified as high-extraction-ratio compounds based on the *in vitro* monkey microsomal stability data. From the results of this study, it appears that the parallel-tube model provided a slightly better classification than the well-stirred model. The parallel-tube model correctly classified all 10 compounds with high extraction ratios, whereas the well-stirred model classified 7 out of 10 compounds into the high-extraction-ratio category.

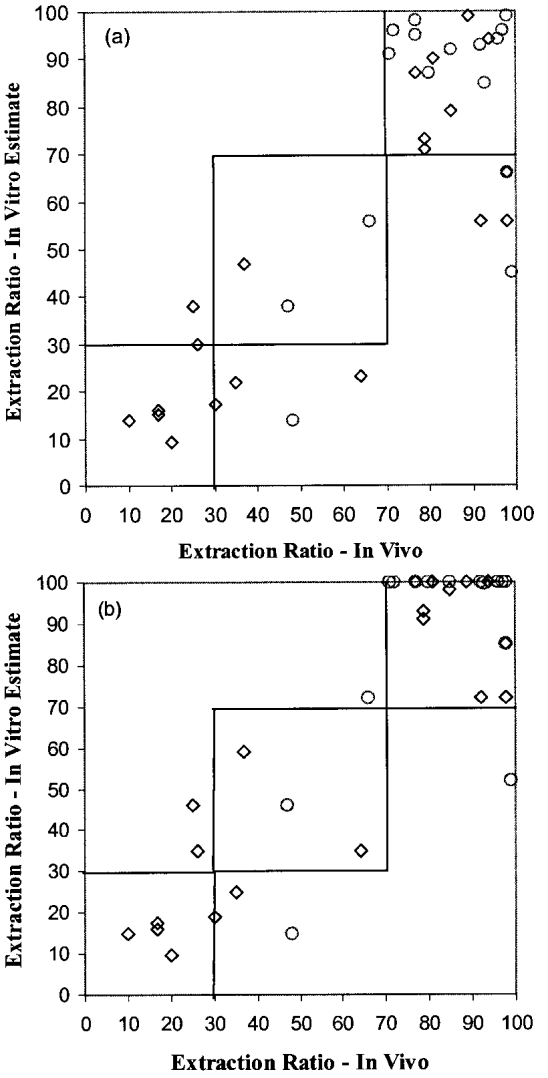

Fig. 1. Correlation of predicted (*in vitro*) and observed *in vivo* extraction ratios using **(a)** the well-stirred model and **(b)** the parallel-tube model. Symbols are as follows: f_a from *in vivo* data **(♦);** f_a from Caco-2 model **(○).**

For nine compounds from a therapeutic area (Table II), the lack of radioactive materials precluded the determination of *in vivo* fraction absorbed. For these compounds, only bioavailability data were available from monkey IV and PO studies. Therefore, *in vivo* extraction ratios for these compounds were calculated from Eq. (5) using f_a estimated from the Caco-2 model. The P_{app} for mannitol and propranolol in the Caco-2 permeability experiments ranged from 0.93 ± 0.17 to 2.36 ± 0.17 nm/s and 8.25 ± 1.56 to 12.3 ± 0.45 nm/s, respectively. The P_{app} of Schering compounds, %TI relative to propranolol permeability, and estimated f_a are shown in Table II. For another five compounds from three therapeutic areas, the absorption data based on *in vivo* as well as *in vitro* Caco-2 monolayer were available, and hence, both *in vivo* and Caco-2 estimate of f_a were used to calculate the extraction ratio. The

Table I. Summary of Predicted (*in Vitro*) and Observed *in Vivo* Extraction Ratios Where *in Vivo* Extraction Ratios Were Determined from ^{3}H-Labeled Studies in Monkeys

		Extraction ratio		
			In vitro	
Therapeutic area	Compound	*In vivo*	Well-stirred	Parallel-tube
M2	68901	0.98	0.66	0.85
M2	211334	0.92	0.56	0.72
M2	211803	0.85	0.79	0.98
M2	217443	0.79	0.71	0.91
M2	210010	0.79	0.73	0.93
Thrombin RA	205831	0.35	0.22	0.25
NK	206272	0.77	0.87	1.00
NK	209119	0.89	0.99	1.00
FPT	66336	0.25	0.38	0.46
FPT	74909	0.94	0.94	1.00
FPT	226374	0.81	0.90	1.00
D4	44643	0.26	0.30	0.35
D1	39166	0.98	0.59	0.76
PDE	351591	0.37	0.26	0.30
CCR5	350634	0.17	0.16	0.17
CCR5	351125	0.20	0.09	0.10
CCR5	351867	0.30	0.17	0.19
CCR5	352437	0.17	0.15	0.16
H3	201690	0.64	0.23	0.35
H3	79687	0.10	0.14	0.15

in vivo and *in vitro* extraction ratio relationship using both well-stirred and parallel-tube models are summarized in Table II and Fig. 1. All except three (two from well-stirred and one from parallel-tube models) of the compounds with high extraction ratio (>70%) *in vivo* were successfully classified as high-extraction-ratio compounds based on the *in vitro* monkey microsomal stability and Caco-2 absorption data.

DISCUSSION

Accurate prediction of *in vivo* metabolic clearance in monkeys using *in vitro* preparations can greatly reduce the time-consuming and expensive in-life studies and increase the efficiency of drug discovery. Such predictions in various species including humans can be used to prioritize and perhaps exclude compounds that are likely to have high hepatic extraction and low oral bioavailability in monkeys. In the present study, a simple validated *in vitro* method that allows the classification of new chemical entities according to their *in vivo* extraction ratio in monkey is reported. The use of *in vitro* microsomal stability data to predict the extraction ratio depends on the following assumptions: (a) the major clearing organ is the liver, and (b) phase I oxidative metabolism is the predominant clearance mechanism.

Table II. Summary of the *in Vitro* and *in Vivo* Extraction Ratio Based on f_a Estimated from the Caco-2 Monolayer Model

				Extraction ratio		
					In vitro	
SCH No.	P_{app} (nm/s)	%TI	Estimated f_a	*In vivo*	Well-stirred	Parallel-tube
211478	3.75 ± 0.23	32	0.5	0.93	0.85	1.0
214472	1.83 ± 0.13	22	0.15	0.72	0.96	1.0
211784	5.75 ± 0.44	47	0.5	0.98	0.99	1.0
218829	1.84 ± 0.08	21	0.15	0.99	0.42	0.52
224380	4.09 ± 0.54	40	0.5	0.97	0.96	1.0
220236	2.11 ± 0.57	24	0.15	0.77	0.95	1.0
214468	3.34 ± 0.09	32	0.5	0.85	0.92	1.0
221000	2.65 ± 1.10	24	0.15	0.71	0.91	1.0
213949	2.19 ± 0.54	22	0.15	0.92	0.93	1.0
66336	12.3 ± 0.6	144	0.85	0.47	0.38	0.46
74909	8.9 ± 0.6	104	0.85	0.96	0.94	1.00
68901	7.1 ± 1.6	83	0.85	0.98	0.66	0.85
79687	15.9 ± 0.23	140	0.85	0.48	0.14	0.15
211334	1.5 ± 0.16	15	0.15	0.66	0.56	0.72

During screening in the discovery phase, the metabolites of the compounds studied are not known; therefore, the *in vitro* measurement of the rate of metabolism was based on substrate disappearance rather than the appearance of metabolite. In this study, the *in vitro* intrinsic clearance was calculated from metabolic stability data after normalization for liver weight and cytochrome P450 content. This approach resulted in good classification of compounds according to their extraction ratio. As indicated by Houston (14) and Lavé *et al.* (7), *in vitro* intrinsic clearance is a pure measure of enzyme activity toward a compound, which should be used for the extrapolation of *in vitro* data to the *in vivo* situation as based on a scaling factor.

In this study, protein binding has not been included; however, disregarding this factor yielded excellent predictions of hepatic extraction ratio. Since in-house compounds were in early discovery phase from nine different programs, protein-binding data were determined for only a few of the compounds used in this study. The compounds used in this study were randomly chosen from nine different programs; therefore, a very wide range (25% to 99.9% bound) of protein binding was observed. Protein binding is significant only for highly protein-bound (>90%), low-clearance drugs (6,15). Because the purpose of this study is to screen for the high-clearance drugs, this factor is not important. Obach *et al.* (1) also indicated that excluding plasma protein binding resulted in superior predictions of clearance from *in vitro* microsomal data. Lavé *et al.* (7) suggested that difference in the relative binding in plasma and hepatocytes might result in discrepancies in observation for certain drugs. However, data from our own laboratory showed that compounds that were highly bound to plasma protein were also highly bound to the liver microsomes.

Naritomi *et al.* (10) demonstrated that selection of mathematical model was very important in the case of predicting oral clearances for high-clearance drugs. Previous studies by Naritomi *et al.* (10) showed that parallel-tube and dispersion models could overestimate oral clearance for high-clearance drugs because of the exponential correlation between *in vitro* intrinsic clearance and oral clearance. However, in the present study, both well-stirred and parallel-tube models appear to provide similar results, and no protein binding data were needed for successful prediction or classification by this method. The parallel-tube model successfully classified the 10 compounds with high hepatic extraction into the correct category, whereas the well-stirred model successfully classified 7 out of 10 into the correct category (high extraction ratio).

The present study demonstrates that extraction ratios predicted from *in vitro* microsomal stability studies can be used to classify compounds into low-, intermediate-, and high-hepatic-extraction ratio categories. Ten compounds that were predicted to have high extraction ratio (>70%) by the *in vitro* systems were actually found to have high *in vivo* extraction ratios in monkeys. There were false positives but no false negatives, meaning that this approach does not screen out low-hepatic-extraction compounds. Therefore, the present *in vitro* method appears to be valuable in screening and prioritizing compounds with respect to liver first-pass metabolism in monkeys at an early phase of drug discovery and decrease the demand for in-life monkey screening.

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