Prediction of Drug Clearance by Glucuronidation from in Vitro Data: Use of Combined Cytochrome P450 and UDP-Glucuronosyltransferase Cofactors in Alamethicin-Activated Human Liver Microsomes

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ABSTRACT:

Glucuronidation via UDP-glucuronosyltransferase (UGT) is an increasingly important clearance pathway. In this study intrinsic clearance (CLint) values for buprenorphine, carvedilol, codeine, diclofenac, gemfibrozil, ketoprofen, midazolam, naloxone, raloxifene, and zidovudine were determined in pooled human liver microsomes using the substrate depletion approach. The in vitro clearance data indicated a varying contribution of glucuronidation to the clearance of the compounds studied, ranging from 6 to 79% for midazolam and gemfibrozil, respectively. The CLint was obtained using either individual or combined cofactors for cytochrome P450 (P450) and UGT enzymes with alamethicin activation and in the presence and absence of 2% bovine serum albumin (BSA). In the presence of combined P450 and UGT cofactors, CLint ranged from 2.8 to 688 μl/min/mg for zidovudine and buprenorphine, respectively; the clearance was approximately equal to the sum of the CLint values obtained in the presence of individual cofactors. The unbound intrinsic clearance (CLint, u) was scaled to provide an in vivo predicted CLint; the data obtained in the presence of combined cofactors resulted in 5-fold underprediction on average. Addition of 2% BSA to the incubation with both P450 and UGT cofactors reduced the bias in the clearance prediction, with 8 of 10 compounds predicted within 2-fold of in vivo values with the exception of raloxifene and gemfibrozil. The current study indicates the applicability of combined cofactor conditions in the assessment of clearance for compounds with a differential contribution of P450 and UGT enzymes to their elimination. In addition, improved predictability of microsomal data is observed in the presence of BSA, in particular for UGT2B7 substrates.

Glucuronidation is an important reaction in the metabolism of drugs (Williams et al., 2004). It is catalyzed by UDP-glucuronosyltransferase (UGT), a superfAMILY of membrane-bound enzymes that catalyze the conjugation of d-glucuronic acid to various endo- and xenobiotics. Known UGT enzymes are classified into two families, UGT1 and UGT2 (Mackenzie et al., 2005). Of the hepatically expressed enzymes UGT1A1, 1A4, 1A9, 2B7, and 2B15 appear to be of greatest significance in drug elimination (Miners et al., 2004; Kiang et al., 2005). UGTs are primarily involved in conjugation of metabolites from oxidation reactions. However, if a suitable electrophilic acceptor group is present, UGTs can also conjugate drugs directly without any prior oxidation step, as seen in the case of buprenorphine (Picard et al., 2005) and diclofenac (Kumar et al., 2002).

Microsomes are commonly used as an in vitro system to predict the metabolic clearance of new chemical entities (Obach, 1999; Soars et al., 2002; Rawden et al., 2005; Rostami-Hodjegan and Tucker, 2007). They offer an advantage over other in vitro systems (e.g., human cryopreserved hepatocytes) because of ease of preparation, wide availability at a low cost, and ease of transport and storage. However, incubations require cofactors for cytochrome P450 (P450) and UGT reactions to be added. The active site of UGTs is on the luminal side of the endoplasmic reticulum, resulting in an in vitro latency. To overcome this phenomenon in vitro, detergents or pore-forming agents (e.g., alamethicin) are added to allow UGT activation (Fisher et al., 2000; Boase and Miners, 2002). Assessment of glucuronidation clearance is further complicated by a lack of glucuronide standards; therefore, substrate depletion offers an alternative approach. However, this approach for the assessment of glucuronidation clearance has been applied in only a limited number of studies (Mohutsky et al., 2006).

Prediction of clearance from microsomal in vitro data has shown a general trend toward underprediction in the case of both P450 and glucuronidated drugs (Mistry and Houston, 1987; Soars et al., 2002; Ito and Houston, 2005; Riley et al., 2005; Miners et al., 2006), with studies reporting 10- to 30-fold underprediction of clearance (Mistry and Houston, 1987; Miners et al., 2006). Investigation into these studies showed that incubation conditions greatly affect the clearance

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; P450, cytochrome P450; BSA, bovine serum albumin; UDPGA, UDP-glucuronic acid; afe, average-fold error; rmse, root mean squared prediction error.
prediction for the glucuronidated drugs (Boase and Miners, 2002; Soars et al., 2003). Although alamethicin has been shown to enhance UGT activity without having any detrimental effect on P450 enzymes (Fisher et al., 2000), the general utility of alamethicin-activated microsomes for the prediction of glucuronidation clearance is still arguable (Engtrakul et al., 2005).

Glucuronidation clearance has been investigated recently in the presence of bovine serum albumin (BSA) in the microsomal incubations (Rowland et al., 2007, 2008b). The authors reported that long-chain fatty acids (linoleic and arachidonic acid) released during microsomal incubations competitively inhibit UGT2B7 and UGT1A9 enzymes, with no effect on UGT1A1, UGT1A6, and UGT1A4 (Rowland et al., 2007, 2008b). The addition of BSA to incubations sequesters the fatty acids, resulting in a 9- to 10-fold increase in the intrinsic clearance (\(C_{\text{Lmax}}\)) of propofol (Rowland et al., 2008b) and zidovudine (Rowland et al., 2007). A comparable “albumin effect” on the CYP2C9 substrate phenytoin was also observed (Rowland et al., 2008a).

In this study, \(C_{\text{Lmax}}\) values for 10 drugs with differential contribution of P450 and UGT pathways were determined in pooled human liver microsomes using the substrate depletion approach. The data set included buprenorphine, carvedilol, codeine, diclofenac, gemfibrozil, ketoprofen, midazolam, naloxone, raloxifene, and zidovudine. The aim of the current study was to investigate the utility of alamethicin-activated human liver microsomes to estimate the fraction metabolized (\(f_{\text{CYP}}\)) of either P450 (\(f_{\text{CYP}+\text{UGT}}\)) or UGT (\(f_{\text{UGT}}\)) using individual P450 and UGT cofactors. In addition, the utility of combined cofactor conditions (P450 + UGT) for the prediction of clearance was investigated, either in the absence or presence of 2% BSA. The general implications of these findings on the suitability of alamethicin-activated microsomes for predicting the clearance for compounds with parallel P450 and UGT pathways are discussed.

Materials and Methods

Chemicals. Buprenorphine, codeine, diclofenac, gemfibrozil, ketoprofen, midazolam, naloxone, raloxifene, NADP\(^+\), UDPGA, isocitric acid, EDTA, alamethicin (from Trichoderma viride), BSA, and isocitric acid dehydrogenase were purchased from Sigma Chemical (Poole, Dorset, UK). Carvedilol and zidovudine were purchased from Sequoia Research Products (Pangbourne, West Berkshire, UK).

P450 Incubation Conditions. Pooled human liver microsomes (n = 22; BD Gentest, Woburn, MA) were diluted to a final concentration of 0.2 mg of protein/ml for codeine and zidovudine. Microsomes were diluted in 0.1 M phosphate buffer with 2% BSA and the drug investigated, were added to the donor side, and phosphate buffer was added to the acceptor side. After reaching equilibrium, aliquots were taken from both sides after 6 h and quenched in ice-cold acetonitrile containing the relevant internal standard.

LG-MS/MS. The LC-MS/MS system used consisted of a Waters 2790 with a Micromass Quattro Ultima triple quadruple mass spectrometer (Waters, Milford, MA). Samples from the microsomal incubations were centrifuged at 2500 rpm for 10 min, and an aliquot of 10 \(\mu\)l was injected into the LC-MS/MS system. Varying gradients of four mobile phases were used, the composition of which were as follows: A, 90% water and 0.05% formic acid with 10% acetonitrile; B, 10% water and 0.05% formic acid with 90% acetonitrile; C, 90% water and 10 mM ammonium acetate with 10% acetonitrile; and D, 10% water and 10 mM ammonium acetate with 90% acetonitrile. For buprenorphine, diclofenac, gemfibrozil, midazolam, naloxone, and raloxifene a Luna C18 column (Phenomenex, Torrance, CA) (3 \(\mu\)m, 50 \(\times\) 4.6 mm) was used for chromatographic separation of analytes. For carvedilol a Luna phenyl-hexyl column (Phenomenex) (5 \(\mu\)m, 30 \(\times\) 4.6 mm) was used for chromatographic separation of analytes. The flow rate was set at 1 ml/min, and this was split to 0.25 ml/min before entering the mass spectrometer. The details on the internal standards, mass transitions, and retention times have been outlined previously (Gertz et al., 2008).

Data Analysis. The \(C_{\text{Lmax}}\) value determined with both P450 and UGT cofactors present in the incubation and in the presence and absence of 2% BSA was corrected for the corresponding fraction unbound in the incubation (\(f_{\text{unb}}\)) and scaled to a whole body clearance (milligrams per minute per kilogram) using eq. 1 (Houston, 1994; Obach, 1999):

\[
C_{\text{Lmax}} = \frac{0.693}{\text{in vitro } t_{1/2}} \times \frac{\text{mg microsomes}}{\text{g liver}} \times \frac{\text{g liver}}{\text{kg body weight}}
\]

\(1\) with a mean scaling factor for a 30-year-old individual of 40 mg protein/g liver (range of 13–54 mg protein/g liver) (Barter et al., 2007) and a liver weight of 21.4 g liver/kg body weight (Ito and Houston, 2005). The observed hepatic clearance from in vivo intravenous data were converted to an in vivo \(C_{\text{Lmax}}\) value using the well-stirred and parallel tube liver models, defined in the eqs. 2 and 3, respectively (Ito and Houston, 2005):

\[
C_{\text{Lmax}} = \frac{C_{\text{Lmax}}}{f_{\text{unb}}} = \frac{f_{\text{unb}}}{R_b} \times \ln \left(1 - \frac{C_{\text{Lmax}}}{Q_{\text{H}}}ight)
\]

\(2\)

\[
C_{\text{Lmax}} = - \frac{Q_{\text{H}}}{f_{\text{unb}}/R_b} \times \ln \left(1 - \frac{C_{\text{Lmax}}}{Q_{\text{H}}}ight)
\]

\(3\)

where \(f_{\text{unb}}\) is the fraction unbound in the plasma, \(C_{\text{Lmax}}\) is hepatic blood clearance, \(R_b\) is the blood to plasma concentration ratio, and \(Q_{\text{H}}\) is the hepatic blood flow (20.7 ml/min/kg) (Brown et al., 2007; Yang et al., 2007).

For buprenorphine and zidovudine, the calculated observed \(C_{\text{Lmax}}\) values exceeded the \(Q_{\text{H}}\) therefore, due to the sensitivity of the well-stirred liver model to the \(Q_{\text{H}}\), the \(C_{\text{Lmax}}\) value was set at 90% of hepatic blood flow for these two drugs. For consistency, a 90% cutoff was applied for these compounds for both liver models used. For raloxifene, no intravenous clearance data were...
available; therefore, the observed CL_in is calculated from an oral clearance using eq. 4:

$$\text{CL}_\text{in} = \frac{\text{CL}_H}{\mu g/R_h}$$  \hspace{1cm} (4)

When the $R_h$ was not available a value of 1 was assumed for basic compounds (buprenorphine, codeine, and raloxifene) and a value of 1 − hematocrit (i.e., 0.55) was assumed for acidic compounds (e.g., gemfibrozil and ketoprofen).

The in vitro $fm_{UGT}$ and $fm_{CYP}$ were determined from the CL_in obtained in the presence of individual P450 (CL_in, CYP) and UGT (CL_in, UGT) cofactors using eqs. 5 and 6, respectively:

$$fm_{UGT} = \frac{(\text{CL}_\text{in, UGT})}{(\text{CL}_\text{in, UGT} + \text{CL}_\text{in, CYP})} \hspace{1cm} (5)$$

$$fm_{CYP} = \frac{(\text{CL}_\text{in, CYP})}{(\text{CL}_\text{in, UGT} + \text{CL}_\text{in, CYP})} \hspace{1cm} (6)$$

The predicted CL_in values (from in vitro data obtained with combined cofactors and in the presence or absence of BSA) for the current data set ($n = 10$) were compared with the observed CL_in values obtained from the literature. The bias in the predicted CL_in was assessed from the geometric mean of the ratio of the predicted and the actual value [average-fold error (afe), eq. 7]. The root mean squared prediction error (rmse; eqs. 8 and 9) provided a measure of precision for the predictions of the CL_in values (Sheiner and Beal, 1981; Obach et al., 1997):

$$\text{afe} = 10^{\frac{1}{n} \sum \log \frac{\text{predicted}}{\text{observed}}}$$  \hspace{1cm} (7)

$$\text{mse} = \frac{1}{n} \sum (\text{predicted} - \text{observed})^2$$  \hspace{1cm} (8)

$$\text{rmse} = \sqrt{\text{mse}}$$  \hspace{1cm} (9)

**Results**

Clearance via UGT and P450 enzymes was investigated for 10 selected compounds in human liver microsomes using either individual or combined cofactors for these enzymes. The depletion plots for all of the compounds showed a linear time profile, with the exception of diclofenac for which depletion was best described by a biphasic profile; in this case the initial linear phase of depletion plots was used to calculate the CL_in. Figure 1 shows the unbound CL_in obtained for three compounds, buprenorphine (CL_in, CYP > CL_in, UGT), raloxifene (CL_in, UGT > CL_in, CYP), and ketoprofen (CL_in, CYP = CL_in, UGT) as representative examples of clearance data obtained with either individual P450 or UGT cofactors or in the presence of combined cofactors (P450 + UGT). For buprenorphine, carvedilol, zidovudine, and midazolam the clearance by P450 enzymes was greater than the clearance by UGT enzymes, whereas the opposite trend was observed for the remaining six compounds. For all the drugs in the data set, the CL_in with combined cofactors present was comparable with the sum of the individual CL_in, CYP and CL_in, UGT (Tables 1 and 2).

Table 1 shows the unbound in vitro CL_in obtained in the incubations with individual cofactors and the estimated in vitro $fm_{UGT}$ and $fm_{CYP}$ values for the 10 compounds studied. The unbound $CL_H$ for codeine and buprenorphine ranged from 2.4 to 472 $\mu l/min/mg$, whereas raloxifene had the highest CL_in, UGT (444 $\mu l/min/mg$). The CL_in calculated with the individual cofactors was used to estimate an in vitro contribution of UGT and P450 enzymes, as described in eqs. 5 and 6, respectively. The in vitro clearance data indicated a varying contribution of glucuronidation to the clearance of the compounds studied, ranging from 6 to 79% for midazolam and gemfibrozil, respectively. The $fm_{CYP}$ ranged from 0.21 to 0.94 for gemfibrozil and midazolam, respectively (Table 1).

In the presence of both cofactors in the incubation, the CL_in ranged from 2.8 to 688 $\mu l/min/mg$ for zidovudine and buprenorphine, respectively (Table 2). The unbound intrinsic clearance (CL_in, u) obtained under these incubation conditions were scaled using the mean human microsomal scaling factor of 40 mg protein/g liver (Ito and Houston, 2005; Barter et al., 2007) (Fig. 2A). The observed CL_in covered a 500-fold range with codeine and raloxifene at the lower and upper end of the clearance (Table 3). With use of the well-stirred liver model, the predicted CL_in obtained from data in the presence of both cofactors gave a bias of 8.8 and poor precision (rmse of 4566). However, use of the parallel tube model reduced bias by approximately 50% with no significant effect on the precision (Fig. 2A). Independent of the model used, zidovudine and codeine were poorly predicted, with an 18-fold underprediction of clearance observed for these compounds when the parallel tube model was used. The range of scaling factors (13–54 mg protein/g liver) had a marginal effect on the prediction of clearance; however, they influence the extent of underprediction observed.

When the well-stirred liver model was used, variability in the $Q_H$ had a pronounced effect on highly cleared compounds such as buprenorphine. An exponential increase in the estimated in vivo CL_in was observed when the hepatic clearance approached hepatic blood flow (>95% of $Q_H$), resulting in a significant underprediction of clearance from in vitro data. For example, in the case of buprenorphine, a 52-fold underprediction of buprenorphine clearance was observed when the hepatic clearance was set at 99% of $Q_H$, whereas setting the hepatic clearance at 90% of $Q_H$ resulted in only 5-fold underprediction. Therefore, for drugs for which the observed clearance approached hepatic blood flow, the CL_in was limited to 90% of the $Q_H$. For consistency, an analogous approach was also applied when the parallel tube liver model was used.

The experiments with alamethicin-activated human liver microsomes using individual and combined P450 and UGT cofactors were also performed in the presence of 2% BSA. Table 2 shows the

![Fig. 1. Intrinsic clearance for buprenorphine, raloxifene, and ketoprofen obtained in human liver microsomes using either individual (P450 and UGT) or combined (P450 + UGT) cofactor incubation conditions.](image-url)
clearance values (combined cofactors) obtained in the presence and absence of BSA corrected for the extent of nonspecific binding. In the absence of BSA, the \( \text{fm}_{\text{mic}} \) ranged from 0.1 to 0.99 for buprenorphine and codeine, respectively. In the presence of BSA, the \( \text{fm}_{\text{mic}} \) ranged from 0.008 to 0.99 for diclofenac and codeine, respectively, with the largest decrease in \( \text{fm}_{\text{mic}} \) observed for diclofenac (approximately 100-fold). The increase in the individual P450 and UGT \( \text{Cl}_{\text{int, u}} \) estimates in the presence of BSA ranged from 0.9 to 12.1 for buprenorphine and gemfibrozil, respectively. On average, a 50% increase in \( \text{fm}_{\text{UGT}} \) was observed in the presence of BSA for UGT2B7 substrates (6 of 10 drugs), whereas a negligible effect was observed for drugs glucuronidated via UGT1A1, with the exception of raloxifene for which a 3.3-fold increase in \( \text{Cl}_{\text{int, u}} \) was observed. The effect was minimal on all drugs with predominant P450 pathways that are not CYP2C9-mediated. For example, a 1.5-fold increase was observed for midazolam, for which UGT1A4 and CYP3A4 are involved in the metabolism.

The impact of 2% BSA on the prediction of \( \text{Cl}_{\text{int, u}} \) was also assessed, as shown in Fig. 2B. Prediction of clearance from the in vitro data obtained in the presence of BSA reduced the bias and extent of underprediction, resulting in 8 of 10 compounds within 2-fold of in vivo values when the parallel tube liver model was used (Fig. 2B). Incorporation of BSA in the incubation resulted in significant overprediction (18-fold) of gemfibrozil clearance, in contrast to data obtained without BSA, for which predicted and observed clearances were in very good agreement (Table 2). For raloxifene, clearance was underpredicted even after the addition of BSA.

**Discussion**

In recent years an increasing number of studies have been performed to ascertain the suitability of microsomes to accurately assess the glucuronidation of drugs in vitro (Boase and Miners, 2002; Soars et al., 2002; Miners et al., 2004; Mohutsky et al., 2006). Incubations with microsomes are often carried out to investigate P450 and UGT metabolism individually; however, both pathways of metabolism are not commonly evaluated for a single compound (Engtrakul et al., 2005; Mohutsky et al., 2006). Methods for studying glucuronidation in microsomes have varied considerably, which has led to questions about the suitability of this in vitro system to accurately predict the

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**TABLE 1**

Clearance obtained for 10 drugs in the presence of individual P450 and UGT cofactors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Major P450</th>
<th>Major UGT</th>
<th>( \text{Cl}_{\text{int, u}} )</th>
<th>( \text{fm}_{\text{MIC}} )</th>
<th>( \text{fm}_{\text{MIC}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>( \mu\text{ml/min/mg} )</td>
<td>( \text{S.D.} )</td>
<td>( \text{References} )</td>
</tr>
</tbody>
</table>

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**TABLE 2**

Clearance obtained for 10 drugs in the presence and absence of BSA using combined P450 and UGT cofactors and scaled to in vivo

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \text{Cl}_{\text{int, u}} ) (P450 + UGT)</th>
<th>-Fold Difference: ( \text{Cl}<em>{\text{int, u}} ) + BSA/( \text{Cl}</em>{\text{int, u}} ) - BSA</th>
<th>( \text{fm}_{\text{MIC}} )</th>
<th>( \text{S.D.} )</th>
<th>( \text{Scaled Cl}_{\text{int, u}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu\text{ml/min/mg} )</td>
<td></td>
<td>( \text{mll/min/kg} )</td>
<td>( \text{S.D.} )</td>
<td>( \text{S.D.} )</td>
</tr>
</tbody>
</table>

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* In vitro \( \text{fm}_{\text{UGT}} \) and \( \text{fm}_{\text{CYP}} \) were calculated using eqs. 5 and 6, respectively.

* \( \text{Cl}_{\text{int, u}} \) was scaled using a human microsomal scaling factor of 40 mg protein/g liver (Ito and Houston, 2005; Barter et al., 2007) and an average liver weight of 21.4 g liver/kg.
glucuronidation clearance (Engtrakul et al., 2005). The current study assesses the use of microsomes in the prediction of clearance for compounds with parallel P450 and UGT elimination pathways and the utility of this system to obtain estimates of \( f_{\text{m,UGT}} \) and \( \text{CL}_{\text{int, UGT}} \) in vitro. The impact of the addition of 2% BSA to microsomal incubations on the clearance prediction was also investigated.

Depletion of parent compound is a common method to determine \( \text{CL}_{\text{int}} \), and it has been shown to be comparable with values obtained by metabolite formation (Boase and Miners, 2002; Soars et al., 2002), low clearance compounds were comparable with values obtained by metabolite formation (Boase and Miners, 2002; Soars et al., 2002), indicating the suitability of depletion data. To ensure that substrates have access to the active site of the UGT enzyme on the luminal side of the endoplasmic reticulum, alamethicin is commonly used as an alternative to detergents (Fisher et al., 2000; Barter et al., 2007) and a range of scaling factors on the \( x \)-axis from 17 to 25.5 ml/min/kg (Kato et al., 2003).

### Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>( f_{\text{m,UGT}} )</th>
<th>( R_u )</th>
<th>( f_{\text{bio}} )</th>
<th>Observed CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine</td>
<td>0.04</td>
<td>0.60</td>
<td>0.07</td>
<td>19</td>
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<tr>
<td>Carvedilol</td>
<td>0.02</td>
<td>1.00</td>
<td>0.02</td>
<td>7.8</td>
</tr>
<tr>
<td>Codeine</td>
<td>0.70</td>
<td>1.00</td>
<td>0.70</td>
<td>15</td>
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<tr>
<td>Diclofenac</td>
<td>0.005</td>
<td>0.55</td>
<td>0.00</td>
<td>3.5</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>0.03</td>
<td>0.55</td>
<td>0.05</td>
<td>1.7</td>
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<tr>
<td>Ketoprofen</td>
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<td>0.55</td>
<td>0.01</td>
<td>1.6</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.017</td>
<td>0.53</td>
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<td>Naloxone</td>
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<tr>
<td>Raloxifene</td>
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<td>1.00</td>
<td>0.05</td>
<td>735</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>0.8</td>
<td>0.98</td>
<td>0.82</td>
<td>25</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fold Difference: ( \text{CL}<em>{\text{int, UGT}} + \text{BSA} / \text{CL}</em>{\text{int, UGT}} - \text{BSA} )</th>
<th>( \text{In Vitro} f_{\text{m,UGT}} + \text{BSA}^a )</th>
<th>( \text{In Vivo} f_{\text{m,UGT}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine</td>
<td>0.7</td>
<td>0.44</td>
<td>0.50</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>1.1</td>
<td>0.50</td>
<td>N.A.</td>
</tr>
<tr>
<td>Codeine</td>
<td>0.8</td>
<td>0.81</td>
<td>0.82</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>4.1</td>
<td>0.65</td>
<td>N.A.</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>7.4</td>
<td>0.86</td>
<td>0.40</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>1.8</td>
<td>0.71</td>
<td>0.90</td>
</tr>
<tr>
<td>Midazolam</td>
<td>3.7</td>
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<td>N.A.</td>
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<tr>
<td>Naloxone</td>
<td>0.6</td>
<td>0.89</td>
<td>0.65</td>
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<tr>
<td>Raloxifene</td>
<td>15.7</td>
<td>0.44</td>
<td>N.A.</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>0.8</td>
<td>0.78</td>
<td>0.85</td>
</tr>
</tbody>
</table>

N.D., data not obtained in vitro; N.A., \( f_{\text{m,UGT}} \) was not available from renal excretion data.

\( a \) In vitro \( f_{\text{m,UGT}} \) was calculated using eq. 5. The \( f_{\text{m,UGT}} \) estimate obtained in the absence of BSA is shown in Table 1.

In vivo clearance values for 10 drugs investigated and the main parameters used in in vitro-in vivo extrapolation

References for plasma clearance, \( R_u \), and \( f_{\text{bio}} \) are available at http://www.pharmacy.manchester.ac.uk/capkr/.

\[ \text{CL}_{\text{int, UGT}} \] obtained for low clearance compounds were comparable with values obtained by metabolite formation (Boase and Miners, 2002; Soars et al., 2002), indicating the suitability of depletion data.

To ensure that substrates have access to the active site of the UGT enzyme on the luminal side of the endoplasmic reticulum, alamethicin was found not to affect P450 activity, which is particularly apparent when individual and combined cofactor studies are compared, as the sum of the combined P450 and UGT cofactors and in the absence (A) and presence (B) of 2% BSA. 

\( \Delta \), buprenorphine; \( \nabla \), carvedilol; \( \Box \), codeine; \( \square \), diclofenac; \( \blacklozenge \), gemfibrozil; \( \blacksquare \), ketoprofen; \( \square \), midazolam; \( \bigtriangledown \), naloxone; \( \bullet \), raloxifene; \( \bigcirc \), zidovudine. Error bars indicate range of scaling factors on the \( y \)-axis from 13 to 54 mg/g liver (Barter et al., 2007) and a range of \( Q_{\text{el}} \) on the \( x \)-axis from 17 to 25.5 ml/min/kg (Kato et al., 2003).

For most of the compounds, the formation approach (Obach, 2001; Jones and Houston, 2004). Due to a lack of glucuronide standards available, a depletion approach was used to obtain the \( \text{CL}_{\text{int}} \) for this data set. For most of the compounds, the protein concentration and time course used were greater than the proposed optimal values of 0.5 mg/ml and an incubation time of 30 min (Jones and Houston, 2004). These conditions ensured that greater than 20% metabolism was obtained during the incubations to distinguish from any baseline variability in the analytical methodology. For low clearance compounds codeine and zidovudine (zidovudine has high hepatic clearance in vivo), microsomal protein concentrations were >1 mg/ml. However, because these two compounds are not highly bound to the microsomal incubation matrix (Gertz et al., 2008), binding did not restrict clearance estimation. \( \text{CL}_{\text{int}} \) for all of the drugs investigated (Tables 1 and 2). This result is supported by Fisher et al. (2000), who showed that alamethicin had minimal effects on P450 activity when the CYP3A substrate testosterone was studied. Determination of \( \text{CL}_{\text{int}} \) in the presence of either P450 or UGT cofactors allowed the calculation of the in vitro fraction metabolized by the corresponding pathways. In this study we determined the \( f_{\text{m,CYP}} \) and \( f_{\text{m,UGT}} \) for 10 compounds with varying success, compared with renal excretion data (Tables 1 and 4). Improved correlation was observed when BSA was included in the incubation for 4 of 6 drugs for which the in vivo \( f_{\text{m,UGT}} \) data were available. Discrepancy between in vitro and in vivo data (Fig. 3) may have arisen because of the methods for determining the in vivo \( f_{\text{m,UGT}} \), the estimates were obtained from the amount of glucuronide excreted in the urine, which does not take into account the glucuronide metabo-
lites excreted in the bile/feces. The discrepancy in gemfibrozil estimates may be a result of the stability issues affecting the quantification of the acyl-glucuronide metabolites in vivo (Spahn-Langguth and Benet, 1992), which may lead to an underestimation of the fraction glucuronidated. The $fm_{UGT}$ in vitro may represent a useful initial estimate of the importance of metabolism via glucuronidation and can be incorporated in the prediction of clearance or drug-drug interactions. However, in the absence of information on the potential contribution of renal and biliary clearance to drug elimination, caution is needed in interpretation of these in vitro estimates.

For three compounds investigated metabolism was observed via both direct P450 and UGT pathways although the literature indicates only glucuronidation. In the case of naloxone and raloxifene, a P450 component accounting for approximately 30% of the total clearance was identified. In the case of zidovudine, a significant depletion was also observed in the presence of P450 cofactor (Table 1). However, when BSA is added to the incubation, this trend is reversed, with glucuronidation accounting for 78% of zidovudine metabolism (Table 4), in agreement with the previously reported UGT contribution (Blum et al., 1988). In the case of midazolam, direct glucuronidation accounted for a small proportion of the total clearance (6%), consistent with recent reports on $N$-glucuronidation via UGT1A4 (Klieber et al., 2008). Therefore, cautious interpretation of the clearance data obtained in the presence of individual cofactors is required, especially if the pathways of metabolism are unknown.

The presence of both cofactors for P450 and UGT in the incubation allowed the assessment of direct oxidation and glucuronidation simultaneously in alamethicin-activated microsomes. This approach has previously been reported for 7-hydroxycoumarin (Fisher et al., 2000) and buprenorphine (Mohutsky et al., 2006) and may provide a useful
alternative to the individual cofactor assays for new chemical entities. Predicted $CL_{int}$ obtained from data in the presence of both cofactors correlated well with the observed total $CL_{int}$; however, a 5.0-fold underprediction was observed. This was mainly driven by the significant underprediction seen with zidovudine, codeine, and raloxifene (predicted $CL_{int}$ represents only 3–6% of observed $CL_{int}$). The poor prediction observed for low clearance compounds (e.g., codeine) may be confounded by the use of the depletion approach to determine $CL_{int}$. Over the time course used in this study, 20% depletion of the parent compound was only just reached for these two drugs, adding a potential error in the $CL_{int}$ estimates. For this data set, the parallel tube liver model gave an improved prediction of clearance compared with the well-stirred liver model, reducing the bias by 50%. The improved prediction accuracy with the use of the parallel tube liver model was in good agreement with the observations by Ito and Houston (2005).

It has recently been reported that the addition of BSA to microsomal incubation decreases the $K_m$ and consequently increases the clearance estimates for UGT2B7 and UGT1A9 substrates (Rowland et al., 2007, 2008b); an analogous effect was observed on CYP2C9 (Carlile et al., 1999; Rowland et al., 2008a). The rationale is that BSA sequesters the inhibitory effect of unsaturated long-chain fatty acids released during the incubation on certain UGTs. Therefore, zidovudine was included as a control substrate in this study, and formation studies (data not shown) showed a decrease in the $K_m$ in the presence of 2% BSA from 1357 to 204 $\mu$M, in agreement with findings by Rowland et al. (2007). Within the current data set there was no substantial decrease in clearance observed in the presence of BSA. An improvement in the prediction of clearance was observed for 8 of 10 compounds when 2% BSA was added to the incubation, resulting in a bias of 1.7 (Fig. 2B). This improvement in the clearance prediction in comparison with the data obtained in the absence of BSA is predominantly driven by the increased clearance for the 6 compounds that are metabolized by UGT2B7 (Table 2; Fig. 4). In contrast to the increase in $CL_{int}$ observed for UGT2B7 substrates in the data set, a negligible effect was seen for most drugs glucuronidated by UGT1A1 with the exception of raloxifene for which a 3.3-fold increase in $CL_{int}$ was observed. This increase could be attributed to the effect on the P450-mediated pathway (exact P450 not defined) that contributes 31% to the total clearance of this drug. Raloxifene is also metabolized by UGT1A9 (Kiang et al., 2005), and the 3-fold increase in $CL_{int}$ could be caused by a decrease in the $K_m$ for the UGT1A9 enzyme in the presence of BSA, as reported in the case of propofol (Rowland et al., 2008b). The increase in $CL_{int}$ values for ketoprofen and diclofenac is most likely due to the combined effect of BSA on UGT2B7 and CYP2C9-mediated pathways (Table 4). For diclofenac, this assumption is confirmed by the comparable affinities of this drug for both CYP2C9 and UGT2B7 (Carlile et al., 1999; Kiang et al., 2005). The -fold change in $CL_{int}$ is in good agreement with a 5-fold increase in phenytoin $CL_{int}$ observed in the presence of 2% BSA (Rowland et al., 2008b). Diclofenac and phenytoin are reported to be metabolized at the same CYP2C9 binding site (Kumar et al., 2006); therefore, the effects on diclofenac are probably caused by a decrease in the CYP2C9 $K_m$ value in a manner similar to that of phenytoin (Rowland et al., 2008a), resulting in an increased $CL_{int}$.

In conclusion, the current study indicates the applicability of combined cofactor conditions in the assessment of clearance for compounds with a differential contribution of P450 and UGT enzymes to their elimination. Addition of 2% BSA improved the clearance predictability of alamethicin-activated microsomal data, in particular for UGT2B7 substrates. General application of this approach in vitro-in vivo extrapolation is promising, although the BSA effect is enzyme-specific. Underprediction observed for certain compounds (e.g., raloxifene and naloxyone) regardless of the incubation conditions or models used may be attributed to potential metabolism by cytosolic enzymes or contribution of extrahepatic glucuronidation.

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