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Prediction of hepatic clearance using cryopreserved human hepatocytes: a comparison of serum and serum-free incubations

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Abstract

Cryopreserved human hepatocytes have been used to predict hepatic in-vivo clearance. Physiologically-based direct scaling methods generally underestimate human in-vivo hepatic clearance. Cryopreserved human hepatocytes were incubated in 100% serum and in serum-free medium to predict the in-vivo hepatic clearance of six compounds (phenazone (antipyrine), bosentan, mibefradil, midazolam, naloxone and oxazepam). Monte Carlo simulations were performed in an attempt to incorporate the variability and uncertainty in the measured parameters to the prediction of hepatic clearance. The intrinsic clearance (CL_{int}) and the associated variability of the six compounds decreased in the presence of serum and the values were reproducible across donors. The predicted $CL_{hep,\ in\text{-}vivo}$ obtained with hepatocytes from donors incubated in serum was more accurate than the prediction obtained in the absence of serum. For example, the $CL_{hep,\ in\text{-}vivo}$ of mibefradil in donor GNG was 4.27 mL min^{-1} kg^{-1} in the presence of serum and 0.46 mL min^{-1} kg^{-1} in the absence of serum (4.88 mL min^{-1} kg^{-1} observed in-vivo). Using the results obtained in this study together with an extended data set (26 compounds), the clearance of 77% of the compounds was predicted within a 2-fold error in the absence of serum. In the presence of serum, 85% of the compounds were successfully predicted within a 2-fold error. In conclusion, cryopreserved human hepatocyte suspensions represented a convenient and predictive model to assess human drug clearance.

Introduction

The prediction of human pharmacokinetic properties of new chemical entities at an early phase of drug discovery and development is of utmost importance. In this context, prediction of hepatic clearance is critical as the liver is the most important organ for metabolism of endogenous and exogenous compounds. Various in-vitro models are available for metabolism studies, such as microsomes, S9 and cellular systems. Hepatocytes are considered to be the method of choice for clearance predictions as a result of their broad spectrum of enzyme activity, physiological cofactor-enzyme levels, active gene expression and cellular integrity (Carlile et al 1998; Li et al 1999b; Roymans et al 2004). It has been shown that human hepatic clearance can be predicted reasonably well from in-vitro data (Obach 2001; Lau et al 2002; Soars et al 2002; Niro et al 2003; Salonen et al 2003; McGinnity et al 2004). Excellent reviews that cover all aspects of the in-vitro to in-vivo scaling procedure have been published (Houston 1994; Iwatsubo et al 1997). Although successful predictions of in-vivo clearance using in-vitro data have been reported in a number of cases, some issues still need to be clarified to further improve prediction accuracy. Approaches based on physiologically-based direct scaling tend to lead to a systematic (approximately 2-fold) underestimation of in-vivo clearance in man (Zuegge et al 2001). Such underestimation may result from extra-hepatic metabolism, the involvement of active transport processes through the sinusoidal membrane and/or inappropriate considerations of in-vitro and in-vivo protein binding (Ito et al 1998). To address the latter issue, a "novel" in-vitro method using rat and human hepatocytes incubated in serum was developed and showed promising results for predicting in-vivo hepatic clearance in rat and man (Shibata et al 2000, 2002; Bachmann et al 2003;

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McGinnity et al 2004). The results reported in those studies were very encouraging and, depending on the analytics, could be used in a high throughput manner with either single donors or pooled cryopreserved hepatocytes. Although freshly isolated human hepatocytes have been shown to be a good model for the prediction of in-vivo metabolism in man (Lave et al 1999), cryopreserved human hepatocytes are becoming more widely used for routine screening (Li et al 1999a, b; Hewitt et al 2001; Naritomi et al 2003). Cryo-preserved human hepatocytes are easily available and have been reported to quantitatively retain most of the phase I metabolic activity observed in the fresh liver (Li et al 1999b; Hengstler et al 2000). However it has been observed that phase II metabolic activity is lower in cryopreserved preparations compared with intact human liver (Li et al 1999b; Steinberg et al 1999; Hengstler et al 2000; Rialland et al 2000). A new thawing technique has been developed which has improved cell recovery and viability by dramatically decreasing the thawing time and the osmotic stress on the cells. This thawing method has resulted in an increased attachment efficiency of hepatocytes from certain donors (Roymans et al 2004) suggesting that overall cell vitality has been improved using this new method.

Information used in the in-vitro–in-vivo scaling procedure for clearance is subject to variability and uncertainty. This may be as a result of variability in expression and activity of certain enzymes or uncertainty related to the assumptions made or the experimental procedure. In general, mean values are used when scaling hepatic clearance; however, incorporation of the variability and uncertainty may help to predict the existing variation and any extremes in the population, resulting in an increased confidence in the prediction. Methodology to incorporate variability and uncertainty into the prediction of in-vivo hepatic clearance have been evaluated (Nestorov et al 2002).

The aim of this study was: to gain more insight into the impact of serum in the in-vitro incubation medium on clearance prediction; to confirm the improvement in the prediction accuracy with human hepatocytes incubated in serum as observed in the rat; to estimate the variability in predicted clearance and the related confidence interval by considering the variability associated with the in-vitro parameters; and to extend the compound data set for which serum or serum-free media were tested with human hepatocyte suspensions. For this purpose, a series of six compounds, phenazone (antipyrine), oxazepam, bosentan, mibefradil, midazolam and naloxone, encompassing a 50-fold range of clearance, a range of protein binding from 1% to 100% and metabolized by a variety of phase I and phase II enzymes, were investigated with three different donors of cryopreserved human hepatocytes in the absence and presence of serum. The six compounds were well known reference compounds.

Materials and Methods

Chemicals and hepatocytes

Twenty-four-well plates (Falcon Cat N° 351147) and cryopreserved hepatocytes (Lots 86, GNG, 107) were purchased from In Vitro Technologies, Inc. (Baltimore, MD, USA). The enzyme characterization data and donor demographics are shown in Tables 1 and 2. The selection of the donors was based purely on enzyme characteristics. Low, medium and high CYP3A4 were chosen and other enzyme activities were of variable ranges.

William's E medium without L-glutamine, without phenol red (041-94198M), penicillin/streptomycin 10 000 IU mL^{-1} (15140-106) and glutamine 200 mM (25030-024) were obtained from Gibco (Life Technologies AG, Basel, Switzerland). Human serum was obtained from EFS Strasbourg and heat inactivated at 56°C for 30 min. All other chemicals were obtained from Sigma (Division of Fluka Chemie AG, Buchs, Switzerland).

Incubation with compounds

Cryopreserved hepatocytes were thawed in William's E medium (supplemented with 10% foetal calf serum, 0.5% streptomycin/penicillin (50 IU mL^{-1}), insulin (1.2×10^{-6} M), glutamine (400×10^{-6} M)), according to the up-dated protocol described by In Vitro Technologies (www.invitrotech.com). Briefly, the cells were thawed in a 37°C water bath and then transferred to a Falcon tube containing 45 mL warmed William's E medium. The cells were centrifuged for 5 min at 60 *g*, at room temperature. After

Table 1 Donor enzyme characterization

Lot	Viable cell recovery (10^6 cells/vial)	Viability after thawing	3A4 (0–250)	2D6 (0–47)	2C9 (0–135)	2A6 (0–135)	2C19 (0–177)	1A2 (0–82)	2E1 (0–87)	Ph II G (0–300)	Ph II S (0–77)
107	8	65%	234	28	41	60	16	7	48	21	BLQ
86	8.7	62%	38	23	13	51	1	BLQ	18	50	9
GNG	8.5	56%	95	17	22	57	5	16	22	54	8

The viability was measured using Trypan blue after thawing. The viable cell recovery refers to the number of viable cells thawed. The activities of CYP 3A4, 2D6, 2C9, 2A6, 2C19, 1A2, 2E1 were characterized using testosterone, dextromethorphan, tolbutamide, coumarin, mephenytoin, phenacetin and chlorzoxazone, respectively, and were expressed as pmol min^{-1}/million cells. Phase II (Ph II) enzyme activity (glucuronidation (G) and sulfatation (S)) was determined using 7-hydroxycoumarin as a substrate and was expressed in pmol min^{-1}/million cells. For each enzyme, the range of activity measured in human hepatocytes obtained from the In Vitro Tech website was stated in parentheses (www.invitrotech.com).

Table 2 Donor demographics and medical history

Lot	Age	Sex	Race	Cause of death	Tobacco use	Alcohol use	Substance abuse	Medical history
107	62	F	C	MVA	No	Yes	No	None provided
86	73	F	C	Cerebrovascular accident	No	No	No	Heart valve replacement, anaemia
GNG	45	F	C	Subarachnoid haemorrhage	Yes	Yes	No	Hypertension, kidney stones

F denotes female. C denotes Caucasian and age is expressed in years. The cause of the death and the medical history are given. All donors were negative for HIV, hepatitis B and C. Additional information as to whether the donor smoked, drank alcohol or used drugs is indicated. MVA, motor vehicle accident.

centrifugation, the supernatant was removed and the pellet was resuspended in William's E medium. The viable recovery and viability of cells from each donor was assessed using 0.4% trypan blue exclusion (the values are shown in Table 1). Thawed hepatocytes were re-centrifuged at 60 *g* for 5 min and re-suspended at a density of 3×10^6 cells mL^{-1} in either 0% serum (William's E medium with 0.5% streptomycin/penicillin (50 IU mL^{-1}), insulin (1.2×10^{-6} M), and glutamine (400×10^{-6} M)) or in 100% human serum. The hepatocyte suspensions were sampled into 24-well plates in half of the final incubation volume (250 μL) and incubated in a thermomixer at 37°C, 300 rev min^{-1} for 30 min (to allow the cells to recover and equilibrate) until the addition of compounds. The final cell density was 1.5×10^6 cells mL^{-1} for all test compounds.

Stock solutions of compounds were prepared in dimethyl sulfoxide (DMSO) at 100 mM for phenazone, and 10 mM for oxazepam, bosentan, mibefradil, midazolam and naloxone. The final concentration of DMSO in the incubation medium was always below 1%.

After a 30-min pre-incubation, 250 μL serum-free medium or human serum, containing a given compound at twice the final concentration, was added to 250 μL hepatocytes suspended in the corresponding medium (serum-free medium or human serum). Phenazone was incubated at a final concentration of 100 μM. Bosentan and midazolam were incubated at a final concentration of 5 μM, and mibefradil, naloxone and oxazepam were incubated at a final concentration of 1 μM. In all cases the concentrations chosen were below the K_m concentrations. Samples were taken up to 300 min, the timing of which was based on the known in-vivo clearance of the compounds. At each time point, 250 μL hepatocyte suspensions were removed from the incubate and added to 500 μL methanol to stop the reaction and to precipitate hepatocellular proteins. The amount of each unchanged parent compound remaining was determined by LC-MS/MS.

LC/MS-MS analysis of parent compounds

Phenazone, bosentan, mibefradil, midazolam, naloxone and oxazepam levels were determined by high performance liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS). Concentrations were calculated against a calibration curve of parent compounds mixed in the sample matrices. The system consisted of a Shimadzu binary gradient HPLC system, a Waters C18 Symmetry column (2.1×30 mm, particle size 3.5 μm) and a Sciex API 3000 mass spectrometer.

A two-component mobile phase, pumped at 0.2 mL min^{-1}, contained the following solvents: A, 20 mM ammonium acetate in Merck KGaA water, pH 3; and B, 100% acetonitrile. An initial isocratic period of 0.5 min at 5% B was followed by a gradient from 5 to 95% B in 2 min, followed by an isocratic period of 95% B for 1 min. Detection was performed in a positive mode for all six compounds. The quantification limit of the assay was 0.26 μM for phenazone, 0.042 μM for bosentan, 0.01 μM for mibefradil, 0.015 μM for midazolam, 0.0031 μM for naloxone and 0.054 μM for oxazepam.

Protein binding

The free fraction in serum and any non-specific binding to plates under the experimental conditions were determined by ultrafiltration. ^{14}C-labelled test compounds (except for oxazepam, which was unlabelled because the ^{14}C derivative was not available) were incubated in 0% or 100% serum (without cells). Measurements were performed at 5 μM, except for phenazone, which was tested at 100 μM. Ultrafiltration was performed in a micropartition system MPS-1 (Centrifree, Amicon) at 37°C by centrifugation at 2000 *g* for 10 min. The resulting ultrafiltrates were assayed either for total radioactivity or by LC/MS-MS (for oxazepam). The percentage protein and non-specific binding for each compound is shown in Table 3. These data were used in the scaling of serum-free in-vitro data to in-vivo data, according to equation 1.

Data analysis

Intrinsic clearance

CL_{int} for the in-vitro hepatocyte incubations was calculated from the ratio of the initial amount (obtained from the actual concentration measured at t = 2 min) of test compound in the incubation medium (A_{TC}) to the number of cells in the incubation medium (n) and the

Table 3 Extent of protein binding of each compound to serum and non-specific binding to the plastic incubation plates (n = 3)

Test compound	% unbound in 100% serum	% unbound in 0% serum
Phenazone	100.0 ± 0.0	100.0 ± 0.0
Oxazepam	5.0 ± 0.0	80 ± 0.0
Bosentan	2.6 ± 1.2	100.0 ± 6.0
Mibefradil	1.2 ± 0.1	100.0 ± 0.0
Midazolam	2.1 ± 0.1	81.7 ± 3.1
Naloxone	59.6 ± 5.0	100.0 ± 0.0

Test compounds were incubated in 0% or 100% serum (both without cells) and the unbound fraction was measured.

corresponding AUC values (its area under the concentration vs time curve in the incubation medium). The AUC during the incubation period (AUC_t) was calculated using the method of linear trapezoids, and then extrapolated to time infinity (AUC_∞) by adding C_t/β to the AUC, where C_t is the predicted concentration at the last sampling time and β is the slope of the terminal phase of the log concentration–time curve, determined by linear regression of the last three or four data points.

Thus: hepatocyte CL_{int} ($\mu L\ min^{-1}/10^6$ cells) = $A_{TC}/n.AUC_\infty$

In-vitro-to-in-vivo scaling
Physiologically-based direct scaling was used to scale the in-vitro clearance (CL_{int}) to the hepatic blood clearance (CL_{hep}) in-vivo using the well-stirred model (equation 1):

$$CL_{hep,\ in\ vivo} = \frac{LBF \cdot \left(\frac{Fu}{Fu'}\right) \cdot CL_{int,invitro} \cdot SF_{dir} \cdot LW}{LBF + \left(\frac{Fu}{Fu'}\right) \cdot CL_{int,invitro} \cdot SF_{dir} \cdot LW} \quad (1)$$

For the calculation of $CL_{hep,\ in\text{-}vivo}$, the values for CL_{int} were converted to $\mu L\ min^{-1}$/million cells. The average liver weight (LW) and liver blood flow (LBF) values used in man were 1800 g and $20\ mL\ min^{-1}\ kg^{-1}$, respectively, for a body weight of 70 kg (Zuegge et al 2001). $SF_{dir} = 1.2 \times 10^8$ cells (g liver)$^{-1}$ corresponded to the hepatocellularity scaling factor to correct the in-vitro hepatocyte intrinsic clearance for the number of cells per gram of liver (Zuegge et al 2001). The parameter Fu represents either the free fraction measured in 100% serum (Fu, to reflect the binding of the compound in the blood) or non-specific binding to plastic (Fu′, in the absence of serum). When incubations were performed in serum it was assumed that Fu = Fu′.

The in-vivo blood clearance values of the various compounds were obtained from literature and compared with the predicted hepatic blood clearance values. When binding to erythrocytes was not known, the blood/plasma ratio was assumed to be unity.

Monte Carlo simulations to incorporate variability into the in-vitro–in-vivo scaling of clearance
A log-normal statistical distribution was assigned to all parameters involved in the scaling of clearance (eqn 1). Information on the variability of these parameters was obtained from the in-vitro data in the case of in-vitro CL_{int} and plasma Fu; and from the literature (Price et al 2003; Wilson et al 2003) in the case of hepatocellularity, liver weight and hepatic blood flow. Each of these distributions was randomly sampled and hepatic clearance was calculated using equation 1. This sampling process was repeated 1000-times to generate a statistical distribution of predicted hepatic clearance. These simulations were all performed in Microsoft Excel.

Statistics
Observed and predicted clearance values were compared using a paired *t*-test. The significance level was set at 0.05.

Results

Figure 1 shows the depletion of all six compounds in hepatocytes from one donor (Lot 107). Phenazone (Figure 1A) was metabolized very slowly with very little of the compound being depleted during the entire 5-h-incubation (only the first 300 min are shown). In contrast, the metabolism of midazolam (Figure 1E) and naloxone (Figure 1F) was complete by 30 min. There was a marginal increase in the concentration of phenazone, bosentan and oxazepam after 2 min and depletion was not seen until after this time point. Incubating the hepatocytes in 100% serum resulted in a slower rate of depletion of all compounds, except for bosentan and phenazone (which was unaffected).

For all compounds tested, the hepatocytes' CL_{int} values from each donor were determined. For each compound, the CL_{int} varied between the donors, especially when the incubation was carried out in serum-free media. For example, naloxone CL_{int} in serum-free medium varied from 6.6 $\mu L\ min^{-1}$/million cells in donor 86 to 200 $\mu L\ min^{-1}$/million cells in donor 107. When these cells were incubated in serum, the CL_{int} values were less variable (20, 3.25 and 25 $\mu L\ min^{-1}$/million cells in donors 107, 86 and GNG, respectively). The effect of incubating compounds in serum was not donor specific or compound specific. In some cases serum caused a decrease in CL_{int} (for example mibefradil and phenazone in donor GNG), whereas other CL_{int} values were unaffected (for example oxazepam, midazolam and naloxone in donor 86). The changes in CL_{int} were not related to the extent of protein binding, as this would be evident in all donors for each compound bound to plasma proteins.

The CL_{int} of midazolam in serum-free media was 37.5, 1.25 and 22.5 $\mu L\ min^{-1}$/million cells in donors 107, 86 and GNG, respectively. CL_{int} was scaled to $CL_{hep,\ in\text{-}vivo}$ using the well-stirred model (Table 4). The predicted $CL_{hep,\ in\text{-}vivo}$ obtained with hepatocytes from donors incubated in serum was more accurate than the prediction

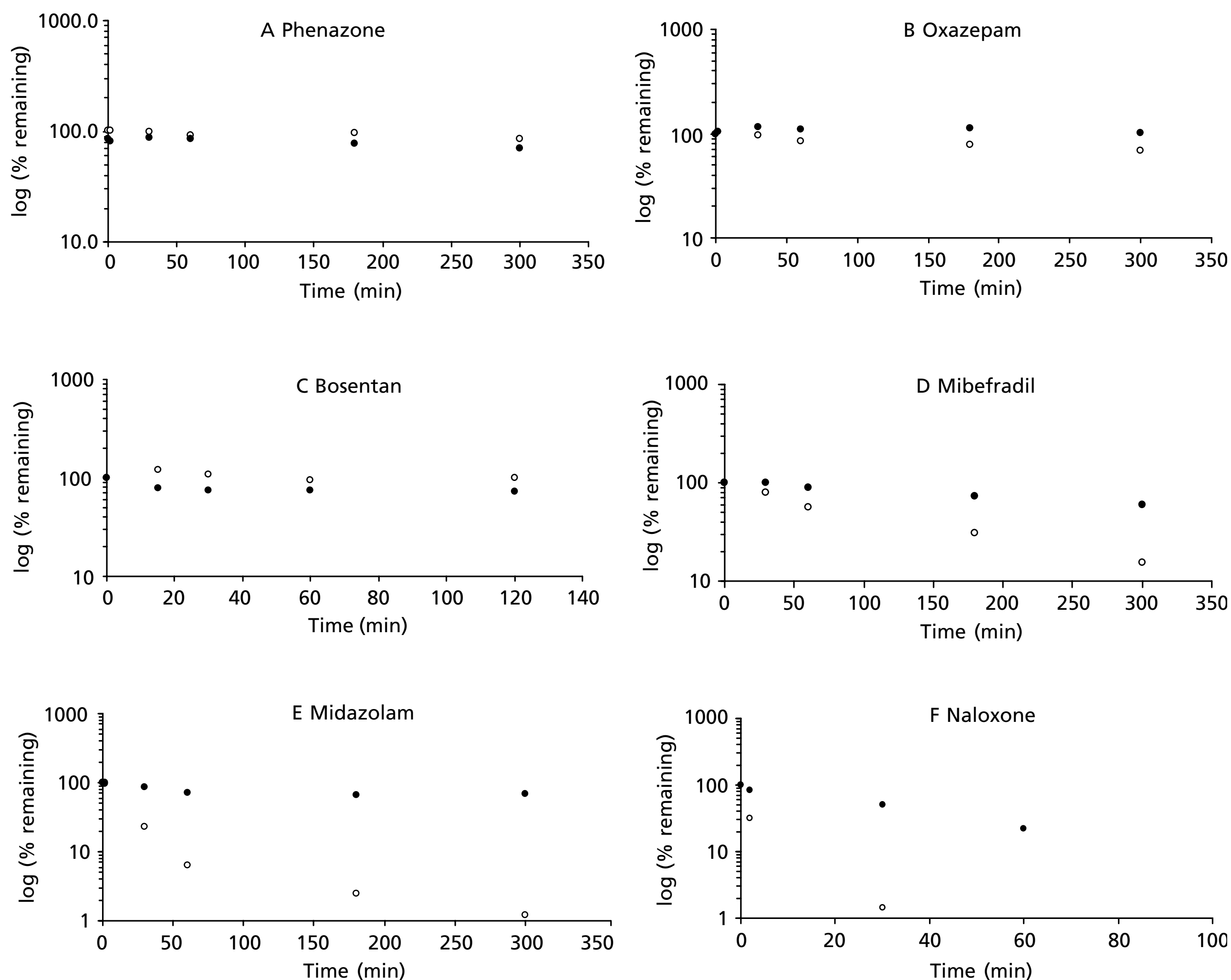

Figure 1 Depletion of parent compounds in cryopreserved human hepatocytes from donor 107 incubated in 0% serum (○) and 100% serum (●).

obtained in the absence of serum. In this case the observed and predicted clearance values were statistically not different whereas in the absence of serum the clearance values were significantly lower than the observed values. For example, the $CL_{hep,\ in\text{-}vivo}$ of mibefradil in donor GNG was $4.27\,mL\,min^{-1}\,kg^{-1}$ in the presence of serum and $0.46\,mL\,min^{-1}\,kg^{-1}$in the absence of serum. The in-vivo CL_{hep} was reported to be $4.88\,mL\,min^{-1}\,kg^{-1}$. However, the two exceptions to this were phenazone and naloxone, two compounds with low plasma binding, whose $CL_{hep,\ in\text{-}vivo}$ were predicted accurately with serum-free and 100% serum. The prediction of $CL_{hep,\ in\text{-}vivo}$ for bosentan, mibefradil and midazolam obtained in serum-free media were all approximately 10-fold lower than the observed $CL_{hep,\ in\text{-}vivo}$. By incubating hepatocytes in 100% serum, the CL_{hep} predictions were improved. The in-vivo $CL_{hep,\ in\text{-}vivo}$ of midazolam was $11\,mL\,min^{-1}\,kg^{-1}$, whereas the predicted CL_{hep} obtained in the presence and absence of serum was 4.83 and $1.44\,mL\,min^{-1}\,kg^{-1}$, respectively.

Figure 2 shows the correlation of the CL_{hep} predicted using cryopreserved human hepatocytes vs the observed in-vivo CL_{hep} (both expressed on a log scale) for all six compounds, combined with data taken from the literature (Shibata et al 2002; Bachmann et al 2003). In these studies, hepatocyte clearance in the absence and presence of serum was compared and full experimental details are included in these literature references. In the absence of serum, six of the 26 compounds considered were underestimated by more than a 2-fold factor. These outliers were bosentan, mibefradil, carbamazepine, phenazone, oxazepam and midazolam. More than 8-fold deviations were observed in the case of bosentan, mibefradil and midazolam. In the presence of serum, only four compounds were predicted with more than a 2-fold error of the observed value (diazepam, midazolam, naloxone, oxazepam) but all compounds were correctly predicted within 3-fold. With the serum incubation method the observed and predicted clearance values were statistically not different whereas in the absence of serum the clearance values were significantly lower than the observed values.

Figure 3 shows the predicted CL_{hep} in-vivo distribution curves in the presence and absence of serum for the six

Table 4 A comparison of predicted and observed clearance values obtained using cryopreserved human hepatocytes

Compound	Percentage of serum in the incubation medium	CL_{hep} predicted ($mL\,min^{-1}\,kg^{-1}$)	CL_{hep} observed ($mL\,min^{-1}\,kg^{-1}$)
Phenazone	0	1.53 ± 0.34	0.58 ± 0.22
100 μM	100	0.69 ± 0.603	0.58 ± 0.22
Oxazepam	0	0.44 ± 0.2	1.52 ± 0.99
1 μM	100	2.9 ± 1.53	1.52 ± 0.99
Bosentan	0	0.14 ± 0.12	3.95 ± 0.76
5 μM	100	2.54 ± 0.81	3.95 ± 0.76
Mibefradil	0	0.46 ± 0.174	4.88 ± 1.08
1 μM	100	4.27 ± 2.18	4.88 ± 1.08
Midazolam	0	1.44 ± 1.25	11.1 ± 3.9
5 μM	100	4.83 ± 1.26	11.1 ± 3.9
Naloxone	0	14.26 ± 6.4	25
1 μM	100	11.54 ± 5.99	25

Test compounds were incubated in 0% or 100% serum, and the observed and measured hepatic clearance for the six compounds.

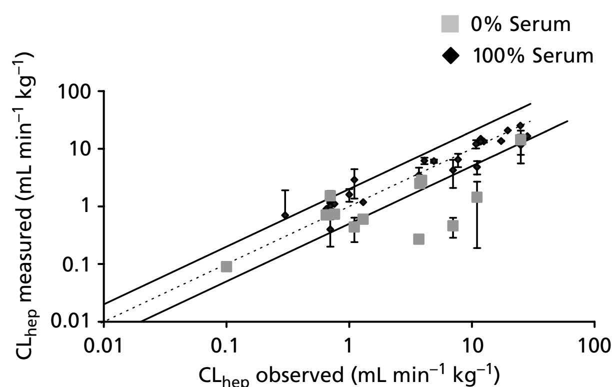

Figure 2 Hepatic clearance measured vs observed of compounds incubated in the absence or presence of human serum. The dashed lines represent lines of unity and the area between the solid line represents an area within a 2-fold error (over or under-prediction of the clearance). The literature data included in this figure were obtained from Shibata et al (2002) and Bachmann et al (2003).

compounds, compared with the distribution curves of the CL_{hep} observed in-vivo. The overlap between in-vivo and in-vitro distributions of CL_{hep} was greatest for phenazone, oxazepam, bosentan and mibefradil. For example, the range of CL_{hep} for oxazepam was between 0.36 and 10 mL min^{-1} kg^{-1} and 0 and 8 mL min^{-1} kg^{-1}for in-vivo and in-vitro (serum) distributions, respectively. The peak frequency was approximately 2 mL min^{-1} kg^{-1} in both in-vivo and in-vitro distributions, showing a good overlap (Figure 3B). The range of CL_{hep} in-vivo for mibefradil was between 2 and 10 mL min^{-1} kg^{-1}, with a peak frequency at 5 mL min^{-1} kg^{-1}. Although the in-vitro distribution was much broader (between 0 and 18 mL min^{-1} kg^{-1}, Figure 3D) it covered the in-vivo range and had a similar peak frequency (approximately 4 mL min^{-1} kg^{-1}). The distribution of CL_{hep} for midazolam and naloxone was under-predicted using the serum and serum-free models, the peak frequencies of CL_{hep} in-vitro being approximately 4-fold lower than the in-vivo peak frequencies. The serum-free in-vitro predictions resulted in narrower ranges of CL_{hep} than corresponding serum predictions and, with the exception of phenazone and naloxone, a large shift of the range to the lower clearance values.

Discussion

With the increasing number of compounds that are being synthesized, there is growing pressure, not only to test them at much higher speeds, but to do this with a high degree of accuracy. Clearance data has traditionally been generated from human and rat liver microsomal incubations, however subcellular fractions do not represent the whole metabolic process that occurs in the liver and there is a need to move towards hepatocyte incubations (Shibata et al 2000, 2002; Bachmann et al 2003). Although it is necessary to determine the clearance in rats to interpret in-vivo data produced in toxicology and pharmacokinetic studies, these data cannot be relied upon to predict clearance in man. Therefore, human clearance predictions are best carried out in hepatocytes from the most relevant species i.e. man. Fresh cells are in greater demand but their sporadic supply does not allow for scheduling of experiments. Cryopreserved human hepatocytes are the best alternative to fresh hepatocytes. These cells have been shown to have equivalent phase I and II enzyme activity to freshly isolated cells and are now used routinely by many researchers as an alternative to fresh cells (Li et al 1999a, b; Hewitt et al 2001; Naritomi et al 2003). Suspensions of rat (Shibata et al 2000; Blanchard et al 2004) and human hepatocytes (Shibata et al 2002; Bachmann et al 2003; Blanchard et al 2005) were shown to represent a predictive in-vitro model for hepatic metabolic clearance. Moreover, we have shown previously that CL_{hep} obtained with suspensions of fresh or cryopreserved hepatocytes from a given donor provided similar results.

The data obtained in this study supported the high predictive capacity of the serum method and confirmed previous findings (Shibata et al 2002; Bachmann et al 2003). Using the results obtained in this study together with an extended data set (26 compounds), the clearance of 85% of the compounds was successfully predicted within a 2-fold error (and 100% within 3-fold). In the absence of serum, 77% of the compounds were predicted within a 2-fold error. Interestingly, compounds such as bosentan and mibefradil, which were poorly predicted in previous studies (Zuegge et al 2001), where hepatocytes were incubated in the presence of foetal calf serum (10%) as well as with the serum-free method, were accurately predicted with the serum incubation method. In this study, the predictions of average clearances and its associated variability was made using three individual batches of human hepatocytes. One of the goals of this study was to go beyond predictions of average parameters and give some idea about the variability and uncertainty of the predicted

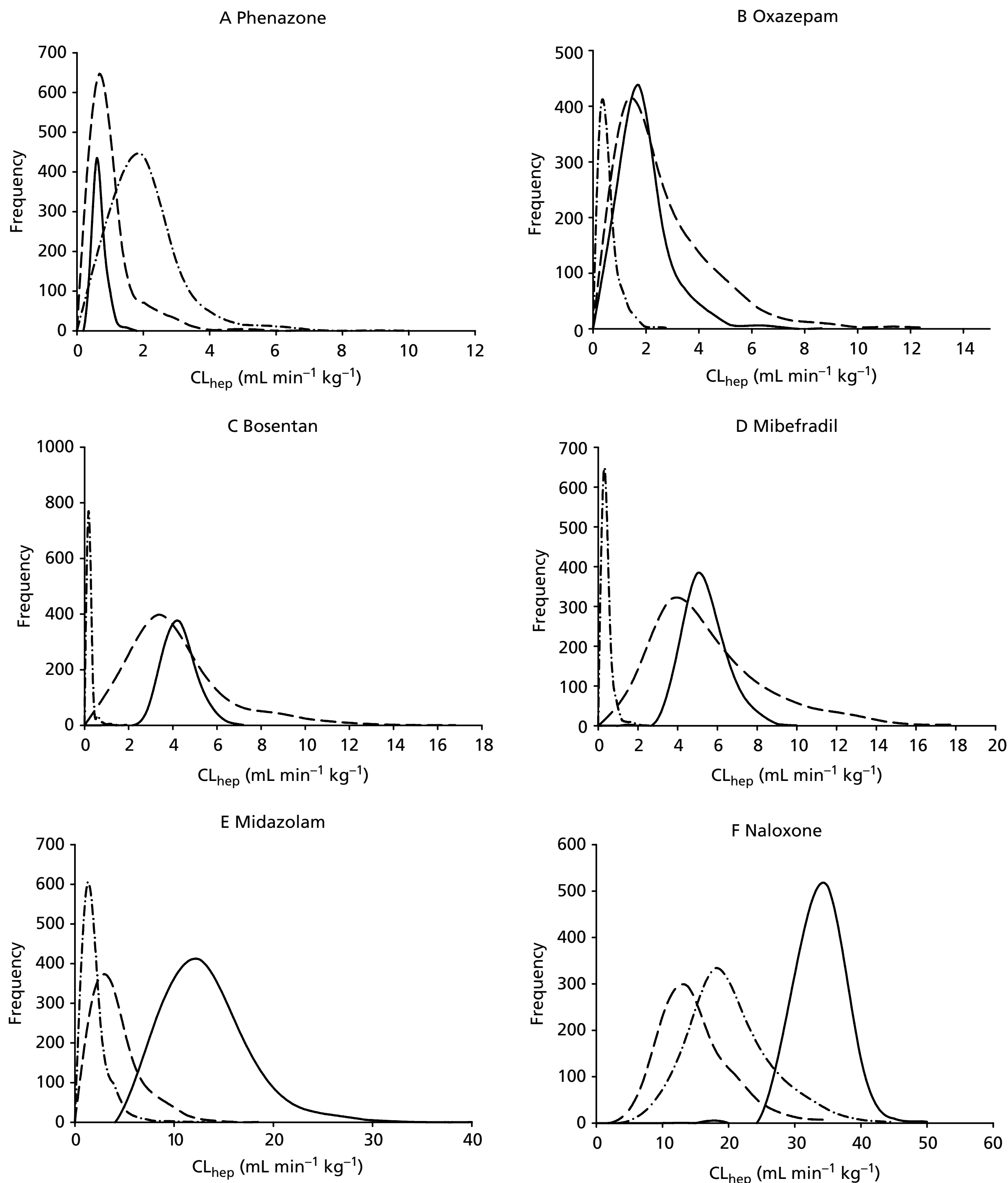

Figure 3 Distribution curves of hepatic clearance observed in-vivo (—) or scaled from in-vitro data either in absence of serum (–·–·–·) or in presence of serum (– – –) obtained by incorporating measures of variability and uncertainty. A log-normal statistical distribution was assigned to in-vitro CL_{int}, plasma Fu, liver weight and hepatic blood flow. Each of these distributions was randomly sampled and hepatic clearance was calculated using equation 1. This sampling process was repeated 1000 times to generate a statistical distribution of predicted hepatic clearance.

CL_{hep}. We have shown that, with the exception of midazolam and naloxone, the distribution curves of CL_{hep} obtained in the presence of serum showed considerable overlapping with the distribution curves of CL_{hep} in-vivo. The under-predictions observed for naloxone, in serum and serum-free incubations, was explained by the fact that the scaling of CL_{int} to predict in-vivo CL_{hep} was limited by the liver blood flow (20 mL min^{-1} kg^{-1}), whereas the observed

in-vivo CL_{hep} was markedly higher than the liver blood flow. The well-stirred model was used for scaling in this study. It was shown that this model tended to under-predict clearances of high clearance substrates and that improved predictions could be achieved using the dispersion model for example (Iwatsubo et al 1996). The in-vitro prediction of CL_{hep} in the absence of serum generally under-predicted the CL_{hep} obtained in-vivo, even when taking into consideration variability and uncertainty. In this study, the in-vitro experiments were carried out in human hepatocytes from only three donors, which were carefully selected to cover a wide range of metabolic activity. Without such information and/or with a limited number of donors (three in total), the relevance of a distribution curve must be considered cautiously.

By incubating hepatocytes in 100% serum, the CL_{hep} predictions were much improved. Values were increased from 2- to 18-fold as compared with CL_{hep} predictions based on serum-free hepatocyte medium, making them closer to the observed $CL_{hep,\ in\text{-}vivo}$. Taking into consideration hepatocyte binding might represent an opportunity to improve the predictions in the serum-free incubation conditions (Riley et al 2005). This was not expected to be the case when hepatocytes were incubated in serum since hepatocyte binding is deemed to be much less than plasma protein binding (Riley et al 2005). The impact of serum on the clearance estimate might involve mechanisms going beyond plasma binding of the drug and the free drug concentration hypothesis. Iwatsubo et al (1996) reported that the rate of hepatic uptake of compounds that were highly bound (Fu $< 10\%$) to albumin in blood (as for bosentan, mibefradil and midazolam) did not necessarily depend upon the unbound drug concentration in the extracellular space. It was suggested that the protein-bound drug was in some way "intimately" involved in the hepatic uptake process beyond its role of passive storage and "replenishing" the unbound fraction (Burczynski et al 2001). In a number of cases, the uptake rate appeared to be determined more by the bound than the unbound ligand concentrations (Forker et al 1982; Forker & Luxon 1983; Burczynski et al 1989). Independently of the presence of serum in the incubation medium, the same total concentration of the various reference compounds was used in the incubation medium. Thus, for highly bound compounds, the different unbound concentrations used in the presence and absence of serum may account to a certain degree for the differences in CL_{int} for highly protein-bound compounds. However, for highly bound compounds, such as midazolam and mibefradil (with free fractions of 0.02 and 0.01, respectively), the unbound concentration in the presence of serum was 50- to 100-fold lower than in absence of serum. The CL_{int} values obtained in these different concentration ranges might be associated with the involvement of different metabolic processes, therefore perhaps accounting partly for the differences in CL_{int} and CL_{hep}. Another possible explanation might be that some processes (facilitated, active transport) are activated in the presence of serum. For example, in the absence of serum, CL_{int} will be determined mainly by metabolism. In contrast, other processes might be activated in the presence of serum, such as active uptake, which might lead to different V_{max}, K_m and CL_{int} values. Without any mechanistic information on the liver disposition of the compound and given the unknown status of the transporters in human cryopreserved hepatocyte suspensions, it was difficult to draw any further conclusions from this study.

Conclusion

Cryopreserved human hepatocytes represent a convenient and predictive tool for drug metabolism studies, whichever incubation medium is used. The accurate determination of the drug depletion profile could be problematic for highly bound and/or low clearance compounds in the presence of serum. In these cases, the rate of depletion will be slower, therefore the sensitivity of the analytics must be high. However, the presence of serum in the incubation for most compounds appeared to be beneficial, resulting in an improved prediction accuracy, avoiding the need for protein binding measurements.

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