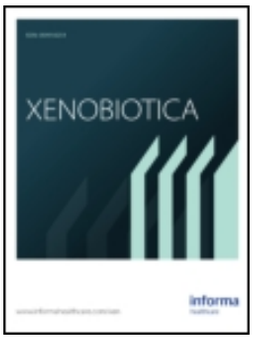

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RESEARCH ARTICLE

Evaluation of the human prediction of clearance from hepatocyte and microsome intrinsic clearance for 52 drug compounds

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Abstract

1. We compare three different approaches to scale clearance (CL) from human hepatocyte and microsome CL_{int} (intrinsic CL) for 52 drug compounds.
2. By using the well-stirred model with protein binding included only 11% and 30% of the compounds were predicted within 2-fold and the average absolute fold errors (AAFE) for the predictions were 5.9 and 4.1 for hepatocytes and microsomes, respectively.
3. When predictions were performed without protein binding, 59% of the compounds were predicted within 2-fold using either hepatocytes or microsomes and the AAFE was 2.2 and 2.3, respectively.
4. For hepatocytes and microsomes there were significant correlations ($P = 8.7 \times 10^{-13}$, $R^2 = 0.72$; $P = 2.8 \times 10^{-9}$, $R^2 = 0.61$) between predicted $CL_{int\ in\ vivo}$ (obtained from *in vitro* CL_{int}) and measured $CL_{int\ in\ vivo}$ (obtained using the well-stirred model). When CL was calculated from the regression, 76% and 70% of the compounds were predicted within 2-fold and the AAFE was 1.6 and 1.8 for hepatocytes and microsomes, respectively. We demonstrate that microsomes and hepatocytes are in many cases comparable when scaling of CL is performed from regression.
5. By using the hepatocyte regression, CL for 82% of the compounds in an independent test set ($n = 11$) were predicted within 2-fold (AAFE 1.4). We suggest that a regression line that adjusts for systematic under-predictions should be the first-hand choice for scaling of CL.

Keywords: *Liver; metabolism; scaling; well-stirred model; in vitro; in vivo*

Introduction

Most drugs are eliminated from the body by hepatic metabolism and the prediction of *in vivo* clearance (CL) from *in vitro* systems therefore plays an important role in the drug discovery process. Much effort has been made to understand the basis of CL prediction, but a consistency within this field is still needed.

Several different approaches for the prediction of CL from hepatocytes and microsomes are reported in the literature. Prediction of human hepatic CL from hepatocyte intrinsic CL (CL_{int}) using the well-stirred model is a standard approach that has been described by several investigators (Hoener, 1994; Obach et al., 1997; Obach, 1999; Blanchard et al., 2005). Studies by De Buck et al. (2007a,b) claim that the highest prediction success is obtained by disregarding non-specific binding both to blood and hepatocytes.

Alternatively, several investigators have shown a correlation between the predicted $CL_{int\ in\ vivo}$ (from *in vitro* systems) and the measured $CL_{int\ in\ vivo}$ (Houston and Carlile, 1997; McGinnity et al., 2004; Riley et al., 2005). For example, by using this approach, McGinnity et al. (2004) studied the *in vivo* CL_{int} of marketed drugs using human hepatocytes and demonstrated that 33 out of 45 drugs (73%) were predicted within 2-fold of the actual *in vivo* CL.

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Hepatocytes contain a complete system of active metabolic enzymes with a natural cellular localization of organelles and cofactors (Brandon et al., 2003; Donato and Castell, 2003; Hewitt et al., 2007). In contrast to microsomes, intact hepatocytes contain both phase I (P450) and phase II (conjugative) enzymes and drug transporters. Previous studies have shown that intrinsic metabolic CLs in fresh and cryopreserved hepatocytes are in many cases comparable (Li et al., 1999; Naritomi et al., 2003; McGinnity et al., 2004; Floby et al., 2009). It has also been demonstrated that certain drug transporter activities are preserved in cryopreserved hepatocytes (Houle et al., 2003; Li, 2007), while further studies are necessary to reveal the full effect of cryopreservation on hepatic drug transport. The *in vivo*-like properties make hepatocytes a valuable tool for the prediction of *in vivo* hepatic CL of xenobiotics (Houston, 1994; Obach et al., 1997; McGinnity et al., 2004; Hallifax et al., 2008). However, when the metabolism is mediated mainly by phase I enzymes, microsomes may be equally well-suited.

In this study, 52 drug compounds, mainly metabolized by the liver, were used to evaluate the prediction success of CL from CL_{int} (in human cryopreserved hepatocytes and microsomes), using three different scaling approaches and all predicted CL values were compared with the corresponding *in vivo* values obtained from the literature. The CL was predicted by using the classic well-stirred model considering both blood protein binding (fu_{bl}) and hepatocyte or microsomal binding (fu_{inc}). Moreover, like DeBuck et al. (2007a,b) we investigated the effect of excluding fu_{inc} and fu_{bl} from the CL prediction calculations. Finally, the correlation between log predicted $CL_{int\ in\ vivo}$ (obtained from *in vitro* CL_{int} by using scaling factors and fu_{bl} and fu_{inc}) and log measured $CL_{int\ in\ vivo}$ (obtained after rearranging the well-stirred model) was investigated and used for scaling of individual compounds, in principal as it has been described by others (Houston and Carlile, 1997; McGinnity et al., 2004; Riley et al., 2005). By using the obtained correlation for hepatocytes, CL was predicted for an additional test set of 11 reference compounds.

Predicted CL from human hepatocytes was compared with predicted CL from human microsomes. This comparison also included 173 AstraZeneca new chemical entities.

Material and methods

Chemicals

The reference compounds acetaminophen, amitryptyline, atenolol, betaxolol, bufuralol, buspirone, carvedilol, chlorpheniramine, chlorpromazine, cimetidine, clozapine, desipramine, dexamethasone, diazepam, diclofenac, diflunisal, diltiazem, diphenhydramine, etodolac, fenoprofen, furosemide, gemfibrozil, glipizide, granisetron, hydrocortisone, ibuprofen, imipramine, ketoprofen, lorazepam, lidocaine, naproxen, methylprednisolone, metoprolol, midazolam, nadolol, naloxone, nifedipine, omeprazole, ondansetron, oxaprozin, oxazepam, phenacetin, pindolol, prazosin, prednisolone, propafenone, propranolol, quinidine, ranitidine, risperidone, sildenafil, tenoxicam, theophylline, tolbutamide, verapamil, warfarin, zolpidem, and William´s medium E were obtained from Sigma Chemical Co. (St. Louis, Mo). Bosentan, cervastatin, dofetilide, lorcainide, montelukast, and irbesartan were from the AstraZeneca compound collection. AZ compounds were synthesized at AstraZeneca R&D Södertälje (Södertälje, Sweden). All other chemicals were of analytical grade and obtained from commercial suppliers.

Pools of cryopreserved hepatocytes

Cryopreserved hepatocytes were from CellzDirect Inc (www.cellzdirect.com) (Durham, NC). Each vial was thawed according to the manufacturer's instructions. Each experiment was performed with pools of hepatocytes from two to five individuals (i.e. pooled after thawing) in order to represent an average human donor pool. Hepatocyte viabilities (as determined by Trypan blue exclusion) after thawing and pooling were routinely ~80–90%. All hepatocyte incubations were performed in 96-well deep microplates (Treff Ag, Degersheim, Switzerland). The hepatocytes were diluted in Williams medium E and 90 µl of the cell suspension was added to 10 µl of a 10 µM solution of each compound so that the final hepatocyte concentration was 1×10^6 cells/ml, and the concentration of compound was 1 µM. Each experiment was in general repeated at least two times and the incubations performed in duplicate at 37°C under an atmosphere of 5% CO_2/95% air and the plates were shaken gently. The hepatocytes were incubated with the compounds for 0, 15, 30, 60, and 90 min and the reactions were stopped by the addition of 200 µl ice-cold acetonitrile. The plates were centrifuged (1900*g* for 5 min) and aliquots of the supernatant analyzed by LC-MS/MS.

Human microsomes

A pool of microsomes from 33 donors (28 male and 5 female), purchased from BD Gentest (Woburn, MA), was incubated at a concentration of 0.5 mg/ml in 96-well microplates in the presence of 1 µM of the drug compound. The reaction was initiated by the addition of NADPH (final concentration 1.5 mM). The final volume was of 100 µl and the incubations were performed in duplicate for 5, 15, 30, and 60 min. The reactions were stopped by the addition of 100 µl acetonitrile, after

which the samples were mixed and centrifuged and the supernatant thus obtained, analyzed. Control incubations (without microsomes) were performed for all compounds.

Determination of plasma protein binding

Plasma protein binding was determined by equilibrium dialysis. Human plasma was obtained from AstraZeneca's internal blood tapping at Clinical Pharmacology and Drug Metabolism and Pharmacokinetics. Plasma from three individuals was pooled and mixed with up to four compounds with a concentration of 10 µM each (in 0.1% dimethylsulfoxide). The compounds were chosen so that their analysis would not interfere. A dialysis membrane obtained from AstraZeneca R&D Mölndal was soaked in Milli/Q water and thereafter placed between two dialysis plate halves with 48 wells obtained from the same source. A volume of 180 µl 0.122 M phosphate buffer, pH 7.4 with 75 mM NaCl was added to each well on one side of the dialysis plate side, and 180 µl of the plasma/compound mix to the opposite side. After incubation on an orbital shaker (4 mm in diameter and 100 rpm) at 37°C for 18 h the samples on both sides of the membrane were analyzed as described below. The fraction unbound (fu) in plasma was calculated from the ratio of the MS-area of the compound in the buffer to the MS-area of the compound in the plasma. Recovery was measured from the ratio of the sum of the mass spectrometry peak areas (MS-areas) in buffer and sample to the MS-area in the plasma/compound mix at zero time. The stability of the compounds was studied, and all compounds were stable during the 18 h incubation. The volume change over time was negligible (<10%).

LC-MS/MS analysis

LC-MS/MS analyses were performed with a Micromass Quattro Micro triple quadrupole (Micromass, Manchester, UK) coupled to two Shimadzu LC-10AD pumps working together as a binary pump (Shimadzu Corporation, Kyoto, Japan), a divert valve (Waters Corporation, Milford, MA) and a CTC HTS PAL autosampler (CTC analytics, Zwingen, Switzerland) equipped with a 20 µl sample loop and a 100 µl syringe (SyrX G100, Hamilton Bonaduz AG, Bonaduz, Switzerland). The software MassLynx (which controls the LC system and the mass spectrometer), which includes QuanLynx (quantification) and QuanOptimize (MS/MS optimization), was used.

A HyPurity C-18, (3 µm, 2.1 × 30 mm, Thermo Electron Corporation, Waltham, MA) analytical column was used. Chromatography was performed using a generic gradient at a flow rate of 0.4 ml/min. The mobile phases consisted of A: 2:98 acetonitrile: 0.1% acetic acid (v/v) and B: 80:20 acetonitrile:0.1% acetic acid (v/v). The gradient conditions were as follows: 0–0.2 min 0% B, 0.2–1.0 min 0–100% B, 1.0–2.5 min 100% B. The total time between injections was 3 min. The mobile phase was eluted to waste for the first 1.3 min, whereas data collection occurred between 1.3 and 2.5 min.

Electrospray ionization in both positive and negative mode (ESI^+/ESI^-) with multiple reaction monitoring (MRM) was used. The capillary voltage was set to 3 kV and the source block and desolvation temperatures were set at 150°C and 350°C, respectively. The cone and desolvation gas flow rates were set to 20 l/h and 910 l/h, respectively (high purity nitrogen). The analyzers were set as follows: the low mass and high mass resolutions on both quadrupoles were set to 13.0 (~0.9 amu FWHM) with ion energies of 0.5 V and 2.5 V. The collision entrance and exit lenses were –1 V and 1 V, respectively. High purity argon was used as a collision-induced dissociation (CID) gas. The CID gas was adjusted to give a collision cell pressure of $\sim 4.5 \times 10^{-3}$ mBar.

The dwell time for each transition was 50 ms. QuanOptimize was used to obtain MRM transitions for all the compounds.

Determination of CL_{int}

The Clint values were obtained from disappearance curves where the substrate concentration was plotted against the time. The concentration of each reference compound in the hepatocyte incubation was fitted to a first-order elimination equation

$$C = C_0 \cdot e^{-k \cdot \Delta t} \quad (1)$$

where C is the measured concentration at any time, C_0 is the concentration at zero-time and k is the elimination constant. The decrease in substrate was exponential over time for all reported Clint values. Curve fit was performed after natural logarithm transformation of the concentration data. Therefore, data could be fit to equation 1 by linear regression and data was considered to fit the equation well when the *P* value was < 0.05. For most compounds the *P* value was < 0.01, whereas for stable compounds with $P > 0.05$ no Clint value was reported (i.e. <LOQ).

CL_{int} was calculated as follows:

$$CL_{int} = k \cdot V \quad (2)$$

where V is the volume of the hepatocyte suspension.

The calculations were performed by employing a Microsoft® Excel 2000 based standardized protocol and XLFit (version 2.1.2, Guildford, UK). All regressions were evaluated by correlation coefficients and *P* values for type I errors.

Calculations of fraction unbound in the blood

The fraction unbound in the blood (fu_{bl}) was predicted as described by others (Masimirembwa et al., 2003; Fagerholm, 2007)

$$fu_{bl} = \frac{(1 - Hct) \times fu_{pl}}{(C_{bl}/C_{pl})} \quad (3)$$

where the haematocrit value (Hct) in man is 0.44, fu_{pl} is the fraction unbound in plasma and C_{bl}/C_{pl} is the blood-to-plasma partition ratio. When the latter was unavailable, a value of 1 was assumed for neutral and basic compounds and 0.55 for acidic compounds.

Prediction of CL using the well-stirred model

Prediction of CL with the well-stirred model (with protein binding in blood and incubation included) was performed as follows:

$$\text{Predicted CL} = \frac{Q_h \times CL_{\text{int } in\ vitro} \times scaling\ \ factors \times fu_{bl}/fu_{inc}}{Q_h + (CL_{\text{int } in\ vitro} \times scaling\ \ factors \times fu_{bl})/fu_{inc}} \quad (4)$$

Prediction of CL was also performed using the well-stirred model disregarding binding (in blood and incubation):

$$\text{Predicted CL} = \frac{Q_h \times CL_{\text{int } in\ vitro} \times scaling\ \ factors}{Q_h + CL_{\text{int } in\ vitro} \times scaling\ \ factors} \quad (5)$$

The liver blood flow (Q_h) was 20.7 ml/min/kg (Davies and Morris, 1993). The other scaling factors were (120×10^6 cells/g liver)×(1500 g liver/70 kg body weight) for hepatocytes and (45 mg protein/g liver)×(1500 g liver/70 kg body weight) for microsomes (Bayliss et al., 1990; Obach et al., 1997; Sohlenius-Sternbeck, 2006).

Establishment of regression lines for the determination of CL

The scaling factors for hepatocytes and microsomes (shown above) were used to determine the predicted $CL_{\text{int } in\ vivo}$:

$$\text{Predicted } CL_{\text{int } in\ vivo} = \frac{CL_{\text{int } in\ vitro} \times scaling\ \ factors \times fu_{bl}}{fu_{inc}} \quad (6)$$

Measured $CL_{\text{int } in\ vivo}$, was calculated after rearrangement of the well-stirred model:

$$\text{Measured } CL_{\text{int } in\ vivo} = \frac{CL_{in\ vivo} \times Q_h}{Q_h - CL_{in\ vivo}} \quad (7)$$

where $CL_{in\ vivo}$ for the 52 compounds were obtained from the literature.

Log measured $CL_{\text{int } in\ vivo}$ was plotted versus log predicted $CL_{\text{int } in\ vivo}$. The reason for not including fu_{bl} in the calculation of measured $CL_{\text{int } in\ vivo}$ (i.e. to have unbound $CL_{\text{int } in\ vivo}$ values on both axes) was that by introducing the same measured value (i.e. fu_{bl}) on both axes of the graph, the uncertainty in the predictions would increase.

Prediction of CL using a regression line

The slope and intercept from the established regression line was used to calculate a predicted measured $CL_{in\ vivo}$ from the predicted $CL_{\text{int } in\ vivo}$. The predicted CL was then calculated as follows:

$$\text{Predicted CL} = \frac{Q_h \times \text{predicted measured CL}_{\text{int } in\ vivo}}{Q_h + \text{predicted measured CL}_{\text{int } in\ vivo}} \quad (8)$$

Fraction unbound in the incubations

The fraction unbound in the hepatocyte incubation was predicted according to Kilford et al. (2008):

$$fu_{hep} = \frac{1}{1 + 125 \times V_R \times 10^{0.072 \times \log P/D^2 + 0.067 \times \log P/D - 1.126}} \quad (9)$$

where V_R is the ratio between the cell volume and the incubation volume and it has a value of 0.005 at the cell concentration of 10^6 cells/ml.

The fraction unbound in the microsome incubation was predicted according to Hallifax and Houston (2006):

$$fu_{mic} = \frac{1}{1 + P \times 10^{0.072 \times \log P/D^2 + 0.067 \times \log P/D - 1.126}} \quad (10)$$

where P is the microsomal protein concentration (0.5 mg/ml).

Statistical analysis

As a measure of the bias, the average fold error (AFE), i.e. the geometric mean fold error, was calculated as follows (Tang et al., 2007):

$$AFE = 10^{\frac{1}{N}\sum log\left(\frac{observed}{predicted}\right)} \quad (11)$$

The average absolute fold error (AAFE), as a measure of the precision, was calculated by using the following equation (Tang et al., 2007):

$$AAFE = 10^{\frac{1}{N}\sum \left|log\left(\frac{observed}{predicted}\right)\right|} \quad (12)$$

The precision was also quantified by the root mean squared prediction error (RMSE), as described by Sheiner and Beal (1981):

Table 1. Hepatocyte and microsome CL_{int}, fu_{pl}, R_b, predicted CL from hepatocytes and microsomes using the regression model, *in vivo* blood CL and fold under-prediction.

Compound and chemical class	Hepatocyte CL_{int} (µl/min/10^6 cells)	Microsome CL_{int} (µl/min/mg protein)	fu_{pl}	R_b	Predicted CL from hepatocytes (ml/min/kg)	Predicted CL from microsomes (ml/min/kg)	*In vivo* blood CL^i (CL_{pl}/R_b) (ml/min/kg)	Fold under-prediction (hepatocytes)	Fold under-prediction (microsomes)
Acids									
Bosentan	<LOQ	14	0.018	0.55[b]	NV	2.1	3.8[a]	NV	1.8
Cerivastatin	6.1	22	0.010[a]	0.55[b]	2.1	2.0	5.3[a]	2.5	2.7
Diclofenac	19	207	0.005[a]	0.55[c]	2.9	4.5	6.4[a]	2.2	1.4
Furosemide	<LOQ	30	0.017	0.55[c]	NV	3.1	2.9[a]	NV	0.9
Gemfibrozil	16	45	0.002	0.55[c]	1.3	1.2	3.1[c]	2.3	2.6
Glipizide	1.0	<LOQ	0.020[a]	0.55[b]	0.95	NV	1.0[a]	1.1	NV
Ibuprofen	6.7	35	0.006[a]	0.55[c]	1.6	1.9	1.5[a]	0.95	0.78
Ketoprofen	3.7	8.1	0.008[a]	0.55[c]	1.2	0.95	2.9[a]	2.4	3.1
Naproxen	2.0	21	0.001	0.55[c]	0.16	0.48	0.19[a]	1.2	0.43
Tolbutamide	1.1	2.1	0.016	0.75[c]	0.89	0.65	0.28[a]	0.31	0.40
Warfarin	<LOQ	2.2	0.008	0.55[c]	NV	0.42	0.10[a]	NV	0.24
Bases									
Amitryptyline	1.9	17	0.041	0.86[d]	7.8	8.0	7.1[a]	0.91	0.89
Atenolol	1.9	14	0.492	1.07[e]	10	9.9	2.3[a]	0.23	0.24
Betaxolol	<LOQ	2.6	0.224[a]	1.0[b]	NV	3.5	3.4[a]	NV	0.98
Bufuralol	5.1	18	0.106[a]	0.80[c]	10	7.5	11[a]	1.1	1.5
Carvedilol	29	167	0.011[a]	0.7[e]	10	9.0	11[a]	1.1	1.2
Chlorpheniramine	1.0	<LOQ	0.302[a]	1.34[e]	6.7	NV	1.9[a]	0.28	NV
Chlorpromazine	17	58	0.027[a]	1.17[c]	16	12	14[a]	0.84	1.1
Cimetidine	13	10	0.520	0.97[e]	17	9.0	8.4[a]	0.49	0.9
Clozapine	7.6	16	0.030	0.87[d]	6.8	3.8	2.9[a]	0.42	0.75
Desipramine	3.3	23	0.093	0.96[c]	11	9.6	11[a]	1.1	1.2
Diltiazem	6.8	31	0.244	1.0[c]	12	4.2	13[a]	1.1	3.1
Diphenhydramine	2.0	9.6	0.258	0.65[d]	9.3	7.7	15[a]	1.6	1.9
Dofetilide	1.9	<LOQ	0.298	0.62[k]	8.4	NV	8.4[a]	1.0	NV
Imipramine	6.6	32	0.062	1.066[c]	15	12	12[a]	0.83	1.0
Lidocaine	6.2	NV	0.254	0.89[c]	12	NV	18[a]	1.5	NV
Lorcainide	5.9	192	0.109[a]	0.77[d]	14	16	21[a]	1.5	1.3
Metoprolol	1.4	4.3	0.436[a]	1.13[c]	8.6	6.0	11[a]	1.3	1.9
Nadolol	3.0	20	0.078[a]	1.0[b]	5.0	5.4	2.9[a]	0.6	0.53
Naloxone	25	14	0.175	1.22[c]	16	NV	19[a]	1.2	NV
Ondansetron	1.0	<LOQ	0.189[a]	0.83[e]	4.4	NV	7.0[a]	1.6	NV
Propafenone	25	194	0.074	0.7[d]	15	14	23[a]	1.6	1.7
Propranolol	7.8	22	0.135	0.83[c]	11	7.7	14[a]	1.3	1.9
Quinidine	4.5	25	0.114	0.87[c]	8.8	7.7	4.6[a]	0.52	0.60
Risperidone	9.7	43	0.089	0.67[e]	11	8.5	8.1[a]	0.77	1.0
Verapamil	13	142	0.072	1.0[b]	15	15	18[a]	1.2	1.2

Table 1. continued on next page

Table 1. Continued.

Compound and chemical class	Hepatocyte CL_{int} (µl/min/10^6 cells)	Microsome CL_{int} (µl/min/mg protein)	fu_{pl}	R_b	Predicted CL from hepatocytes (ml/min/kg)	Predicted CL from microsomes (ml/min/kg)	*In vivo* blood CL^i (CL_{pl}/R_b) (ml/min/kg)	Fold under-prediction (hepatocytes)	Fold under-prediction (microsomes)
Neutrals									
Acetaminophen	1.0	8.1	0.498	1.0[b]	7.6	8.2	4.9[a]	0.64	0.59
Dexamethasone	1.6	10	0.198	0.93[d]	6.2	6.2	3.6[a]	0.57	0.57
Diazepam	1.5	2.2	0.008	0.55[c]	0.74	0.48	0.69[a]	0.72	1.1
Hydrocortisone	8.1	49	0.112[a]	1.0[b]	10	9.3	5.7[a]	0.56	0.6
Lorazepam	<LOQ	23	0.058	1.0[c]	NV	5.4	1.0[a]	NV	0.19
Methylprednisolone	2.2	28	0.143	1.0[c]	6.2	8.3	6.1[a]	1.0	0.7
Midazolam	14	353	0.023	0.55[c]	8.8	13	9.6[a]	1.1	0.8
Nifedipine	13	96	0.030	0.59[c]	7.5	7.8	12.4[a]	1.7	1.6
Omeprazole	3.0	18	0.169	0.62[g]	7.9	7.4	13.6[a]	1.7	18
Phenacetin	6.3	27	0.263	1.0[h]	13	10	21[a]	1.7	2.1
Prazosin	4.3	8.1	0.030	0.7[e]	3.5	2.1	6.7[a]	1.9	3.2
Prednisolone	4.9	<LOQ	0.140[a]	1.0[c]	9.0	NV	2.9[a]	0.32	NV
Sildenafil	8.1	60	0.022[a]	1.0[b]	4.6	11	9.1[a]	2.0	0.29
Tenoxicam	1.0	<LOQ	0.007[a]	0.67[d]	0.29	NV	0.04[a]	0.15	NV
Theophylline	<LOQ	3.1	0.412[a]	0.83[c]	NV	5.0	1.0	0.21	NV
Zolpidem	2.8	13	0.045	0.76[c]	4.0	3.7	5.7[a]	1.4	1.5

[a]Obtained from Obach et al. (2008).
[b]Estimated (0.55 for acids, 1.0 for bases and neutrals).
[c]Obtained from Brown et al. (2007).
[d]Obtained from Obach et al. (1999).
[e]Obtained from Paixao et al. (2009).
[f]Obtained from Youdim et al. (2008).
[g]Obtained from Naratomi et al. (2003).
[h]Obtained from Shibata et al. (2002).
[i]Blood CL calculated from plasma CL (obtained from the indicated references) by dividing by the blood plasma ratio.
NV, no value; LOQ, limit of quantification.

Table 2. Statistical analysis of CL predictions from human hepatocytes.

Statistics ($n = 46$)[a]	Regression model	Well-stirred with protein binding	Well-stirred without protein binding
Within 2-fold (%)	76	11	59
Within 2- to 3-fold (%)	13	13	20
Within 3- to 5-fold (%)	9	26	11
>5-fold (%)	2	50	11
AFE	1.0	5.9	0.8
AAFE	1.6	5.9	2.2
RMSE	3.9	8.0	5.4

[a]Clint was below quantification for 6 of the 52 drug compounds.
AFE, average fold error; AAFE, average absolute fold error; RMSE, root mean squared prediction error.

Table 3. The CL prediction success from hepatocytes for acids, bases, and neutrals.

Chemical class	Regression model	Well-stirred with protein binding	Well-stirred without protein binding
Acids ($n = 8$)			
Within 3-fold (%)	87	0	50
AFE	1.4	21	0.2
AAFE	1.8	21	4.2
RMSE	1.9	3.2	6.0
Bases ($n = 23$)			
Within 3-fold (%)	92	33	79
AFE	0.9	3.6	1.2
AAFE	1.5	3.6	1.8
RMSE	4.3	9.1	5.6
Neutrals ($n = 14$)			
Within 3-fold (%)	86	14	86
AFE	0.9	6.5	0.7
AAFE	1.8	6.5	2.1
RMSE	4.1	7.7	4.5

AFE, average fold error; AAFE, average absolute fold error; RMSE, root mean squared prediction error.

$$\mathrm{MSE} = \frac{1}{N}\sum(\text{predicted} - \text{observed})^2, \mathrm{RMSE} = \sqrt{\mathrm{MSE}} \qquad (13)$$

In all linear regressions, α was set at 5% to avoid false positive results. Regressions were considered statistical significant when $P < \alpha$.

Results

Predictions of CL with the well-stirred model with fu_{bl} and fu_{inc} included

By using the well-stirred model with fu_{bl} and fu_{inc} included, most of the drug compounds with measurable CL_{int} values were under-predicted by 2-fold or more when hepatocytes were used (Tables 1 and 2). With this approach, only 11% of the compounds were predicted within 2-fold. The high degree of under-predictions resulted in an AFE of 5.9. The same pattern was observed for microsomes, where 30% of the compounds were predicted within 2-fold and the AFE was 3.8 (Table 4). The AAFE was 5.9 and 4.1 for hepatocytes and microsomes, respectively.

Using hepatocytes, 0% of the acids, 33% of the bases, and 14% of the neutrals were predicted within 3-fold and the AAFE was 21, 3.6, and 6.5, respectively (Table 3). With microsomes, 0% of the acids, 68% of the bases, and 47% of the neutrals were predicted within 3-fold and the AAFE was 12, 2.6, and 3.6, respectively (Table 5).

Table 4. Statistical analysis of CL predictions from human microsomes.

Statistics ($n = 44$)[a]	Regression model	Well-stirred with protein binding	Well-stirred without protein binding
Within 2-fold (%)	70	30	59
Within 2- to 3-fold (%)	14	16	14
Within 3- to 5-fold (%)	14	14	16
>5-fold (%)	2	41	11
AFE	1.0	3.8	0.5
AAFE	1.8	4.1	2.3
RMSE	4.2	5.8	6.1

[a]Clint was below quantification for 8 of the 52 drug compounds.
AFE, average fold error; AAFE, average absolute fold error; RMSE, root mean squared prediction error.

Table 5. The CL prediction success from microsomes for acids, bases, and neutrals.

Chemical class	Regression model	Well-stirred with protein binding	Well-stirred without protein binding
Acids ($n = 10$)			
Within 3-fold (%)	80	0	40
AFE	1.0	12	0.2
AAFE	2.1	12	5.5
RMSE	1.6	3.0	8.1
Bases ($n = 19$)			
Within 3-fold (%)	95	68	84
AFE	1.1	2.4	0.9
AAFE	1.5	2.6	1.5
RMSE	4.6	7.0	4.5
Neutrals ($n = 15$)			
Within 3-fold (%)	73	47	80
AFE	0.8	3.1	0.5
AAFE	2.0	3.6	2.1
RMSE	4.8	6.3	6.3

AFE, average fold error; AAFE, average absolute fold error; RMSE, root mean squared prediction error.

Predictions of CL with the well-stirred model without regard to fu_{bl} and fu_{inc}

When the prediction of CL from hepatocytes was performed without any binding (*in vivo* and *in vitro*) 59% of the compounds were predicted within 2-fold (Table 2). In this case the AFE was 0.8 and the AAFE 2.2. When the

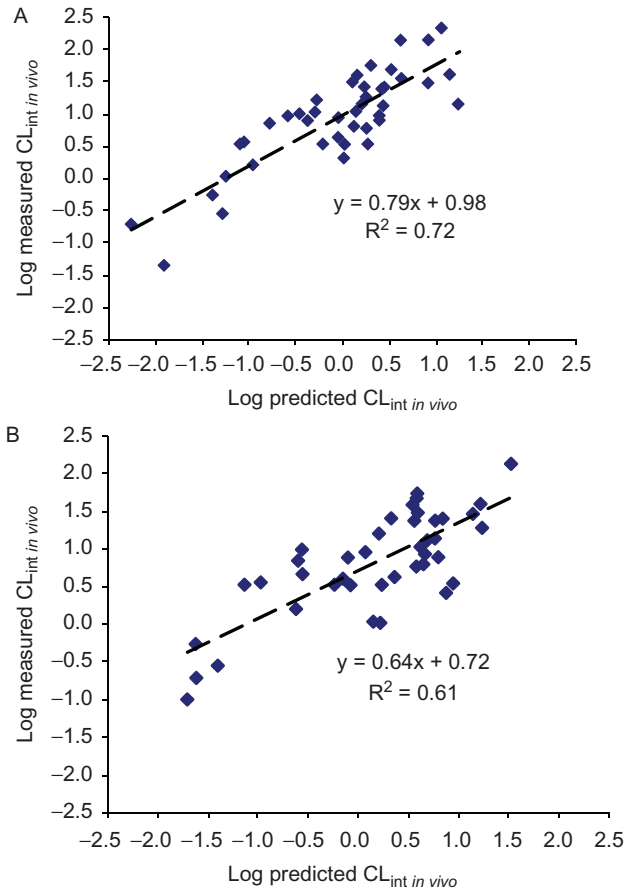

Figure 1. Correlation between the logarithms of measured and predicted $CL_{int\ in\ vivo}$ for (A) human hepatocytes and (B) human microsomes. Measured $CL_{int\ in\ vivo}$, was calculated after rearrangement of the well-stirred model: $\text{Measured } CL_{int\ in\ vivo} = \frac{CL_{in\ vivo} \times Q_h}{Q_h - CL_{in\ vivo}}$

In vitro CL_{int}, fu_{bl}, fu_{inc} and scaling factors (see the Method section) were used to determine the predicted $CL_{int\ in\ vivo}$: $\text{Predicted } CL_{int\ in\ vivo} = \frac{CL_{int\ in\ vitro} \times \textit{scaling factors} \times fu_{bl}}{fu_{inc}}$

same approach was used for microsomes, 59% of the compounds were predicted within 2-fold, and AFE was 0.5 and AAFE 2.3 (Table 4).

Using hepatocytes, 79% of the bases, and 86% of the neutrals were predicted within 3-fold, whereas the corresponding value for the acids was 50%. The AAFE was 1.8 and 2.1 for bases and neutrals, respectively, and 4.2 for the acidic compounds (Table 3). For microsomes, 40% of the acids, 84% of the bases, and 80% of the neutrals were predicted within 3-fold and the AAFE was 5.5, 1.5, and 2.1, respectively (Table 5).

Predictions of CL from a regression line with predicted and measured $CL_{int\ in\ vivo}$

Figure 1A shows a graph with the regression line for predicted $CL_{int\ in\ vivo}$ data from hepatocytes and measured $CL_{int\ in\ vivo}$ data (obtained after rearranging the well-stirred model) for the reference compounds. There was a significant correlation between log predicted $CL_{int\ in\ vivo}$ and log measured $CL_{int\ in\ vivo}$ ($P = 8.7 \times 10^{-13}$, $R^2 = 0.72$) with an intercept and slope of 0.98 ± 0.06 and 0.79 ± 0.08, respectively. When CL was calculated from the regression line, 76% of the compounds were predicted within 2-fold, 13% within 2- to 3-fold, 9% within 3- to 5-fold and 2% of the compounds were predicted over 5-fold (Table 2). The AAFE for all compounds was 1.6. With this approach, 87% of the acids, 92% of the bases, and 86% of the neutrals were predicted within 3-fold and the AAFE was 1.8, 1.5, and 1.9, respectively (Table 3).

Eleven reference compounds were tested using the established regression to predict CL from hepatocytes. Nine of these (82%) were within 2-fold of the actual CL and the AFE was 0.8 and the AAFE was 1.4 (Tables 6 and 7).

In Figure 1B the regression line for predicted $CL_{int\ in\ vivo}$ data from microsomes vs. measured $CL_{int\ in\ vivo}$ data is demonstrated. There was a significant correlation between log predicted $CL_{int\ in\ vivo}$ and log measured $CL_{int\ in\ vivo}$ ($P = 2.8 \times 10^{-9}$, $R^2 = 0.61$) and the intercept was 0.72 ± 0.07 and the slope 0.64 ± 0.08. When CL was predicted from the regression line, 70% of the compounds were predicted within 2-fold and the AAFE was 1.8 (Table 4). Naloxone had a CL_{int} in hepatocytes that was more than twice as high as the corresponding CL_{int} in microsomes, and since this compound is mainly metabolized by glucuronidation (Mistry and Houston, 1987) it was excluded from the regression. For microsomes, 80% of the acids, 95% of the bases, and 73% of the neutrals were predicted within 3-fold and the AAFE was 2.1, 1.5, and 2.0, respectively (Table 5).

Comparisons of prediction of CL from microsomes and hepatocytes

Figure 2A, 2B, and 2C demonstrates comparisons of scaling of CL from CL_{int} in hepatocytes and microsomes for the drug reference compounds using the well-stirred model with or without protein binding (fu_{bl} and fu_{inc}) or using the regression method. All three methods show statistically significant ($P < 0.001$) correlations between hepatocytes and microsomes. The well-stirred model with or without protein binding gives systematically lower predicted CL from hepatocytes than from microsomes, with slopes lower than unity (0.46 ± 0.07 and 0.66 ± 0.08, respectively), whereas the regression method gives a slope close to unity (0.97 ± 0.09). Furthermore, predictions of CL from hepatocytes and microsomes agree better using the regression method ($R^2 = 0.75$) than using the well-stirred model with or without fu_{bl} and fu_{inc} ($R^2 = 0.52$ and 0.65, respectively).

Figure 2D shows the relation between scaled CL from human hepatocytes and microsomes using the regression

Table 6. Hepatocyte CL_{int}, fu_{pl}, R_b, predicted CL from hepatocytes for a test set of compounds using the regression model, *in vivo* blood CL and fold under-prediction.

Substance	Chemical Class	CL_{int} (µl/min/10^6 cells)	fu_{pl}	R_b	Predicted CL (ml/min/kg)	*In vivo* blood CL[e] (CL_{pl}/R_b) (ml/min/kg)	Fold under-prediction
Etodolac	Acid	4.2	0.008	0.60[b]	1.2	1.3[a]	0.95
Fenoprofen	Acid	4.2	0.009[a]	0.55	1.4	1.7[a]	1.2
Montelukast	Acid	8.9	0.001[a]	0.55	2.3	1.3[a]	0.54
Oxaprozin	Acid	2.1	0.001[a]	0.55	0.18	0.07[a]	0.40
Irbesartan	Acid	8.3	0.1[c]	0.55	9.6	4.2[c]	0.44
Oxazepam	Neutral	3.2	0.033	1.11	2.0	0.99[c]	0.50
Buspirone	Base	21.5	0.190	1.0	13.8	16[a]	1.2
Granisetron	Base	2.9	0.35[c]	1.0	8.2	9.1[c]	1.1
Pindolol	Base	2.0	0.840	0.69[b]	11.7	11[c]	1.0
Ranitidine	Base	1.6	0.95[c]	1.0	9.5	9.6[c]	1.0
Timolol	Base	4.1	0.90[c]	0.84[d]	13.8	10.2[c]	0.74

[a]Obtained from Riley et al. (2005).
[b]Obtained from Paixao et al. (2009).
[c]Obtained from Obach et al. (2008).
[d]Obtained from "Brown, et. al., (2007).
[e]Blood CL calculated from plasma CL (obtained from the indicated references) by dividing by the blood plasma ratio.

Table 7. Statistical analysis of CL predictions from human hepatocytes using a test set of compounds.

Statistics ($n = 11$)	Regression model	Well-stirred with protein binding	Well-stirred without protein binding
Within 2-fold (%)	82	11	27
AFE	0.8	6.5	2.3
AAFE	1.4	6.5	3.3
RMSE	2.1	5.4	5.6

AFE, average fold error; AAFE, average absolute fold errors; RMSE, root mean squared prediction error.

method for 173 AstraZeneca new chemical entities (this data set consists of all in house compounds that have measured values for CL_{int} in hepatocytes and microsomes, and fu_{plasma} measured with equilibrium dialysis). Also in this larger dataset, the agreement is good ($R^2 = 0.64$) and the slope is close to unity (0.90 ± 0.10).

Discussion

The prediction of CL has become an important approach for discovery and development of new chemical entities within the pharmaceutical industry. The use of hepatocytes and microsomes in the prediction of CL requires that hepatic CL is the major CL mechanism.

In this study, three different approaches to scale CL from CL_{int} in cryopreserved human hepatocytes and microsomes were employed for 52 drug compounds with known *in vivo* CL and with hepatic CL as the main elimination pathway. The classic well-stirred model was investigated taking into consideration the non-specific binding both in blood as well as the *in vitro* incubation. Moreover, this method was also tested without protein binding considerations. Finally, the correlation between log predicted $CL_{int\ in\ vivo}$ (obtained from *in vitro* CL_{int} by using scaling factors and fu_{bl} and fu_{inc}) and log measured $CL_{int\ in\ vivo}$ (obtained after rearranging the well-stirred model) was investigated and used for scaling of the individual compounds.

Predictions of CL from CL_{int} in hepatocytes or microsomes have been reported using the well-stirred model with fraction unbound in both blood and incubation included in the calculations (Obach et al., 1997; Obach, 1999; Blanchard et al., 2005). A problem with this approach is under-predictions of CL, especially for acids that are highly protein bound (Obach, 1999). When protein binding (in blood and incubation) was included in the calculations we demonstrated that 11% and 30% of the compounds are predicted within 2-fold using hepatocytes and microsomes, respectively. The effect of high protein binding in blood on the prediction of CL is visualized in Figure 3A, where the CL prediction errors are plotted against the fu_{bl} for compounds with fu_{bl} below 0.2.

When predictions were preformed for the well-stirred model disregarding protein binding in blood and incubation, 59% of the compounds with CL_{int} from human hepatocytes and microsomes were predicted within 2-fold. In this case there was a tendency for over-predictions when the fu_{bl} value was very low (Figure 3B), as is often the case for acids. It has also been shown by other groups that the well-stirred model which disregards both blood and incubation protein binding works for bases and neutrals, but not for acids (Obach, 1999). De Buck et al. (2007a,b) have previously claimed that the best prediction of CL is obtained with the classic well-stirred method disregarding both blood and incubation binding. They investigated the prediction success for

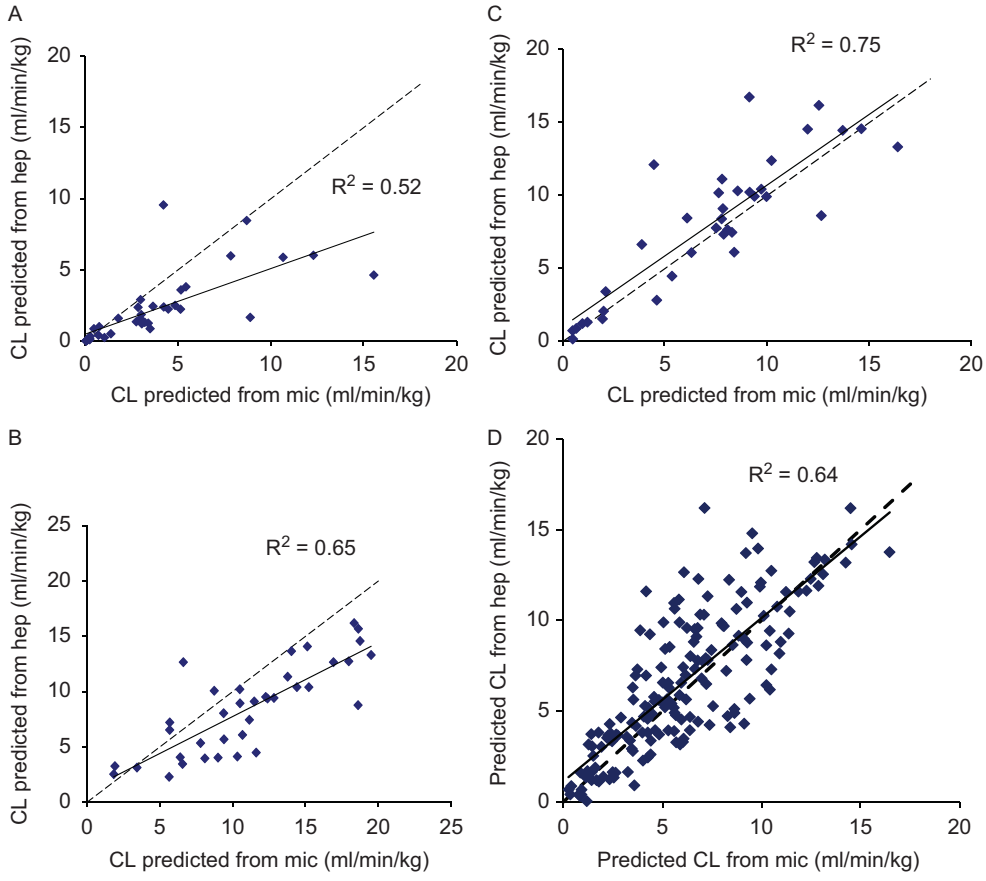

Figure 2. Comparison between predicted CL from hepatocytes and microsomes for the drug compounds using A) the well-stirred model with protein binding incorporated; B) the well-stirred model without protein binding; C) the regression model; and D) the regression model with 173 AstraZeneca new chemical entities. Dotted line, line of identity; solid line, trend line.

21 drug compounds, of which the majority were bases, using human *in vitro* metabolism data (De Buck et al., 2007b) and 50 basic compounds using rat *in vitro* data (De Buck et al., 2007a) and demonstrated good agreement between predicted and observed CL. Thus, their results are in agreement with our results for the basic and neutral compounds using the well-stirred model disregarding all binding. However, it appears that this method is less suited for compounds with high protein binding.

Several investigators have previously demonstrated the power of comparing a predicted $CL_{int\ in\ vivo}$ with the corresponding measured $CL_{int\ in\ vivo}$ (Houston and Carlile, 1997; McGinnity et al., 2004; Riley et al., 2005). For example, Riley et al. (2005) have previously demonstrated a correlation between predicted and measured $CL_{int\ in\ vivo}$ for 56 compounds in human hepatocytes and 37 compounds in human microsomes. Such regressions obtained with reference compounds may be used as tools for scaling of CL in order to adjust for the systematic under-predictions observed when the well-stirred model is employed (Houston and Carlile, 1997; McGinnity et al., 2004; Riley et al., 2005).

In this study, the logarithm of measured $CL_{int\ in\ vivo}$ was plotted versus the logarithm of predicted $CL_{int\ in\ vivo}$. In the calculations of predicted $CL_{int\ in\ vivo}$ (shown on the x-axis) we incorporated $CL_{int\ in\ vitro}$, fu_{bl} and fu_{inc} (predicted), while the only measured parameter incorporated in the calculations of measured $CL_{int\ in\ vivo}$ (shown on the y-axis) was *in vivo* CL. The reason for not including fu_{bl} in the calculation of measured $CL_{int\ in\ vivo}$ (i.e. to have unbound $CL_{int\ in\ vivo}$ values on both axes) was that by introducing the same measured value (i.e. fu_{bl}) on both axes of the graph, the uncertainty in the predictions would increase.

In agreement with other investigators, we demonstrated a good correlation between predicted $CL_{int\ in\ vivo}$ and measured $CL_{int\ in\ vivo}$, both when scaling is performed from hepatocyte and microsome CL_{int}. When CL was calculated from the regression lines, a majority of the compounds with measurable CL_{int} were predicted within 2-fold (76% and 70% for hepatocytes and microsomes, respectively). Moreover, CL for 82% of the compounds in an independent test set ($n = 11$) were predicted within 2-fold using the hepatocyte regression line. These results suggest that a regression line established with reference compounds can be used for the prediction of CL

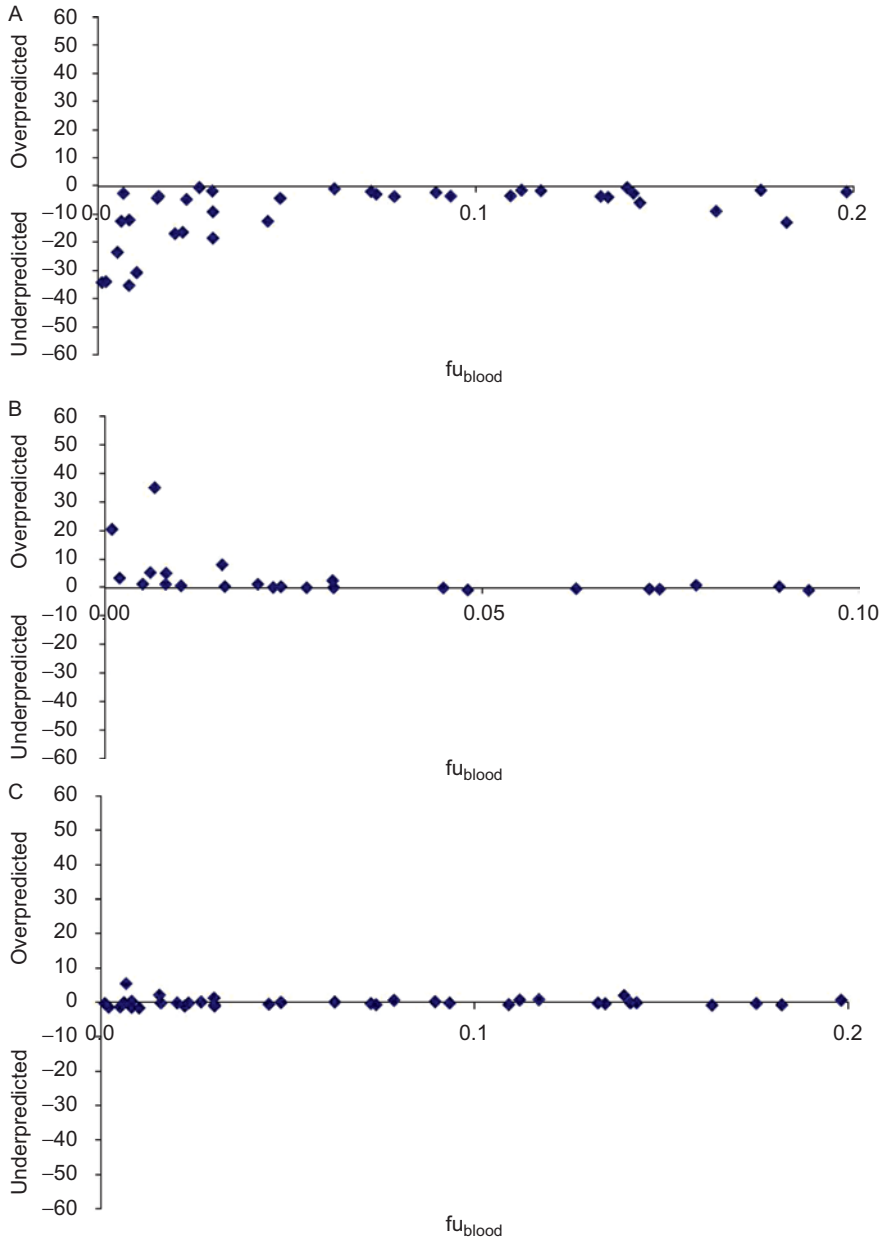

Figure 3. Comparison between prediction errors for hepatocytes and fu_{bl} by using (A) the well-stirred model with protein binding incorporated; (B) the well-stirred model without protein binding, and (C) the regression model.

for independent compounds. A prerequisite is that the compounds are cleared by the liver.

For all compounds in our study, fraction unbound in the incubation was predicted according to Kilford et al. (2008). Using this prediction model, fu_{inc} for compounds with logP/D ≤ 2.5 are predicted with high accuracy. Of the 52 compounds in our data set, 17 had logP/D above 2.5 (data not shown), but the accuracy of the prediction of fu_{inc} for these compounds has not been considered here. However, of the 17 compounds, 15 predicted CL within 2-fold and the remaining two within 3- and 5-fold, respectively, using human hepatocytes and the regression method (data not shown).

A significant correlation and a slope close to unity was observed when scaling from hepatocytes is compared with scaling from microsomes, both for the reference compounds and for 173 AstraZeneca new chemical entities by using the regression method (Figure 2C and 2D). Thus, it appears that predictions of CL from hepatocytes and microsomes are in many cases comparable when using the regression line. This is advantageous, since experiments employing hepatocytes are much more costly than experiments employing microsomes. The prerequisite is, however, that phase II reactions are not major metabolic pathways. For this reason, naloxone which is mainly metabolized by glucuronidation (Mistry

and Houston, 1987) was omitted from the microsomal regression.

We suggest that scaling of CL with a regression line that adjusts for systematical under-predictions should be the first-hand choice for scaling of hepatic CL. However, the slope and intercept of the regression line may be affected by incubation conditions, and it is therefore important that the same assay conditions are used for all included compounds. In other words, any compound that is scaled by using the regression method should be incubated under these same assay conditions. Comparisons of CL_{int} values for microsomes or hepatocytes across different laboratories often demonstrate large differences in the measured values and each lab should presumably use its own regression.

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Declaration of interest

The authors report no conflicts of interests. The authors alone are responsible for the content and writing of the paper.

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