Evaluation of Recombinant Cytochrome P450 Enzymes as an in Vitro System for Metabolic Clearance Predictions

Rowan A. Stringer, Claire Strain-Damerell, Paul Nicklin, and J. Brian Houston

Novartis Institutes for Biomedical Research, Horsham, West Sussex, United Kingdom (R.A.S., C.S.-D., P.N.); and Centre for Applied Pharmacokinetic Research, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom (J.B.H.)

Received September 24, 2008; accepted January 29, 2009

ABSTRACT:
The aim of this study was to explore the potential of recombinant cytochrome P450 (P450) enzymes for human metabolic clearance prediction. The relative abundance and relative activity approaches were compared as methods to bridge the gap between catalytic activities in recombinant P450 enzymes and human liver microsomes (HLMs). Relative activity factors were measured by determining the intrinsic clearance (CL\text{int}) of probe substrates (butyraldehyde-CYP2D6, diclofenac-CYP2C9, midazolam-CYP3A4, and phenacetin-CYP1A2) in recombinant P450s and 16 HLM donors. Simultaneous determination of drug depletion and metabolite formation profiles has enabled a direct comparison of these methods for CL\text{int} determination. Of the 110 drugs tested, 66% were metabolized by one or more P450 enzymes; of these 44% were metabolized by CYP3A4 (0.3–21 μl/min/pmol of P450), 41% by CYP2D6 (0.6–60 μl/min/pmol of P450), 26% by CYP2C19 (0.4–8.1 μl/min/pmol of P450), 9% by CYP1A2 (0.4–2.5 μl/min/pmol of P450), and 4% by CYP2C9 (0.9–6.4 μl/min/pmol of P450). Recombinant enzymes demonstrated improved prediction reliability relative to HLMs and hepatocytes. The most reliable correlations in terms of lowest bias and highest precision were observed by comparing in vivo CL\text{int} calculated using the parallel-tube model and incorporating fraction unbound in blood, with in vitro CL\text{int} determined using relative activity factors and adjusted for nonspecific binding. Predictions were less reliable using the relative abundance approach. For these drugs, recombinant P450 enzymes offer improved assay sensitivity compared with HLMs and cryopreserved hepatocytes for CL\text{int} determination using the drug depletion method.

Standard in vitro approaches for metabolic clearance prediction using microsomes and hepatocytes have been reasonably successful (Obach, 1999; Ito and Houston, 2005; Riley et al., 2005; Brown et al., 2007). Recombinant cytochrome P450 (P450) enzymes offer an alternative in vitro system for human metabolic clearance predictions; potential advantages include the incorporation of interindividual variation in P450 expression and added value in terms of an early understanding of drug metabolism enzymology. There is a need to test the clearance prediction reliability of this in vitro system with a substantial set of reference drugs.

Systems for heterologous expression of recombinant P450 enzymes include bacterial expression in *Escherichia coli*, expression in yeast cells, mammalian expression systems, and baculovirus-driven expression in insect cells (Crespi, 1995; Gonzalez and Korzekwa, 1995; Clarke, 1998). To achieve quantitative metabolic clearance predictions with cDNA-expressed P450 enzymes it is good to compare P450 activity in the recombinant systems with that in human liver microsomes (HLMs). Two main approaches have been adopted for bridging the gap between drug catalytic rates observed in expression systems and HLMs. The first approach is based on adjustment of recombinant P450 enzyme activities according to levels of immunoquantified protein (Becquemont et al., 1998). Alternatively relative activity factors (RAFs), which describe the ratio of specific activity of a probe substrate in HLMs to the activity in recombinant P450 enzymes may be used (Crespi, 1995). In general, these methods are used to characterize human P450 enzymology (Soars et al., 2003); however, metabolic clearance predictions can be achieved by incorporating recombinant derived microsomal intrinsic clearance (CL\text{int}) values into established liver models. The use of recombinant P450 enzymes for quantitative predictions has been investigated using the RAF and relative abundance approaches; metabolic clearance predictions for five benzodiazepines were within 2-fold, and a marked underprediction was observed using immunoquantified protein levels (Galetin et al., 2004).

Different measures of enzyme activity have been considered for RAF determinations. Nakajima et al. (1999) demonstrated, using azelastine N-demethylation as a model reaction, that CL\text{int} rates were the most appropriate approach for estimating RAFs compared with either an arbitrary velocity or V\text{max}; furthermore, the RAF method was found to be preferable to that based on abundance of P450 proteins. Further studies with azelastine indicate that differences in levels of
NADPH-cytochrome P450 reductase and cytochrome b<sub>5</sub> in recombinant P450s from baculovirus-infected insect cells are not critical for quantitative predictions using RAFs (Nakajima et al., 2002). Because P450 enzymes are known to demonstrate a high degree of interindividual variability, it is recommended that RAFs be determined using a panel of individual microsome donors (Venkataraman et al., 2001). Intersystem extrapolation factors, which extend the RAF approach to incorporate P450 abundance data, have been proposed to account for differences in activity per unit amount of P450 in expression systems and liver (Proctor et al., 2004), and this approach has been successfully applied to achieve human metabolic clearance predictions in adult (Howgate et al., 2006) and pediatric populations (Johnson et al., 2006).

In this study, we have evaluated the reliability of recombinant P450 enzymes for quantitative predictions of in vivo metabolic clearance using a large set of reference compounds. RAFs were measured by determining the CL<sub>int</sub> of probe substrates (bufuralol-CYP2D6, diclofenac-CYP2C9, midazolam-CYP3A4, and phenacetin-CYP1A2) in both recombinant P450s and a panel of 16 HLM donors. Simultaneous determination of drug depletion and metabolite formation profiles has enabled a direct comparison of these methods for CL<sub>int</sub> estimation. This approach has been validated by testing 110 reference drugs for which human pharmacokinetic data were available. These reference compounds were screened across the panel of recombinant P450 enzymes, and CL<sub>int</sub> determinations were scaled to microsomal values and compared with observed values. Various scaling parameters were considered for their affect on the overall correlation including 1) relative abundance versus RAF, 2) omission or inclusion of fraction unbound in blood (f<sub>ub</sub> for in vivo CL<sub>int</sub> estimation, 3) adjustment of in vitro CL<sub>int</sub> values for nonspecific binding to microsomal protein, and 4) comparison of the well stirred (WS) and parallel-tube (PT) models for in vivo CL<sub>int</sub> estimations.

**Materials and Methods**

**Chemicals.** The reference drugs, reagents, and chemicals were of the highest grade available and were purchased from Sigma-Aldrich Ltd. (Poole, UK), Apin Chemicals Ltd. (Abingdon, UK), Sequoia Research Products Ltd. (Cambridge, UK), LGC Promochem (Teddington, UK), and Fisher Scientific UK Ltd. (Loughborough, UK). Levoprotezine was synthesized in house. Physicochemical properties were calculated for the reference compounds: molecular weight ranging between 151 and 645 and lipophilicity, estimated by calculated octanol/water partition coefficients, ranged between 0 and 6 (cLogP) and between -2 and 4 (cLogD<sub>7.4</sub>). Polar surface area ranged between 0 and 140 Å<sup>2</sup> with molecules containing between 0 and 6 H-bond donors and 0 and 10 H-bond accepters. Pooled hepatic microsome preparations from a panel of 16 donors were obtained from Xenotech LLC (Lexena, KS). Insect cell-derived recombinant P450 enzymes CYP3A4 (P450 concentration = 10 000 pmol/ml, total protein = 10.1 mg/ml), CYP2D6 (P450 concentration = 1000 pmol/ml, total protein = 8.5 mg/ml), CYP2C9 (P450 concentration = 910 pmol/ml, total protein = 5.9 mg/ml), CYP1A2 (P450 concentration = 1100 pmol/ml, total protein = 11.2 mg/ml), and CYP2C19 (P450 concentration = 1060 pmol/ml, total protein = 11.9 mg/ml) were supplied by Invitrogen (Paisley, UK). Each P450 enzyme was coexpressed with rabbit P450 reductase.

**General Incubation Conditions.** Drug stock solutions were prepared in dimethyl sulfoxide and frozen as aliquots at -80°C. Before incubations stock solutions were thawed and then diluted 83-fold in acetonitrile-water (30:70 v/v). This solution was diluted 4-fold with 100 mM potassium phosphate buffer (pH 7.4) before addition to incubation mixture. The final solvent concentration was consistent for all incubations, 0.01% dimethyl sulfoxide and 0.2% acetonitrile. Reference drugs were added to either HLMs or recombinant P450s in a total volume of 300 μl of phosphate buffer. After a 10-min preincubation period a 0-min time point was prepared; 25 μl of incubate was removed, quenched with 100 μl of ice-cold acetonitrile, and diluted with 25 μl of cofactor solution containing 100 mM potassium phosphate buffer, 1 mM EDTA, 10 mM MgCl<sub>2</sub>, and 2.6 mM NADPH. Reactions were then initiated with 275 μl of cofactor solution and mixed thoroughly. At predetermined time points, 50 μl of incubate was dispensed into 100 μl of ice-cold acetonitrile and diluted with 150 μl of water, and 30 μl of an appropriate internal standard was added. Before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis samples were centrifuged for 15 min at 2500 rpm.

**RAF Determination.** For each P450 probe substrate preliminary incubations were undertaken over a range of substrate (1.0–0.1 μM) and microsomal protein concentrations (0.2–2.0 mg/ml for HLMs) or P450 concentrations (10–100 pmol/ml for recombinant P450s) to achieve appropriate experimental conditions for CL<sub>int</sub> determination. Substrate concentrations were based on reported Κ<sub>i</sub> values taken from the literature (Marcucci et al., 2002; Walsky and Obach, 2004). Catalytic rates were measured by both depletion of parent compound and the formation rate of specific metabolites. For drug depletion experiments CL<sub>int</sub> was calculated by dividing the amount of drug added to the incubation by the total area under the concentration-time curve (AUC) calculated using the log-linear trapezoidal method. The extrapolated AUC was determined by dividing the last observed concentration time point by the elimination rate constant (k), calculated using eq. 1. Metabolite formation CL<sub>int</sub> calculation was achieved by fitting an appropriate function describing the metabolite concentration-time curve. For hydroxybufuralol, 4'-hydroxydiclofenac, and acetaminophen a monoeponential function was used (eq. 2), for 1'-hydroxy- and 4'-hydroxymidazolam a biphasic function was required describing both the formation and subsequent elimination of the metabolites (eq. 3):

\[
C = C(0) \cdot e^{-kt}
\]  

(1)

where C is the drug concentration, C(0) is the concentration determined from the equation at zero time, and k is the elimination rate constant.

\[
M = M'(1 - e^{-kt})
\]  

(2)

\[
M = M(1 - e^{-k1t} - e^{-k2t})
\]  

(3)

where M' and M are metabolite concentrations from the equation and k<sub>1</sub> and k<sub>2</sub> are rate constants for metabolite formation and elimination, respectively. CL<sub>int</sub> was calculated by multiplying the formation rate constant k<sub>e</sub> by the incubation volume and normalizing for microsomal protein concentration.

For diclofenac, midazolam, and bufuralol, microsomal CL<sub>int</sub> values were dependent on total protein concentration, and values were back-extrapolated to a theoretical zero protein concentration (eq. 4):

\[
CL_{int(i)} = \frac{CL_{int}}{1 + K \cdot P}
\]  

(4)

where K is the binding constant determined by fitting this equation to the observed data points, P is the microsomal protein concentration (in milligrams or microsomal protein per milliliter), and CL<sub>int(i)</sub> is the CL<sub>int</sub> rate determined from the equation at zero protein concentration.

For RAF determination, microsomal CL<sub>int</sub> values were determined for each probe substrate using tissue from a panel of 16 human livers; specific aspects of the incubation conditions for each probe substrate, including protein concentration, incubation time, and substrate concentration, are defined in Table 1. Microsomal CL<sub>int</sub> values for each donor were normalized for microsomal protein concentration using eq. 4. CYP2C19 RAF determination based on S-mephentoin metabolism was not possible because of the low depletion rate for this compound in our assay, and microsomal and recombinant CL<sub>int</sub> values were taken from the literature (Walsky and Obach, 2004). The RAF for each P450 enzyme i (in picomoles per milligram of microsomal protein) was calculated as the microsomal rate of the probe substrate reaction divided by the corresponding rate of metabolism in the recombinant P450 enzyme (eq. 5):

\[
RAF_i = \frac{CL_{int,HLM}}{CL_{int,recCYPi}}
\]  

(5)

A prediction of microsomal CL<sub>int</sub> contribution for each P450 enzyme (CL<sub>int,CYPi</sub>) for the reference drugs was determined (eq. 6):

\[
CL_{int,CYPi} = CL_{int,recCYPi} \cdot RAF
\]  

(6)
where CL_{int recCYP} is the CL_{int} rate for recombinant enzyme i for each test compound.

Alternatively, microsomal CL_{int} estimates were calculated from recombinant P450 enzymes using the relative abundance method. With this method, projections of microsomal CL_{int} contributions for test compounds were based on immunounquantified P450 protein levels for each enzyme. Relating abundance scaling factors were calculated using a P450 percentage contribution (to total P450 content) of 30% for CYP3A4 (Shimada et al., 1994; Bello et al., 1996; Becquemont et al., 1998), 2% for CYP2D6 (Shimada et al., 1994; Imaoka et al., 1996; Becquemont et al., 1998), 20% for CYP2C9 (Shimada et al., 1994; Bello et al., 1996), 13% for CYP1A2 (Guengerich and Turvy, 1991; Shimada et al., 1994; Inoue et al., 1997; Becquemont et al., 1998; Lasker et al., 1998; Wester et al., 2000), 1% for CYP1A2 (Guengerich and Turvy, 1991; Shimada et al., 1994; Bello et al., 1996), and 4% for CYP2C19 (Guengerich and Turvy, 1991; Shimada et al., 1994; Bello et al., 1996). These values were applied to the total P450 content values from the individual donors (data provided by supplier). Relative abundance scaling factors (in picomoles per milligram of microsomal protein) were calculated using the total HLM P450 concentration (in units of picomoles per milligram of microsomal protein) and the percent content of each P450 enzyme in HLMs (A) for enzyme i (eq. 7).

\[
\text{Relative abundance} = \frac{\text{Total HLM P450 concentration}}{100} \cdot A_i \tag{7}
\]

An estimation of microsomal CL_{int} contribution for each P450 enzyme (CL_{int,CYP}) for the reference drugs was determined (eq. 8):

\[
\text{CL}_{\text{int,CYP}} = \frac{\text{CL}_{\text{int recCYP}} \cdot \text{Relative abundance}}{} \tag{8}
\]

where CL_{int recCYP} is the CL_{int} rate for recombinant enzyme i for each test compound.

### Analysis of Reference Drugs

CL_{int} values for 110 commercially available drugs were measured with CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (protein concentration 20 pmol/ml and substrate concentration 1 μM) using a similar method to that described under general incubation conditions; with the exception that a smaller total reaction volume was used (300 μl), fewer time points (0, 5, 15, and 30 min), and ice-cold methanol was used to stop the reaction instead of acetonitrile. To minimize bioanalysis time, samples were centrifuged at 2500 rpm for 15 min before LC-MS/MS analysis. The samples were evaporated to dryness under a nitrogen stream and reconstituted in 100 μl of methanol, followed by 200 μl of water and an internal standard (doxepin, at 2 μM, for basic and neutral compounds, sulfaphenazole, at 2 μM, for acidic compounds). The samples were centrifuged at 2500 rpm for 15 min before LC-MS/MS analysis.

Total CL_{int} estimated by sum of the metabolic rates for each P450 enzyme, was scaled to in vivo CL_{int} values by incorporating average liver weight, body weight, and a microsomal recovery factor. This calculation is described in eq. 9:

\[
\text{CL}_{\text{int}} = \frac{\left(S / \text{AUC}\right) \cdot (1/P) \cdot \text{SF1} \cdot \text{LW}}{\text{BW}} \tag{9}
\]

where S is the initial substrate concentration (micromolar), AUC is the total area under the curve (micromoles per minute), P is the concentration of microsomal protein (milligrams per milliliter), SF1 is the microsomal recovery factor of 40 mg/microsomal protein/g of liver (Hakooz et al., 2006), LW is liver weight (1400 g) and BW is body weight (70 kg).

Where appropriate, in vitro CL_{int} values were adjusted for nonspecific binding to the incubation components using methods based on drug distribution coefficients in octanol and water. The fraction of unbound drug in plasma was calculated using the equation:

\[
\text{fu} = \frac{Q_p - Q_{m}}{Q_p} \cdot 100 \%
\]

where Q_{m} is the clearance, the fraction unbound in blood (fu), and hepatic blood flow (Q_{H} ml/min/kg (Boxenbaum, 1980)).

### Comparison with In Vivo Clearance Data

The scaled CL_{int} values were evaluated relative to in vivo human clearance data collated from the literature. Because of its size the full in vivo database is included as Supplemental Data. In vivo CL_{int} values were calculated from data of intravenous hepatic clearance, the fraction unbound in blood (fu), and hepatic blood flow (Q_{H}). According to either the WS or PT model (eqs. 11 and 12, respectively), where Q_{H} is 25.4 ml/min/kg (Boxenbaum, 1980):
micromere donor panel were in good agreement (2-fold) with literature data determined by enzyme kinetic analysis. In the recombinant P450 assay system, results for diclofenac and midazolam were in good agreement with literature values. For phenacetin-CYP1A2 CL_{int} estimations by drug depletion exceeded those observed for the enzyme kinetic assessment based on acetaminophen formation (2.6-fold).

For the relative abundance method the average and S.D. of the total P450 concentration across the microsomal liver samples were 435 ± 210 (range, 189–921 pmol/mg protein). Based on average immunooquantified P450 levels, relative abundance scaling factors were 130 ± 63 pmol/mg protein for CYP3A4, 9 ± 4 pmol/mg protein for CYP2D6, 84 ± 42 pmol/mg protein for CYP2C9, 57 ± 27 pmol/mg protein for CYP1A2, and 17 ± 18 pmol/mg protein for CYP2C19.

**Drug Depletion Versus Metabolite Formation.** To examine intrassay variability associated with either depletion or formation CL_{int}, coefficient of variation (percentage) for CL_{int} estimates determined on three separate days were plotted against CL_{int} (Fig. 2B). Greater assay variation was observed for CL_{int} values determined using metabolite formation compared with drug depletion with a trend for higher variation at lower CL_{int} values. Drug depletion and metabolite formation were compared as methods for CL_{int} estimation; to achieve a suitable dynamic range for correlation analysis values were not normalized for microsomal protein concentration. Regression results (Fig. 3) show good overall agreement between the two methods (r^2 = 0.98); representation of the data set as a log residual plot reveals increased deviation between the two methods at lower CL_{int} rates (< 100 μl/min).

**Quantitative Predictions of in Vivo Metabolic Clearance.** The metabolism of 72 compounds (65% of the compound set) was detectable by one or more of the P450 enzymes. Of these, the majority were metabolized by CYP3A4, CYP2D6, and CYP2C19. The range of catalytic activities for each enzyme was consistent, and the mean of the values was lowest for CYP1A2 (1.1 μl/min/pmol of P450) compared with CYP2D6, which showed the highest catalytic activity (5.7 μl/min/pmol of P450) (Table 3). The largest proportion of reference compounds were metabolized by a single P450 enzyme, most often either CYP3A4 or CYP2D6, 37% of the reference compounds were metabolized by a single P450 enzyme, less often metabolism was catalyzed by two P450 enzymes (24%), and the number of drugs metabolized by three or four enzymes was lower still (Table 4).

For 72 drugs predicted CL_{int} values were compared with observed in vivo CL_{int} values to assess the reliability of recombinant P450 enzymes for metabolic clearance prediction; the remaining 38 drugs were not included in this analysis because they were not turned over by P450s under the conditions studied. Various scaling parameters were considered for their affect on the overall correlation including 1) relative abundance versus RAFs, 2) omission or inclusion of f_{int} for in vivo CL_{int} estimation, 3) adjustment of in vitro CL_{int} values for nonspecific binding to microsomal protein using calculated parameters, and 4) comparison of the WS and PT model for in vivo CL_{int} estimations. Table 5 shows the reliability of in vitro predictions using various combinations of scaling parameters; the following section describes these results. The most reliable correlations in terms of bias (lowest afe) and precision (lowest rmse) were observed by comparing in vivo CL_{int} calculated using the PT model incorporating f_{int}, with in vitro CL_{int} determined using RAFs and adjusted for nonspecific binding (Table 5, method 1). Relative activity and abundance factors were compared for their ability to scale in vitro data (Table 5, method 1 versus method 2); values generated using the relative abundance method showed increased bias and lower precision compared with those generated using the former method. The exclusion of f_{int}
values for estimations of in vivo CL_{int} was considered (Table 5, method 1 versus method 3); when fu values were omitted from the liver model, a very poor correlation was observed. The impact of nonspecific binding (Table 5, method 1 versus method 4) was less marked; however, improved bias and precision were observed when CL_{int} values were normalized for nonspecific binding using calculated values. A comparison of liver models demonstrated that more reliable results were achieved with the PT than with the WS model (Table 5, method 1 versus method 5). In vivo-in vitro correlations for scaling methods 1 to 5 are illustrated in Fig. 4.

Clearance prediction reliability using recombinant P450 enzymes was compared with the predictive performance of human microsomes and hepatocytes determined previously (Stringer et al., 2008); these results are presented in Table 6. For this comparison a consistent scaling method was applied for each in vitro system, specifically in vivo CL_{int} was calculated using the PT model and incorporating fu, with in vitro CL_{int} adjusted for microsomal binding. For these reference compounds recombinant P450 enzymes provided the most reliable correlations in terms of bias (afe 1.58) and precision (rmse 1528). Use of recombinant P450 enzymes was the most sensitive method for CL_{int} determinations; using this method values were generated for 65% of the reference compounds compared with 52% for hepatocytes and 37% for microsomes.

Discussion

The prediction of clearance from in vitro data follows a well established stepwise scheme incorporating scaling factors to account for enzyme recovery with use of microsomes and hepatocytes and liver models to relate these data to the in vivo situation by accounting for physiological factors such as blood flow and blood binding. To achieve clearance predictions with recombinant P450 enzymes, a less frequently used in vitro system, a further scaling step is required to
In this study, we present a comprehensive evaluation of clearance prediction reliability using recombinant P450 enzymes. RAFs and the relative abundance method were evaluated for scaling catalytic activities between recombinant enzymes and HLMs, with the former method requiring determination of CLint values for P450 probe substrates in both in vitro systems.

**Determination of Relative Activity Factors.** The drug depletion approach offers advantages over full enzyme kinetic analysis for CLint determination because kinetic characterization and measurement of specific drug metabolites are not required. These advantages formed the rationale for using the drug depletion approach in this study; the availability of literature \( V_{\text{max}}/K_m \) ratios in both recombinant P450s and microsomes for midazolam, diclofenac, and phenacetin enabled comparison of these methods for CLint determination. For both recombinant enzymes and HLMs CLint (from both depletion and formation methods) and \( V_{\text{max}}/K_m \) ratios were in good agreement, providing confidence for the application of the drug depletion method for this study. Assay sensitivity is clearly an issue for the drug depletion approach; for instance, CYP2C19 RAF determination using S-mephenytoin was not possible because of the low depletion rate of this substrate in recombinant CYP2C19 and HLMs. On the basis that literature \( V_{\text{max}}/K_m \) ratios and depletion CLint values were in good agreement for midazolam, diclofenac, and bufuralol, the RAF for CYP2C19 was based on \( V_{\text{max}}/K_m \) ratios taken from the literature. The issue of assay sensitivity is further exemplified by

---

**TABLE 2**

Relative activity factor estimates for major human cytochrome P450 enzymes in human liver microsomes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzyme</th>
<th>Current Study</th>
<th></th>
<th></th>
<th>Literature</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin</td>
<td>CYP1A2</td>
<td>27 ± 20</td>
<td>4.9 ± 0.3</td>
<td>5.6 ± 4.1</td>
<td>15</td>
<td>1.9</td>
<td>8</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>CYP2C9</td>
<td>487 ± 262</td>
<td>24 ± 1</td>
<td>20 ± 11</td>
<td>418</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Midazolam</td>
<td>CYP3A4</td>
<td>1073 ± 860</td>
<td>23 ± 3</td>
<td>47 ± 37</td>
<td>537</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Bufuralol</td>
<td>CYP2D6</td>
<td>93 ± 58</td>
<td>27 ± 6</td>
<td>3.4 ± 2.1</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Mephenytoin</td>
<td>CYP2C19</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>1.0</td>
<td>0.5</td>
<td>2</td>
</tr>
</tbody>
</table>

N.A., not available; N.T., not tested.

---

**FIG. 2.** A, variation in P450 enzyme activity using drug depletion profiles of P450 probe substrates in human hepatic microsomes from 16 individual livers. Donors are ordered for increasing CYP3A4 activity to illustrate independent activity of each isoform. Data points are the mean of triplicate determinations. B, interassay variation determined by the coefficient of variation (CV) for triplicate determinations by either drug depletion (●) or metabolite formation (○).

**FIG. 3.** A, correlation of cytochrome P450 enzyme activity measured by either drug depletion or metabolite formation in hepatic microsomes from 16 human donors. B, log residual plot of depletion/formation intrinsic clearance ratio against depletion intrinsic clearance. Dotted lines at −0.3 and 0.3 indicate data points that are within 2-fold.
the apparent low activity of CYP2C9 in this study. For reference compound selection, care was taken to include a significant proportion of acidic compounds (19% of total drugs tested), many of which are known CYP2C9 substrates. Despite this, recombinant \( CL_{int} \) values were only determined for a small proportion of these compounds. Therefore, the apparent lack of CYP2C9 activity in this study more likely reflects the challenge of determining in vitro \( CL_{int} \) values for low clearance acids, and, in addition, many of these acidic compounds are known UDP-glucuronosyltransferase substrates.

It is relevant to consider the relative sensitivity of recombinant P450s compared with other in vitro systems for drug depletion studies. For example, the elimination half-life of bufuralol in HLMs ranged between 40 and 75 min, depending on microsomal protein concentration. In comparison, elimination rates were faster for recombinant CYP2D6 (\( t_{1/2} \approx \) 5 min at 10 pmol/mg protein). This difference in metabolic rate would be expected because HLMs contain only a small proportion (\( \approx 2\% \)) of CYP2D6.

If we consider the entire set of reference compounds and compare our results to previous studies with HLMs and cryopreserved hepatocytes, it is apparent that \( CL_{int} \) rates were quantified for 66% of the reference drugs using recombinant P450s (0.2 mg/ml total protein and 30-min incubation time), 38% using HLMs (0.5 mg/ml total protein and 60-min incubation time), and 52% using cryopreserved human hepatocytes (1 million cells/ml and 2-h incubation time). This result demonstrates the enhanced resolving capacity of the recombinant test system, enabling quantification of \( CL_{int} \) estimates over a wider range without the need to resort to high protein/cell concentrations and the associated risks of extensive nonspecific binding and poor protein stability.

Probe substrate selectivity could potentially be compromised using the drug depletion approach as the probability of multiple enzymes contributing to the overall metabolism of a drug is increased compared with the formation of a specific metabolite. Simultaneous measurement of drug depletion and associated metabolite formation concentration-time profiles has enabled a direct comparison of these methods for \( CL_{int} \) estimation. Regression analysis of metabolite formation and drug depletion \( CL_{int} \) estimates indicate that data from these two methods are highly correlated with a tendency for greater deviation at lower \( CL_{int} \) values. Because \( CL_{int} \) values were not normalized to microsomal protein concentration, values are directly proportional to rate constants for either drug depletion or metabolite formation. Deviation between drug depletion and metabolite formation rates could be attributed to involvement of additional pathways. We have observed good agreement between formation and depletion \( CL_{int} \) values for fast reactions (\( k > 0.1 \) min\(^{-1}\)), supporting probe substrate selectivity in the depletion assay format. However, for slower reactions (\( k < 0.1 \) min\(^{-1}\)) the higher interassay variation is most likely to be attributed to inaccuracies in fitting single-exponential or biexponential functions to the metabolism formation profiles. For the drug depletion approach, evidence of probe substrate selectivity, consistency with \( V_{max}/K_m \) ratios, and low intra-assay variability provided confidence to apply this method for both RAF determination and method validation.

The production of metabolites that modulate enzyme activity is clearly a drawback of the drug depletion approach; phenacetin metabolism in recombinant CYP1A2 provides a clear example of depletion of substrate that leads to the nonlinearity of the plot after more than 90% of the substrate is lost (Fig. 1). In this case rapid time point sampling provided a suitable method to measure the initial first-order elimination rate. In this example the time-dependent loss of CYP1A2 activity is most likely to be attributed to the P450-mediated formation of the N-acetyl-p-benzoquinonimine moiety via acetylamino (James et al., 2003). Inclusion of 5 mM glutathione in the assay incubate failed to reverse the time-dependent loss of CYP1A2 activity (data not shown). This example demonstrates the importance of sampling adequate time points to accurately define concentration-time profiles, ensuring detection of nonlinear depletion profiles. Oxidation of midazolam has also been associated with time- and concentration-dependent inactivation of recombinant CYP3A4 (Khan et al., 2002). In this study, depletion profiles in both HLMs and recombinant enzyme were log-linear; lack of inactivation may be attributed to the lower substrate concentrations used in our test system.

### TABLE 3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Drugs Metabolized</th>
<th>( CL_{int} )</th>
<th>Mean ± S.D.</th>
<th>Range (-Fold Difference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>41</td>
<td>4.5 ± 5.6</td>
<td>0.3–21 (70)</td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>37</td>
<td>5.7 ± 9.9</td>
<td>0.6–60 (100)</td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>21</td>
<td>1.6 ± 1.7</td>
<td>0.4–8.1 (21)</td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>9</td>
<td>1.1 ± 0.8</td>
<td>0.4–2.5 (6)</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>3</td>
<td>2.8 ± 3.1</td>
<td>0.9–6.4 (7)</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 4

| P450 Enzyme Contribution to the metabolism of 72 reference drugs |

<table>
<thead>
<tr>
<th>P450 Enzyme Contribution</th>
<th>No. of Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>One enzyme</td>
<td>41</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>21</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>14</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>2</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>2</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>2</td>
</tr>
<tr>
<td>Two enzymes</td>
<td>17</td>
</tr>
<tr>
<td>CYP3A4, -2D6</td>
<td>6</td>
</tr>
<tr>
<td>CYP2D6, -2C19</td>
<td>5</td>
</tr>
<tr>
<td>CYP3A4, -2C19</td>
<td>4</td>
</tr>
<tr>
<td>CYP2C9, -3A4</td>
<td>1</td>
</tr>
<tr>
<td>CYP2D6, -1A2</td>
<td>1</td>
</tr>
<tr>
<td>Three enzymes</td>
<td>9</td>
</tr>
<tr>
<td>CYP3A4, -2D6, -2C19</td>
<td>7</td>
</tr>
<tr>
<td>CYP3A4, -2D6, -1A2</td>
<td>1</td>
</tr>
<tr>
<td>CYP2D6, -1A2, -2C19</td>
<td>1</td>
</tr>
<tr>
<td>Four enzymes</td>
<td>5</td>
</tr>
<tr>
<td>CYP3A4, -2D6, -1A2, -2C19</td>
<td>5</td>
</tr>
</tbody>
</table>

The production of metabolites that modulate enzyme activity is clearly a drawback of the drug depletion approach; phenacetin metabolism in recombinant CYP1A2 provides a clear example of depletion of substrate that leads to the nonlinearity of the plot after more than 90% of the substrate is lost (Fig. 1). In this case rapid time point sampling provided a suitable method to measure the initial first-order elimination rate. In this example the time-dependent loss of CYP1A2 activity is most likely to be attributed to the P450-mediated formation of the N-acetyl-p-benzoquinonimine moiety via acetylamino (James et al., 2003). Inclusion of 5 mM glutathione in the assay incubate failed to reverse the time-dependent loss of CYP1A2 activity (data not shown). This example demonstrates the importance of sampling adequate time points to accurately define concentration-time profiles, ensuring detection of nonlinear depletion profiles. Oxidation of midazolam has also been associated with time- and concentration-dependent inactivation of recombinant CYP3A4 (Khan et al., 2002). In this study, depletion profiles in both HLMs and recombinant enzyme were log-linear; lack of inactivation may be attributed to the lower substrate concentrations used in our test system.
Comparison of Scaling Methods. Correlations using RAFs have demonstrated more reliable predictions of in vivo CL\textsubscript{int} compared with the relative abundance method. The relatively poor correlation observed with the relative abundance method may be, at least in part, attributed to limitations in the immunological techniques used for measuring P450 concentrations in HLMs. In addition, the use of RAFs accounts for both differing quantities of enzymes between the two systems and also variation in activities between the natural and recombinant P450 enzymes. Overall, the most successful combination of scaling parameters for reliable predictions of clearance were observed by a statistical comparison of in vitro CL\textsubscript{int} scaled using the RAF method and adjusted for microsomal binding against in vivo CL\textsubscript{int} estimated using the PT liver model incorporating \(f_{\text{bc}}\). Incorporation of blood-binding parameters was essential to achieve the most reliable in vivo CL\textsubscript{int} predictions, supporting our previous observations with HLMs (Stringer et al., 2008), and statistical analysis revealed that omission of this parameter produced a very poor correlation and thus blood distribution measurements are considered essential for reliable predictions of in vivo CL\textsubscript{int}. Likewise, less reliable predictions of in vivo CL\textsubscript{int} were observed when calculated

<table>
<thead>
<tr>
<th>Scaling methods</th>
<th>Activity</th>
<th>Abundance</th>
<th>Activity</th>
<th>Activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood distribution</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver model</td>
<td>PT</td>
<td>PT</td>
<td>PT</td>
<td>PT</td>
<td>WS</td>
</tr>
<tr>
<td>Nonspecific binding</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistics
- \(r^2\): 0.42, 0.44, 0.05, 0.35, 0.37
- afe: 1.53, 2.08, 5.41, 2.08, 2.15
- rmse: 1528, 2352, 1820, 2548, 17920
- % Inside 2-fold error: 32, 34, 12, 26, 27
- % Inside 5-fold error: 73, 71, 45, 68, 66

Table 5: Statistical analysis of clearance predictions for 72 drugs using recombinant cytochrome P450 enzymes

Scaling methods 1 and 2 compare the relative activity and abundance methods for clearance predictions. Methods 1 and 3 assess the importance of blood distribution parameters, specifically the free fraction in blood for in vivo intrinsic clearance calculation. Methods 1 and 4 assess the importance of in vitro binding and methods 1 and 5 compare the PT and WS models for in vivo intrinsic clearance estimation.

Fig. 4. Correlation between observed and predicted human intrinsic clearance values for 72 reference compounds using recombinant cytochrome P450 enzymes with various scaling methods. —, line of identity; ·····, values within 2-fold; — —, values within 5-fold. Scaling methods 1 to 5 are described in Table 5.
Reliability of in vitro systems for metabolic clearance predictions

For each method, in vivo intrinsic clearance values were calculated using the parallel-tube model incorporating the free fraction in blood. In vitro intrinsic clearance values were adjusted for either microsomal or hepatocyte binding using the methods of Gertz and Hallifax.

<table>
<thead>
<tr>
<th>Recombiant Enzymes</th>
<th>Microsomes</th>
<th>Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>72</td>
<td>41</td>
</tr>
<tr>
<td>r²</td>
<td>0.43</td>
<td>0.28</td>
</tr>
<tr>
<td>afe</td>
<td>1.53</td>
<td>2.32</td>
</tr>
<tr>
<td>rmse</td>
<td>1528</td>
<td>3501</td>
</tr>
<tr>
<td>% Inside 2-fold error</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>% Inside 5-fold error</td>
<td>73</td>
<td>66</td>
</tr>
</tbody>
</table>

Data taken from the literature (Stringer et al., 2008).

Conclusions

Recombinant P450 enzymes have provided improved prediction reliability compared with HLMs and cryopreserved hepatocytes, with more accurate predictions achieved using the relative activity rather than the relative abundance method. In addition, with recombinant incubations artificially high enzyme levels and lower total protein amounts enhanced assay sensitivity for drug depletion assays. With recombinant enzymes the most reliable predictions were achieved by comparison of in vivo CLint calculated using the PT model (incorporating fmicros) with in vitro CLrat adjusted for fmicros. This study has provided realistic precision and accuracy for this diverse set of reference drugs with approximately 40% of predictions within 2-fold and 60% within 5-fold of observed values using optimized scaling methods.

Taken together, subcellular fractions, hepatocytes, and recombinant P450 enzymes each play a key quantitative or qualitative role in understanding the disposition of new drugs. Although subcellular fractions assist in the understanding of relative contributions of different enzyme families to drug metabolism (e.g., P450, UDP-glucuronosyltransferases, or sulfotransferases), hepatocytes with an intact cell membrane and complete complement of drug-metabolizing enzymes provide the most relevant information on permeability limitations or multiple enzyme interactions. For drugs elminated primarily by P450 isoforms, recombinant enzymes provide a valuable alternative in vitro system for metabolic clearance predictions, in particular for lower clearance drugs, for which it may not be possible to define metabolic rates using the aforementioned methods. For new chemical classes preliminary studies are essential to evaluate the suitability of these methods for clearance predictions; appropriate selection of in vitro methods and application of optimized scaling practices will ensure that human tissue may be used with confidence.

References


TABLE 6

<table>
<thead>
<tr>
<th>TABLE 6</th>
<th>Predicting Human Clearance Using Cytochrome P450 Enzymes</th>
</tr>
</thead>
</table>

[^Data taken from the literature (Stringer et al., 2008).]


Address correspondence to: Dr. Rowan A. Stringer, Novartis Institutes for Biomedical Research, Wimblehurst Road, Horsham, West Sussex, RH12 5AB, UK. E-mail: rowan.stringer@novartis.com