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Prediction of drug clearance by glucuronidation from in vitro data: Use of combined P450 and UGT cofactors in alamethicin activated human liver microsomes

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Running title: Prediction of glucuronidation clearance using microsomes

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Abbreviations used: UGT, UDP-glucuronosyltransferase; P450, cytochrome P450; CL_{int}, intrinsic clearance; $CL_{int,u}$, unbound intrinsic clearance; BSA, bovine serum albumin; fm_{CYP}, fraction metabolised by cytochrome P450; fm_{UGT}, fraction metabolised by glucuronidation; fu_p, fraction unbound in plasma; Q_H, hepatic blood flow; R_B, blood to plasma concentration ratio; fu_{inc}, fraction unbound in the incubation; afe, average fold error; rmse, root mean squared prediction error

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Abstract

Glucuronidation via UDP-glucuronosyltransferase (UGT) is an increasingly important clearance pathway. In this study the CL_{int} for buprenorphine, carvedilol, codeine, diclofenac, gemfibrozil, ketoprofen, midazolam, naloxone, raloxifene and zidovudine was 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 CL_{int} was obtained using either individual or combined cofactors for 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, CL_{int} ranged from 2.8 to 688 µl/min/mg for zidovudine and buprenorphine, respectively; the clearance was approximately equal to the sum of the CL_{int} obtained in the presence of individual cofactors. The $CL_{int,u}$ was scaled to provide an in vivo predicted CL_{int}; the data obtained in the presence of combined cofactors resulted in 5-fold under-prediction on average. Addition of 2%BSA to the incubation with both P450 and UGT cofactors reduced the bias in the clearance prediction, with 8/10 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, an 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 catalysed by UDP- glucuronosyltransferase (UGT), a superfamily of membrane bound enzymes that catalyse 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 directly conjugate drugs 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) due to 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 the 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, a limited number of studies have applied this approach for the assessment of glucuronidation clearance (Mohutsky et al., 2006).

Prediction of clearance from microsomal in vitro data has shown a general under-prediction trend 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 a 10 to 30-fold under-prediction of clearance (Mistry and Houston, 1987; Miners et al., 2006). Investigation into these studies showed that incubation conditions greatly affect the clearance 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 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 recently investigated in the presence of bovine serum albumin (BSA) in the microsomal incubations (Rowland et al., 2007; Rowland et al., 2008a). The authors have 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, 1A6 and 1A4 (Rowland et al., 2007; Rowland et al., 2008a). The addition of BSA to incubations sequesters the fatty acids resulting in 9-10 fold increase in propofol (Rowland et al., 2008a) and zidovudine (Rowland et al., 2007) intrinsic clearance (CL_{int}). A comparable 'albumin effect' was also observed on the CYP2C9 substrate phenytoin (Rowland et al., 2008b).

In this study, CL_{int} for ten drugs with differential contribution of P450 and UGT pathways was determined in pooled human liver microsomes (HLM) using the substrate depletion approach. The dataset 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 metabolised 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 to predict 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 Chemicals Co. (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 protein/mL for raloxifene and midazolam, 1 mg protein/mL for buprenorphine, carvedilol, diclofenac, gemfibrozil, ketoprofen and naloxone, and 1.5 mg protein/mL for codeine and zidovudine. Microsomes were diluted in 0.1M phosphate buffer (pH 7.4) and added to drug solutions to give a final concentration of 5 µM (incubation volume 0.12 mL) for all the compounds in the dataset with the exception of midazolam where a concentration of 1 µM was used. Drug solutions and microsomes were pre-incubated for 5 minutes and the incubation was initiated by the addition of a NADPH regenerating system containing $NADP^+$ (1mM), isocitric acid (7.5mM), magnesium chloride (10mM) and isocitric acid dehydrogenase (1.2 units/mL).

UGT Incubation conditions. Pooled human liver microsomes (n=22) were diluted as above in 0.1M phosphate buffer containing magnesium chloride (3.4mM), the chelating agent, EDTA (1.15mM) and saccharic acid lactone (115µM) (conditions adapted from (Ogilvie et al., 2006)). The microsomes were activated by the addiction of alamethicin at a final concentration of 50 µg/mg microsomal protein and left on ice for 15 minutes. Drug solutions and microsomes were then pre-incubated for 5 minutes and the reaction was initiated by the addition of UGT cofactor solution containing UDPGA (5mM).

Combined P450 and UGT incubation conditions. Microsomes and drug solutions were treated as described for UGT conditions. The reaction was initiated by the addition of a combined cofactor solution containing $NADP^+$ (1mM), isocitric acid (7.5mM), magnesium chloride (10mM), isocitric acid dehydrogenase (1.2 units/mL) and UDPGA (5mM). For all incubations the reaction was terminated at the required time points (0, 2.5, 5, 10, 20, 30, 45,

60) by the addition of 120µL of acetonitrile containing the relevant internal standard. For experiments in the presence of BSA (both individual and combined cofactors) the following additions were made to the method. Microsomes were diluted in the incubation buffer containing BSA (final 2%). Alamethicin was then added to the microsomes in buffer and left on ice for 15 minutes. All the clearance data represent the mean ± sd of 3 separate experiments.

Microsomal binding. Microsomal binding was determined using high-throughput dialysis as described previously (Gertz et al., 2008). The extent of binding in the presence of BSA was determined following a slightly modified method using high-throughput dialysis kit (LLC, Gales Ferry, CT) with membranes with a molecular weight cut off of 12-14Kda. Microsomes, diluted in buffer with 2% BSA and the drug investigated was 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 hours and quenched in ice cold acetonitrile containing the relevant internal standard.

LC-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 µL was injected into the LC-MS/MS. Varying gradients of four mobile phases were used, the composition of which were; 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 10mM ammonium acetate with 10% acetonitrile, D- 10% water and 10mM ammonium acetate with 90% acetonitrile. For buprenorphine, diclofenac, gemfibrozil, midazolam, naloxone and raloxifene a Luna C18 column (Phenomenex, Torrance, CA) (3µ, 50 x 4.6mm) was used for chromatographic separation of analytes. For carvedilol a Luna phenyl-hexyl column (Phenomenex, Torrance, CA) (5µ, 30 x 4.6mm) 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 (Gertz et al., 2008).

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Data Analysis. The CL_{int} 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 fu_{inc} and scaled to a whole body clearance (ml/min/kg) using equation 1 (Houston, 1994; Obach, 1999).

(1)

$$CL_{int} = \frac{0.693}{\text{in vitro } t_{1/2}} \times \frac{\text{ml incubation}}{\text{mg microsomes}} \times \frac{\text{mg microsomes}}{\text{g liver}} \times \frac{\text{g liver}}{\text{kg body weight}}$$

using 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 was converted to in vivo CL_{int} value using the well-stirred and parallel tube liver models, defined in the equations 2 and 3, respectively (Ito and Houston, 2005).

$$CL_{int} = \frac{CL_b}{\frac{fu_p}{R_B} \times \left(1 - \frac{CL_b}{Q_H}\right)} \quad (2)$$

$$CL_{int} = -\frac{Q_H}{fu_p/R_B} \times \ln\left(1 - \frac{CLb}{Q_H}\right) \quad (3)$$

where fu_p is the fraction unbound in the plasma, CL_b hepatic blood clearance, R_B is the blood to plasma concentration ratio and Q_H is the hepatic blood flow (20.7 ml/min/kg) (Brown et al., 2007; Yang et al., 2007).

In the case of buprenorphine and zidovudine, the calculated observed CL_b values exceeded the Q_H; therefore, due to the sensitivity of the well-stirred liver model to the Q_H the CL_b value was set at 90% of hepatic blood flow for these two drugs. For consistency, a 90% cut-off was applied for these compounds for both liver models used. For raloxifene no intravenous clearance data were available; therefore, the observed CL_{int} was calculated from an oral clearance using equation 4.

$$CL_{int} = \frac{CL_H}{fu_p/R_B} \quad (4)$$

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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-haematocrit (i.e., 0.55) was assumed for acidic compounds (e.g., gemfibrozil, ketoprofen).

The in vitro fm_{UGT} and fm_{CYP} was determined from the CL_{int} obtained in the presence of individual P450 ($CL_{int,\ CYP}$) and UGT ($CL_{int,\ UGT}$) cofactors using equation 5 and equation 6, respectively.

$$fm_{UGT} = \frac{CL_{int,\ UGT}}{\left(CL_{int,\ UGT} + CL_{int,\ CYP}\right)} \quad (5)$$

$$fm_{CYP} = \frac{CL_{int,\ CYP}}{\left(CL_{int,\ UGT} + CL_{int,\ CYP}\right)} \quad (6)$$

The predicted CL_{int} values (from in vitro data obtained with combined cofactors and in presence/ absence of BSA) for the current dataset (n = 10) were compared to the observed CL_{int} obtained from the literature. The bias in the predicted CL_{int} 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*, eq. 9) provided a measure of precision for the predictions of the CL_{int} values (Sheiner and Beal, 1981; Obach et al., 1997):

$$afe = 10^{\left|\frac{1}{n}\sum \log\frac{Predicted}{Observed}\right|} \quad (7)$$

$$mse = \frac{1}{n}\sum\left(Predicted - Observed\right)^2 \quad (8)$$

$$rmse = \sqrt{mse} \quad (9)$$

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 the compounds showed a linear time profile with the exception of diclofenac where depletion was best described by a biphasic profile; in this case the initial linear phase of depletion plots was used to calculate the CL_{int}. Figure 1 shows the unbound CL_{int} obtained for three compounds, buprenorphine ($CL_{int,CYP}$>$CL_{int,UGT}$), raloxifene ($CL_{int,UGT}$ > $CL_{int,CYP}$) and ketoprofen ($CL_{int,CYP}$=$CL_{int,UGT}$) 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 opposite trend was observed for the remaining six compounds. For all the drugs in the dataset the CL_{int} with combined cofactors present was comparable to the sum of the individual $CL_{int,CYP}$ and $CL_{int,UGT}$ (Table 1, 2).

Table 1 shows the unbound in vitro $CL_{int,}$ 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 $CL_{int,CYP}$ ranged from 2.4 to 472µl/min/mg for codeine and buprenorphine, respectively. Zidovudine had the lowest clearance by UGT enzymes (2.2µl/min/mg), whereas raloxifene had the highest $CL_{int,UGT}$ (444µl/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 equations 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 688µl/min/mg for zidovudine and buprenorphine, respectively (Table 2). The $CL_{int,u}$ 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) (Figure 2A). The

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observed CL_{int} covered a 500-fold range with codeine and raloxifene at the lower and upper end of the clearance (Table 3). Using 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 4566). However, use of the parallel tube model reduced bias by approximately 50% with no significant effect on the precision (Figure 2A). Independent of the model used, zidovudine and codeine were poorly predicted, with an 18-fold under-prediction of clearance observed for these compounds when using the parallel tube model. The range of scaling factors (13 – 54mg protein/g liver) had a marginal effect on the prediction of clearance; however, they influence the extent of under-prediction observed.

When using the well-stirred liver model variability in the Q_H had a pronounced effect on highly cleared compounds like 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 under-prediction of clearance from in vitro data. For example, in the case of buprenorphine, a 52-fold under-prediction of buprenorphine clearance was observed when the hepatic clearance was set at 99% of Q_H, whereas setting the hepatic clearance at 90% of the Q_H resulted in only 5-fold under-prediction. Therefore, for drugs where the observed clearance approached hepatic blood flow the CL_B was limited to 90% of the Q_H. For consistency, an analogous approach was also applied when using the parallel-tube liver model.

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 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 fu_{mic} ranged from 0.1 to 0.99 for buprenorphine and codeine, respectively. In the presence of BSA the fu_{mic} ranged from 0.008 to 0.99 for diclofenac and codeine, respectively, with the largest decrease in fu_{mic} 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 to the experimental values (Figure 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 estimated in vivo. In contrast, the fm_{UGT} obtained in the presence of 2% BSA was more comparable to the in vivo estimates for these drugs (Figure 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 µl/min/mg protein for zidovudine and diclofenac, respectively. Addition of 2% BSA resulted in 0.84 to 13-fold increase in CL_{int} in the case of buprenorphine and gemfibrozil, respectively, as illustrated in Figure 4. When 2% BSA was added there was an increase in CL_{int} by more than 2-fold for all UGT2B7 substrates in the dataset (six out of ten drugs), whereas a negligible effect was observed for drugs glucuronidated via UGT1A1, with the exception of raloxifene where 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 in the case of midazolam, where 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 the Figure 2B. Prediction of clearance from the in vitro data obtained in the presence of BSA reduced the bias and extent of under-prediction resulting in 8 out of 10 compounds within 2-fold of in vivo values when using the parallel tube liver model (Figure 2B). However, incorporation of BSA in the incubation resulted in significant over-prediction (18-fold) of gemfibrozil clearance, in contrast to data without BSA, where predicted and observed clearances were in very good agreement (Table 2). In the case of raloxifene, clearance was under-predicted even after the addition of BSA.

Discussion

Over recent years an increasing number of studies have been carried out 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 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 fm_{CYP} and fm_{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 CL_{int} and it has been shown to be comparable to a metabolite formation approach (Obach, 2001; Jones and Houston, 2004). Due to a lack of glucuronide standards available a depletion approach was used to obtain the CL_{int} for this dataset. 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 minutes (Jones and Houston, 2004). This 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 >1mg/mL. However, as these two compounds are not highly bound to the microsomal incubation matrix (Gertz et al., 2008), binding did not restrict clearance estimation. $CL_{int,UGT}$ obtained for low clearance compounds were comparable to values obtained by metabolite formation (Boase and Miners, 2002; Soars et al., 2002), indicating the suitability of depletion data.

To ensure 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 and this is particularly apparent when comparing the individual to the combined cofactor studies, as the sum of the individual CL_{int} is approximately equal to the combined cofactor CL_{int} for all the drugs investigated (Tables 1 and 2). This is supported by a study by Fisher et al. (2000) where alamethicin was shown to have minimal effects on the CYP activity when studying the CYP3A substrate testosterone. Determination of CL_{int} in the presence of either P450 or UGT cofactors allowed the calculation of the in vitro fraction metabolised by the corresponding pathways. This study determined the fm_{CYP} and fm_{UGT} for ten compounds with varying success when compared to the renal excretion data (Tables 1, 4). An improved correlation was observed when BSA was included in the incubation for 4/6 drugs for which the *in vivo* fm_{UGT} data were available. Discrepancy between in vitro and in vivo data (Figure 3) may have arisen due to the methods for determining the in vivo fm_{UGT}, where the estimates are obtained from the amount of glucuronide excreted in the urine, which does not take into account the glucuronide metabolites excreted in the bile/faeces. 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 under-estimation of 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 to 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.

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 under-prediction was observed. This was mainly driven by significant under-prediction seen in the case of zidovudine, codeine and raloxifene (predicted CL_{int} represents only 3-6% of the observed). The poor prediction observed for low clearance compounds (e.g., codeine) may be confounded by the use of depletion approach to determine the CL_{int}. Over the time course used in this study 20% depletion of parent compound was only just reached for these two drugs adding a potential error in the CL_{int} estimates. For this dataset the parallel tube liver model gave an improved prediction of clearance when compared to the well-stirred liver model, reducing the bias by 50%. The improved prediction accuracy with the use of parallel tube liver model was in the good agreement with the observations by Ito and Houston (2005).

Recently, it has been reported that the addition of BSA to microsomal incubation decreases the Km and consequently increases the clearance estimates for UGT2B7 and UGT1A9 substrates (Rowland et al., 2007; Rowland et al., 2008a); analogous effect was observed on CYP2C9 (Carlile et al., 1999; Rowland et al., 2008b). 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 Km in the presence of

2% BSA from 1357 to 204 µM, in agreement with findings by Rowland et al. (2007). Within the current dataset there was no substantial decrease in clearance observed in the presence of BSA. An improvement in the prediction of clearance was observed for 8/10 compounds when 2% BSA was added to the incubation resulting in a bias of 1.7 (Figure 2B). This improvement in the clearance prediction in comparison to the data obtained in the absence of BSA is predominantly driven by the increased clearance for the six compounds that are metabolised by UGT2B7 (Table 2, Figure 4). In contrast to increase in CL_{int} observed for UGT2B7 substrates in the dataset, negligible effect seen for most drugs glucuronidated by UGT1A1 with the exception of raloxifene where a 3.3-fold increase in CL_{int} was observed. This increase could be attributed to the effect on P450-mediated pathway (exact CYP not defined) that contributes 31% to the total clearance of this drugs. Raloxifene is also metabolised by UGT1A9 (Kiang et al., 2005) and the 3-fold increase in CL_{int} could be caused by a decrease in the Km for the UGT1A9 enzyme in the presence of BSA, as reported in the case of propofol (Rowland et al., 2008a). Increase in ketoprofen and diclofenac CL_{int} 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., 2008a). Diclofenac and phenytoin are reported to be metabolised at the same CYP2C9 binding site (Kumar et al., 2006); therefore the effects on diclofenac are likely to be caused by a decrease in the CYP2C9 Km value in a similar manner to phenytoin (Rowland et al., 2008b), 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 clearance predictability of alamethicin activated microsomal data, in particular for UGT2B7 substrates. General application of this approach in the in vitro-in vivo extrapolation is promising, although the BSA effect is enzyme specific. Under-prediction observed for certain

compounds (e.g., raloxifene, naloxone) 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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FOOTNOTES

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Figure Legends

Figure 1 Intrinsic clearance for buprenorphine, raloxifene and ketoprofen obtained in HLM using either individual (P450 and UGT) or combined (P450+UGT) cofactor incubation conditions.

Figure 2 Prediction of clearance from in vitro data obtained in the presence of combined P450 and UGT cofactors in the absence (A) and presence (B) of 2% BSA. ▲ represents buprenorphine, ▼ carvedilol, ▲ codeine, □ diclofenac, ■ gemfibrozil, ■ ketoprofen, ● midazolam, ▼ naloxone, ● raloxifene, ○ 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_H on the x axis from 17 to 25.5 ml/min/kg (Kato et al. 2003).

Figure 3 Comparison of the in vitro fm_{UGT} –BSA (grey bar) and fm_{UGT} +BSA (black bar) to the in vivo fm_{UGT} obtained from renal excretion data (white bar) for six compounds investigated.

Figure 4 Fold difference in $CL_{int,u}$ for 10 compounds obtained in the presence and absence of 2% BSA. The CL_{int} was obtained in the presence of P450 and UGT cofactors in both cases. Solid line indicates the $CL_{int,u}$ +BSA/CL_{int}-BSA ratio of 1.

TABLE 1

Clearance (mean ± sd) obtained for 10 drugs in the presence of individual P450 and UGT cofactors

Compound	Major P450	Major UGT	$CL_{int,u}$ (µl/min/mg)		In vitro[a]	
			P450	UGT	fm_{UGT}	fm_{CYP}
Buprenorphine	CYP3A4	UGT1A1	472±40	279±28	0.37	0.63
Carvedilol	CYP2D6	UGT1A1	252±17.6	36.0±9.8	0.13	0.87
Codeine	CYP3A4	UGT2B7	2.44± 0.06	2.53±0.01	0.51	0.49
Diclofenac	CYP2C9	UGT2B7	129±32.2	214±46.1	0.62	0.38
Gemfibrozil	CYP3A4	UGT2B7	18.4±9.3	70±9.3	0.79	0.21
Ketoprofen	CYP2C9	UGT2B7	9.35±3.6	9.35±4.3	0.50	0.50
Midazolam	CYP3A4	UGT1A4	144±41.8	8.83±1.9	0.06	0.94
Naloxone	-	UGT2B7	5.52±3.2	10.2±2.9	0.65	0.35
Raloxifene	-	UGT1A9/ 1A1	197±25.8	444±86.4	0.69	0.31
Zidovudine	-	UGT2B7	3.84±0.7	2.21±1.4	0.37	0.63

[a]In vitro fm_{UGT} and fm_{CYP} were calculated using equations 5 and 6, respectively.
References for major UGT enzymes are available at http://www.pharmacy.manchester.ac.uk/capkr/

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

Compound	**$CL_{int,u}$ (µl/min/mg)**		**Fold difference**	**fu_{mic}**		**Scaled $CL_{int,u}$[a]**	
	(P450 + UGT)		**Cl_{int}+BSA/CL_{int}-BSA**			**(ml/min/kg)**	
	- BSA	**+ BSA**		**- BSA**	**+ BSA**	**- BSA**	**+ BSA**
Buprenorphine	688	577	0.84	0.10	0.17	588.9	494
Carvedilol	356	246	0.69	0.10	0.04	305	210
Codeine	3.4	16.9	5.0	0.96	0.99	2.9	14.5
Diclofenac	369	2143	5.8	0.87	0.008	316	1834
Gemfibrozil	97.3	1274	13.0	0.77	0.01	83.3	1090
Ketoprofen	26.0	115	4.4	0.92	0.11	22.2	98.5
Midazolam	159	241	1.5	0.54	0.04	137	207
Naloxone	14.4	35.1	2.4	0.87	0.99	12.3	30.1
Raloxifene	544	1812	3.3	0.08	0.009	465	1551
Zidovudine	2.8	13.2	4.7	0.60	0.49	2.4	11.3

[a]$CL_{int,u}$ 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.

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TABLE 3

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

Compound	**fu_p**	**R_B**	**fu_b**	**Observed CL (ml/min/kg)**
Buprenorphine	0.04	0.60	0.07	19
Carvedilol	0.02	1.00	0.02	7.8
Codeine	0.70	1.00	0.70	15
Diclofenac	0.005	0.50	0.009	3.5
Gemfibrozil	0.03	0.55	0.05	1.7
Ketoprofen	0.008	0.55	0.01	1.6
Midazolam	0.017	0.53	0.03	5.3
Naloxone	0.54	1.22	0.44	23
Raloxifene	0.05	1.00	0.05	735
Zidovudine	0.8	0.98	0.82	25

References for plasma clearance, R_B, and fu_p are available at http://www.pharmacy.manchester.ac.uk/capkr/

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TABLE 4

Fold difference and fm_{UGT} estimates obtained for 10 drugs obtained in the presence of BSA using individual P450 and UGT cofactors

Compound	**Fold difference**		***In vitro***	**In vivo**
	Cl_{int}+BSA/CL_{int}-BSA		**$fm_{UGT+BSA}$** [a]	**fm_{UGT}** [b]
	P450	**UGT**		
Buprenorphine	0.7	0.9	0.44	0.50
Carvedilol	1.1	7.9	0.50	-
Codeine	0.8	3.2	0.81	0.82
Diclofenac	4.1	4.7	0.65	-
Gemfibrozil	7.4	12.1	0.86	0.40
Ketoprofen	1.8	4.5	0.71	0.90
Midazolam	3.7	-	-	-
Naloxone	0.6	2.6	0.89	0.65
Raloxifene	15.7	5.4	0.44	-
Zidovudine	0.8	4.8	0.78	0.85

[a]In vitro fm_{UGT} was calculated using equation 5. The fm_{UGT} estimate obtained in the absence of BSA is shown in Table 1. [b]In vivo fm_{UGT} values were estimated from renal excretion data.

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Figure 1

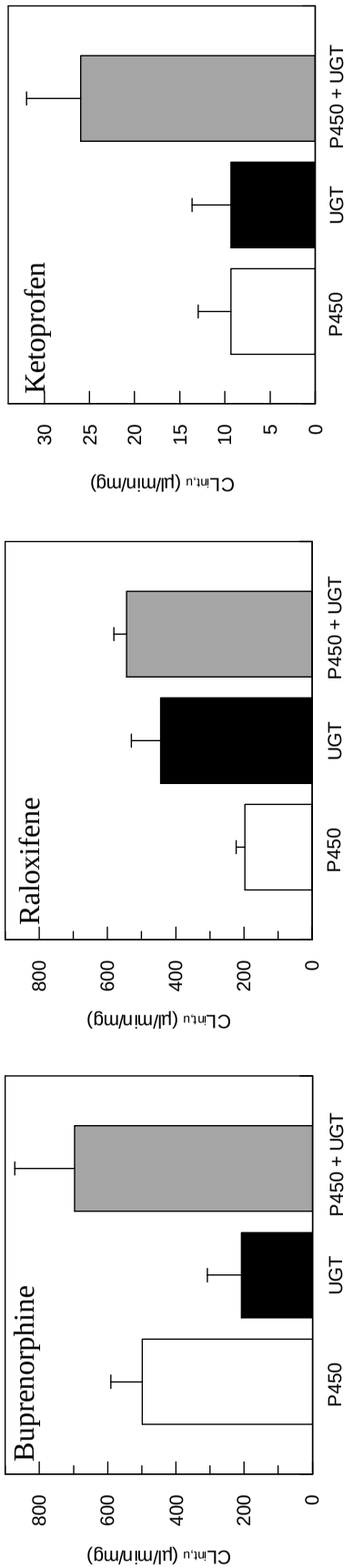

Figure 2A

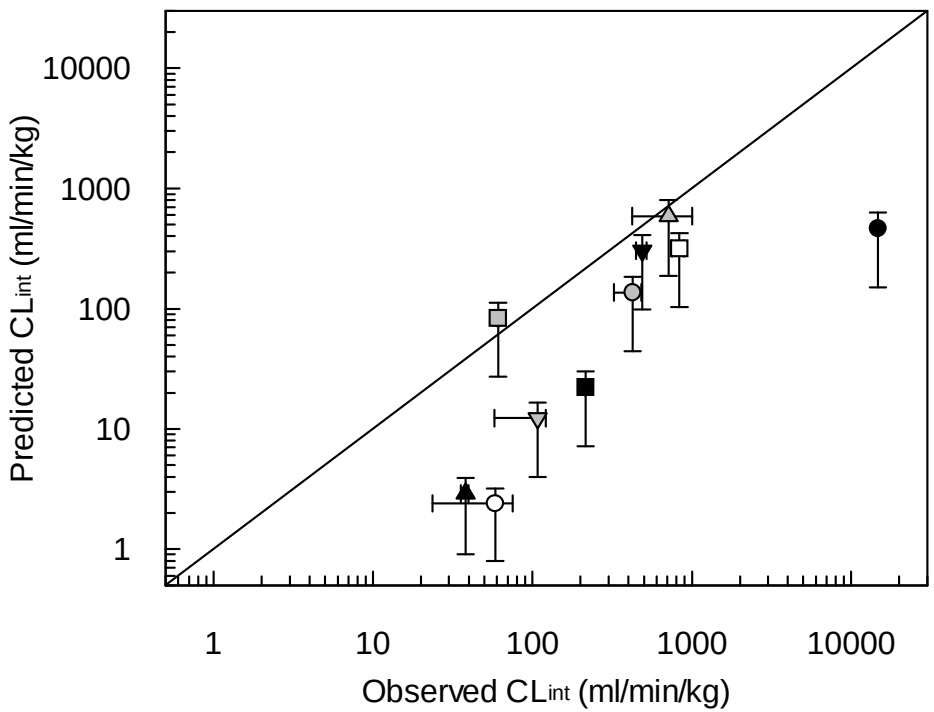

Figure 2B

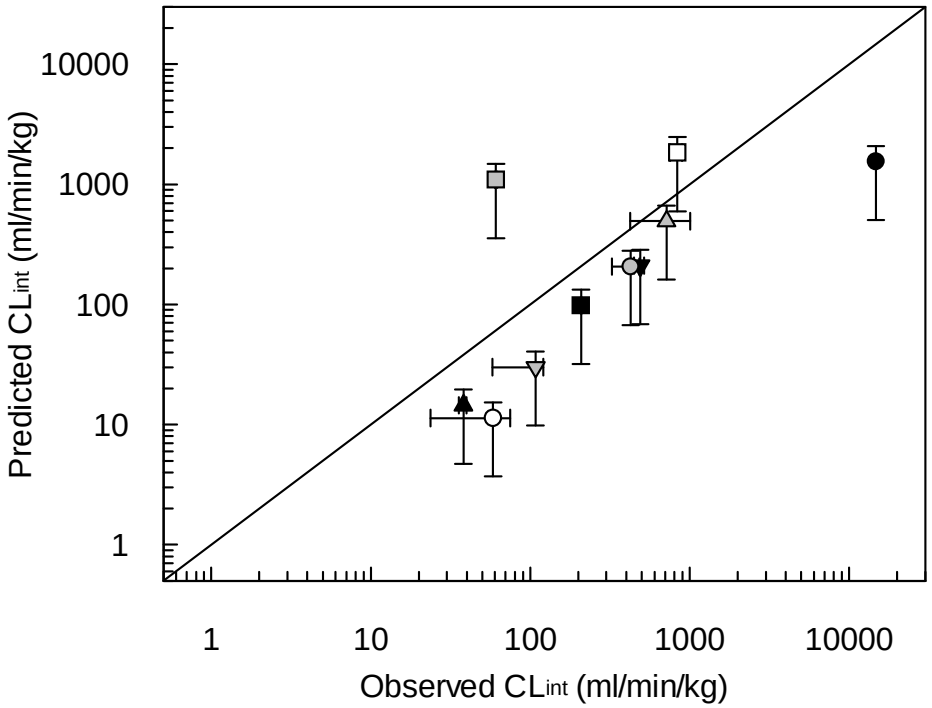

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Figure 3

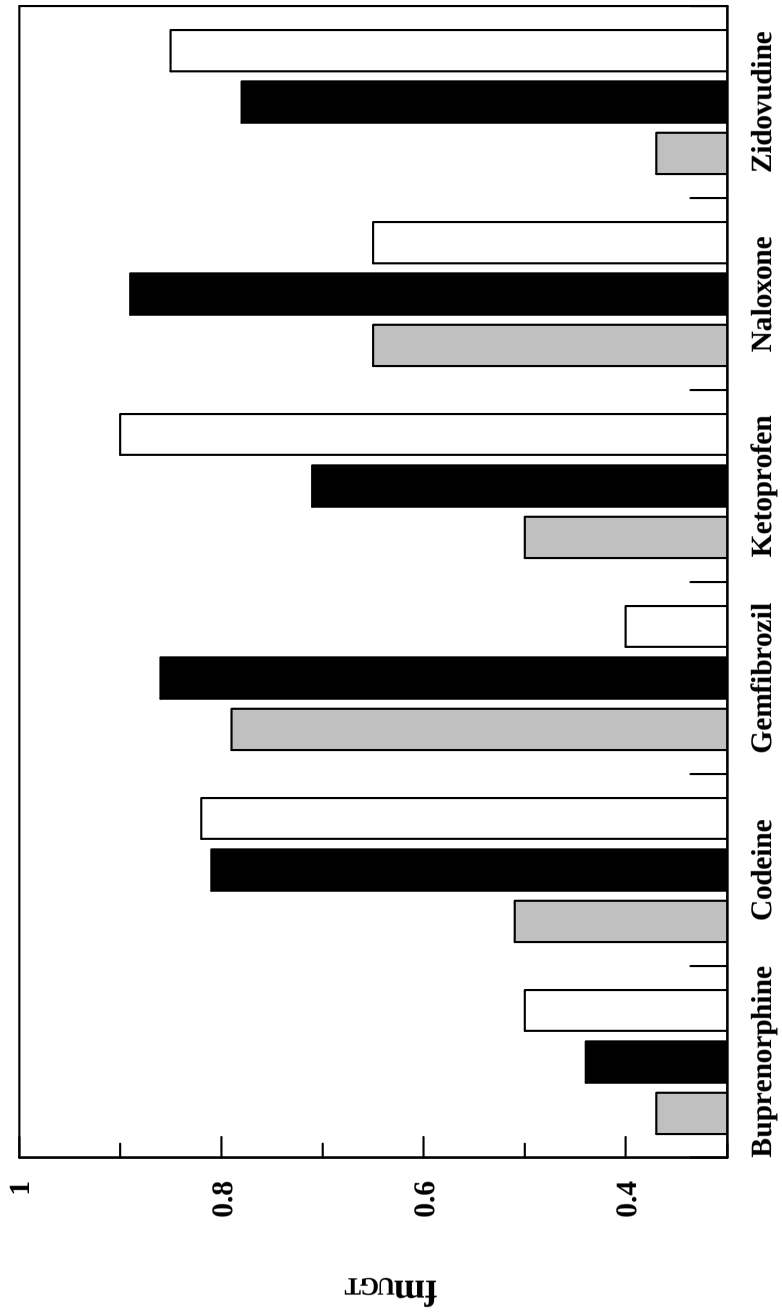

Figure 4