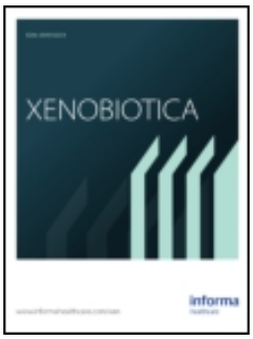

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Comparison of clearance predictions using primary cultures and suspensions of human hepatocytes

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Abstract

Various incubation conditions of human hepatocytes were compared for their accuracy in predicting the *in vivo* hepatic clearance (CL_H) of model compounds. The test compounds were the highly cleared, low protein bound naloxone (*in vivo* $CL_H = 25\,ml\,min^{-1}\,kg^{-1}$; free fraction = 0.6), the medium clearance, highly protein bound midazolam ($CL_H = 12\,ml\,min^{-1}\,kg^{-1}$; free fraction = 0.04) and the low clearance, highly protein bound bosentan ($CL_H = 3.9\,ml\,min^{-1}\,kg^{-1}$; free fraction = 0.02). Each compound was tested in three 'hepatocyte systems', using resections from three donors, in the presence and absence of human serum. Those hepatocyte systems were: conventional primary cultures, freshly isolated suspensions and cryopreserved suspended hepatocytes. Except for a twofold overestimated CL_H for bosentan from conventional primary cultures, and despite variable cryopreservation recoveries, similar predictions of CL_H were recorded with all hepatocyte systems. Moreover, the CL_H values obtained with cryopreserved suspended hepatocytes were similar to those obtained with freshly isolated suspensions. For midazolam and bosentan, the predicted *in vivo* CL_H was markedly higher in the presence of serum, whereas serum had little influence on the scaled-up CL_H of naloxone. *In vivo*, CL_H was properly approached for naloxone and bosentan (particularly from experiments in the presence of serum), but it was strongly underestimated for midazolam (particularly in the absence of serum). Additional compounds need to be investigated to confirm the above findings as well as to assess why the clearances of some highly protein-bound compounds are still considerably underestimated.

Keywords: *Human hepatocytes, in vitro models, clearance prediction, cryopreservation, Viaspan, serum, bosentan, midazolam, naloxone*

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Introduction

Predicting the human pharmacokinetics of new chemical entities at an early phase of drug discovery/development is becoming increasingly important. At this stage, since the liver is the main organ of elimination, various hepatic *in vitro* models are used to investigate the metabolism of xenobiotics. Freshly isolated human hepatocytes, however, are being increasingly used for these studies (Gomez-Lechon et al., 2003; Richert et al., 2003). Due to their broad spectrum of enzyme activities, their physiological cofactor-enzyme levels, active gene expression and cell integrity (particularly the intact plasma membrane), these have been described as one of the best *in vitro* models for the prediction of *in vivo* metabolism in humans (Lave et al., 1999). However, although successful predictions of the *in vivo* hepatic metabolic clearance (CL_H) have been reported in several cases (Fabre et al., 1990), a number of issues still need to be clarified to improve the accuracy of these predictions. Thus, physiologically based direct scaling tends to underestimate systematically the *in vivo* CL_H in humans (Zuegge et al., 2001). Such underestimations might be explained by a number of factors, which include extra-hepatic metabolism, the involvement of active transport processes through the sinusoidal membrane, and/or inappropriate considerations of both the *in vitro* and *in vivo* binding. Recently, a novel *in vitro* method based on hepatocytes suspended in serum was developed and it showed promising results for predicting the *in vivo* CL_H in rat (Shibata et al., 2000) and man (Shibata et al., 2002; Bachmann et al., 2003). For physiologically based direct scaling, the use of rat hepatocyte suspensions has recently been shown to improve the prediction of *in vivo* clearance (Blanchard et al., 2004). Moreover, the current study confirmed earlier observations (Shibata et al., 2000, 2002; Bachmann et al., 2003) that the presence of serum in the incubation medium greatly improved the accuracy of clearance predictions.

Compared with animal models, however, a number of factors complicate the predictions of *in vivo* rates of metabolism in humans from *in vitro* data. For example, inter-individual differences in the drug-metabolizing enzyme activities in man are more marked than in laboratory animals, and these variations are reflected in the *in vitro* metabolic clearances of standard compounds (Ponsoda et al., 2001). In addition to differences between donors (genetic polymorphism, liver disease, medications, etc.), the experimental conditions used to collect the liver samples (such as the time between liver resection and dissociation, or the duration of warm ischaemia) also markedly affect the metabolic activities *in vitro* (Sahi et al., 2000). Furthermore, the availability of human liver samples is erratic and unpredictable, and in certain countries is still limited by legal and ethical considerations.

In the past decade, one approach used to offset the erratic availability of human liver samples has been to cryopreserve freshly isolated human hepatocytes as they become available. Although numerous laboratories have reported satisfactory cryopreservation, the recovery of viable hepatocytes and the relative maintenance of phase I and II metabolic activities after thawing are still controversial (Gomez-Lechon et al., 2003). It is commonly accepted that thawed hepatocytes loose their capacity to attach to a substratum (Alexandre et al., 2002b; Gomez-Lechon et al., 2003). For this reason, cryopreserved hepatocytes are generally used in suspension (Li, 2001). Concerns about the maintenance of metabolic capacities after thawing, however, still exist, and comparing the metabolic activities between different batches is complicated by the inter-individual differences in drug metabolism. Together these factors complicate the comparisons of fresh and cryopreserved hepatocytes (Gomez-Lechon et al., 2003).

With the above points in mind, the primary objectives of the present study was to compare the ability of conventional primary cultures (CPC), suspensions of freshly

isolated human hepatocytes (FSH) and cryopreserved human hepatocytes (CSH) from the same donors to predict the *in vivo* CL_H in humans. A secondary objective was to assess the impact of the presence of serum on the accuracy of these predictions.

Materials and methods

Test compounds

Batch numbers of the material used in these studies were recorded on file. Samples of midazolam, bosentan (Roux et al., 1999) (supplied by F. Hoffmann-La Roche Ltd, Basel, Switzerland) and naloxone (N-7758; Sigma, Division of Fluka Chemie AG, Buchs, Switzerland), were of 99.9, 99.9 and 98% minimum chemical purity, respectively. For protein binding studies, ^{14}C midazolam (170 μCi mg^{-1}), ^{3}H-naloxone (130 mCi mg^{-1}) and ^{14}C-bosentan (40 μCi mg^{-1}) were obtained from F. Hoffmann-La Roche AG.

Reagents

William's E w/o L-glutamine w/o phenol red (Gibco 041-94198M) and Dubelcco's modified Eagle's medium (DMEM) (Gibco 31053-028) were from Life Technologies AG (Basel, Switzerland) or Sigma W1878 (Division of Fluka Chemie AG, Buchs, Switzerland). Insulin was purchased from Sigma. Penicillin/streptomycin 10 000 UI ml^{-1} (Gibco 15140-106), foetal calf serum (Gibco 10108-165) and glutamine 200 mM (Gibco 25030-024) were from Life Technologies. Human serum (obtained from EFS Strasbourg, France), was decomplemented by heating for 30 min at 56°C. University of Wisconsin medium, VIASPAN (UW) solution was furnished by the hospital of Strasbourg.

Methods

Isolation of human hepatocytes. Human hepatocytes were isolated as described (Alexandre et al., 2002a). Hepatocytes were either:

- seeded at 0.3×10^6 cells/wells/0.5 ml on precoated BIOCOAT 24-well plates and incubated for 24 h at 37°C with 5% CO_2; or
- kept at 4°C in culture medium (donors 1 and 2) or shipped in UW (donor 3) for about 3 h; or
- cryopreserved as previously (Alexandre et al., 2002b). Briefly, 5×10^6 cells were suspended in 1 ml ice cold medium (Alexandre et al., 2002b). Cells were placed in 'isopropanol progressive freezing containers' (CRYOFREEZE, VWR, Strasbourg, France). The container was placed at −80°C for 18 h thus allowing for a temperature decrease at 1°C min^{-1}. Cryopreserved hepatocytes were stored at −196°C in liquid nitrogen.

Incubation of human hepatocytes. The test compounds were used at three concentrations. Stock solutions of compounds were prepared in DMSO at 10 mM. The final concentration of DMSO in the incubation medium was always below 1%. The corresponding unchanged parent compounds were analysed by LC/MS-MS.

Conventional primary cultures of human hepatocytes (CPC). After a 24-h attachment in a humidified chamber at 37°C with 5% CO_2 in DMEM-glutamax-I containing 5% foetal

calf serum and 50 μg ml^{-1} gentamicin, the medium was replaced by 200 μl of the incubation medium consisting of William's E medium 0.5% streptomycin/penicillin (50 UI ml^{-1}), insulin (1.2×10^{-6} M), glutamine (400×10^{-6} M) and containing the test compounds (midazolam, naloxone or bosentan) at 1, 10 or 50 μM. At each of the following time points: 2, 5, 15, 30, 60, 120, 180 and 300 min, the extracellular medium was collected. The intracellular medium was then obtained by scraping the cell monolayer with 200 μl methanol-H_2O (50:50, v/v) and combined with the extracellular medium.

Suspensions of freshly isolated human hepatocytes (FSH). Hepatocytes were suspended in 0 or 100% serum, at 4×10^6 viable cells ml^{-1} in 250 μl, in 24-well plates and incubated on a thermomixer at 37°C at 300 rpm. Following a 30-min pre-incubation, 250 μl of an appropriate dilution of each of the test compounds prepared either in 0 or 100% serum were added to reach final concentrations of 1, 10 and 50 μM. At each of the following time points: 2, 5, 15, 30, 60, 120, 180 and 300 min, 250 μl of the hepatocyte incubate were collected and added to 500 μl methanol to inactivate the cells.

Suspensions of cryopreserved human hepatocytes (CSH). The vials of cryopreserved hepatocytes were thawed by immersion for 2 min in a 37°C water bath. After thawing, William's E medium with 10% foetal calf serum, 0.5% streptomycin/penicillin (50 UI ml^{-1}), insulin (1.2×10^{-6} M), and glutamine (400×10^{-6} M) was added to hepatocytes. Hepatocytes were centrifuged at 50 *g* for 5 min at room temperature. The pellet was resuspended in William's E medium with 10% foetal calf serum, 0.5% streptomycin/penicillin (50 UI ml^{-1}), insulin (1.2×10^{-6} M), glutamine (400×10^{-6} M). The cell concentration and the viability of the preparations were assessed. Thawed hepatocytes were suspended at a density of 4×10^6 viable cells ml^{-1} in 0% serum (William's E medium with 0.5% streptomycin/penicillin (50 UI ml^{-1}), insulin (1.2×10^{-6} M), glutamine (400×10^{-6} M)) or in 100% human serum. The hepatocyte suspension was distributed in 24-wells plates in half of the final incubation volume (250 μl) and incubated in thermomixer at 37°C, 300 rpm for 30 min. Thereafter, an equivalent volume of incubation medium (250 μl) containing the test compound was added to achieve final concentrations of 1, 10 and 50 μM. At each of the following time points: 2, 5, 15, 30, 60, 120, 180 and 300 min, 250 μl of the hepatocytes incubate were collected and added to 500 μl methanol to inactivate the cells.

Protein binding

The free fractions of midazolam, naloxone and bosentan were determined in both serum-free medium and in human serum by ultrafiltration. Triplicates of ^{14}C-midazolam, ^{3}H-naloxone and ^{14}C-bosentan were prepared in serum-free medium and human serum and adjusted to pH 7.4. Samples were incubated for 10 min at 37°C in the 24-well plates and in glass vials. Ultrafiltration was performed in a micropartition system MPS-1 (Centrifree, Amicon) at 37°C by centrifugation (2000*g*) for 10 min. The radioactivity of the resulting ultrafiltrates was measured and compared with the unfiltered solutions to determine the free fractions of each test compound.

LC/MS-MS analysis

The concentrations of bosentan, midazolam and naloxone in the various *in vitro* samples were determined by high-performance liquid chromatography coupled with tandem

mass spectrometry (LC/MS-MS). 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} was made of solvent A (20 mM ammonium acetate pH 3) and solvent B (acetonitrile). An initial isocratic step of 0.5 min at 5% B was followed by a gradient from 5 to 95% B within 2 min, again followed by an isocratic phase of 1 min. Detection was performed in a positive mode for the three compounds. The quantification limit of the assay was 0.042, 0.015 and 0.0031 μM for bosentan, midazolam and naloxone, respectively.

Data analysis

Intrinsic clearance (CL_{int}). This was estimated as described for rat hepatocytes (Blanchard et al., 2004), from the ratio of V_{max} (maximal rate of metabolism) over K_m (Michaelis–Menten constant for the substrate–enzyme interaction). V_{max} and K_m were determined by simultaneously fitting the drug-depletion profiles for the three concentrations of each compound using model maker 4.0 (ModelKinetix, Reading, UK).

In vitro–in vivo scaling. 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 as follows (Zuegge et al., 2001):

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

The average liver weights (LW) and liver blood flows (LBF) in humans were 1800 g and 20 ml min^{-1} kg^{-1}, respectively, based on a body weight of 70 kg (Zuegge et al., 2001). The direct scaling factor (SF_{dir}) used to correct the *in vitro* hepatocyte clearance from the number of cells/g liver was 1.2×10^8 cells/g liver. The parameters Fu and Fu′ represent the free fraction measured in serum and the non-specific binding to the *in vitro* matrix, respectively. When incubations were performed in serum, it was assumed that Fu = Fu′. Thus, non-specific binding was considered to be negligible in the presence of serum. In addition, Fu in presence of serum was considered to be similar *in vitro* and *in vivo* (Shibata et al., 2000).

Note that the lower CL_{int} values obtained *in vitro* in the presence of serum (as compared with those obtained in the absence of serum) will predict higher CL_H *in vivo* when correction for protein binding is considered (see the above equation).

Results

Protein binding

The ultrafiltration results indicated that midazolam and bosentan are highly bound to plasma proteins under the conditions used in the present study, with mean free fractions of 0.04 and 0.02, respectively (data not shown); by contrast, naloxone exhibited low protein binding (Fu = 0.6). The results for midazolam agree with published data (Lave et al., 1997), as also do those for naloxone (Asali and Brown, 1984).

Characteristics of the human hepatocyte preparations

The characteristics of the donors and the viabilities of the initial cell preparations are given in Table I.

Results for the attachment of freshly prepared hepatocytes and their viability after storage under different conditions are shown in Table II. For freshly isolated hepatocytes, the cell attachment in culture ranged from 62 to 92%. After a 3-h cold preservation as suspensions at 4°C in the modified Eagle medium, 90% recoveries of viable hepatocytes were recorded for donors 1 and 2. In contrast, by 3 h after shipment at 4°C in UW medium, the recovery in the preparation from donor 3 had dropped to 53% (Table II). Following cryopreservation, the recovery of viable hepatocytes for all three donors decreased markedly and varied between subjects, from 50% recovery for donor 2 to 0% recovery for donor 3.

Determination of in vitro CL_{int} using isolated human hepatocytes

V_{max} and K_m were determined for the incubations of midazolam, naloxone and bosentan (each compound was incubated at concentrations of 1, 10 and 50 μM) in conventional primary cultures (CPC), suspensions of freshly isolated hepatocytes (FSH) and suspensions of cryopreserved hepatocytes (CSH), both with and without serum. These data are shown in Table III, together with the corresponding derived values for intrinsic clearance (CL_{int}). Since all of the hepatocytes from donor 3 were lost during the cryopreservation/thawing process, only those from donor 1 and 2 were included in the third part of the study. Overall, the CL_{int} for midazolam, naloxone and bosentan were of the same order of magnitude in FSH, CPC and CSH.

Despite the high protein binding of both midazolam and bosentan, the addition of serum to the incubations appeared to produce different effects on their CL_{int} values. For all three hepatocyte systems, the addition of serum generally reduced the CL_{int}

Table I. Characteristics of donors and preparations of human hepatocytes shortly after isolation.

Donor number	Age (years)	Gender	Disease	Size of resection (g)	Yield[1]	Hepatocyte viability (%)
1	55	Female	Colorectal adenocarcinoma	34	62×10^6	92
2	62	Female	Gastroduodenal metachronous metastasis	100	25×10^6	74
3	56	Male	Pancreatic adenocarcinoma	150	16×10^6	80

[1]Viable hepatocytes/g liver.

Table II. Extent of attachment of freshly isolated hepatocytes after seeding and recoveries of viable hepatocytes after cold preservation and cryopreservation.

	Donor number		
	1	2	3
Extent of attachment of freshly isolated hepatocytes in culture (%)	62	65	92
Recovery of viable cells after cold preservation (%)	90[1]	90[1]	53[2]
Recovery of viable cells after cryopreservation (%)	31	50	0

[1]Cold preservation in culture medium at 4°C for 3 h.
[2]Cold preservation during transport in UW at 4°C for 3 h.
Data were derived from *n* determinations.

Table III. Intrinsic clearance (CL_{int}) of midazolam, naloxone and bosentan in human hepatocytes from three donors either as conventional primary cultures (CPC), freshly suspended hepatocytes (FSH) or in suspensions of cryopreserved hepatocytes (CSH).

	Serum (%)		Midazolam			Naloxone			Bosentan		
			Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
CPC	0	V_{max} (nmol min^{-1}/10^{6}cells)	0.03	0.03	0.10	0.13	0.18	0.56	0.008	0.010	0.007
		K_m (μM)	6.9	3.3	2.4	8.6	2.1	5.2	4.3	4.7	4.0
		CL_{int} (μl min^{-1}/10^{6}cells)	4.6	10	42	15	90	108	1.6	2.0	1.8
	100	V_{max} (nmol min^{-1}/10^{6}cells)	0.01	0.01	0.02	0.12	0.13	0.28	0.030	0.007	0.009
		K_m (μM)	11	4.9	4.0	9.5	7.3	7.1	2.9	5.6	3.1
		CL_{int} (μl min^{-1}/10^{6}cells)	0.8	2.6	4.9	12	17	40	10	1.3	2.8
FSH	0	V_{max} (nmol min^{-1}/10^{6}cells)	0.03	0.05	0.10	0.32	0.03	0.99	0.007	0.012	0.007
		K_m (μM)	9.1	9.3	4.1	5.8	5.2	3.4	6.2	4.5	5.1
		CL_{int} (μl min^{-1}/10^{6}cells)	3.1	5.7	25	55	5.8	293	1.1	2.6	1.3
	100	V_{max} (nmol min^{-1}/10^{6}cells)	0.03	0.002	0.02	0.18	0.02	0.59	0.008	0.009	0.006
		K_m (μM)	7.6	14	7.3	6.7	8.0	4.7	4.8	5.9	5.92
		CL_{int} (μl min^{-1}/10^{6}cells)	4.2	1.2	2.1	26	1.9	113	1.6	1.5	1.5
CSH	0	V_{max} (nmol min^{-1}/10^{6}cells)	0.04	0.01		0.19	0.25		0.010	0.008	
		K_m (μM)	1.7	4.8	n.r.	2.4	3.1	n.r.	5.8	5.9	n.r.
		CL_{int} (μl min^{-1}/10^{6}cells)	23	2.3		78	81		1.6	1.4	
	100	V_{max} (nmol min^{-1}/10^{6}cells)	0.01	0.01		0.21	0.11		0.012	0.006	
		K_m (μM)	2.7	7.8	n.r.	2.1	6.4	n.r.	4.5	6.7	n.r.
		CL_{int} (μl min^{-1}/10^{6}cells)	4.2	0.3		104	17		2.6	0.8	

n.r., No result was available for donor 3 since no viable hepatocytes were recovered following cryopreservation.
Data were derived from a single determination for the three donors, incubated with the three compounds, in each condition and configuration of incubation.

of midazolam by between five- to tenfold, with the decrease apparently being to due to a lower V_{max}, as the K_m values based on total concentrations were not markedly affected by serum. By contrast, for bosentan, the addition of serum to the incubation appeared to have little effect on either its V_{max} or K_m. For the weakly protein bound naloxone, a decrease in CL_{int} was recorded for one subject (donor 2), which appeared to be more related to K_m than to V_{max}, but the addition of protein had little effect on the values recorded for the other two donors.

The physiologically based *in vitro* to *in vivo* direct scaling approach (Zuegge et al., 2001) was then used to scale up the CL_{int} values to CL_H. Figure 1 compares the hepatic clearances predicted by simultaneously fitting depletion profiles at the various concentrations to derive V_{max} and K_m for the various experimental conditions with the values reported in the literature for *in vivo* hepatic clearances of the three test compounds. These are respectively: $12.0 \pm 1.6\,ml\,min^{-1}\,kg^{-1}$ for midazolam (Greenblatt, 1980; Allonen et al., 1981; Amrein et al., 1981; Greenblatt et al., 1981; Smith et al., 1981), $25\,ml\,min^{-1}\,kg^{-1}$ for naloxone (Zuegge et al., 2001) and $3.9 \pm 0.7\,ml\,min^{-1}\,kg^{-1}$ for bosentan (F. Hoffmann-La Roche, data on file).

Predicted in vivo CL_H from isolated human hepatocytes

For each type of the hepatocyte preparations, in the absence of serum, the predictions of *in vivo* CL_H for midazolam were markedly underestimated (Figure 1). By contrast, the values predicted for all three donors were higher in the presence of serum. Nevertheless, overall these predictions were still two- to threefold lower than the published value.

For naloxone, the presence of serum in the incubation medium had little/no influence on the *in vivo* CL_H predictions (Figure 1). For donors 1 and 3, all three types of cultures gave CL_H values close to the observed *in vivo* CL_H of approximately $25\,ml\,min^{-1}\,kg^{-1}$ (Zuegge et al., 2001). With respect to donor 2, the predicted CL_H derived from the FSH preparation ($4-6\,ml\,min^{-1}\,kg^{-1}$) was approximately one-quarter of the published value, whereas the values that were obtained from the CPC and CSH cultures ($14-16\,ml\,min^{-1}\,kg^{-1}$) were closer to the true value. Nevertheless, all the predictions for *in vivo* CL_H were still slightly less than the literature value.

In the absence of serum, all of the predicted *in vivo* CL_H values for bosentan were substantially underestimated, whereas higher values were recorded in the presence of serum (Figure 1). For the hepatocyte suspensions (FSH) in the presence of serum, all three batches predicted a CL_H close to the value observed *in vivo*. By contrast, two of the three CPC results overestimated the *in vivo* CL_H by two- to threefold, whereas one of the two CSH samples gave a value approximately half of the *in vivo* CL_H.

Discussion

Liver preparations such as microsomes and more complex cellular systems such as human CYP-engineered cells or hepatoma cell lines are currently used as *in vitro* models to investigate drug hepatic metabolism. Nevertheless, hepatocytes have been recognized as the most reliable model to predict the hepatic metabolic clearance (CL_H) in humans (Fabre et al., 1990; Lave et al., 1999). Also, suspensions of rat hepatocytes were reported to predict *in vivo* hepatic metabolic clearance in the rat (Shibata et al., 2000), and similar results have been reported with suspensions of human hepatocytes (Shibata et al., 2002; Bachmann et al., 2003).

The inter-individual variability in liver metabolism is higher in humans than in laboratory animals. For example, using preparations from different subjects, a three- to fivefold

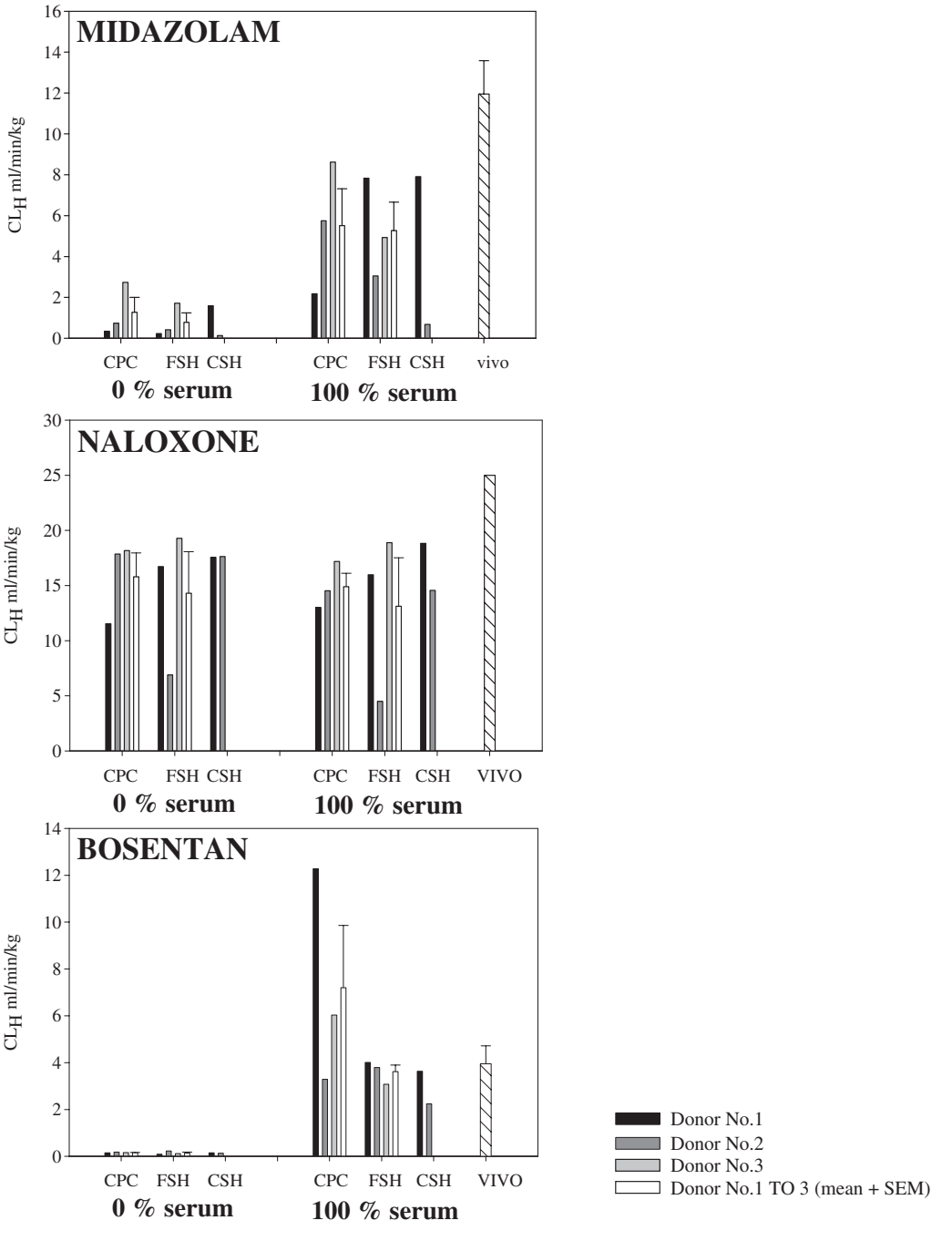

Figure 1. Predicted *in vivo* CL_H of midazolam, naloxone and bosentan using human hepatocytes prepared from three donors. Hepatocytes were used either as fresh conventional primary cultures (CPC) or fresh suspensions (FSH), or as suspended hepatocytes following cryopreservation (CSH). Each 'hepatocyte system' was assessed in the presence (100% serum) or absence (0%) of serum. White bars represent the mean + SEMs for each of the test conditions; hatched bar represents the observed *in vivo* hepatic clearance. CSH values are not shown for donor 3 since no viable hepatocytes were recovered after cryopreservation.

variation was found in the *in vitro* metabolic clearance for some standard compounds (Shibata et al., 2002). This inter-individual variability in the metabolic capacity of human liver reportedly reflects differences that are due to, for example: genetic polymorphism, liver diseases and/or co-administrations of other medications (Gomez-Lechon et al., 2003). However, factors that affect the quality of hepatocyte preparations, such as the time elapsed between liver resection and dissociation, or the duration of warm ischaemia (Sahi et al., 2000; Alexandre et al., 2002b), or differences in configuration (i.e. culture versus suspension; Waring et al., 2003), or the time elapsed before use (Skett et al., 1995), can all contribute to the variations in metabolic capacity.

In the present study, human hepatocytes were isolated from liver resections of three donors under conditions that have recently been reported to be optimal (Richert et al., 2004). These were used both as suspensions and as conventional primary cultures in order to assess their ability to predict the *in vivo* CL_H in humans. Both the yield of viable human hepatocytes/g liver and the viability of the preparations were in the range of those previously described by our group for such biopsies (Alexandre et al., 2002a; Richert et al., 2004). Also, the average 60–90% attachment of plated human hepatocytes seeded immediately after isolation was in agreement with literature data (LeCluyse, 2001).

After 3 h of storage at 4°C in culture medium (DMEM), the recoveries of viable hepatocytes from donors 1 and 2 were approximately 90%. By contrast, after a 3-h shipment in UW at 4°C, the recovery of hepatocytes isolated from donor 3 was only 50% (Table II). To investigate the impact of cold-storage conditions further, we assessed the recoveries of viable hepatocytes kept in suspension for up to 24 h at 4°C, either in culture medium containing 5% foetal calf serum or in UW.

Data shown in Figure 2 represent the three sets of hepatocytes for which both types of medium were evaluated. They confirmed that the recovery of viable hepatocytes was lot dependent and varied between 60 and 100% after 3 h, irrespective of the medium used. Thereafter, the viability of the preparations was relatively well maintained for up to 18 h of cold storage. These data are in agreement with others that described a 50% loss of hepatocyte viability when the hepatocytes are kept as suspensions in UW for 24 h (Olinga et al., 1997).

Following cryopreservation, the loss of viable hepatocytes for donors 1–3 was 70, 50 and 100%, respectively. The high attachment (90%) obtained for donor 3 directly after isolation indicates that it was a good preparation. However, the low recovery obtained after cold preservation in UW (60%) and the loss of 100% of the viable cells after cryopreservation confirm that 'resistance' to storage (cold or cryopreservation) is highly dependent on the donor. Such a high variability in the resistance of human hepatocytes to cryopreservation is well known and has been shown to be independent of the protocol used (Gomez-Lechon et al., 2003). Also following cryopreservation/thawing, the values obtained for *in vitro* CL_{int} (and thus the predicted *in vivo* CL_H) were similar to those recorded after storage for 3 h at 4°C. This is consistent with previous results that after cryopreservation/thawing, the viable human hepatocytes not only maintain their major cytochrome P450 isoenzyme activities, but also the UDP-dependent glucuronyl- and sulfo-transferase activities (Steinberg et al., 1999; Alexandre et al., 2002a).

Based upon the three batches of human hepatocytes investigated, both the *in vitro* CL_{int} and the predicted *in vivo* CL_H showed relatively low variabilities between the donors for all three test compounds. Bosentan and midazolam are known to be metabolized mainly by CYP2C9 and CYP3A4 (Ubeaud et al., 1995; Lave et al., 1996; Van Giersbergen et al., 2002), and CYP3A4 (Gorski et al., 1994), respectively, whereas naloxone is metabolized by phase II enzymes (Wahlstrom et al., 1989; Weaver 2001). Although CYP3A4 and the

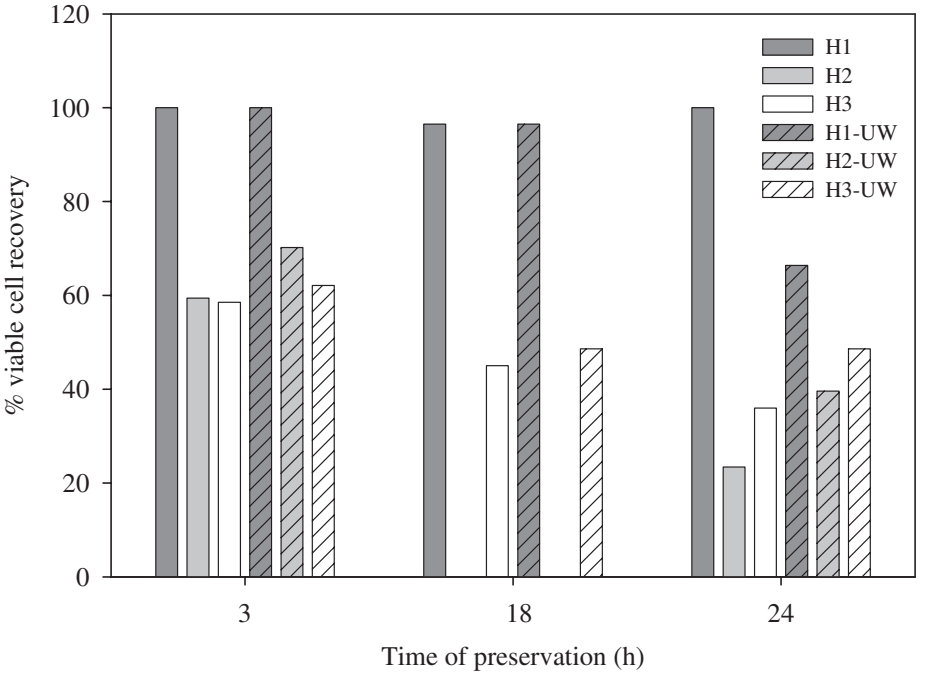

Figure 2. Viability of human hepatocytes from three donors during storage. Individual donors are identified as H1, H2 or H3. The value '100%' represents the viability of the cells immediately after isolation. Samples were then tested after 3, 18 and 24 h of storage in either DMEM medium containing 5% foetal calf serum ('H1', 'H2', 'H3') or UW medium ('H1-UW', 'H2-UW', 'H3-UW'). Data were derived from $n =$ determinations.

subfamily of CYP2C enzymes are quantitatively the most important in human livers (e.g. CYP3A4 accounts for 50–80% of total hepatic CYP enzymes), their inter-individual variability does not exceed 20-fold for 3A4 and eightfold for 2C9, in contrast to >1000-fold variability for the polymorphic CYP2D6 (Weaver, 2001).

The primary objective of the present study was to assess whether or not the values of *in vitro* CL_{int} (and thus the predicted *in vivo* CL_H) vary with the type of hepatocyte preparation used. In this respect, the critical factor is likely to be the relative efficiency of the diffusion, transport and elimination processes in the suspension compared with primary cultures. For a compound such as naloxone that is highly cleared *in vivo*, the factors that determine its rate of metabolism by hepatocytes are likely to be dispersion through the incubation medium as well as the diffusion processes that control its transport into the cells (in contrast to the *in vivo* situation, when liver blood flow is the rate-limiting factor). In this case, CL_{int} might be expected to be higher in suspensions than in cultures. Such a higher CL_{int} in suspensions was observed for naloxone in at least two of three preparations (Table III). The same observation has been made with hepatocytes from the rat (Blanchard et al., 2004), a species in which naloxone is also a high clearance compound (Weinstein et al., 1973; Mistry & Houston 1987). As the differences between cultures and suspensions do not affect the apparent membrane surface area, this is therefore unlikely to account for the observed differences in the kinetic parameters. However, the uptake of a drug into hepatocytes in suspension is probably enhanced by continuous mixing, thus resulting in a more rapid availability of the test compounds to the intracellular enzymes.

If so, the mechanisms that lie behind the small differences that were recorded in CL_{int} for the suspensions and primary cultures are unlikely to be directly relevant to the *in vivo* CL_H of a highly cleared compound (for which liver blood flow is the rate-limiting step). Nevertheless, when the *in vitro* data are extrapolated to predict the clearance *in vivo*, the slightly higher CL_{int} values recorded for the suspensions might be expected to provide a more accurate prediction of *in vivo* CL_H.

Conversely, in humans, midazolam and bosentan are, respectively, medium- ($12\,ml\,min^{-1}\,kg^{-1}$) and low- ($4\,ml\,min^{-1}\,kg^{-1}$) clearance compounds (Lave et al., 1997; Zuegge et al., 2001). In these cases, the hepatocyte configurations (FSH versus CPC) did not influence the CL_{int} values (and thus the predictions of *in vivo* CL_H). For these compounds, the limiting steps are probably the rate of transfer across the membrane (through passive or active diffusion) and/or the intrinsic capacities of the CYPs involved. Therefore, for both medium- and low-clearance compounds, the metabolic capacities of human hepatocytes as conventional cultures at 24 h after attachment are likely to be equivalent to those of suspensions, either after a short period of cold preservation or after cryopreservation. In this case, when their CL_{int} values are extrapolated to predict *in vivo* CL_H, all three test systems are likely to be equally accurate (or inaccurate).

The secondary objective of the present study was to assess whether the addition of serum to the hepatocyte cultures would alter the values of *in vitro* CL_{int} (and thus the predictions of *in vivo* CL_H). For each hepatocyte configuration (CPC, FSH or CSH), CL_{int} was determined both in the presence and absence of human serum. For naloxone, which exhibits a low protein binding, CL_{int} was not much altered by the presence of serum. Conversely, for midazolam (which is highly bound to serum proteins), the CL_{int} in both cultures and suspensions would be expected to be (and actually were; Table III) considerably lower in the presence than in the absence of serum. Surprisingly, however, the CL_{int} of bosentan (which is also highly bound to serum proteins) was similar with and without serum.

When appropriate corrections for the specific and non-specific binding of midazolam and bosentan were applied to their CL_{int} values, the predicted CL_H values in the presence of serum were both higher and closer to the corresponding observed *in vivo* CL_H. These results confirm our previous observation that the presence of serum in the incubation medium for hepatocytes is a key parameter for the clearance determination of some compounds, particularly those like midazolam and bosentan that are highly protein bound (Blanchard et al., 2004). Research activities to confirm this surprising effect of serum on the determination of CL_{int} and CL_H particularly for compounds such as bosentan are being performed. However, for both midazolam and bosentan, the CL_{int} values recorded in the absence of serum correspond to total CL_{int} and those in the presence of serum to $CL_{int,u}$. Thus, after correction for specific and non-specific binding, the $CL_{int,u}$ values in the presence and absence of serum were expected to be similar. However, for both these highly bound compounds, the $CL_{int,u}$ obtained in serum-free medium was significantly lower than in presence of serum (data not shown). Similar findings were recorded in our previous study with rat hepatocytes (Blanchard et al., 2004). The explanations proposed for this effect included the possibility that the rate of hepatic uptake of compounds that are highly bound to serum albumin ($Fu < 10\%$, which is the case for both bosentan and midazolam) might not necessarily depend upon the unbound drug concentration in the extracellular space (Forker et al., 1982; Burczynski et al., 1989). The observation for naloxone (which exhibits a low protein binding; $Fu = 0.6$) that serum did not have any impact on the predicted CL_H might support this suggestion.

Moreover, the decrease in the CL_{int} for midazolam in the presence of serum was apparently due to a lower V_{max}. Such an effect was also recorded in our other studies with rat hepatocytes (Blanchard et al., 2004). Hypotheses to explain this effect might include the following: (1) addition of serum to the incubation medium alters the disposition of compounds within the hepatocytes; (2) for highly bound compounds, the unbound concentrations will vary with the amount of serum in the incubation medium; or (3) the addition of serum might activate certain processes (such as facilitated/active transport). Thus, in the absence of serum, the transport processes might not be activated and CL_{int} is determined by metabolism. By contrast, in the presence of serum, activation of the uptake might lead to a different V_{max} and/or K_m, and thus to a change in CL_{int}. If so, the V_{max} and K_m obtained in the presence and absence of serum would not be directly comparable since they describe different processes. Whatever the explanation for these effects, however, even though the addition of serum to the incubation medium improved the accuracy of the predicted *in vivo* CL_H for both of the highly bound drugs, the values for midazolam were still significantly underestimated. Further studies are, therefore, needed to define a hepatocyte system that can provide truly reliable values for predicting *in vivo* CL_H from *in vitro* data.

Overall, based on a limited number of test compounds ($n = 3$) and donors ($n = 3$), both *in vitro* hepatocyte models (culture and suspension) provided similar results in terms of clearance predictions in humans. However, because of the small number of subjects and compounds used, a number of findings need to be confirmed. The potential advantage of using suspensions for high-clearance compounds, particularly obvious for the determination of intrinsic clearance, needs to be investigated further. Also, for cryopreserved human hepatocytes, a profiling of CYP activities (using cocktails of CYP-specific substrates) is likely to be needed before and after freezing/thawing in order to differentiate between inter-individual variability and variability in the 'resistance' to the cryopreservation process. In addition, having shown that the use of serum as incubation medium was a key parameter to improve the accuracy of clearance predictions for highly protein-bound compounds, a number of technical issues still need to be addressed (including the supply of human serum). Moreover, limitations could arise for very highly bound/low-clearance compounds, such as an insufficient sensibility to assess their intrinsic *in vitro* clearance.

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