DMD Fast Forward. Published on November 28, 2006 as doi:10.1124/dmd.106.011569
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Title Page

Evaluation of Cryopreserved Human Hepatocytes as an Alternative In Vitro System to Microsomes for the Prediction of Metabolic Clearance

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DMD Fast Forward. Published on November 28, 2006 as DOI: 10.1124/dmd.106.011569
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Running title page

Running title: Predicting human clearance using cryopreserved hepatocytes

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Number of text pages: **33**
Number of tables: **6**
Number of figures: **5**
Number of references: **28**

Words in abstract: **242**
Words in introduction: **622**
Words in discussion: **1414**

Non-standard abbreviations:

CL_{int}, total intrinsic clearance, $CL_{int,u}$, unbound intrinsic clearance, CL_{max}, clearance at maximal activation, CL_H, hepatic clearance, fu_b, fraction unbound in blood, fu_p, fraction unbound in plasma, R_B, blood-to-plasma ratio, Q_H, hepatic blood flow, K_a, the affinity constant for the drug-protein interaction, $fu_{mic,}$ fraction unbound in microsomal incubation, fu_{inc}, fraction unbound in hepatocyte incubation, K_p, tissue-plasma partition coefficient, V_{cell}, volume of the hepatocyte, V_{inc} volume of the incubation, IS, internal standard.

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Abstract

Human liver microsomes have typically resulted in marked under-prediction of in vivo human intrinsic clearance (CL_{int}), therefore the utility of cryopreserved hepatocytes as an alternative in vitro system has become an important issue. In this study, 10 compounds (tolbutamide, diclofenac, S-warfarin, S-mephenytoin, dextromethorphan, bufuralol, quinidine, nifedipine, testosterone and terfenadine) were selected as substrate probes for CYP2C9, 2C19, 2D6 and 3A4, and the kinetics of metabolite formation (n=14 pathways) were investigated in three individual lots of cryopreserved hepatocytes, and in a pool of human liver microsomes. For the majority of the compounds, lower unbound K_M or S_{50} values were observed in hepatocytes compared to microsomes, on average by 50% over a 200-fold range (0.5-140μM). Expressed on an equivalent liver weight basis, a good correlation between microsomal and hepatocyte V_{max} values were observed for most pathways over 5-orders of magnitude (0.16-216 nmoles/min/g liver). Unbound hepatocyte CL_{int} ($CL_{int,u}$) when scaled to the whole liver, (range 0.38-4000 ml/min/kg) were on average 2.5-fold higher than microsomal $CL_{int,u}$ values, with the exception of tolbutamide and diclofenac where lower hepatocellular $CL_{int,u}$ values were observed. Hepatocyte predicted CL_{int} values were compared with human in vivo CL_{int} values and in order to supplement our data, in vitro data from cryopreserved hepatocytes were collated from four other published sources. These data show that for 37 drugs, there is on average a 4.5-fold under-prediction of the in vivo CL_{int} using cryopreserved hepatocytes, representing a significant reduction in prediction bias compared to human microsomes.

Introduction

Human liver microsomes have traditionally been the most commonly used in vitro system for the prediction of metabolic clearance, in particular for new chemical entities within drug discovery programmes in the pharmaceutical industry. However, the use of this system has typically resulted in an under-estimation of clearance, as illustrated by a dataset of 55 compounds where a 9-fold under-prediction of the in vivo intrinsic clearance (CL_{int}) was observed (Ito and Houston, 2005). As a result of this, attention in recent years has been placed on the use of alternative in vitro systems for clearance prediction. Fresh human hepatocytes are envisaged as a potentially more accurate system due to the full complement of both phase I and phase II metabolising enzymes, along with the presence of transporter proteins which should result in drug concentrations within the hepatocyte that are equivalent to in vivo concentrations within the liver. However, the limited availability of fresh human tissue and the cost implications involved in the preparation of freshly isolated human hepatocytes have resulted in cryopreserved hepatocytes emerging as the favoured alternative, which also have the added advantage of being readily available commercially and more convenient to use (Li et al., 1999).

Two major issues concerning the use of cryopreserved hepatocytes are whether the metabolic activities of specific CYP enzymes are conserved post-thawing after cryopreservation (Li et al., 1999), and whether these hepatocytes retain their functional transporter activity, both of which could confound accurate clearance prediction. Recent studies however both in rat (Naritomi et al., 2003; Griffin and Houston, 2004) and in human (Soars et al., 2002; McGinnity et al., 2004) have shown that CYP activities are comparable in both cryopreserved and fresh hepatocytes. Studies have also shown that thawed cryopreserved hepatocytes show no difference in transporter activities when compared to fresh hepatocytes in both rat and human (Houle et al., 2003; 2004).

These results in combination with metabolic stability studies (Soars et al., 2002; Shibata et al., 2002; Lau et al., 2002) illustrate the potential of cryopreserved hepatocytes as an alternative system to microsomes. However, the majority of CL_{int} data from cryopreserved hepatocytes have been obtained using the substrate depletion approach rather than that of metabolite formation, and the availability of K_M and V_{max} data is limited to only one study (Hallifax et al., 2005).

We have previously characterised metabolite kinetic profiles for a series of five benzodiazepines in cryopreserved hepatocytes, and have compared data to that obtained in microsomes to ultimately assess the utility of hepatocytes for the prediction of human clearance (Hallifax et al., 2005). Overall a good correlation between microsomal and hepatocyte CL_{int} was observed, therefore the aims of the present study were to extend this dataset to include four additional CYP3A4 substrates, along with six other P450 substrates to investigate whether this correlation is observed across multiple P450 enzymes. We have determined the metabolite kinetic profiles for 14 pathways involved in the metabolism of quinidine, nifedipine, testosterone, terfenadine, tolbutamide, diclofenac, S-warfarin, S-mephenytoin, bufuralol and dextromethorphan in three individual lots of cryopreserved hepatocytes and in a pool of human liver microsomes, to enable a more detailed comparison of both the affinity constant and P450 activity in both systems. We have compared the scaled CL_{int} values in both systems following correction for the fraction unbound within the incubation, since the importance of accounting for microsomal and hepatocyte binding has recently been stressed (Austin et al., 2005; Riley et al., 2005; Hallifax and Houston, 2006). Given that the accuracy of metabolic clearance predictions is dependent on the selection of in vivo data that the predictions are validated against, plasma clearance data for each compound has been compiled from a number of studies, to enable robust comparison of the hepatocyte predictions with observed in vivo values.

Materials and Methods

Chemicals

(3S)-3-Hydroxyquinidine, oxidized nifedipine, S-warfarin, 7-hydroxywarfarin, S-mephenytoin, 4-hydroxymephenytoin, bufuralol and 1-hydroxybufuralol was purchased from Ultrafine Chemicals (Manchester, UK). Azacyclonol was purchased from Acros Organics. Terfenadine alcohol was a gift from Pfizer (Sandwich, Kent, UK). All other chemicals and reagents were purchased from Sigma Chemical (Poole, Dorset, UK).

Hepatocyte Thawing Procedure

Cryopreserved human hepatocytes (lots 091, RNG/TPZ, KSE, 104) were purchased from In Vitro Technologies (Baltimore, MD, USA) and were stored in liquid nitrogen until required. Hepatic tissue was obtained from non-transplantable livers, however there was no information available regarding health status of the donors. Immediately before use, hepatocytes were thawed according to the recommended protocol. Briefly, vials of hepatocytes were rapidly thawed in a shaking waterbath at 37°C; the contents of the vial were emptied into 48 ml pre-warmed thawing media (Williams Medium E, pH 7.4) and the suspension centrifuged at 60g for 5 min at room temperature. The hepatocyte pellet was resuspended in 2.0mL pre-warmed Krebs-Henseleit buffer (pH 7.4) by gentle inversion, and the cell number and viability was assessed using the trypan blue exclusion test.

Hepatocyte Incubations

Hepatocyte lots were incubated with each compound over a substrate concentration range which was sufficiently wide enough to detect both the expected K_M and V_{max} values (see Table 1). For terfenadine experiments, lot KSE was replaced by lot 104. Incubations were performed in

duplicate (except for terfenadine which was performed in triplicate) and were optimised with respect to linearity with incubation time (Table 1) and hepatocyte density (see Table 3).

Briefly, cells were resuspended in fresh medium (Krebs-Henseleit Buffer, pH 7.4) to twice the required concentration and pre-warmed at 37°C. Drug was diluted in Krebs-Henseleit Buffer (pH 7.4) (125µl/well), placed into 24-well polypropylene plates (Hampton Research Corp, CA, USA) and pre-incubated for 5 min at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. Reactions were initiated by the addition of pre-warmed hepatocytes (125µl cell suspension/well) followed by gentle swirling of the plates and incubated as described above. Reactions were terminated by immersing the plates in liquid nitrogen. Samples were thawed and incubated with 250µl β-glucuronidase with sulphatase activity (200 units/ml in sodium acetate, 60mM, pH 4.5) for 60 min in a plate shaker at 37°C to hydrolyse any glucuronide/sulphate conjugates. The reactions were terminated by the addition of 0.5ml ice-cold acetonitrile containing 1µM of the appropriate internal standard. For studies involving tolbutamide and terfenadine no hydrolysis step was performed. In order to minimise CYP inhibition or induction potential, the concentration of organic solvent (either dimethylformamide or methanol) was kept below 0.2% (v/v), except for testosterone, nifedipine and S-mephenytoin where the solvent concentrations were 1%, 1% and 0.8%, respectively. However, even at these higher concentrations, no inhibitory effect is expected using the recommended solvent for each CYP enzyme (Easterbrook et al., 2001).

Microsomal Incubations

Pooled human liver microsomes (n=22 livers) were purchased from BD Gentest (Woburn, MA, USA). All incubations were carried out in duplicate (or triplicate in the case of terfenadine) and were performed under initial rate conditions with respect to incubation time (see Table 1) and microsomal protein concentration (see Table 3). Substrates were pre-incubated with microsomal protein in phosphate buffer (0.1M, pH 7.4) for 5 min in a shaking waterbath at 37°C. Reactions were initiated by the addition of a cofactor regenerating system (1mM $NADP^+$, 7.5mM isocitric acid, 10mM magnesium chloride, 0.2 units isocitric dehydrogenase) to give a final incubation volume of 0.25ml. Incubations were terminated by the addition of 0.25ml of ice-cold acetonitrile containing 1µM of the appropriate internal standard. For the terfenadine studies, incubations were performed in 96-well polypropylene plates, and were terminated by the addition of 0.07ml acetonitrile containing 0.2µM internal standard. The final substrate concentration range studied was the same as that used in the hepatocyte incubations for all compounds (Table 1). Kinetic parameters for tolbutamide and dextromethorphan were taken from previously published data from this laboratory (Brown et al., 2006).

Determination of Metabolite Concentration

Samples were centrifuged for 10 min at 11600g (Eppendorf Centrifuge 5413) and an aliquot (10µL) of the supernatant was analysed by LC-MS/MS. The metabolites of each substrate were analysed by LC-MS/MS (Waters 2795 with Micromass Quattro Ultima triple quadrapole mass spectrometer) with atmospheric pressure electrospray ionisation. The LC column eluate was split and one quarter was delivered into the mass spectrometer where the desolvation gas (nitrogen) flow rate was 600 L/hr, the cone gas (nitrogen) flow rate was 100 L/hr, and the source

temperature was 125°C. Product ions were formed in argon at a pressure of 2×10^{-3} mbar and detection was performed by multiple reaction monitoring (MRM) of the parent and selected daughter ion. The mass transitions of each substrate, metabolite and internal standard along with the cone voltage and collision energies are shown in Table 1. For each assay, twelve calibration standards with a blank were prepared in a matrix identical to the incubation extracts and included levels at below and above the expected concentrations. The ion chromatograms were integrated and quantified by quadratic regression of standard curves using Micromass QuanLynx 3.5 software.

Data Analysis

All data were analysed by nonlinear regression analysis using Grafit 4 (Erithacus Software, Horley, Surrey, UK) using models for either Michaelis-Menten kinetics, biphasic kinetics, substrate inhibition kinetics or sigmoidal kinetics. The goodness of fit criteria used to select the model with the most appropriate fit comprised visual inspection, consideration of the randomness of residuals and the standard error of the parameters.

Determination of Microsomal Binding and Hepatic Uptake

The binding of each compound was determined in both microsomal and hepatocyte incubations. The microsomal binding of S-mephenytoin, testosterone, nifedipine and quinidine were performed using the method of microfiltration as has been described previously (Carlile et al., 1999). Microsomal binding data for the other substrates were obtained from published literature (Obach et al., 1999; Margolis and Obach, 2003; Carlile et al., 1999 and Baba et al., 2002), and the fraction unbound in microsomes (fu_{mic}) calculated using equation 1 where K_a represents the

affinity constant for the drug-protein interaction and [P] represents the microsomal protein concentration.

$$fu_{mic} = \frac{1}{1 + K_a[P]} \qquad \text{Equation 1}$$

In the absence of human microsomal binding data, fu_{mic} values for dextromethorphan were taken from rat data (Witherow and Houston, 1999). For five of the 10 compounds (dextromethorphan, S-warfarin, tolbutamide, diclofenac and quinidine), hepatic uptake experiments had previously been performed using isolated rat hepatocytes to determine the tissue-plasma partition coefficient (K_p) using methods as described in Witherow and Houston, 1999. This value was used to calculate the fraction unbound within the hepatocyte incubation (fu_{inc}) using equation 2 (Witherow and Houston, 1999) where V_{cell} and V_{inc} represent the volume of the cell and the volume of the incubation, respectively, and is equal to 5µl/1000µl for 1×10^6 cells/ml. The use of equation 2 however assumes that active transport processes are not involved in the hepatic uptake of these compounds.

$$fu_{inc} = \frac{1}{1 + \frac{V_{cell}}{V_{inc}} \bullet K_p} \qquad \text{Equation 2}$$

For compounds for which hepatocyte uptake data were unavailable, the binding within the hepatocyte incubation was calculated by assuming that the extent of binding to microsomes at a concentration of 1mg/ml was equivalent to binding at 1×10^6 cells/ml (Austin et al., 2005).

Calculation of Intrinsic Clearance

$CL_{int,u}$ was determined from the ratio of $V_{max}/K_{M,u}$ for substrates displaying Michaelis-Menten, biphasic kinetics and substrate inhibition kinetics. For substrates displaying sigmoidal kinetics, the equivalent of CL_{int}, the clearance at maximal activation (CL_{max}) was calculated (Witherow

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and Houston, 1999). Hepatocyte CL_{int} (µL/min/10^6 cells) and microsomal CL_{int} (µL/min/mg protein) were scaled to *in vivo* CL_{int} (ml/min/kg) using a hepatocellularity of 120×10^6 cells g^{-1} liver, a microsomal recovery value of 40mg microsomal protein g^{-1} liver (Hakooz et al., 2006) and a human liver weight of 21.4 g liver kg^{-1} bodyweight. After normalising both data sets to per gram of liver, the unbound CL_{int} values from cryopreserved hepatocytes and human liver microsomes were compared. CL_{int} predictions were assessed from the prediction error (difference between the predicted and observed *in vivo* value). The bias of CL_{int} prediction was assessed from the geometric mean of the ratio of predicted and observed value (average fold error – *afe*), and the fold under-prediction calculated using equations 3 and 4, respectively.

$$afe = 10^{[\frac{1}{n}\sum \log \frac{Predicted}{Observed}]} \qquad \text{Equation 3}$$

$$\text{Fold under-prediction} = \frac{1}{afe} \qquad \text{Equation 4}$$

Comparison with In Vivo Clearance Data

The scaled CL_{int} values were evaluated relative to in vivo human clearance data. In vivo CL_{int} values were calculated from the published values of intravenous hepatic clearance (CL_H), the fraction unbound in plasma (fu_p) and the blood to plasma concentration ratio (R_B), according to Equation 5, where Q_H represents hepatic blood flow (20.7 ml min^{-1} kg^{-1}). Values of hepatic blood clearance (CL_b) were calculated by correcting the CL_H for the R_B value.

$$CL_{int} = \frac{CL_b}{\frac{fu_p}{R_B} \bullet (1 - \frac{CL_b}{Q_H})} \qquad \text{Equation 5}$$

For the majority of compounds, metabolic clearance data were compiled from intravenous studies, however when such data were unavailable, oral clearance data were used. Therefore for three

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compounds (S-mephenytoin, dextromethorphan, terfenadine) the CL_{int} was calculated by correcting the oral plasma clearance (assuming complete absorption from the gastro-intestinal tract) for the fraction unbound in blood (fu_b) as shown in equation 6.

$$CL_{int} = \frac{CL_H}{fu_p / R_B} \qquad \text{Equation 6}$$

For several CYP3A4 compounds, clearance values following intravenous administration were unavailable, so these studies were removed from the database. Oral clearance values could not be used since the fraction of drug escaping intestinal extraction (F_G) could not be assumed to be 1, due to the high levels of CYP3A4 in the intestine, resulting in a substantial contribution to first-pass metabolism following oral administration. In the case of two compounds, propofol and naloxone, intravenous plasma clearance values after intravenous dosing were higher than hepatic blood flow, so in the absence of oral clearance data, the CL_{int} values were calculated using equation 6 assuming CL_H was just below the value of Q_H. The in vivo clearance of propofol is very high and this may reflect a significant renal component in metabolic clearance (Al-Jahdari et al., 2006).

The fu_p and R_B values were compiled from two major sources (Thummel et al., 2005; Clarke 2004). In vivo plasma clearance data was compiled from various literature sources using The University of Washington Metabolism and Transport Drug Interaction Database (http://www.druginteractioninfo.org/DatabaseInfo.aspx). When the R_B was unavailable, a value of 1 was assumed for basic and neutral compounds. A value of 0.55 (1-hematocrit) was assumed for acids as acidic compounds are usually highly bound to plasma proteins, therefore restricting blood cell uptake. For dextromethorphan and nifedipine, the R_B value obtained in rats was used, and for bufuralol a value of 0.80 was assumed since this correlates to that observed with other

structurally similar beta-blockers with a similar lipophilicity. Predictions of in vivo human CL_{int} were supplemented by using other data previously obtained in this laboratory for five benzodiazepines (Hallifax et al., 2005) and other literature data using cryopreserved hepatocytes (Soars et al., 2002; Shibata et al., 2002 and Lau et al., 2002).

Results

Metabolite Formation Kinetics

The kinetics of metabolite formation were determined for each of the 10 probe substrates in three individual lots of cryopreserved human hepatocyte suspensions and in a pool of human liver microsomes. For most substrates the kinetic profiles were described using classic one-site Michaelis-Menten kinetics (hydroxytolbutamide, 3-hydroxyquinidine, 4-hydroxydiclofenac, 4-hydroxymephenytoin, 7-hydroxywarfarin, 3-methoxymorphinan and azacyclonol). However two pathways displayed biphasic kinetics (1-hydroxybufuralol and dextrorphan) indicative of both a high-affinity low-capacity site, and a low-affinity high-capacity site. For three CYP3A4 substrates, atypical Michaelis-Menten kinetics were evident with substrate-inhibition kinetics (oxidized nifedipine, terfenadine alcohol and fexofenadine) and sigmoidal kinetics (6β-hydroxytestosterone). The kinetic profiles of each compound were best described using the same models in both systems and were assigned as previously described in the methods.

Determination of In vitro CL_{int}

The in vitro kinetic parameters, V_{max}, unbound K_M or S_{50} (in the case of sigmoidal kinetics) obtained in cryopreserved hepatocytes were compared with those obtained in microsomes (Table 2). In order to calculate these parameters, the binding of each compound was determined in both microsomes and hepatocytes to give the fu_{mic} and fu_{inc}, respectively (Table 3). From these data it can be observed that the majority of the 10 compounds studied displayed relatively little binding at the protein concentrations employed within the incubations. Substrates of CYP2C displayed the least binding with fu values in the range of 0.9 and 1, as would be expected due to their acidic nature, whereas the range of fu values for substrates of CYP3A4 and CYP2D6 ranged between 0.58 to 0.85 for microsomal incubations, and 0.48 to 0.93 for hepatocyte incubations. The only

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exception was terfenadine which displayed significant binding in both systems, with fu values of 0.04 and 0.1 in microsomal and hepatocyte incubations, respectively. Regardless of the extent of binding, all data were corrected to give the unbound value. Figure 1 compares the unbound affinity constants ($K_{M,u}$ or $S_{50,u}$ values) for each individual pathway of metabolism (14 pathways) in both human liver microsomes and in cryopreserved human hepatocytes. It was observed that for the majority of the pathways, hepatocyte values were typically lower than the corresponding values in microsomes, by an average of 50%. The only exceptions to this were azacyclonol, nifedipine, diclofenac and the low affinity bufuralol pathway, where higher $K_{M,u}$ values were observed in hepatocytes.

When expressed on an equivalent liver weight basis (using scaling factors as indicated in Methods), a good correlation was observed between microsomal and hepatocyte V_{max} values (Figure 2), with the exception of diclofenac and tolbutamide where lower hepatocellular activities were observed compared to microsomes. For some CYP3A4 substrates, notably nifedipine and terfenadine, higher cellular V_{max} values were obtained, with a 10-fold higher value observed for terfenadine in hepatocytes compared to in microsomes. The $CL_{int,u}$ for each pathway was determined by the ratio of $V_{max}/K_{M,u}$ or CL_{max} in the case of testosterone, and when values were scaled to the whole liver, overall there was a good correlation between both systems. In fact, for 12 pathways involved in the metabolism of 8 compounds (dextromethorphan, S-mephenytoin, quinidine, nifedipine, testosterone, terfenadine, bufuralol and S-warfarin), hepatocellular $CL_{int,u}$ values were higher than the corresponding microsomal values, on average by 2.5-fold (Figure 3). Hepatocyte $CL_{int,u}$ values for tolbutamide and diclofenac however were on average 30% of the $CL_{int,u}$ observed in microsomes.

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Comparison with In Vivo CL_{int}

The CL_{int} values in cryopreserved hepatocytes were compared to human in vivo CL_{int} values. In vivo CL_{int} was calculated from published values of total plasma clearance, the R_B and the fu_p (as described in Materials and Methods). In order to ensure the accurate estimation of CL_{int}, plasma clearance data were collated from various in vivo studies in a number of different individuals to ensure that a representative value was obtained. These in vivo data for nine of the probe substrates are shown in Table 4. No in vivo plasma clearance data for testosterone were available. For some compounds such as bufuralol, S-warfarin and diclofenac, limited intravenous data were available, therefore plasma clearance values for these substrates should not be regarded as definitive compared to other compounds such as dextromethorphan, where in vivo data were obtained from a larger population dataset. Also, the lack of intravenous studies involving S-mephenytoin meant that the oral bioavailability could not be estimated, therefore this in vivo clearance value must be regarded as an upper estimate as complete systematic availability cannot be ensured.

In order to supplement our data, in vitro parameters using cryopreserved hepatocytes were also included from four other published literature sources (Hallifax et al., 2005; Soars et al., 2002; Shibata et al., 2002; Lau et al., 2002), resulting in an additional 27 compounds. In some instances, compounds had been studied by more than one laboratory, so for seven substrates, the mean in vitro CL_{int} was determined. In vivo CL_{int} values were calculated from in vivo data as previously described in Materials and Methods, and are displayed in Table 5.

Table 6 compares the observed and predicted CL_{int} values using cryopreserved hepatocytes for compounds in the present study and the literature (n=37). Compounds were grouped into four

categories according to the accuracy of CL_{int} predictions (≤ 2-fold, 2-fold ≥ 5-fold, 5-fold ≥10-fold, ≥ 10-fold). The major P450s responsible for metabolism of the 37 compounds along with the contribution of glucuronidating phase II metabolizing enzymes are also presented in order to assess whether prediction accuracy is systematic for a certain group of P450s. Comparison of CL_{int} values for the 9 compounds in this study involved the predicted $CL_{int,u}$. The extent of binding within the incubation for the five benzodiazepines involved in the study of Hallifax et al., (2005) had previously been determined, and fu values in both microsomal and hepatocyte incubations ranged between 0.69 and 0.94 for the protein concentrations employed within the incubations (Rawden et al., 2005). Therefore, the $CL_{int,u}$ for these five compounds were also compared with the in vivo CL_{int}. Corrections for binding with the other literature data were also possible. In the study of Shibata et al., 2002, incubations were performed in 100% human serum, therefore the CL_{int} in vitro was corrected for the fraction unbound in plasma, since it was assumed that the impact of binding to plasma proteins would be greater than that of binding to hepatocytes in reducing the concentration available within the incubation for metabolism. In the study of Soars et al., 2002, microsomal binding values at a concentration of 2mg/ml were reported, and since hepatocyte incubations were performed at a concentration of 2×10^6cells/ml, the extent of binding was assumed to be the same (Austin et al., 2005). Hepatocyte binding values for the compounds studied by Lau et al., 2002, were reported by Riley et al., 2005. Analysis of these data show that for the 37 compounds, there is a systematic under-prediction of in vivo human clearance using cryopreserved hepatocytes, with an average 4.5-fold under-prediction observed.

Twenty five compounds were identified for whom in vivo predictions of CL_{int} could be made from both microsomes (Figure 5, panel A) and hepatocytes (Figure 5, panel B). From this dataset,

a 9-fold and a 6-fold under-prediction was observed based on data from microsomes and hepatocytes, respectively.

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Discussion

Recent investigations have focused on the utility of cryopreserved hepatocytes as an alternative in vitro system for the prediction of human clearance (Soars et al., 2002; Shibata et al., 2002; Lau et al., 2002; Naritomi et al., 2003; Hallifax et al., 2005). However these studies, with one exception (Hallifax et al., 2005), have determined in vitro CL_{int} via substrate depletion rather than metabolite formation assays, resulting in very limited K_M and V_{max} data. Frequently the CL_{int} determined is based upon the total rather than the unbound drug concentration, with the assumption that the fraction unbound in blood and within the incubation are equal, and therefore binding corrections are unnecessary (Lau et al., 2002). This is clearly not the case for acidic compounds where extensive binding to albumin is usually observed, compared to negligible binding to microsomal protein or hepatocytes (Ito and Houston, 2005). Therefore the aim of the present study was to provide a comprehensive analysis of CL_{int} predictions obtained using the metabolite formation approach for a range of P450 substrates, and also to assess the effects of non-specific binding within the incubation matrix on CL_{int} predictions.

The $K_{M,u}$ (or $S_{50,u}$) values for the 14 pathways of metabolism in the present study were typically lower in hepatocytes than those observed in microsomes, by an average of 50% over a 200-fold range (Figure 1). This observation is consistent with previous data obtained with five benzodiazepines, where K_M values were lower for all nine pathways of metabolism (Hallifax et al., 2005). The lower K_M values in hepatocytes would suggest that an increased unbound drug concentration is available to the enzyme compared to that in a microsomal incubation, which may be indicative of some membrane uptake transporter involvement. However, there is little evidence to suggest that any of these 10 compounds are in fact substrates for hepatic transporters, so currently there is no explanation for this phenomenon. The data obtained in the present study

and in the study of Hallifax et al., (2005) enable comparisons for 23 pathways of metabolism and represent the only studies to date for which CL_{int} has been determined in cryopreserved hepatocytes via the use of metabolite formation.

P450 activities, when scaled to per gram of liver show good agreement between systems over 5-orders of magnitude (Figure 2). In only two cases values in hepatocytes were higher than in microsomes (CYP3A4 substrates, nifedipine and terfenadine), but lower CYP2C activities were observed for both tolbutamide and diclofenac. For the CYP2C9 substrates, this could suggest the possibility of differential P450 loss through the cryopreservation process as there is some evidence in rat to suggest that the CYP2C isoforms may be compromised during cryopreservation (Hewitt and Utesch, 2004). However the fact that S-warfarin activity (also a CYP2C9 probe) is comparable in both microsomes and hepatocytes would appear to rule out this possibility, and suggest that these discrepancies are associated with specific issues related to tolbutamide and diclofenac, such as the contribution of acyl-glucuronidation to the elimination of diclofenac (Kumar et al., 2002). Phase I oxidative metabolism is often followed by phase II conjugation of metabolites, but it is clear that for a significant number (at least 15) of the 37 compounds in this database, direct conjugation catalysed by UGT enzymes is also a major pathway of metabolism (Table 6). Although the data presented in Table 6 suggests that poor CL_{int} predictions ($\geq$ 10-fold in vivo CL_{int}) are observed for compounds that are directly conjugated, this is clearly not a systematic trend since good predictions ($\leq$ 2-fold in vivo CL_{int}) are also observed for the UGT substrates, codeine, naloxone and propofol. There does not appear to be any trend between the accuracy of CL_{int} predictions and metabolism by any particular UGT enzyme (Table 6). It is also interesting to note that for three compounds that only undergo phase I oxidative metabolism with no sequential conjugation processes (caffeine, theophylline and

terfenadine), that microsomes do not predict as well as hepatocytes. However, tolbutamide is an exception to this trend. Conversely, for those compounds that do undergo sequential metabolism, e.g. dextromethorphan, hepatocytes predict more accurately than microsomes for around 50% of the compounds (data not shown). It is also evident that there is no bias in prediction accuracy towards compounds metabolised by a particular P450 or indeed compounds of a certain chemical class (acid/base/neutral).

Overall higher $CL_{int,u}$ values (range 0.38 – 4000 ml/min/kg liver) were observed in hepatocytes, and were on average two-fold higher than those observed in microsomes for eight of the 10 compounds (Figure 3). The higher CL_{int} values in hepatocytes appear to result from the combined effect of comparable P450 activities in both systems, and an apparent higher P450 affinity using hepatocyte preparations. The CL_{int} values of diclofenac and tolbutamide were lower in hepatocytes, resulting from a higher cellular K_M and a lower cellular V_{max} value, respectively. Previous studies with five benzodiazepines showed that comparable CL_{int} values were obtained in both systems (Hallifax et al., 2005) in contrast to the higher CL_{int} values in hepatocytes observed in this study. Lower V_{max} values in hepatocytes were the main difference to the present study however sources of both microsomes and hepatocytes were different. This highlights the difficulties in comparing in vitro methods due to variability between the livers investigated in each particular study (Rawden et al., 2005). It is unlikely that the lower hepatocyte activities observed for the five benzodiazepines occur as a result of their atypical kinetics, since this was not evident with other 3A4 substrates in the present study, such as testosterone and nifedipine, whose kinetic profiles were described by sigmoidal and substrate inhibition kinetics, respectively. Although it has recently been emphasised that the experimentally determined CL_{int} is not necessarily the same as the unbound CL_{int} (Austin et al., 2005), correction for non-specific

binding had little effect on CL_{int} predictions for those compounds investigated in the present study, with the exception of terfenadine where the observed CL_{int} was 10-fold lower than the unbound CL_{int}. Consideration of the data presented by Riley et al., (2005) would suggest that this is a common situation.

The CL_{int} values predicted from cryopreserved hepatocytes in the present study were supplemented with other in vitro data from the published literature (Soars et al., 2002, Shibata et al., 2002; Lau et al., 2002; Hallifax et al., 2005) to enable in vivo clearance predictions for a total of 37 studies (Figure 4). Although these 37 studies include CL_{int} data obtained from both metabolite formation (n=14) and substrate depletion (n=23) approaches, the overall trend of a 4.5-fold under-prediction was the same in all datasets. This systematic under-prediction using both methods supports the claim that for the metabolite formation studies, the major metabolites were indeed analysed. Also, the fact that a similar general trend was observed between published data from three different laboratories where studies were performed with different livers is encouraging. However due to the complexities of inter liver differences alluded to earlier, plus inter laboratory issues confounding the comparison, we believe detailed comparison must be cautious.

The 4.5-fold under-prediction illustrated in Figure 4 represents a significant improvement in the accuracy of clearance predictions compared to the 9-fold under-prediction observed in the microsomal dataset discussed previously (Ito and Houston, 2005). Both datasets are relatively extensive, containing 55 compounds in the microsomal database and 37 compounds in the hepatocyte database. However not all the same compounds were studied in both systems and this improvement in prediction accuracy could be biased as a result of the particular compounds

involved. Subsequent analysis found that 25 compounds were common to both in vitro systems, and in fact a 9-fold under-prediction of in vivo CL_{int} was observed for the microsomes (Figure 5A), compared to a 6-fold under-prediction observed for the hepatocytes (Figure 5B). Therefore it is reasonable to conclude that the use of cryopreserved hepatocytes do represent a significant improvement compared to microsomes for the prediction of in vivo CL_{int}.

Although the results of this study would appear to favour the use of cryopreserved hepatocytes over microsomes for the prediction of clearance due to the reduced bias, it is clear that hepatocytes are still not fully quantitative for prediction, and there are a number of possible explanations for this. For example, like most in vitro systems these hepatocyte studies involved static incubations and will possibly contain excretory products normally cleared from the system. Also, the majority of the in vivo plasma clearance data used to validate the CL_{int} predictions are typically compiled from studies in young healthy volunteers receiving no concurrent medications. Detailed donor information on the livers used in the preparation of hepatocytes are generally incomplete; therefore the donor livers may be unrepresentative of those individuals from whom the in vivo plasma clearance data are obtained. Donor livers used in the preparation of hepatocytes are commonly obtained from waste material from patients undergoing a partial hepatectomy or from multi-organ donors, and the source of the liver will have implications for the preparation of viable and metabolically functional hepatocytes due to differences in the procurement and storage of the tissue. It is unknown to what extent differences in the procurement of the liver, in addition to the processing of liver tissue to prepare hepatocytes, will affect the metabolic and transport functions of hepatocyte preparations and this is clearly an area that requires further investigation.

Acknowledgements

The authors would like to thank Sue Murby for valuable assistance with the LC-MS/MS.

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Footnotes.

This work was funded by a consortium of pharmaceutical companies (GlaxoSmithKline, Novartis, Pfizer and Servier) within the Centre for Applied Pharmacokinetic Research at the University of Manchester. M.G was financially supported by a GlaxoSmithKline studentship. Part of this study was presented at the 9th European ISSX Meeting, June 4-7, 2006, Manchester, UK, and appeared in abstract form in *Drug Metab Rev* **38 (Suppl 1)**: 83-84 (2006).

Legends for figures

Fig 1. Comparison of $K_{M,u}$ (or $S_{50,u}$) values for 14 pathways in human liver microsomes and human cryopreserved hepatocytes. The metabolites represented are (1) hydroxytolbutamide, (2) 4-hydroxydiclofenac, (3) 7-hydroxy-(S)-warfarin, (4) 4-hydroxymephenytoin, (5) 3-hydroxyquinidine, (6) 6β-hydroxytestosterone, (7a) terfenadine alcohol, (7b) azacyclonol, (8) oxidised nifedipine, (9a) 1-hydroxybufuralol (high affinity pathway), (9b) 1-hydroxybufuralol (low affinity pathway), (10a) dextrorphan (high affinity pathway), (10b) dextrorphan (low affinity pathway), (10c) methoxymorphinan. The solid line represents the line of unity. Data are represented as mean ± S.D. of three determinations.

Fig 2. Comparison of 14 V_{max} values obtained in human liver microsomes and human cryopreserved hepatocytes. The metabolites represented are (1) hydroxytolbutamide, (2) 4-hydroxydiclofenac, (3) 7-hydroxy-(S)-warfarin, (4) 4-hydroxymephenytoin, (5) 3-hydroxyquinidine, (6) 6β-hydroxytestosterone, (7a) terfenadine alcohol, (7b) azacyclonol, (8) oxidised nifedipine, (9a) 1-hydroxybufuralol (high affinity pathway), (9b) 1-hydroxybufuralol (low affinity pathway), (10a) dextrorphan (high affinity pathway), (10b) dextrorphan (low affinity pathway), (10c) methoxymorphinan. V_{max} data are expressed on an equivalent weight basis (g liver). The solid line represents the line of unity. Data are represented as mean ± S.D. of three determinations.

Fig 3. Comparison of 14 $CL_{int,u}$ values obtained in human liver microsomes and human cryopreserved hepatocytes. The metabolites represented are (1) hydroxytolbutamide, (2) 4-hydroxydiclofenac, (3) 7-hydroxy-(S)-warfarin, (4) 4-hydroxymephenytoin, (5) 3-

hydroxyquinidine, (6) 6β-hydroxytestosterone, (7a) terfenadine alcohol, (7b) azacyclonol, (8) oxidised nifedipine, (9a) 1-hydroxybufuralol (high affinity pathway), (9b) 1-hydroxybufuralol (low affinity pathway), (10a) dextrorphan (high affinity pathway), (10b) dextrorphan (low affinity pathway), (10c) methoxymorphinan. The solid line represents the line of unity. The dashed line represents the 2.5-fold average higher value (calculated using equations 3 and 4) observed in hepatocytes compared to microsomes when normalized to kg liver weight. Data are represented as a mean of three determinations.

Fig 4. Comparison between observed in vivo CL_{int} and CL_{int} predicted using cryopreserved hepatocytes for 37 substrates. Open symbols represent predictions from literature data – Soars et al., 2002; Shibata et al., 2002; Lau et al., 2002 ♗ represents benzodiazepine data taken from Hallifax et al., 2005 and ♀ represents the 9 compounds in the present study. The solid line represents the line of unity, whereas the dashed line represents a 4.5-fold under-prediction (calculated using equations 3 and 4).

Fig 5. Comparison between observed CL_{int} and CL_{int} predicted using (A) microsomes (from Ito and Houston, 2005) and (B) hepatocytes for the same 25 compounds. The solid line represents the line of unity, whereas the dashed line represents a 9-fold and a 6-fold under-prediction in microsomes and hepatocytes, respectively (calculated using equations 3 and 4).

Tables

Table 1 Incubation and analytical LC-MS/MS conditions for substrates in cryopreserved hepatocytes and human liver microsomes

	In vitro conditions			LC-MS/MS conditions		
Substrate	Substrate conc. in incubation (µM)	Incubation Time (mins)		Mass Transitions (m/z)	Cone voltage (V)	Collision energy (eV)
		Hepatocytes	Microsomes			
Tolbutamide	10-1000	60	60	Tolbutamide 269.05 → 170.15 Hydroxytolbutamide 285.05 → 186.1 Diclofenac (IS) 293.95 → 250.1	50 50 50	15 17 11
Diclofenac	0.25-200	10	20	Diclofenac 293.95 → 250.1 4-hydroxydiclofenac 310 → 266.1 Tolbutamide (IS) 269.05 → 170.15	50 70 50	11 12 15
S-warfarin	0.05-100	45	60	S-warfarin 307.15 → 161.10 7-hydroxywarfarin 323.15 → 177.1	100 130	20 20

				Naproxen (IS) 229.15 → 170.05	60	15
S-mephenytoin	5-1000	60	60	S-Mephenytoin 217.05 → 188.2 Hydroxymephenytoin 233.05 → 190.25 Sulphaphenazole (IS) 313.05 → 156.15	70 75 90	14 14 25
Quinidine	5-400	15	20	Quinidine 325.1 → 307.3 3-hydroxyquinidine341.1 → 226.3 Dextromethorphan (IS) 272.15 → 171.35	49 70 89	20 28 40
Nifedipine	1-200	10	15	Nifedipine 347.15 → 315.2 Oxidised nifedipine 345.05 → 284.2 Dextromethorphan (IS) 272.15 → 171.35	50 70 89	8 20 40
Testosterone	2.5-500	10	10	Testosterone 289.15 → 97.2 6β-hydroxytestoseterone 305.15 → 269.3 Prednisone (IS) 359.15 → 341.4	60 80 60	20 15 10
Terfenadine	0.16-79.5	10	5	Terfenadine 427.3 → 436.2 Terfenadine alcohol 488.25 → 452.25 Fexofenadine 502.3 → 466.15	95 120 115	24 27 25

				Azacyclonol 268.2 → 250.2 Metoprolol (IS) 268.2 → 116.2	75 50	10 18
Bufuralol	0.25-1000	15	20	Bufuralol 262.15 → 188.25 1-hydroxybufuralol 278.15 → 186.1 Metoprolol (IS) 268.2 → 116.2	50 70 50	15 20 18
Dextromethorphan	0.1-1000	30	30	Dextromethorphan 272.15 → 171.35 Dextrorphan 258.2 → 199.5 3-methoxymorphinan 258.2 → 213.5 Levallorphan 284.3 → 157.45	89 70 70 80	40 28 25 40

Table 2 Kinetic parameters obtained for each compound in microsomes and hepatocytes

CYP	Substrate	$K_{M,u}$ (or $S_{50,u}$)		V_{max}		$CL_{int,u}$	
		Cryopreserved Human Hepatocytes	Human Liver Microsomes	Cryopreserved Human Hepatocytes	Human Liver Microsomes	Cryopreserved Human Hepatocytes	Human Liver Microsomes
		µM		*nmol/min/10^6 cells or mg protein*		*µl/min/10^6 cells or mg protein*	
2C9	Tolbutamide methylhydroxylation	112 ± 38	212 ± 90	0.015 ± 0.002	0.37 ± 0.08	0.06 ± 0.03	1.9 ± 0.4
	Diclofenac 4'-hydroxylation	7.4 ± 0.7	3.7 ± 0.4	0.29 ± 0.20	1.8 ± 0.3	38 ± 23	501 ± 28
	S-warfarin 7-hydroxylation	2.7 ± 1.5	4.3 ± 0.02	0.001 ± 0.001	0.004 ± 0.001	1.0 ± 1.0	0.87 ± 0.22
2C19	S-Mephenytoin 4'-hydroxylation	13 ± 4	30.3	0.016 ± 0.004	0.04	1.4 ± 0.7	1.37
3A4	Quinidine 3S)-3-hydroxylation	50 ± 8	78	0.31 ± 0.33	0.88	7.0 ± 7.9	11
	Nifedipine oxidation	16 ± 12	11	1.07 ± 1.15	0.95	56 ± 26	87
	Testosterone 6-β-hydroxylation	24 ± 1[a]	47[a]	1.8 ± 1.3	6.1	31 ± 22	131

	Terfenadine methylhydroxylation	0.49 ± 1.73	1.0 ± 0.5	91 ± 57	43 ± 14	1410 ± 590	11100 ± 275
	Terfenadine N-dealkylation	1.7 ± 1.2	0.8 ± 0.02	21 ± 8	2.8 ± 0.4	154 ± 102	90 ± 13
	Dextromethorphan N-demethylation[d]	139 ± 88	221 ± 50	0.28 ± 0.24	0.61 ± 0.1	2.8 ± 2.5	2.8 ± 0.3
2D6	Bufuralol	2.1 ± 1[b]	2.8[b]	0.028 ± 0.016[b]	0.071[b]	17 ± 15[b]	26[b]
	1-hydroxylation	44 ± 39[c]	43.3[c]	0.027 ± 0.002[c]	0.076[c]	0.98 ± 0.65[c]	1.8[c]
	Dextromethorphan	0.9± 0.3[b]	2 ± 0.4[b]	0.05 ± 0.03[b]	0.134 ± 0.01[b]	63 ± 46[b]	69 ± 16[b]
	O-demethylation[e]	44 ± 15[c]	106 ± 55[c]	0.06 ± 0.017[c]	0.16 ± 0.03[c]	1.6 ± 0.90[c]	1.72 ± 0.5[c]

[a] denotes S_{50} value
[b] represents the high-affinity, low-capacity pathway
[c] represents the low-affinity, high-capacity pathway
[d] represents 3-methoxymorphinan
[e] represents dextrorphan

Table 3 Binding of compounds in both microsomal and hepatocyte incubations

Substrate	[Microsomal Protein] (mg/ml)	fu_{mic}	[Hepatocyte] (1×10^6/ml)	K_p	fu_{inc}	Reference
S-mephenytoin	1	1	0.75	-	1[a]	Unpublished observations
Tolbutamide	0.5	1	0.5	1.1	0.99	Carlile et al., 1999
Diclofenac	0.1	1	0.25	22	0.97	Obach et al., 1999; unpublished observations
S-warfarin	1	0.9	1	15	0.93	Obach et al., 1999; unpublished observations
Nifedipine	0.25	0.85	0.25	-	0.85[a]	Unpublished observations
Quinidine	0.5	0.77	0.25	150	0.84	Brown, 2004
Testosterone	0.5	0.71	0.1	-	0.93[a]	Unpublished observations
Dextromethorphan	0.5	0.68[b]	1	50[b]	0.8	Witherow and Houston, 1999
Bufuralol	0.5	0.58	0.75	-	0.48[a]	Margolis and Obach, 2003
Terfenadine	0.25	0.04	0.1	-	0.1[a]	Baba et al., 2002

[a] represents values calculated using equation 1 assuming binding at 1mg/ml microsomal protein is equivalent to binding at 1×10^6 cells/ml (Austin et al., 2005)
[b] indicates binding data obtained using rat microsomes and hepatocytes

Table 4 In vivo human clearance values for n=9 compounds in this study and the key parameters for prediction[a]

Compound	Observed plasma clearance (ml/min/kg)	Number of in vivo studies	Number of individuals	Blood-to-plasma ratio (R_B)	Fraction unbound in plasma (fu_p)
S-warfarin	0.06	1	10	0.55	0.01
Tolbutamide	0.24	6	35	0.75	0.05
Diclofenac	3.8	1	7	0.55	0.01
Quinidine	5.2	4	30	0.87	0.13
Nifedipine	8.1	9	54	0.59[b]	0.04
Bufuralol	8.8	1	7	0.80	0.19
Mephenytoin	53[c]	3	35	0.84	0.60
Dextromethorphan	415[c]	5	72	1.76[b]	0.50[b]
Terfenadine	1300[c]	5	69	1	0.03

[a] References for plasma CL data available at www.capkr.man.ac.uk
[b] rat data
[c] CL value determined after oral dosing

Table 5 In vivo human clearance values for n=28 compounds with the key parameters for prediction[a]

Compound	Observed plasma clearance (ml/min/kg)	Number of in vivo studies	Number of individuals	Blood-to-plasma ratio (R_B)	Fraction unbound in plasma (fu_p)
Naproxen	0.106	1	10	0.55	0.01
Diazepam	0.38	14	131	0.55	0.022
Antipyrine	0.65	15	216	1	0.97
Ibuprofen	0.84	1	12	0.55	0.01
Theophylline	0.87	43	>339	0.83	0.44
Alprazolam	1.0	20	237	0.78	0.78
Ketoprofen	1.2	1	7	0.55	0.01
Lorazepam	1.2	10	121	1	0.09
Caffeine	1.3	1	8	1.075	0.7
Oxazepam	1.6	1	31	1.11	0.05
Furosemide	1.7	-	-	0.55	0.012
Gemfibrozil	1.7	-	-	0.55	0.03

Prednisolone	2.4	12	123	1	0.1
Flunitrazepam	3.4	8	127	0.78	0.22
Triazolam	4.6	10	127	0.76	0.1
Midazolam	6.0	17	151	0.55	0.042
Methylprednisolone	6.7	8	58	1	0.22
Chlorpromazine	8.6	-	-	1.17	0.05
Codeine	9.5	2	20	1	0.93
Timolol	11	2	18	0.84	0.4
Diltiazem	12	-	-	1	0.22
Desipramine	12	3	>13	0.96	0.18
Lidocaine	13	8	66	0.89	0.3
Propranolol	13	1	6	0.83	0.13
Imipramine	14	3	>24	1.066	0.1
Metoprolol	17	3	31	1.13	0.89
Naloxone	22	-	-	1.22	0.56
Propofol	36	3	41	1.25	0.02

[a] References for plasma CL data available at www.capkr.man.ac.uk

Table 6 Accuracy of CL_{int} predictions for n=37 drugs from human cryopreserved hepatocytes

Substrate	Major P450	Known Direct Glucuronidation	Hepatocyte Predicted CL_{int} (ml/min/kg)	Observed in vivo CL_{int} (ml/min/kg)	Fold under-prediction[a]	Prediction Range
Codeine	2D6, 3A4	Yes (UGT2B7/2B4)	35[b]	19	0.6	
Naloxone	NA[c]	Yes (UGT2B7)	284[b,d,e]	200	0.7	
Theophylline	1A2		2.6[e]	2.1	0.8 (63)	
Prednisolone	3A4	Yes	30[e]	27	0.9	
Caffeine	1A2		2.1[d,e]	2.0	1.0 (4.7)	Within 2-fold of in vivo (n=11)
Antipyrine	Multiple		0.67[d,e]	0.69	1.0 (4.9)	
Methylprednisolone	3A4	Yes	33[e]	45	1.4	
Chlorpromazine	2D6		188[e]	267	1.4 (11)	
Midazolam	3A4		200[f]	314	1.6 (6.9)	
Alprazolam	3A4		2.1[f]	3.7	1.8 (1.8)	
Propofol	2B6	Yes (UGT1A9)	2773[b]	5052	1.8	
Bufuralol	2D6		45[g]	99	2.2 (7.2)	
Desipramine	2D6	Yes	74[e]	167	2.3 (10.5)	
Diazepam	3A4, 2C19		6.6[f]	18	2.7 (4.2)	Within 2 to 5-fold of in vivo (n=7)
S-warfarin	2C9		1.9[g]	6.1	3.3 (9.2)	
Quinidine	3A4		18[g]	61	3.4 (8.1)	
Nifedipine	3A4		146[g]	597	4.1 (9.5)	
Flunitrazepam	3A4		4.5[f]	20	4.4 (3.9)	
Oxazepam	NA[c]	Yes (UGT2B15)	6.9[e]	34	5.0	
Diclofenac	2C9	Yes (UGT2B7/1A9)	98[g]	561	5.7 (1.3)	
Triazolam	3A4		11[f]	66	6.2 (4.9)	
Diltiazem	3A4		19[e]	125	6.7 (1.5)	Within 5 to 10-fold of in vivo (n=9)
Ibuprofen	2C9	Yes (UGT2B7)	12[e]	80	6.8 (9.8)	
Lidocaine	2D6, 3A4		21[d]	157	7.5 (51)	
Naproxen	2C9, 1A2	Yes (UGT2B7)	1.4[b]	11	7.6	
Imipramine	Multiple	Yes	49[b,d,e]	380	7.8 (21)	

Dextromethorphan	2D6, 3A4		172[g]	1482	8.6 (34)	
Terfenadine	3A4		4136[g]	43333	11 (1053)	
Propranolol	Multiple	Yes	39[d,e]	454	12 (5)	
Gemfibrozil	3A4	Yes (UGT1A1)	5.5[b]	67	12	
Tolbutamide	2C9		0.38[g]	4.9	13 (3.1)	
Lorazepam	NA[c]	Yes (UGT2B15)	0.95[e]	14	15	> 10-fold of in vivo (n=10)
Timolol	2D6		4.4[d]	77	17	
Metoprolol	2D6		4.3[d]	75	18 (4.2)	
Mephenytoin	2C19		3.6[g]	64	18 (38)	
Ketoprofen	NA[c]	Yes (UGT2B7)	4.7[b]	129	28	
Furosemide	NA[c]	Yes	0.91[b,e]	162	178	

[a] values in parentheses indicate the fold under-prediction observed in microsomal studies
[b] in vitro data taken from Soars et al., 2002
[c] not available, therefore exclusive metabolism by UGT isoforms is assumed
[d] in vitro data taken from Shibata et al., 2002
[e] in vitro data taken from Lau et al., 2002
[f] in vitro data taken from Hallifax et al., 2005
[g] in vitro data obtained in this laboratory
where in vitro data is taken from more than one published source, the mean in vitro CL_{int} value is calculated

Fig 1.

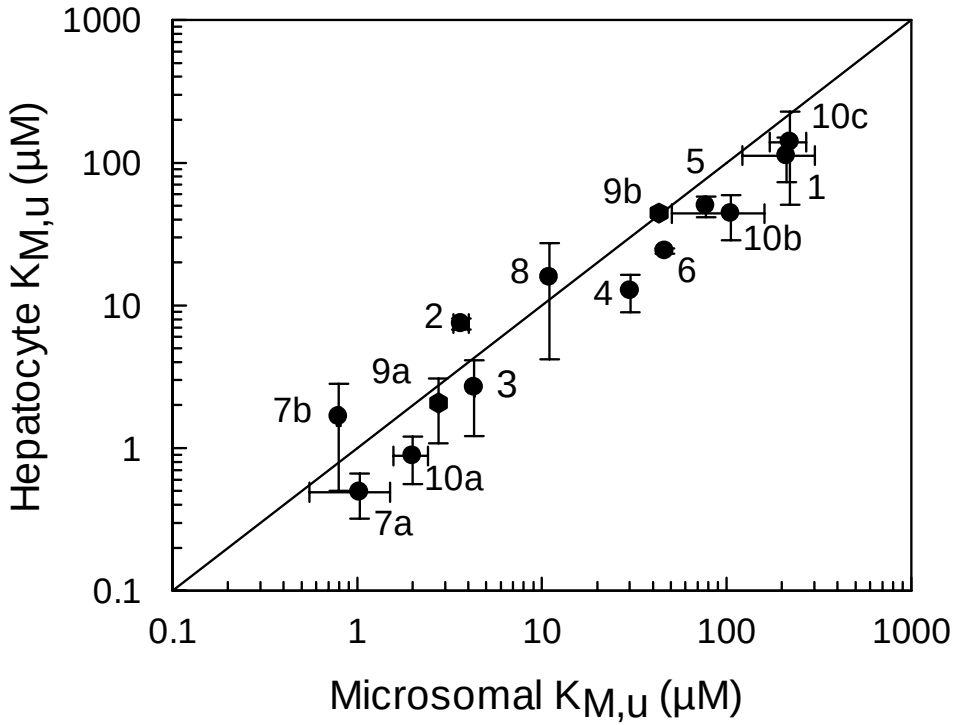


Fig 2.

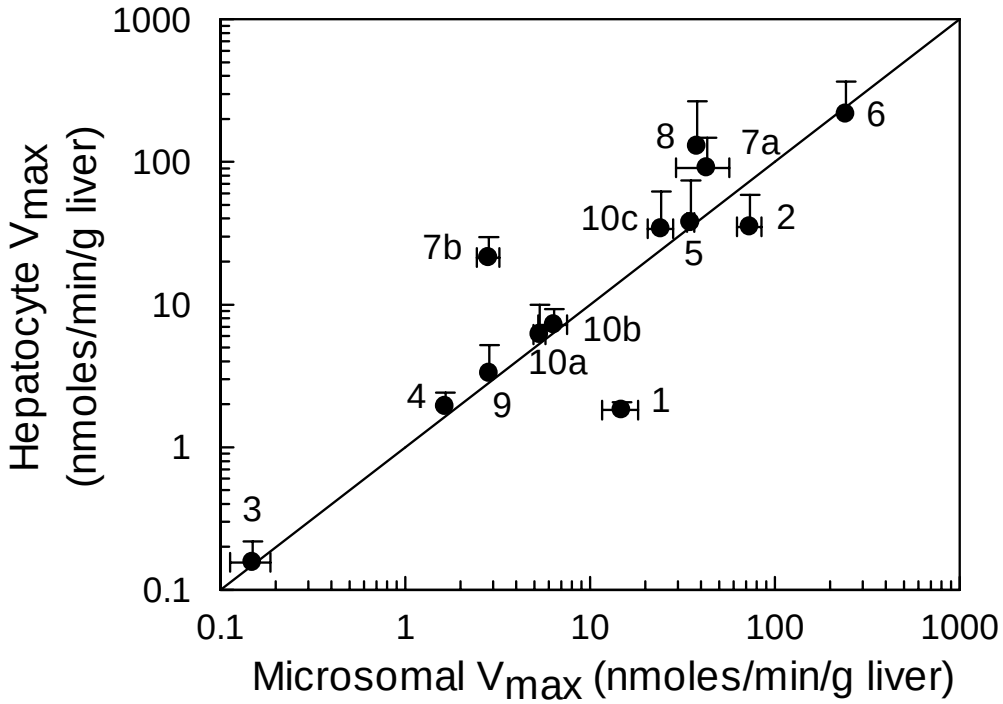


Fig. 3

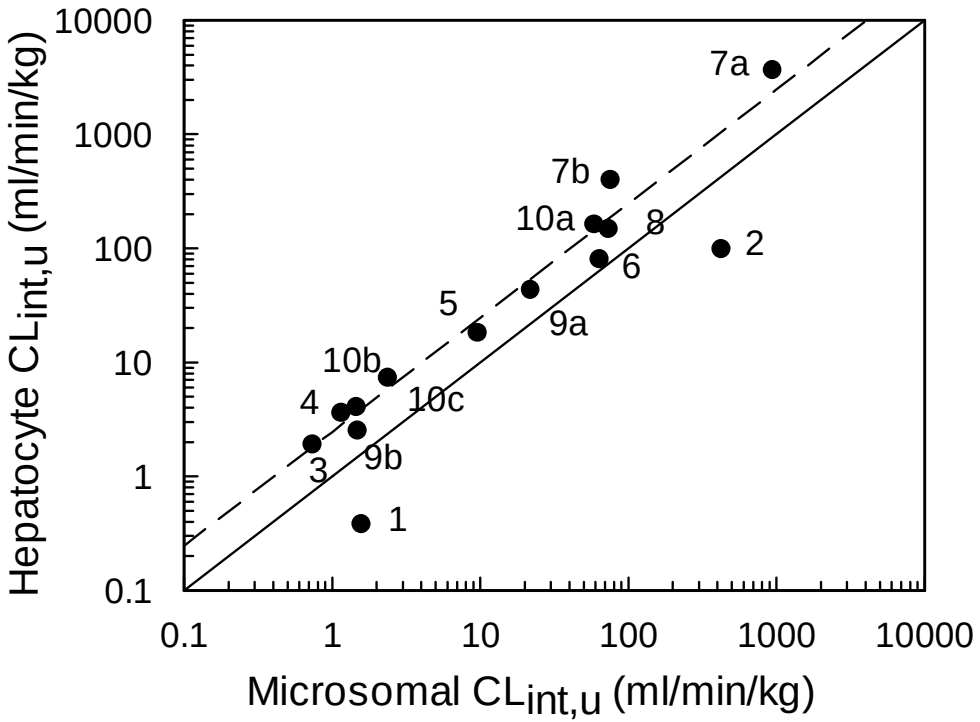
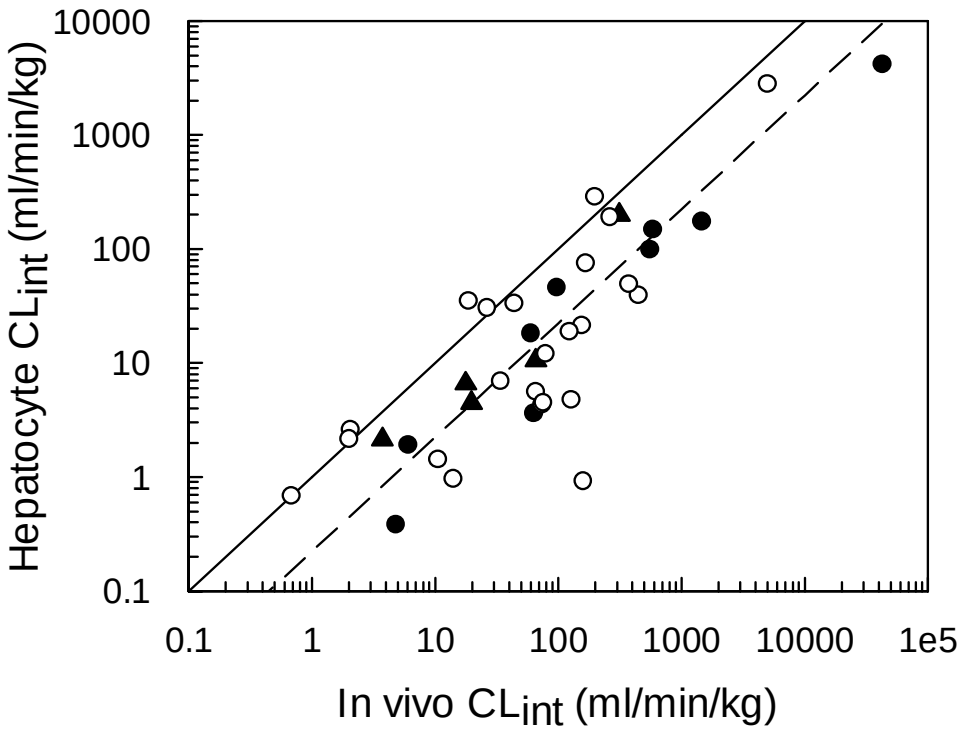


Fig 4.



DMD Fast Forward. Published on November 28, 2006 as DOI: 10.1124/dmd.106.011569
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Fig 5.