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Use of Hepatocytes to Assess the Contribution of Hepatic Uptake to Clearance In Vivo

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Use of Hepatocytes to Assess the Contribution of Drug Uptake

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Abbreviations

afe	Average fold error
$AUC_{0-\infty}$	Area under the drug concentration time curve from time zero to a point where the drug concentration is zero (extrapolated from the final two time points)
CL_h	Hepatic clearance
CL_{int}	Intrinsic clearance
$CL_{int\ ub\ in\ vitro}$	Unbound drug intrinsic clearance *in vitro*
$CL_{int\ ub\ in\ vivo}$	Unbound drug intrinsic clearance *in vivo*

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CYP	Cytochrome P450
DMPK	Drug metabolism and pharmacokinetics
f_{medium}	Fraction of drug in the incubation medium
fu_b	Fraction of drug unbound in the blood
fu_{inc}	Fraction of drug unbound in the hepatocyte incubation
'Media loss'	Loss of parent compound from the incubation medium into hepatocytes
NCE	New chemical entity
OATP	Organic anion transporting polypeptide
PgP	P-glycoprotein
Q_h	Hepatic blood flow

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Abstract

The wealth of information which has emerged in recent years detailing the substrate specificity of hepatic transporters necessitates an investigation into their potential role in the elimination of drugs. Therefore an assay in which the loss of parent compound from the incubation medium into hepatocytes ('media loss' assay) was developed to assess the impact of hepatic uptake on unbound drug intrinsic clearance *in vivo* ($CL_{int\ ub\ in\ vivo}$). Studies using conventional hepatocyte incubations for a sub-set of 36 AZ new chemical entities (NCEs) resulted in a poor projection of $CL_{int\ ub\ in\ vivo}$ (r^2 = 0.25, p = 0.002, average fold error = 57). This significant under-estimation of $CL_{int\ ub\ in\ vivo}$ suggested that metabolism was not the dominant clearance mechanism for the majority of compounds examined. However $CL_{int\ ub\ in\ vivo}$ was described well for this dataset using an initial compound 'disappearance' CL_{int} obtained from 'media loss' assays (r^2 = 0.72, p = 6.3 x 10^{-11}, average fold error = 3). Subsequent studies, using this method for the same 36 NCEs, suggested that the active uptake into human hepatocytes was generally slower (3-fold on average) than that observed with rat hepatocyes. The accurate prediction of human $CL_{int\ ub\ in\ vivo}$ (within 4-fold) for the marketed drug transporter substrates montelukast, bosentan, atorvastatin and pravastatin confirmed further the utility of this assay. This work has described a simple method, amenable for use within a drug discovery setting, for predicting the *in vivo* clearance of drugs with significant hepatic uptake.

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Introduction

Prentis *et al.*, (1988) highlighted the importance of drug metabolism and pharmacokinetics (DMPK) in reducing the attrition of candidate drugs in early clinical trials. This has subsequently led to a realignment of DMPK within the drug discovery process and an increased use of a plethora of high throughput screens early in lead optimisation (Riley and Grime, 2004).

Arguably one of the most critical tasks within DMPK is the accurate prediction of *in vivo* clearance from *in vitro* data (Riley, 2001). Although the theory behind this process was published almost thirty years ago (Rane *et al*., 1977), the potential impact on drug discovery was not fully appreciated until the review by Houston, (1994). Subsequently, hepatic microsomes and hepatocytes prepared from both pre-clinical species and humans have been used to predict *in vivo* clearance successfully (Obach, 1999; Soars *et* al., 2002; Ito and Houston, 2004; McGinnity *et* al., 2004; Ito and Houston, 2005; Riley *et al*., 2005). However, recent studies with hepatocytes have shown a significant under-prediction of *in vivo* clearance for a distinct set of drugs, which has been attributed in some cases to hepatic uptake (Riley et al., 2005; Soars *et al*., in press).

The last decade has seen a rapid increase in the number of publications in which researchers have investigated the role of hepatic uptake in drug clearance (Mizuno *et al*., 2003; Shitara *et al*., 2006). Perhaps the most important superfamily of enzymes for the hepatic uptake of anionic drugs is the organic anion transporting polypeptides (OATPs; Hagenbuch and Meier,

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2003, 2004). The molecular cloning of the major hepatic OATP isoforms and their expression in mammalian cell lines has generated a wealth of knowledge concerning the substrate specificity of OATPs (Mizuno *et al*., 2003; Hagenbuch and Meier, 2003, 2004; Shitara *et al*., 2006). However, the lack of suitable means to quantify data generated from recombinant cell lines directly to obtain hepatic clearance *in vivo* has proved more problematic than for the cytochrome P450 (CYP) enzyme superfamily, for example (Iwatsubo *et* al., 1997; McGinnity *et al*., 2000). Hepatocytes, whether plated or in suspension, have therefore become the system of choice for obtaining quantitative information regarding hepatic drug uptake (Olinga *et al*., 1998; Shitara *et* al., 2003; Hirano *et al*., 2004). Interestingly, most studies to date have obtained a measure of hepatic uptake by investigating the rate of appearance of radiolabelled substrate into cells, determined after a centrifugation step through oil (Olinga *et al*., 1998; Shitara *et al*., 2003; Hirano *et al*., 2004). Indeed, in some instances attempts have been made to use such data to predict the *in vivo* clearance of drugs (Olinga *et al*., 1998; Nezasa *et al*., 2003). Whilst this method provides robust, mechanistic data on individual compounds, it is clearly not amenable for use within an early discovery environment where many NCEs are evaluated in parallel and radiolabelled compounds are not routinely available.

To this end the aims of this study were three-fold: To develop a non-radiolabelled method to assess the impact (on clearance) of hepatic uptake in the rat; to determine if hepatic uptake is responsible for the under-prediction of *in vivo* clearance observed for a number of NCEs in previous studies (Riley *et*

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al., 2005; Soars *et al*., in press); to compare hepatocyte uptake rates *in vitro* between rat and humans for a number of NCEs and key drugs.

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Methods and Materials

Chemicals and human hepatocytes

All chemicals and reagents used were of the highest available grade. Montelukast, bosentan, pravastatin and atorvastatin were sourced from Sequoia Research Products Ltd. (Oxford, UK). [^{3}H]-estrone-3-sulfate (specific activity 2120 GBq/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA, USA). All other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK). AZ compounds were synthesized at AstraZeneca R&D Charnwood (Loughborough, UK).

Freshly isolated human hepatocytes were obtained from the UK Human Tissue Bank following appropriate consent and ethical approval (Leicester, UK). Hepatocyte viability was >80%.

Measurement of $logD_{7.4}$

Partitioning of compounds (40-400 μM) between 1-octanol and 0.02 M phosphate buffer, pH 7.4, at 20 °C was determined using a standard shake flask method (Leo *et al*., 1971). Both layers of the partition mixture were analyzed using HPLC with MS/MS detection as described below.

Preparation of rat hepatocytes

Isolation of rat hepatocytes was performed essentially using the two-step *in situ* collagenase perfusion method of Seglen (1976). Briefly, the hepatic portal vein of an anaesthetized male Sprague-Dawley rat (weight 200-300 g) was cannulated just above the junction of the splenic and pyloric veins. Liver perfusion medium (Invitrogen, Paisley, UK) was perfused via the hepatic

portal vein until the liver cleared to an even tan colour (usually 7-8 min at a perfusion rate of 30 ml/min). Liver digestion medium (Invitrogen, Paisley, UK) was then perfused until the liver displayed evidence of extensive dissociation (usually a further 6-8 min at a perfusion rate of 30 ml/min). The liver was dissected from the rat and cells were gently teased out of the liver capsule into a beaker containing ice cold hepatocyte suspension buffer (2.34 g Na HEPES, 0.4 g D-fructose, 2.0 g bovine serum albumin (BSA), 1 l powder equivalent of Dulbecco's modified Eagle's medium (Sigma, Gillingham, UK) diluted in 1 l of water and adjusted to pH 7.4 with 1 M HCl). The cell suspension was passed through a 250 μm mesh into a pre-cooled tube and centrifuged at 50 g for 2 min at 4 °C. The supernatant was decanted, the cell pellet was resuspended in suspension buffer (without BSA) and the centrifugation step was repeated. The resulting pellet of cells was resuspended in 10 ml of suspension buffer (without BSA) and an estimation of hepatocyte yield and viability was obtained using the trypan blue exclusion method. Only cells with a viability of >80% were used.

Determination of metabolic intrinsic clearance (CL_{int}) using rat and human hepatocytes

NCE stocks were prepared in dimethyl sulfoxide at 100-fold incubation concentration (100 μM). 10 μl of this 100 μM stock was added to a vial containing 490 μl of hepatocyte suspension buffer (without serum). A vial containing either rat or human hepatocytes at a concentration of 2 million viable cells/ml was pre-incubated for 5 min in a shaking (80 oscillations/min) waterbath at 37 °C along with the vial containing the drug/buffer mix.

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Reactions were initiated by adding 500 μl of hepatocyte suspension to the 500 μl of drug/buffer mix (giving a final substrate concentration of 1 μM at 1 % v/v dimethyl sulfoxide). Aliquots (40 μl) were removed at 0, 2, 6, 15, 30, 45, 60 and 90 min and reactions were quenched in 120 μl of ice-cold methanol. Samples were subsequently frozen for 1 h at –20 °C and then centrifuged at 2000 g for 20 min at 4 °C. The supernatants were removed and analysed as described below.

Determination of loss from media CL_{int} using rat and human hepatocytes

Loss from media CL_{int} values were determined essentially as described above except that 1 ml incubations were prepared in duplicate. Aliquots (80 μl) were removed at 0, 0.5, 1, 2, 4, and 6 min from the first incubation and at 15, 30, 45, 60, 75 and 90 min from the second incubation and placed into centrifuge tubes. These aliquots were immediately centrifuged at 7000 g for 30 s using a MSE MicroCentaur® centrifuge (Fisher Scientific, Loughborough, UK) and 40 μl of the supernatant was pipetted into 120 μl of ice-cold methanol. Samples were then frozen for 1 h at –20 °C, and centrifuged at 2000 g for 20 min at 4 °C. The supernatants were removed and analysed as described below.

Determination of CL_{int} for the appearance of [^{3}H]-estrone-3-sulfate into human hepatocytes

CL_{int} values for drug appearance into hepatocytes were determined using a method adapted from the centrifugal filtration technique of Petzinger and Fuckel (1992). A vial containing human hepatocytes at a concentration of 2 million viable cells/ml was pre-incubated for 5 min in a waterbath at 37 °C

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along with a vial containing 500 μl of tritiated and unlabelled estrone-3-sulfate in suspension buffer (final concentration 3 μM, specific activity 2120 mBq/mmol). Reactions were initiated with the addition of 500 μl of hepatocyte suspension to the estrone-3-sulfate/buffer mix. Aliquots (100 μl) were removed at 10, 20, 30 and 40 s and immediately centrifuged at 7000 g for 30 s through 150 μl of oil (density of 1.015 g/ml, containing 1M potassium hydroxide) using a MiniSpin® centrifuge (Eppendorf, Cambridge, UK). During this process the hepatocytes pass through the oil into the alkaline solution. After an overnight incubation in the alkaline solution to dissolve the hepatocytes, each centrifuge tube was frozen in liquid nitrogen and cut, collecting the cell pellet in a scintillation vial. Following the addition of scintillation cocktail, the amount of radioactivity in the cells was determined using a Packard 2200CA Tri-Card liquid scintillation counter (Packard Instrument Co, Pangbourne, UK).

Analysis of hepatocyte and log$D_{7.4}$ samples

Mass spectrometry was conducted on a Micromass Quattro Ultima Platinum triple quadrupole (Waters, Manchester, UK) using a Hewlett Packard 1100 HPLC system (Hewlett Packard, Palo Alto, CA) for separation. Analysis was by multiple reaction monitoring using either positive or negative ion mode. Cone voltage and collision energy were optimised for each compound.

In these analyses, chromatographic separation was achieved using a Hypersil Gold C_{18} (4.6 x 50 mm, 3 μm) column obtained from ThermoElectron Corp. (Basingstoke, UK) using 10 μl of each sample. The mobile phase consisted of

water with 0.1 % (v/v) formic acid with the organic phase being methanol containing 0.1 % (v/v) formic acid. All chromatography was performed using a generic gradient (t = 0 min % organic = 5, t = 0.5 min % organic = 5, t = 2 min % organic = 100, t = 3 min % organic = 100, t = 3.1 min % organic 5, total runtime = 4 min). The flow rate was set at 1.5 ml/min, which was introduced into the mass spectrometer source at 0.4 ml/min.

Data analysis

CL_{int} was estimated using:

$$CL_{int} = Dose/AUC_{0-\infty}$$

and

$$CL_{int} = V \times k$$

Where V is the incubation volume (corrected for non-specific binding-see below) and *k* is the elimination rate constant. For compounds exhibiting a mono-exponential loss, these two equations give equivalent values for CL_{int} since under these conditions, $AUC_{0-\infty}$ is equal to the initial drug concentration (C_0) divided by the elimination rate constant (and dose/C_0 = V). Non-specific binding was determined as the difference in drug concentration between the 0 and 0.5 min time-point. Therefore, the elimination rate concentration was calculated from the initial linear phase from log concentration-time plots starting from the 0.5 min time-point. This method was also used for compounds exhibiting a biphasic profile. Although this represents a potential composite of uptake and metabolism, curve stripping produced similar results for a representative set of compounds (data not shown).

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A schematic highlighting the processes involved in a 'media loss' CL_{int} determination are shown in Figure 1. The overall CL_{int} as viewed from the media is effectively the sum of uptake and metabolism minus any potential efflux out of the cell. Since only free drug is available for transport/metabolism, binding will modify each of these processes.

For the appearance of [^{3}H]-estrone-3-sulfate into hepatocytes, CL_{int} was calculated from:

$$CL_{int} = v/S$$

Where *v* is the initial rate of appearance of drug into the hepatocytes, and S is the initial drug concentration (since the reaction was performed at a low substrate concentration i.e. S << Km). This equation is the differential of the equation, CL_{int} = dose / AUC.

Determination of $CL_{int\ ub\ in\ vitro}$

$CL_{int\ ub\ in\ vitro}$ values were calculated from CL_{int} divided by the unbound fraction of drug in the hepatocyte incubation (fu_{inc}). fu_{inc} was predicted using the method of Austin *et al*., (2005) from a consideration of chemical class and either $logD_{7.4}$ or logP. Since no cells were present in the aliquots obtained from 'media loss' experiments, no fu_{inc} correction was required. Using physiological scaling factors to account for hepatocellularity and liver weight in the rat (Ito and Houston, 2004) and human (Riley *et al*., 2005), predicted $CL_{int\ ub\ in\ vivo}$ values were calculated from the values for $CL_{int\ ub\ in\ vitro}$, derived as described above.

Determination of $CL_{int\ ub\ in\ vivo}$

The unbound drug intrinsic clearance *in vivo* ($CL_{int\ ub\ in\ vivo}$) was calculated from hepatic blood clearance (CL_h) using the parallel tube model (Pang and Rowland, 1977), as shown below:

$$CL_{int} = \frac{Q_h}{fu_b \times \ln\ (Q_h - CL_h) / Q_h}$$

Where CL_h is hepatic blood clearance, fu_b is the fraction of drug unbound in blood and Q_h is blood flow (70 ml/min/kg in the rat and 20 ml/min/kg in human).

Rat plasma clearance was determined following the administration of an intravenous dose (1 mg/kg) to male Sprague-Dawley rats as reported previously (Weaver and Riley, 2006) and converted to CL_h by dividing by the blood to plasma ratio (estimated to be 0.7 for acidic and zwitterionic compounds and 1 for the base and neutral). The fraction of drug unbound in plasma was measured by equilibrium dialysis as detailed previously (Soars *et al*., 2002) and converted to fu_b by dividing by the blood to plasma ratio. Human values for CL_h, and fu_b were obtained from the literature (see Table 2).

Accuracy of predictions

Regression analyses were performed on log transformed data for predicted and observed $CL_{int\ ub\ in\ vivo}$ for each of the rat hepatocyte assays described. The regression equation and correlation coefficient (r^2) were derived and

significance was assessed using the p value (where $p < 0.05$ was considered significant). The accuracy of each prediction method was assessed using the average fold error (*afe*) with the geometric mean of prediction error providing an equal value for both under- and over-predictions.

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Results

Prediction of $CL_{int\ ub\ in\ vivo}$ using a conventional rat hepatocyte assay

A sub-set of 36 AZ NCEs was selected to investigate the potential of a 'media loss' rat hepatocyte assay to predict $CL_{int\ ub\ in\ vivo}$. The compound set comprised 6 acids, 18 zwitterions, one neutral and one basic compound with $logD_{7.4}$ values ranging from -0.2 to 3.5 (Table 1). An initial screen with a conventional rat hepatocyte assay produced a variety of predicted $CL_{int\ ub\ in\ vivo}$ values ranging from a mean value of 9 ml/min/kg for AZ19 to 315 ml/min/kg for AZ7 (see Table 1). Inter-preparation variability in predicted $CL_{int\ ub\ in\ vivo}$ values was acceptable ($\leq$ 3 fold) for the majority of compounds investigated. Figures 2A-C provide example log concentration-time profiles for AZ10, AZ14 and AZ20. However, Figure 3A shows the poor prediction obtained when log predicted $CL_{int\ ub\ in\ vivo}$ values calculated using the conventional rat hepatocyte assay were plotted against log $CL_{int\ ub\ in\ vivo}$ (observed) values. Only 4 compounds (AZ7-AZ10) were predicted within 5-fold and the *afe* of the dataset as a whole was 57.

Prediction of $CL_{int\ ub\ in\ vivo}$ using a 'media loss' rat hepatocyte assay

Figure 3B shows the relationship between predicted $CL_{int\ ub\ in\ vivo}$ obtained using a 'media loss' $AUC_{0-\infty}$ approach and observed $CL_{int\ ub\ in\ vivo}$ (r^2 =0.49, p = 1.9×10^{-6}). Although this method reduced the under-prediction of $CL_{int\ ub\ in\ vivo}$ compared with data generated using the conventional rat hepatocyte assay, the *afe* was still large (16-fold). However, $CL_{int\ ub\ in\ vivo}$ values calculated using an initial disappearance rate from a 'media loss' assay produced an excellent correlation with observed $CL_{int\ ub\ in\ vivo}$ data (r^2 =0.72, p = 6.3×10^{-11}). Figure

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3C also shows that in general this approach produced the most accurate prediction of $CL_{int\ ub\ in\ vivo}$ (*afe* =3). Preliminary experiments using hepatocytes at 4 ºC and inhibitor studies for several compounds confirmed that the uptake observed was active (data not shown).

Determination of f_{medium} using rat hepatocyte incubations

Table 1 shows the fu_{inc} values calculated for 36 AZ compounds as described previously by Austin *et al*., (2005). A ratio of compound concentrations observed during the terminal phase of a 'media loss' assay with those observed at a corresponding time-point from the conventional rat hepatocyte incubation also provides the determination of an f_{medium} (exemplified by Figures 2A-C). This is effectively the fraction of drug in the incubation medium. Table 1 shows that with compounds for which $CL_{int\ ub\ in\ vivo}$ was predicted well using the conventional hepatocyte assay (AZ7-10), there was no discernible difference between fu_{inc} and f_{medium}. This is highlighted by the minimal differences in log concentration for AZ10 observed from conventional and 'media loss' assays (see Figure 2A) However, for compounds that were actively taken up into the cell, the difference in these two values was significant (for example, AZ1 fu_{inc} = 0.93, f_{medium} = 0.04, see Figures 2B and 2C).

Prediction of $CL_{int\ ub\ in\ vivo}$ using a 'media loss' human hepatocyte assay

The uptake rate of [^{3}H]-estrone-3-sulfate was determined with each batch of hepatocytes to assess their suitability for uptake studies (See Table 2). $CL_{int\ ub\ in\ vivo}$ values were predicted for montelukast, prazosin, pravastatin,

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atorvastatin and bosentan using an initial disappearance rate from a 'media loss' assay with three separate human hepatocyte donors. Table 2 shows that for all five drugs studied the calculated $CL_{int\ ub\ in\ vivo}$ values were within 4-fold of the observed $CL_{int\ ub\ in\ vivo}$ estimates obtained from the literature. The dataset with human hepatocytes was extended further to include the 36 AZ compounds investigated previously in the rat (see Table 1). Figure 4 shows the relationship between rat and human uptake CL_{int} for these 36 AZ NCEs determined using an initial disappearance rate constant. This analysis suggests that, in general, hepatic uptake in the rat is more rapid (up to 12-fold, 3-fold on average) than in humans for this compound set.

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Discussion

An understanding and accurate prediction of *in vivo* clearance for pre-clinical species in conjunction with robust human *in vitro* data provides confidence when extrapolating to *in vivo* clearance in humans for a NCE (Grime and Riley, 2006). Rat hepatocytes have been shown previously to be the *in vitro* system of choice for predicting *in vivo* clearance for compounds primarily undergoing metabolic clearance in the rat (Houston, 1994; Soars *et al*., 2002; Ito and Houston 2004). Recent studies have indicated that a comprehensive analysis of the interplay between metabolism and transport in hepatocytes is warranted (Lam *et al*., 2006). For the majority of the sub-set of AZ compounds investigated in this study, CL_{int} estimates determined using conventional rat hepatocyte incubations under-predicted rat $CL_{int\ ub\ in\ vivo}$ significantly (Figure 3A). Studies in bile duct-cannulated rats had shown biliary and renal excretion of parent compound to be minimal for compounds in this dataset (data not shown). It has been noted that hepatic uptake can influence drug disposition ie. modulate the apparent volume of distribution of some drugs and enhance their clearance (Reinoso *et al*., 2001; Shitara *et al*., 2006). It was therefore postulated that hepatic uptake was the rate-determining process in the elimination of these compounds as proposed by other research groups (Yamazaki *et al*., 1996; Shitara *et al*., 2006).

The majority of studies in the literature have investigated the hepatic uptake of compounds into cells using the centrifugation of radiolabelled parent through an oil layer (Olinga *et al*., 1998; Shitara *et al*., 2003; Hirano *et al*., 2004). Indeed *in vitro* uptake data produced with radiolabelled substrates has been

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used to predict the *in vivo* clearance of drugs such as rocuronium, digoxin, rosuvastatin and pravastatin (Olinga *et al*., 1998; Nezasa *et al*., 2003). However, this method is not amenable for work within early drug discovery where radiolabelled compounds are not routinely available. To this end, a simple method of incorporating the effects of hepatic uptake on clearance was sought which obviated the need for either radiolabelled compound or the use of oil. The 'media loss' assay utilised in this work focused on the 'disappearance' of parent drug from the incubation medium which is effectively the inverse of monitoring for the appearance of parent drug within the cells. However by quantifying parent loss from the incubation medium, neither a radiolabelled drug nor an oil centrifugation step were required. An approach for investigating the appearance of non-radiolabelled drug within hepatocytes has been described in the literature recently for a limited dataset (Lam *et al*., 2006; Lu *et al*., 2006) but this method still requires the use of an oil centrifugation step.

When the clearance mechanism is hepatic and metabolic, data from this laboratory has shown there can be a systematic under-prediction of $CL_{int\ ub\ in\ vivo,}$ with an *afe* of 5-fold (Grime and Riley, 2006). If the hepatocyte uptake processes *in vitro* are similarly off-set against those functioning *in vivo*, the dose/$AUC_{0-\infty}$ approach might be expected to under-predict $CL_{int\ ub\ in\ vivo}$ by a similar factor. However, the significant under-prediction of $CL_{int\ ub\ in\ vivo}$ obtained for the majority of the 36 AZ compounds investigated in the rat using this method (see Figure 3B and Table 1) suggests that this is often not the case. A potential explanation for this phenomenon is that passive permeability

in the isolated hepatocyte *in vitro* is substantially greater than that observed *in vivo* (Reinoso *et al*, 2000), artificially increasing $AUC_{0-\infty}$ and hence lowering CL_{int} estimations (see Figure 2B). Upregulation or enhanced activity of efflux transporters in such incubations would also produce a similar result and indeed some compounds in this dataset have been shown to be substrates for P-glycoprotein (PgP). Further, compounds which are actively transported into the bile *in vivo* may be pumped back into the medium in these static hepatocyte experiments which would also artificially increase $AUC_{0-\infty}$. Interestingly, a similar under-prediction of $CL_{int\ ub\ in\ vivo}$ using the $AUC_{0-\infty}$ method was also observed using human hepatocytes (data not shown) for the known PgP substrate atorvastatin (Hochman *et al*., 2004) and montelukast. This is particularly pertinent since Zhao *et al*., (2005) have demonstrated an efficient PgP-mediated efflux using human hepatocytes.

For approximately 20% of the compounds in the rat dataset, $CL_{int\ ub\ in\ vivo}$ was predicted equally well from the initial disappearance rate constant and the $AUC_{0-\infty}$ approach (see Figure 2C). Clearly for these compounds, drug clearance from the media is dominated by hepatocyte uptake since the initial phase contributes the majority of the total AUC. While potential artefacts of the *in vitro* system may contribute to the under-prediction of *in vivo* clearance using an $AUC_{0-\infty}$ method (see Figure 2B), data obtained from the terminal time points of these profiles allow the calculation of f_{medium} (see Results). For drugs exhibiting a significant difference between fu_{inc} and f_{medium} (see Table 1, Figures 2B and 2C), an assessment of hepatic uptake may be required for an

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accurate prediction of *in vivo* clearance. Hence, the calculation of f_{medium} provides a relatively easy initial screen to prompt further uptake studies.

A more simplistic approach for obtaining a CL_{int} estimate from 'media loss' data is to calculate a CL_{int} from 'initial drug disappearance' (see Methods), synonymous with the appearance rates documented previously (Olinga *et al*., 1998; Shitara *et* al., 2003; Hirano *et al*., 2004). Figure 3C highlights the excellent prediction of $CL_{int\ ub\ in\ vivo}$ obtained for the compounds in Table 1 using this method. As anticipated, compounds for which conventional rat hepatocyte incubations accurately predicted *in vivo* clearance (AZ7-AZ10) were also predicted well using CL_{int} estimates determined using a disappearance rate constant (see Figure 2A). These data suggest that the use of a CL_{int} estimate from a 'media loss' assay provides a method suitable to predict *in vivo* clearance accurately, whether mediated by metabolism and/or hepatic uptake. Interestingly, although hepatic uptake has been thought to contribute most appreciably for poorly permeable compounds (Shitara *et al*., 2006), significant levels of uptake were observed in this study for several lipophilic (and highly permeable) compounds (eg. acid AZ5, Table 1).

The use of *in vitro-in vivo* scaling factors derived from studies in pre-clinical species to predict *in vivo* clearance in humans has been proposed by Naritomi *et al*., (2003). However this approach relies on a (quantitative) similarity in clearance route across the pre-clinical species and humans, and has been contested recently (Ito and Houston, 2005). Previous studies from our

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laboratory (Riley *et al*., 2005) have demonstrated an excellent correlation between predicted $CL_{int\ ub\ in\ vivo}$ determined for 57 drugs from conventional human hepatocyte incubations and observed $CL_{int\ ub\ in\ vivo}$ (50% drugs within 3-fold, 74% within 5-fold). However, there were several drugs for which $CL_{int\ ub\ in\ vivo}$ was significantly under-predicted including prazosin and montelukast (30-fold). Therefore the 'media loss' assay was used to predict $CL_{int\ ub\ in\ vivo}$ for these drugs plus the known OATP substrates bosentan (Treiber *et al*., 2004), atorvastatin and pravastatin (Shitara *et al*., 2006). The excellent prediction of $CL_{int\ ub\ in\ vivo}$ for each of the five drugs studied (within four-fold) validates the use of this approach in humans. Although the $CL_{int\ ub\ in\ vivo}$ for prazosin was predicted within 2-fold using the 'media loss' assay, $CL_{int\ ub\ in\ vivo}$ values determined via conventional human hepatocyte incubations (data not shown) also produced similar predictions. This suggests that the original under-prediction in prazosin $CL_{int\ ub\ in\ vivo}$ was due to an underestimation in metabolic CL_{int} rather than hepatic uptake.

Potential differences in hepatic uptake between rat and human were then investigated using the 36 AZ compounds in Table 1. Figure 4 suggests that for this dataset hepatic uptake in the rat was on average three-fold more rapid (up to 12-fold) than in humans, confirming and expanding earlier reports (Sandker *et al*., 1994; Shitara *et al*., 2006). The uptake rate of estrone-3-sulfate, a known OATP1B1 substrate (Hirano *et al*., 2004), obtained in this study agreed with values determined with previous batches of human hepatocytes (Yamashiro *et al*., 2006) suggesting that this inter-species difference in uptake was not due to human tissue quality.

This report has described a ‘media-loss’ method for determining the hepatic uptake of drugs into rat and human hepatocytes. The utility of this method within an early discovery setting has been highlighted with reference to the successful prediction of *in vivo* clearance for over thirty compounds in the rat. Future studies will focus on a more mechanistic approach to enable a thorough understanding into the active uptake process within hepatocytes in addition to the potential for active efflux within hepatocytes to confound clearance predictions. Further work will also aim to build on preliminary investigations into the effect of cryopreservation on active uptake into human hepatocytes by Shitara *et al*., (2003).

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Footnotes

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Figure Legends

Figure 1 Schematic detailing the processes involved in a 'media loss' CL_{int} determination

Figure 2 Representative concentration-time data for AZ10 (A), AZ14 (B) and AZ20 (C)

Concentration-time data was generated for AZ10 (A), AZ14 (B) and AZ20 (c) using either a conventional rat hepatocyte assay (closed symbols) or via a 'media loss' incubation (open symbols). Concentration data has been used for illustration purposes however ln concentration data was used in the calculation of CL_{int} estimates (see methods for details).

Figure 3 Prediction of $CL_{int\ ub\ in\ vivo}$ for 36 AZ compounds using a conventional rat hepatocyte assay (A), 'media loss' $AUC_{0-\infty}$ (B) or initial disappearance approach (C)

CL_{int} estimates were determined using either a conventional rat hepatocyte assay (A) or via a 'media loss' incubation using either an $AUC_{0-\infty}$ (B) or initial disappearance (C) approach (see Methods for details). Each value represents the mean of 2 or 3 determinations in rat hepatocytes (see Table 1) The solid lines represent a regression analysis (A-line of best fit is given by $y = 0.61x + 2.21$, $r^2 = 0.25$ $p = 0.002$, *afe* = 57; B-line of best fit is given by $y = 0.83x + 1.42$, $r^2 = 0.49$ $p = 1.9 \times 10^{-6}$, *afe* = 16; C-line of best fit is given by $y = 0.84x + 0.79$, $r^2 = 0.72$ $p = 6.3 \times 10^{-11}$, *afe* = 3). The dotted lines represent the line of unity.

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Figure 4 Comparison of rat and human CL_{int} estimates for 36 AZ compounds determined using an initial disappearance approach

CL_{int} estimates were determined in rat and human hepatocytes using an initial disappearance approach (see Methods for details). Each value represents the mean of 2 or 3 determinations in rat hepatocytes (see Table 1) and the mean values from three separate donors of human hepatocytes. The solid line represents a regression analysis (line of best fit is given by $y = 0.66x + 09.29$, $r^2 = 0.52$ $p = 0.25$). The dotted line represents the line of unity.

Table 1 Prediction of $CL_{int\ ub\ in\ vivo}$ using rat hepatocytes for 36 AZ compounds

Compound	Charge	$logD_{7.4}$	fu_{inc}	f_{medium}	Predicted $CL_{int\ ub\ in\ vivo}$ (ml/min/kg)			Observed $CL_{int\ ub\ in\ vivo}$ (ml/min/kg)
					Conventional	$AUC_{0-\infty}$	Dis rate	
AZ1	acid	0.6	0.93	0.04	27, 78	360, 700	7000, 3600	3000
AZ2	acid	1.2	0.84	0.10	42, 27	440, 260	6400, 3800	3100
AZ3	acid	1.8	0.95	0.11	37, 44	160, 100	2200, 3300	3500
AZ4	acid	1.3	0.88	0.10	74, 77	540, 190	4500, 2300	15000
AZ5	acid	3.0	0.60	0.16	170, 110	510, 180	4500, 4400	6200
AZ6	acid	1.5	0.86	0.43	50 ± 2.9	150 ± 55	1400 ± 700	1200
AZ7	acid	2.4	0.72	0.70	360, 270	380, 290	380, 290	380
AZ8	acid	2.2	0.76	0.75	200 ± 150	240 ± 170	390 ± 260	830
AZ9	acid	1.7	0.83	0.81	72, 85	60, 110	65, 110	190
AZ10	acid	1.1	0.90	0.84	50, 41	75, 38	75, 39	210
AZ11	acid	2.0	0.79	0.29	190, 83	490, 210	2000, 1500	10000
AZ12	acid	1.3	0.88	0.24	79, 64	290, 200	2000, 1300	5800
AZ13	acid	1.7	0.83	0.20	170, 90	680, 340	2400, 2300	16000
AZ14	acid	2.2	0.77	0.08	52, 120	360, 600	2200, 3100	4600
AZ15	acid	0.7	0.93	0.40	27, 30	65, 50	840, 580	810
AZ16	acid	-0.3	0.97	0.35	10, 42	55, 60	590, 600	790
AZ17	neutral	3.5	0.47	0.06	170, 510	2200, 1000	2200, 1000	4900
AZ18	base	1.8	0.81	0.30	380, 230	1400, 660	4500, 2900	12000
AZ19	zwitterion	0.7	0.93	0.65	11, 7	35, 27	35, 27	340
AZ20	zwitterion	1.1	0.90	0.07	210, 130	1200, 600	2300, 1400	4400
AZ21	zwitterion	1.4	0.87	0.23	16 ± 1.2	110 ± 100	1000 ± 340	630
AZ22	zwitterion	1.0	0.90	0.33	55, 68	120, 140	390, 1100	2500
AZ23	zwitterion	1.4	0.87	0.25	17, 35	80, 100	600, 730	3500
AZ24	zwitterion	0.4	0.94	0.10	31 ± 5.6	340 ± 62	2800 ± 2700	3700
AZ25	zwitterion	1.7	0.83	0.35	48, 34	95, 141	910, 1800	3700
AZ26	zwitterion	1.4	0.87	0.35	23, 9.8	75, 100	640, 360	1200
AZ27	zwitterion	0.2	0.95	0.45	15 ± 8.9	23 ± 24	390 ± 210	480
AZ28	zwitterion	1.5	0.86	0.35	23, 13	40, 80	580, 440	1600
AZ29	zwitterion	1.4	0.86	0.33	52, 42	140, 130	370, 670	1100
AZ30	zwitterion	0.8	0.92	0.32	22, 17	75, 85	200, 220	220
AZ31	zwitterion	1.1	0.90	0.45	11, 8	30, 50	250, 220	1100
AZ32	zwitterion	0.6	0.93	0.30	28 ± 10	42 ± 32	710 ± 280	1200
AZ33	zwitterion	1.2	0.84	0.43	12, 12	45, 60	200, 130	240
AZ34	zwitterion	1.9	0.81	0.37	16, 19	50, 110	520, 360	1100
AZ35	zwitterion	1.3	0.88	0.37	11 ± 6	57 ± 29	250 ± 10	570
AZ36	zwitterion	2.3	0.75	0.34	27, 37	65, 120	750, 1700	2900

Data represent individual experiments or mean ± SD of three experiments

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Table 2 Prediction of $CL_{int\ ub\ in\ vivo}$ using human hepatocytes for 5 marketed drugs

Compound	Mean CL_{int} (μl/min/10^6 cells)	$CL_{int\ ub\ in\ vivo}$ (ml/min/kg)	
		Predicted	Observed
Montelukast	360 ± 250	1100	2700[a]
Prazosin	12 ± 1.2	36	50[a]
Pravastatin	2.1 ± 1.4	6.6	23[b]
Atorvastatin	100 ± 19	320	910[c]
Bosentan	38 ± 6.5	120	340[a]
Estrone-3-sulfate	80 ± 54	NA	NA

CL_{int} values were determined using a 'media loss' incubation via an initial disappearance approach and represent the mean ± SD from three individual human hepatocyte preparations. CL_{int} values for estrone-3-sulfate were determined via an appearance into cells approach. NA = not applicable

[a] As reported in Riley *et al*., 2005. [b] Lennernäs, 2003 and Schachter, 2004. [c] Pan *et al*., 1987

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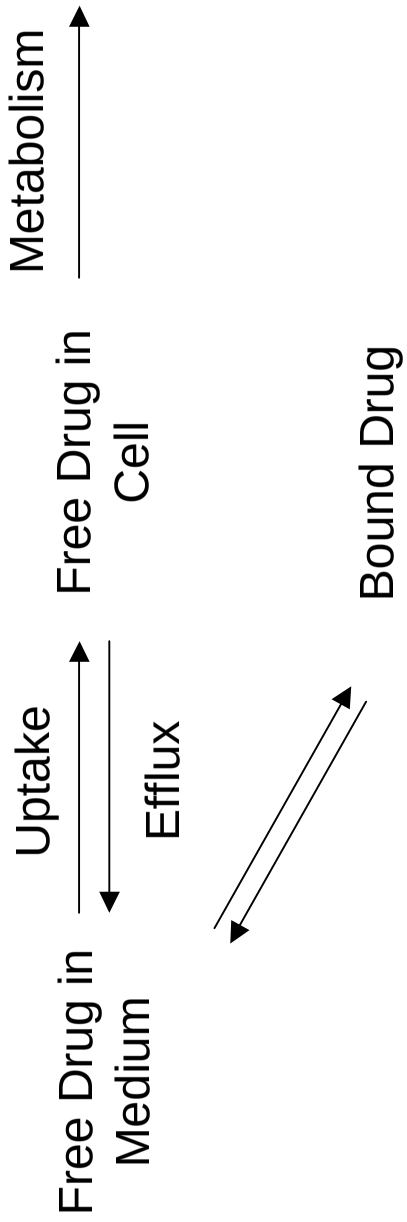
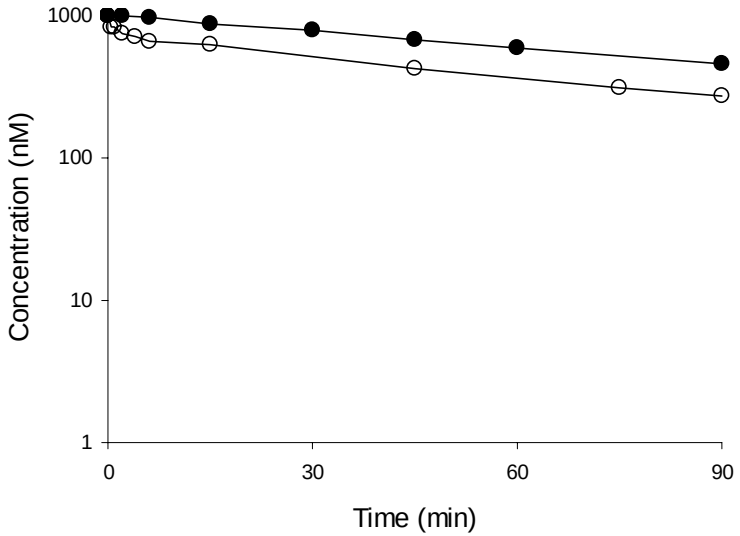


Figure 1

Figure 2A



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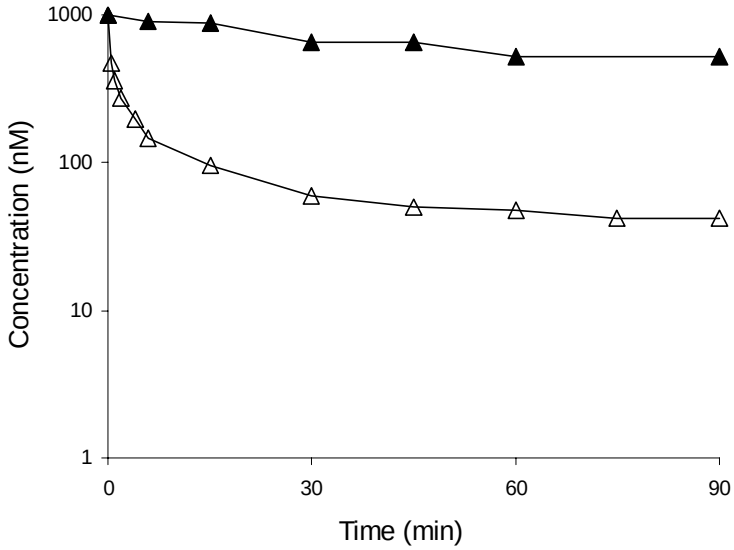

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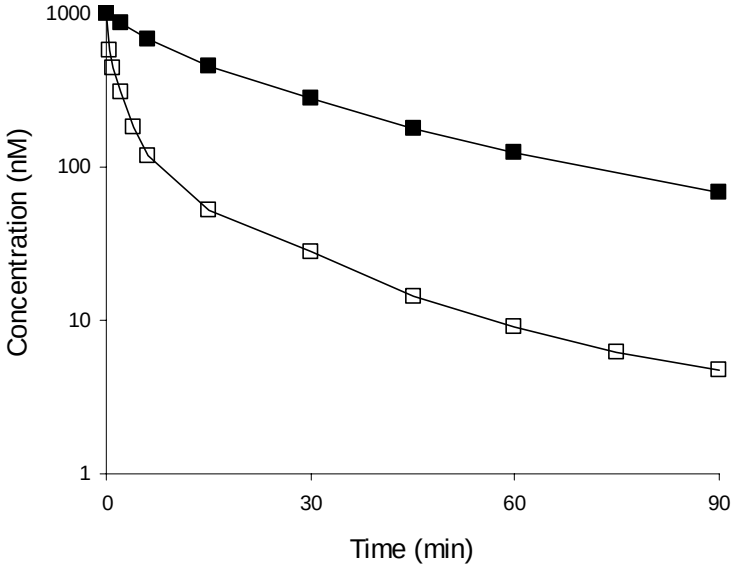
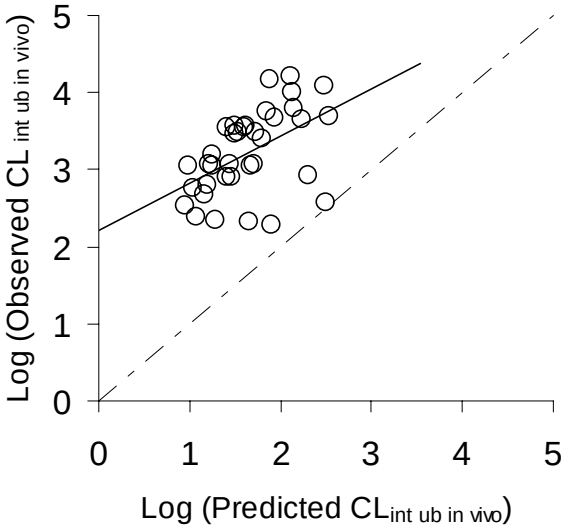


Figure 3A



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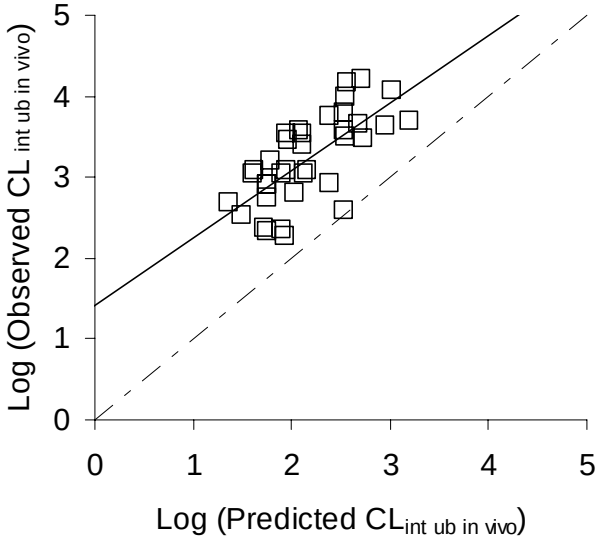

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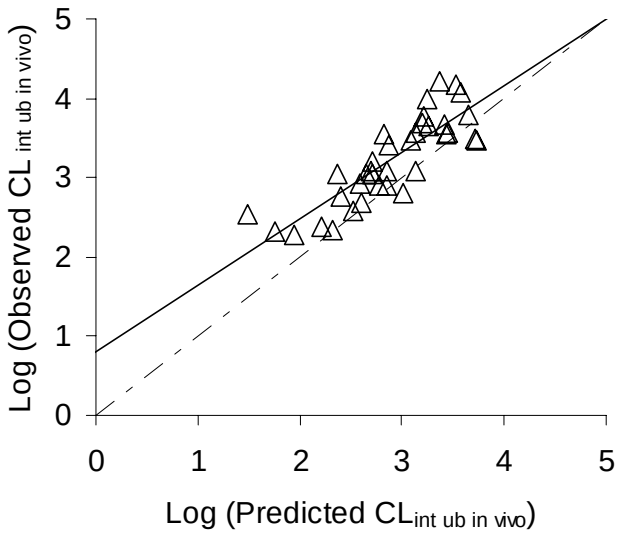

Figure 4