

PREDICTION OF HEPATIC CLEARANCE USING CRYOPRESERVED HUMAN HEPATOCYTES: A COMPARISON OF SERUM AND SERUM-FREE INCUBATIONS



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Abstract

We report the use of cryopreserved human hepatocytes to predict hepatic *in vivo* clearance of six compounds (antipyrine, bosentan, mibefradil, midazolam, naloxone and oxazepam). Hepatocytes from three donors were incubated in 100% serum and in serum-free medium. Monte Carlo simulations were performed 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 presence of serum and the values were more reproducible between donors. The predicted $CL_{H, in vivo}$ obtained with hepatocytes incubated in serum was more accurate than that obtained in the absence of serum. For example, the $CL_{H, in vivo}$ of mibefradil in donor GNG was 4.27 ml/min/kg in the presence of serum and 0.46 ml/min/kg in the absence of serum (4.88 ml/min/kg observed *in vivo*). Using the results obtained in this study together with an extended data set (26 compounds), the clearance of 89% of the compounds was predicted within 2-fold error in the absence of serum. In the presence of serum, all compounds were successfully predicted within a 2-fold error.

In conclusion, cryopreserved human hepatocyte suspensions represent a convenient and predictive model to assess human drug clearance.

Introduction

Hepatocytes are considered to be the method of choice for clearance predictions due to their broad spectrum of enzyme activities, physiological cofactor-enzyme levels, active gene expression and cellular integrity (Li *et al.* 1999). Although successful predictions of *in vivo* clearance based on *in vitro* data have been reported, some issues still need to be clarified to further improve prediction accuracy. A "novel" *in vitro* method of hepatocytes incubated in serum has been developed for predicting *in vivo* hepatic metabolic clearance in rat and man (Shibata *et al.*, 2000; Blanchard *et al.*, 2005).

The aim of the present study was to: (1) determine the impact of serum in the *in vitro* incubation medium on clearance prediction; (2) determine if clearance could be attributed to the enzyme characteristics of the donors; (3) estimate the variability in predicted clearance and the related confidence interval by considering the variability associated with the *in vitro* parameters; and (4) extend the compound data set for which serum or serum-free media were tested with human hepatocyte suspensions. For this purpose, a series of 6 compounds, 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. Bosentan is an in-house F. Hoffmann - La Roche AG compound and the other five are well known reference compounds.

Materials and Methods

Incubation with compounds

Cryopreserved hepatocytes from In Vitro Technologies, Inc. (Baltimore, MD, USA) were thawed in William's E medium (supplemented 0.5% streptomycin/penicillin (50 IU/ml), insulin (1.2 x 10⁻⁶M), glutamine (400 x 10⁻⁶M) with 10% FCS, according to the up-dated protocol described by In Vitro Technologies (www.invitrotech.com). Thawed hepatocytes were incubated in either 0% serum (William's E medium) or 100% human serum. Hepatocyte suspensions were aliquoted into 24-well plates and pre-incubated in a thermomixer at 37°C, 300rpm for 30min before the addition of compounds. The final cell density was 1.5 x 10⁶ cells/ml for all test compounds. Stock solutions of compounds were prepared in DMSO. Final concentrations were: antipyrine = 100µM; bosentan and midazolam = 5µM; mibefradil, naloxone and oxazepam = 1µM. Samples were taken up to 300min. The amount of each unchanged parent compound remaining was determined by LC-MS/MS.

Protein binding

The free fraction in serum and the non-specific binding to the 24-well plates under the experimental conditions were determined by ultrafiltration. Test compounds were incubated in 0% or 100% serum (both without cells).

Data analysis

The intrinsic clearance CL_{int} was estimated using the parent compound depletion profile as the ratio of the initial amount of compound in the incubation medium and the corresponding area under the concentration time curve, $AUC_{(0,inf),in vitro}$. Physiologically-based direct scaling was used to extrapolate the *in vitro* clearance CL_{int} to the hepatic blood clearance $CL_{H, in vivo}$ using the well-stirred model (equation 1):

$$CL_{H, in vivo} = \frac{LBF \times (fu/fu') \times CL_{int, in vitro} \times SF_{dir} \times RLW}{LBF + \{ (fu/fu') \times CL_{int, in vitro} \times SF_{dir} \times RLW \}}$$

Relative liver weight (RLW) = 25.71g/kg body weight, liver blood flow (LBF) = 20 ml/min/kg, SF_{dir} = direct scaling factor = 1.2 x 10⁶ cells/g liver. "fu" = 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'.

Monte Carlo simulations were carried out 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 (see equation 1). Information on the variability of these parameters was obtained from published data on *in vitro* CL_{int} plasma fu; hepatocellularity (Wilson *et al.*, 2003) and physiological parameters (Price *et al.*, 2003). Each of these distributions was randomly sampled and hepatic clearance was calculated. This sampling process was repeated 1000 times to generate a statistical distribution of predicted hepatic clearance. These simulations were all performed in Microsoft Excel.

Results

For each compound, the CL_{int} varied between the donors, but the variability was less when these cells were incubated in serum (Figure 1). The effect of incubating compounds in serum was not donor-specific or compound-specific. The changes in CL_{int} were not related to the extent of protein binding (shown in Table 2).

The CL_{int} and $CL_{H, in vivo}$ of four of the six compounds incubated in serum could be related to the enzyme characteristics of the donors (Table 1). For example, CL_{int} and $CL_{H, in vivo}$ for midazolam was directly correlated to the CYP3A4 activity of the cells (testosterone 6β-hydroxylase activity). Both CL_{int} and $CL_{H, in vivo}$ of mibefradil, RO-X and oxazepam were correlated to the combined activities of more than one enzyme. The CL_{int} of naloxone and antipyrine was not linked to the enzyme activities of the donors.

Figure 3 shows the correlation of the predicted $CL_{H, in vivo}$ determined from cryopreserved human hepatocytes and observed *in vivo* clearance, combined with data taken from the literature (Shibata *et al.*, 2002; Bachmann *et al.*, 2003). The predicted $CL_{H, in vivo}$ obtained with hepatocytes from donors incubated in serum were superior to the prediction obtained in absence of serum. The two exceptions to this were antipyrine and naloxone, two compounds with low plasma binding, whose $CL_{H, in vivo}$ were predicted by incubations in both serum-free media and 100% serum. The prediction of $CL_{H, in vivo}$ for bosentan, mibefradil and midazolam obtained in serum-free media were all approximately 10-fold lower than the observed $CL_{H, in vivo}$. By incubating hepatocytes in 100% serum, the $CL_{H, in vivo}$ predictions were increased from 2- to 18-fold, making them closer to the observed $CL_{H, in vivo}$. In absence of serum, only three compounds of the 26 were underestimated with a more than 2-fold factor. However, in presence of serum, all 26 compounds were within the 2-fold error margin.

Figure 3 shows the distribution curves of the predicted $CL_{H, in vivo}$ in the presence and absence of serum for the six compounds and compares them with the distribution curves of the $CL_{H, in vivo}$ observed *in vivo*. The overlap between *in vitro* and *in vivo* distributions of $CL_{H, in vivo}$ was greatest for antipyrine, oxazepam, bosentan and mibefradil. The distribution of $CL_{H, in vivo}$ of midazolam and naloxone were underpredicted by both serum and serum-free models. The serum-free *in vitro* predictions resulted in narrower ranges of $CL_{H, in vivo}$ than corresponding serum predictions and, with the exception of antipyrine and naloxone, a large shift of the range to the left (reflecting a 2- to 4-fold underprediction). The distributions of $CL_{H, in vivo}$ of antipyrine and naloxone were shifted to the right in serum-free predictions. In the case of antipyrine, this resulted in an overprediction (of about 2-fold) of the $CL_{H, in vivo}$ distribution of naloxone $CL_{H, in vivo}$ was higher than either serum and serum-free distributions of $CL_{H, in vivo}$ (Figure 4F).

Figure 1. Intrinsic clearance of six test compounds, determined in cryopreserved human hepatocytes from three donors in the presence and absence of serum.

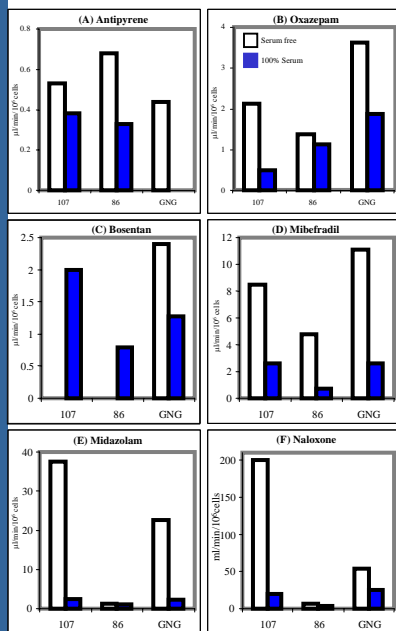


Table 1. Hepatocyte characteristics. The activities of CYP3A4, CYP2D6, CYP2C9, CYP2A6, CYP2C19, CYP1A2, CYP2E1, UGT1A6 and SULT1A1 were characterized using testosterone, dextromethorphan, tolbutamide, coumatil, methylphenytoin, phenacetin, chlorzoxazone and 7-β-hydroxycoumatil, respectively.

Lot	Viability	Enzyme activities (pmo/min/million cells)								
		CYP3A4	CYP2D6	CYP2C9	CYP2A6	CYP2C19	CYP1A2	CYP2E1	UGT1A6	SULT1A1
107	65%	234	28	41	60	16	7	48	21	BLQ
86	62%	38	23	13	51	1	BLQ	18	50	9
GNG	56%	95	17	22	57	5	16	22	54	8

Table 2. Extent of protein binding of each compound to serum and non-specific binding to the plastic incubation plates.

Test compound	% unbound in 100% serum	% unbound in 0% serum
Antipyrine	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

Table 3. A correlation of donor enzyme characteristics with CL_{int} and $CL_{H, in vivo}$.

Compound	Enzyme(s) involved in metabolism	Parameter	R ² value serum free	R ² value 100% serum
Antipyrine	CYP1A2 x CYP3A4 x CYP2A6 x CYP2C9 x CYP2E1 x CYP2D6 x UGT	CL_{int}	0.22	0.09
		$CL_{H, in vivo}$	0.14	0.09
Oxazepam	UGT	$CL_{H, in vivo}$	0.09	0.80
		CL_{int}	0.28	0.80
Bosentan	CYP3A4 x CYP2C9	CL_{int}	0.12	0.95
		$CL_{H, in vivo}$	0.12	0.92
Mibefradil	CYP3A4 x UGT	CL_{int}	0.87	0.93
		$CL_{H, in vivo}$	1.00	1.00
Midazolam	CYP3A4	CL_{int}	0.89	0.65
		$CL_{H, in vivo}$	0.92	0.79
Naloxone	UGT	CL_{int}	0	0
		$CL_{H, in vivo}$	0	0

Figure 2. Hepatic clearance measured versus observed of compounds incubated in the absence or in presence of human serum (extra data sets were from Shibata *et al.*, 2002 and Bachmann *et al.*, 2003).

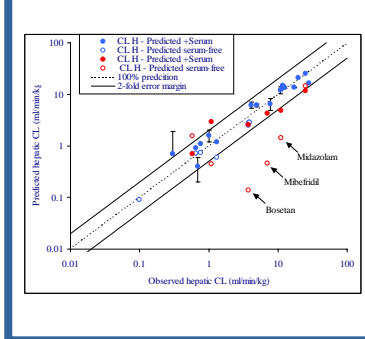
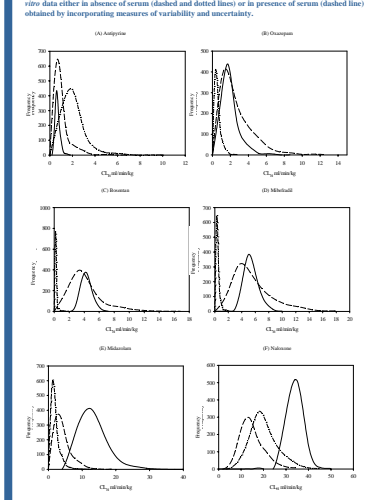


Figure 3. Distribution curves of hepatic clearance observed *in vitro* (solid lines) or scaled from *in vitro* data either in absence of serum (dashed) and in presence of serum (dashed line) obtained by incorporating measures of variability and uncertainty.



Conclusion

- The predicted $CL_{H, in vivo}$ obtained with hepatocytes incubated in serum was more accurate than that obtained in the absence of serum.
- By incubating hepatocytes in serum, the relevant *in vivo* conditions are recreated, thus automatically reflecting the protein binding in plasma. No extra assays to determine protein binding are necessary.
- Clearance values could be linked to enzyme characteristics for four of the six compounds. The failure to correlate naloxone to glucuronyltransferase activity may be because hydroxycoumarin is glucuronidated by UGT1A6 (Lampe *et al.*, 1999), whereas naloxone is selectively metabolized by UGT 2B7 (Wahlstrom *et al.*, 1989). Antipyrine is mainly metabolized by CYP1A2 but in its absence, is metabolized by many other CYP isoforms (CYP3A4, CYP2A6, CYP2C9, CYP2E1, CYP2D6) and is conjugated (Sharer and Wrighton, 1996), all of which make a correlation much more difficult.
- Cryopreserved human hepatocytes represent a convenient, rapid-to-use, easy-to-store and predictive tool for drug metabolism studies, whatever the incubation medium used.

References

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