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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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 human 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., 2001; 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 glucurononlated 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

ABBRéviationS: UGT, UDP-glucuronosyltransferase; P450, cytochrome P450; BSA, bovine serum albumin; UDPGA, UDP-glucuronic acid; afe, average-fold error; rmse, root mean squared prediction error.

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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 micromosomal 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 sequences the fatty acids, resulting in a 9- to 10-fold increase in the intrinsic clearance (CLint) 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, CLint 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 via either P450 (fM,CYP) or UGT (fM,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 raloxifene and midazolam, 1 mg of protein/ml for buprenorphine, carvedilol, diclofenac, gemfibrozil, ketoprofen, and naloxone, and 1.5 mg of protein/ml for codeine and zidovudine. Microsomes were diluted in 0.1 M phosphate buffer (pH 7.4) and added to drug solutions to give a final concentration of 5 mM (incubation volume 0.12 ml) for all of the compounds in the data set with the exception of midazolam for which a concentration of 21.4 mg liver/kg body weight (Ito and Houston, 2005). The observed hepatic clearance from in vivo intravenous data was corrected for the corresponding fraction unbound in the incubation (fu,int) and scaled to a whole body clearance (milligrams per minute per kilogram) using eq. 1 (Houston, 1994; Obach, 1999):

\[
\text{CL}_{\text{int}} = \frac{0.693}{\text{in vitro } t_{1/2}} \times \frac{\text{mg microsomes}}{\text{g liver}} \times \frac{\text{CL}_{\text{b}}}{\text{kg body weight}}
\]

which with a mean scaling factor for a 30-year-old individual of 40 mg protein/liver (range of 13–54 mg protein/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 was converted to an in vivo CLint value using the well-stirred and parallel tube liver models, defined in the eqs. 2 and 3, respectively (Ito and Houston, 2005):

\[
\text{CL}_{\text{int}} = \frac{\text{CL}_{\text{b}}}{\text{fu}_{\text{b}} \times \frac{R_{\text{b}}}{Q_{\text{b}}}} \times \ln \left( \frac{1 - \text{CL}_{\text{b}}}{Q_{\text{b}}} \right)
\]

where \( R_{\text{b}} \) is the fraction unbound in the plasma, \( \text{CL}_{\text{b}} \) is hepatic blood clearance, \( R_{\text{b}} \) is the blood to plasma concentration ratio, and \( Q_{\text{b}} \) 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 CLint values exceeded the CLint values for both the well-stirred liver model to the Qint, the CLint 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

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available; therefore, the observed $CL_{int}$ was calculated from an oral clearance using eq. 4:

$$CL_{int} = \frac{CL_{int}}{f_{mu}/R_b}$$

(4)

When the $R_b$ 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 $CL_{int}$ obtained in the presence of individual P450 ($CL_{int, CYP}$) and UGT ($CL_{int, UGT}$) cofactors using eqs. 5 and 6, respectively:

$$fm_{UGT} = \frac{CL_{int, UGT}}{CL_{int, UGT} + CL_{int, CYP}}$$

(5)

$$fm_{CYP} = \frac{CL_{int, CYP}}{CL_{int, UGT} + CL_{int, CYP}}$$

(6)

The predicted $CL_{int}$ values (from in vitro data obtained with combined cofactors and in the presence or absence of BSA) for the current data set (buprenorphine, codeine, and raloxifene) and a value of 1

The observed $CL_{int}$ ranged from 2.4 to 472 $\mu$L/min/mg for codeine and buprenorphine, respectively. Zidovudine had the lowest clearance by UGT enzymes (2.2 $\mu$L/min/mg), whereas raloxifene had the highest $CL_{int, CYP}$ (444 $\mu$L/min/mg). The $CL_{int}$ 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 $CL_{int}$ ranged from 2.8 to 688 $\mu$L/min/mg for zidovudine and buprenorphine, respectively (Table 2). The unbound intrinsic clearance ($CL_{int, u}$) 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 $CL_{int}$ 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 $CL_{int}$ 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 $CL_{int}$ 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 $CL_{int}$ 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

![Figure 1](https://example.com/figure1.png)

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.
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 \( f_{intrinsic} \) ranged from 0.1 to 0.99 for buprenorphine and codeine, respectively. In the presence of BSA, the \( f_{intrinsic} \) ranged from 0.008 to 0.99 for diclofenac and codeine, respectively, with the largest decrease in \( f_{intrinsic} \) observed for diclofenac (approximately 100-fold). The increase in the individual P450 and UGT \( CL_{int} \) estimates in the presence of BSA is shown in Table 4. The -fold increase in \( CL_{int, UGT} \) in the presence of BSA ranged from 0.9 to 12.1 for buprenorphine and gemfibrozil, respectively. On average, a 50% increase in \( fm_{UGT} \) was observed in the presence of BSA for UGT2B7 substrates. Where available an estimate of the \( fm_{UGT} \) was also obtained from renal excretion data and compared with the experimental values (Fig. 3). For naloxone, in vivo and in vitro \( fm_{UGT} \) estimates were comparable in the absence of BSA. However, for buprenorphine, codeine, zidovudine, and ketoprofen the experimental \( fm_{UGT} \) was lower than the value estimated in vivo. In contrast, the \( fm_{UGT} \) obtained in the presence of 2% BSA was more comparable with the in vivo estimates for these drugs (Fig. 3). In the case of gemfibrozil, the in vitro \( fm_{UGT} \) was higher than the extent of glucuronidation estimated in vivo (0.40), independent of the addition of BSA to the microsomal incubation.

In the presence of BSA, the unbound \( CL_{int} \) obtained with combined P450 and UGT cofactors ranged from 13.2 to 2143 \( \mu \text{mL/min/mg protein} \) for zidovudine and diclofenac, respectively. Addition of 2% BSA resulted in 0.84- to 13-fold increases in \( CL_{int} \) for buprenorphine and gemfibrozil, respectively, as illustrated in Fig. 4. When 2% BSA was added, there was an increase in \( CL_{int} \) by more than 2-fold for all UGT2B7 substrates in the data set (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 \( CL_{int} \) 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 \( CL_{int} \) 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). However, 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, 2004; Soars et al., 2002). 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
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 CLint, and it has been shown to be comparable with a metabolite formation approach (Boase and Miners, 2002). In this study alamethicin was found not to bind to the microsomal incubation matrix (Gertz et al., 2008), 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; Boase and Miners, 2002). In this study 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 individual CLint is approximately equal to the combined cofactor CLint 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 CLint 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 fmcyt and fmuugt 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 fmugt 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 fmugt, 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 $f_{m,UGT}$ 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\textsubscript{int} obtained from data in the presence of both cofactors correlated well with the observed total CL\textsubscript{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\textsubscript{int} represents only 3–6% of observed CL\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{m} and consequently increases the clearance estimates for UGT1A1 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\textsubscript{m} in the presence of 2% BSA from 1357 to 204 µM, 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\textsubscript{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\textsubscript{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\textsubscript{int} could be caused by a decrease in the K\textsubscript{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\textsubscript{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 3-fold change in CL\textsubscript{int} is in good agreement with a 5-fold increase in phenytoin CL\textsubscript{int} observed in the presence of 2% BSA (Rowland et al., 2008b). Diclofenac and phenytoin are reported to be metabolized at CYP2C9 and UGT2B7 (Carlile et al., 1999; Rowland et al., 2008b). Altered AZT (3′-azido-3′-deoxythymidine) glucuronidation kinetics in liver microsomes as an explanation for underprediction of in vivo clearance: comparison to hepatocytes and effect of incubation environment. Drug Metab Dispos 28:1621–1627.


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