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Research paper

Cryopreserved human hepatocytes in suspension are a convenient high throughput tool for the prediction of metabolic clearance

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Abstract

Hepatocyte assays, routinely used to assess the metabolic stability of new chemical entities, were recently improved by using hepatocytes in suspension instead of primary cultures [N. Blanchard, L. Richert, B. Notter, F. Delobel, P. David, P. Coassolo, T. Lavé, Impact of serum on clearance predictions obtained from suspensions and primary cultures of rat hepatocytes, Eur. J. Pharm. Sci. 23 (2004) 189–199].

The aim of the present study was to investigate miniaturising the suspension assay by using cryopreserved human hepatocytes, i.e., 150,000 cells/well in 96-well plates, to predict hepatic clearance (CL_H) in order to increase compound throughput and decrease cost and tissue requirements.

For this, an evaluation was first carried out with rat hepatocytes. Then, human hepatocytes from various donors were used under these predetermined conditions, either immediately after isolation, either after a 20-h-cold storage period in UW or after cryopreservation.

The values of CL_{int} and CL_H determined using human hepatocytes in suspension in 96-well plates, immediately after isolation, after cold storage or after cryopreservation, were comparable to those obtained with hepatocytes in primary culture. In particular, the use of cryopreserved human hepatocytes in suspension in a 96-well format appeared to be largely satisfactory as a tool for screening and ranking of compounds in the early phase of the drug discovery process.

Keywords: Human hepatocytes; Suspension; Cryopreserved; Miniaturisation; High throughput; Clearance prediction

1. Introduction

The pharmaceutical industry is confronted with an increasing number of compounds, which are being synthesised weekly together with the need to test them at high speed and with a high degree of accuracy. In vitro clearance data have, traditionally, been generated from human and rat liver microsomal incubations, but such subcellular fractions do not represent the whole metabolic process that occurs in the liver, prompting researchers to move towards hepatocyte incubations.

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Nowadays, human hepatocytes are recognised as the most reliable model to predict hepatic metabolic clearance (CL_H) in humans [2,3]. Until recently, hepatocytes were routinely used in primary culture. However, there are scientific limitations inherent to this configuration, such as decrease of phase I and phase II activities during culture [1,4,5], at least in part responsible for the observed tendency to under-predict in vivo hepatic clearance [6]. With the aim of improving clearance prediction, in vitro methods using rat [1,7] and human [8–10] (Blanchard et al., submitted[4]) hepatocytes in suspension in 24- and 48-well plates were developed. Cytochrome P450 (CYP)-dependent activities (mainly CYP 3A1) were shown to be more stable during the first 6 h of incubation of rat hepatocytes in suspension than in primary cultures [1]. Moreover, this system presents the advantage of reducing the time required for preparation (cell plating and cell scraping are not required) and for incubation (performed only up to 4–6 h in suspension versus 20 h in primary culture).

A number of factors, such as inter-individual variability and the erratic and unpredictable availability of human liver samples, complicate the prediction of in vivo rates of metabolism from in vitro data in humans. At present, cryopreserved human hepatocytes are easily available and have been reported to retain, quantitatively, most of the phase I and phase II metabolic activities of fresh liver [11,12]. We have shown recently [10] that clearances obtained from suspensions of fresh, cold-preserved (i.e., preserved for 20 h at 4 °C) or cryopreserved human hepatocytes in 24-well plates were similar, making these cells an easy-to-use system for CL determination.

Increasing throughput via automation, decreased cell consumption and the possibility to evaluate variability between humans requires the use of human hepatocytes in suspension in a miniaturised system. The aim of the present study, therefore, was to assess the prediction of hepatic clearance using: (1) freshly isolated rat and human hepatocytes in suspension in 96-well plates, (2) cold- and/or cryopreserved hepatocytes in this miniaturised system and (3) cryopreserved hepatocytes from various donors and from various sources (i.e., in house isolated or commercially available). For this, six reference compounds covering a broad range of clearance, and which were metabolised by a variety of CYP450 and phase II enzymes, namely theophylline, diclofenac, mibefradil, bosentan, bufuralol and midazolam were used.

2. Materials and methods

2.1. Materials

Twenty-four-well plates (Falcon Cat. No 351147), 96-well plates (NUNC Microwell 442587), mixer (IKA HS/KS 260 control), Hepes buffer, William's E w/o L-glutamine w/o phenol red (Gibco 041-94198M) and Dulbecco'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). Leibovitz L-15 medium, Percoll solution and insulin (Sigma I1882) were all purchased at Sigma. Penicillin/streptomycin 10,000 UI/ml (Gibco 15140-106), foetal calf serum (Gibco 10108-165), hydrocortisone (Sigma P 4153) and glutamine 200 mM (Gibco 25030-024) were from Life Technologies. Collagenase (CLS2 LS04176) was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). $CaCl_2$ was purchased from Fluka (Fluka Chemie AG, Buchs, Switzerland). University of Wisconsin medium (U.W.) or VIASPAN solution was furnished by the hospital of Strasbourg. Human serum, obtained from EFS of Strasbourg, was decomplemented by heating for 30 min at 56 °C.

2.2. Animals

Male Wistar rats (200–300 g) supplied by BRL, Biological Research Laboratories Ltd. (Füllinsdorf, Switzerland) were used throughout the studies. They were kept under routine laboratory conditions, given free access to food and water until the experiment.

2.3. Preparation and incubation of hepatocytes

2.3.1. Test compounds

Samples of theophylline, diclofenac, bosentan [13], bufuralol and mibefradil were of 99.9% chemical purity (minimum) and the sample of midazolam were of 98% chemical purity (minimum). Mibefradil, midazolam and bosentan were supplied by F. Hoffmann-LaRoche AG, Basel, Switzerland. Diclofenac was purchased from Sigma (D-6899). Theophylline was purchased from Fluka (88308) and bufuralol was from Ultrafine (UC168, Manchester, England). Batch numbers of the material used in these studies were recorded on file. The test compounds were used at 10 μM. These concentrations, routinely used for the ranking of compounds at the early phase of drug discovery, were chosen so that they were under the Km. Stock solutions of each compound 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. The incubation medium consisted of William's E medium with 0.5% streptomycin/penicillin (50 UI/ml), insulin (1.2×10^{-6} M), glutamine (400×10^{-6} M) and either 10% of FCS for rat hepatocyte incubation or 10% of human serum for human hepatocyte incubation.

2.3.2. Rat hepatocytes

A miniaturisation attempt was performed with four preparations of rat hepatocytes. For this, hepatocytes were isolated from rat liver by a two-step collagenase perfusion

[4] N. Blanchard, N. Hewitt, H. Jones, P. Sibber, P. Coassolo, T. Lave, Impact of serum on clearance predictions using cryopreserved human hepatocytes, submitted.

method as previously described [1,5,14]. Cell viability was determined from exclusion of erythrosine-B by cell membranes. Only preparations with hepatocyte viability over 75% were retained for further studies.

After isolation, rat hepatocytes were either seeded to obtain a primary culture or suspended in 96-well plates.

2.3.2.1. Conventional primary cultures (CPC) of rat hepatocytes. Freshly isolated rat hepatocytes were seeded on precoated BIOCOAT 24-well plates at 0.2×10^6 viable cells/well in 0.5 ml of William's E medium, complemented with 10% foetal calf serum, 0.5% streptomycin/penicillin (50 UI/ml) and glutamine (400×10^{-6} M). After a 2-h attachment period in a humidified chamber at 37 °C with 5% CO_2, the medium was replaced by 200 μl of the incubation medium containing the test compounds (theophylline, diclofenac, bosentan, bufuralol, mibefradil or midazolam) at 10 μM. At each of the following time points: 2, 60, 120, 180, 300, and 1200 min, the extracellular medium was collected; then, the intracellular medium was obtained by scraping the cell monolayer with 200 μl methanol–H_2O (50:50, v/v), combined with the extracellular medium.

2.3.2.2. Suspension (SH) of rat hepatocytes in 96-well plates. Rat hepatocytes were suspended in the incubation medium at 2×10^6 viable cells/ml in 50 μl in 96-well plates and incubated at 37 °C, 5% CO_2 on a mixer at 300 rpm. Following a 30-min pre-incubation, 50 μl of each of the test compounds (theophylline, diclofenac, bosentan, bufuralol, mibefradil or midazolam) prepared at 20 μM in the incubation medium was added to reach a final concentration of 10 μM and a final cell density of 1×10^6 cells/ml. At each of the following time points: 2, 5, 15, 30, 60, 120, 180, and 300 min, 75 μl of the hepatocyte incubation was collected in different wells for each time point and added to 150 μl methanol to inactivate the cells.

2.3.3. Human hepatocytes

Five lots of human hepatocytes were used.

Three lots of human hepatocytes (donors No. 1, No. 2 and No. 3) were obtained and isolated in house as previously described [15].

Donors No. 1 and No. 2 were used either in a primary culture of hepatocytes (CPC for Conventional Primary Culture), either in suspension directly after isolation (FSH for Freshly isolated Suspension of Hepatocytes) or in suspension after 20 h of preservation at 4 °C (CPSH for Cold Preserved Suspension of Hepatocytes).

Donors No. 1 and No. 3, cryopreserved in house as previously described [16], and two lots of cryopreserved human hepatocytes (donor RNG and donor KRM) purchased from In Vitro Technologies, Inc. (Baltimore, MD, USA) were used in suspension after cryopreservation (CSH for Cryopreserved Suspension of Hepatocytes).

The donor demographics and medical histories are reported in Table 1 (http://www.invitrotech.com/donor-demo.cfm?type=hepatocyte for donor RNG and KRM).

The viability of human hepatocytes was from exclusion of erythrosine-B by cell membranes.

2.3.3.1. Conventional primary cultures of human hepatocytes obtained from donors No. 1 and No. 2. After isolation, human hepatocytes were seeded on precoated BIOCOAT 24-well plates at 0.3×10^6 viable cells/well in 0.5 ml of DMEM–glutamax-I containing 5% foetal calf serum and 50 μg/ml gentamicin, and were incubated in a humidified chamber at 37 °C with 5% CO_2 for donor Nos.1 and 2 conventional primary cultures (CPC). After a 24-h attachment period, hepatocytes in the primary culture were incubated separately with each of the six compounds (theophylline, diclofenac, bosentan, bufuralol, mibefradil or midazolam) at 10 μM and processed as described for rat hepatocytes in CPC.

2.3.3.2. Suspensions of freshly isolated (FSH) or cold preserved (CPSH) human hepatocytes obtained from donors No. 1 and No. 2. Human hepatocytes from donor Nos.1 and 2 were used either directly after isolation in suspension (FSH) or kept in U.W. for shipment for about 20 h at 4 °C (CPSH). Viability of these two lots of human hepatocytes was 82.3% and 83.1% of viable cells for donor Nos.1 and 2, respectively, directly after isolation, and 60% and 39% of viable cells for donor Nos.1 and 2, respectively, after cold preservation.

For each condition, hepatocytes were suspended in 50 μl of incubation medium at 3×10^6 viable cells/ml in 96-well plates and incubated at 37 °C, 5% CO_2 under agitation at 300 rpm. Following a 30-min pre-incubation, 50 μl of each of the test compounds (theophylline, diclofenac, bosentan, bufuralol, mibefradil and midazolam) prepared at 20 μM

Table 1
Characteristics of donors and preparations of human hepatocytes shortly after isolation

Donor	Age	Gender	Disease	Size of resection (g)	Yield[a]	Hepatocyte viability[b](%)
No. 1	74	Male	Colic adenocarcinoma	75	31.7×10^6	82.3
No. 2	61	Male	Colic adenocarcinoma	95	7.48×10^6	83.1
No. 3	81	Female	Colic adenocarcinoma	18	3.4×10^6	81.6
RNG	67	Female	Intracranial hemorrhage	NR	NR	NR
KRM	55	Female	Cerebrovascular accident	NR	NR	NR

NR, non reported.
[a] Viable hepatocytes/g liver.
[b] After isolation.

in incubation medium was added, and the suspensions of human hepatocytes were processed as described above for rat hepatocytes in suspension.

2.3.3.3. Suspensions of cryopreserved human hepatocytes obtained from donor Nos. 1, 3, RNG and KRM (CSH). The vials of cryopreserved human hepatocytes were thawed by immersion in a 37 °C water bath. After thawing, William's E medium with 10% of human serum, 0.5% streptomycin/penicillin (50 UI/ml), insulin (1.2×10^{-6} M) glutamine (400×10^{-6} M) was added to the hepatocytes. These were then centrifuged at 50*g* for 5 min at room temperature. The pellet was resuspended in incubation medium. The viabilities obtained after cryopreservation were 46%, 55%, 89.5% and 90.7% for donor Nos.1 and 2, RNG and KRM, respectively. Thawed human hepatocytes were suspended as previously described for fresh preparations.

2.4. LC/MS–MS analysis

Levels of theophylline, diclofenac, bosentan, bufuralol, mibefradil and midazolam 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 contained the following solvents: solvent A (20 mM ammonium acetate in Merck water, pH 3) and a 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 and then again by an isocratic phase of 1 min. Detection was performed in a positive mode for the six compounds. The quantification limit of the assay was 1 μM for theophylline, 0.1 μM for diclofenac, 0.042 μM for bosentan, 0.04 μM for bufuralol, 0.01 μM for mibefradil, and 0.015 μM for midazolam.

2.5. Data analysis

2.5.1. Intrinsic clearance (CL_{int})

The intrinsic clearance CL_{int} was derived from 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-infinity). Depletion was linear with time when plotted on a log scale (data not shown).

2.5.2. In vitro to 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 Eq. (1)

$$CL_{H,\,in\,vivo} = \frac{LBF \cdot CL_{int,in\ vitro} \cdot SF_{dir} \cdot LW}{LBF + CL_{int,in\ vitro} \cdot SF_{dir} \cdot LW}. \quad (1)$$

For the calculation of $CL_{H,\,in\,vivo}$, the values for CL_{int} are converted to μl/min/million cells. The values used for average liver weight (LW) and the liver blood flow (LBF) were 10 g and 60 ml/min/kg for a body weight of 250 g in the rat, respectively, and 1800 g and 20 ml/min/kg in man, respectively, for a body weight of 70 kg. $SF_{dir} = 1.2 \times 10^8$ -cells/g of liver corresponds to the direct scaling factor to correct the in vitro hepatocyte clearance from the number of cells per gram of liver. Incubations were performed in the presence of 10% of human serum or FCS. For these conditions, it was assumed that binding obtained during incubation was similar to binding observed in vivo, and any correction was included for the scaling.

Prediction was considered to be successful, when a less than twofold deviation was observed between the predicted and the observed CL_H.

3. Results

3.1. In vitro CL_{int} and predicted in vivo CL_H obtained using conventional primary cultures (CPC) and suspensions (FSH) of freshly isolated rat hepatocytes

A higher interassay variability of intrinsic clearance was observed with suspensions as compared to primary cultures (except for theophylline, a very low CL compound) (Table 2). However, no major discrepancies were obtained between primary cultures and suspensions with respect to both the in vitro hepatic clearances and the resulting in vivo hepatic clearances (Fig. 1). Thus, both hepatocyte models predict, well, the in vivo hepatic clearances of diclofenac, bosentan, bufuralol and theophylline, while they both under-predict the in vivo observed clearances of midazolam and mibefradil.

Table 2
Recoveries of viable hepatocytes after cryopreservation

Compounds (10 μM)	Rat hepatocytes in primary cultures		Rat hepatocytes in suspension		Rat in vivo
	CL_{int} (μl/min/10^6cells)	CL_H (ml/min/kg)	CL_{int} (μl/min/10^6cells)	CL_H (ml/min/kg)	Plasma CL (ml/min/kg)
Theophylline	0.4 ± 0.2	1.7	0.2 ± 0.14	0.9	1.8 ± 0.1
Diclofenac	5.5 ± 1.3	18	5.6 ± 4.1	17	13 ± 1.3
Bosentan	3.4 ± 1.8	13	8.1 ± 6.8	21	17 ± 5.4
Bufuralol	39 ± 10	45	33 ± 21	44	37 ± 4.5
Mibefradil	1.4 ± 0.5	6.1	2.8 ± 1.7	10	22 ± 1.5
Midazolam	23 ± 4.3	39	26 ± 17	37	82 ± 6

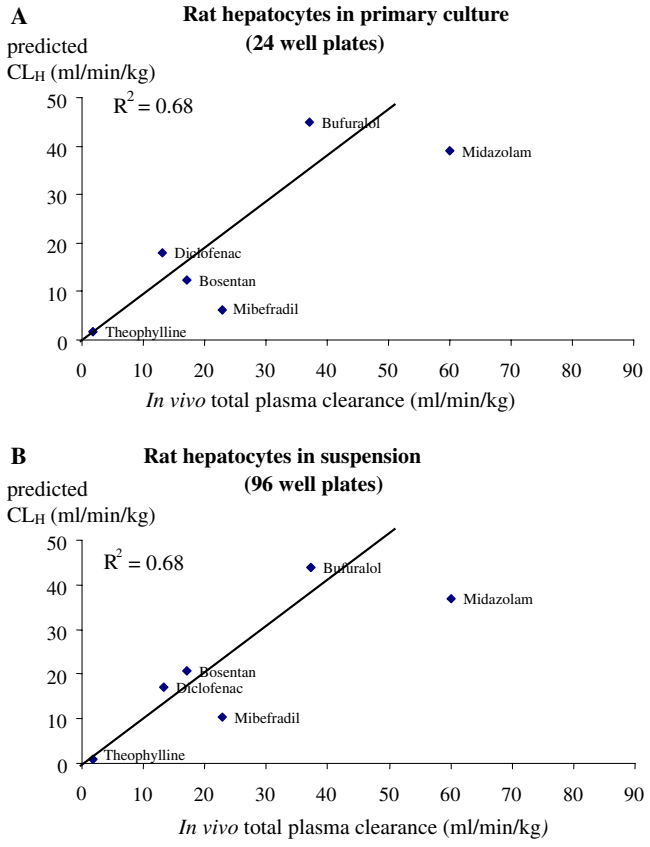

Fig. 1. Predicted hepatic clearance obtained with rat hepatocytes in primary culture (A) or obtained with suspension of rat hepatocytes (B) versus in vivo total plasma observed hepatic clearance.

3.2. Predicted in vivo CL_H using freshly isolated (FSH), cold preserved (CPSH) and cryopreserved (CSH) human hepatocytes

3.2.1. Determination of in vitro CL_{int} using freshly isolated (FSH), cold preserved (CPSH) and cryopreserved (CSH) human hepatocytes from donor Nos.1 and 2

The values of CL_{int} for theophylline, diclofenac, bosentan, bufuralol, mibefradil and midazolam were within a similar range of magnitude in FSH and in CPSH (donor Nos.1 and 2) and in CSH (donor No. 1). As expected, inter-individual variation was observed between donors No. 1 and No. 2. (Fig. 2).

3.2.2. Prediction of in vivo CL_H using freshly isolated (FSH), cold preserved (CPSH) and cryopreserved (CSH) human hepatocytes (donor Nos.1 and 2)

Fig. 3 The direct scaling approach [6] was used to scale up CL_{int} to CL_H. Table 3 compares the predicted hepatic clearance values obtained under the various experimental conditions (FSH vs CPSH vs CSH) with the predicted clearances obtained in conventional primary cultures (CPC). In vivo observed hepatic clearances were collected in the literature for the six test compounds, and the mean values were: 0.61 ml/min/kg for theophylline [17,18], 3.4 ml/min/kg for diclofenac [19,20], 3.9 ml/min/kg for bosentan (F. Hoffmann – La Roche data on file), 6.5 ml/min/kg for bufuralol [21], 4.88 ml/min/kg for mibefradil [22,23] and 12.0 ml/min/kg for midazolam [24–26].

For theophylline and diclofenac, both low clearance compounds, values of in vivo CL_H tended to be equivalent

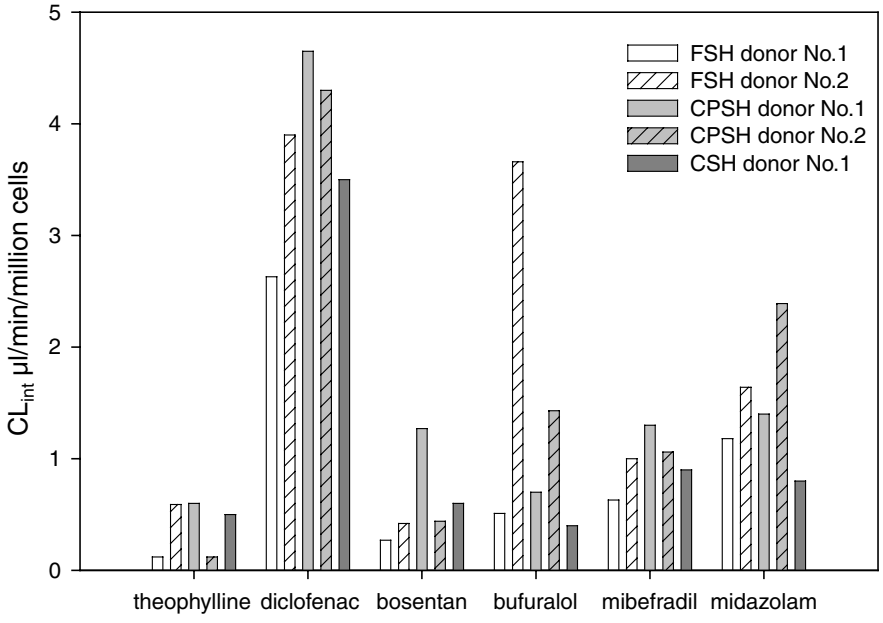

Fig. 2. Values of intrinsic clearance obtained for theophylline, diclofenac, bosentan, bufuralol, mibefradil and midazolam using suspension of human hepatocytes prepared either directly after isolation (FSH) from donors No. 1 and No. 2, either after 24 h of cold preservation (CPSH) from donors No. 1 and No. 2 or after cryopreservation (CSH) from donor No. 1.

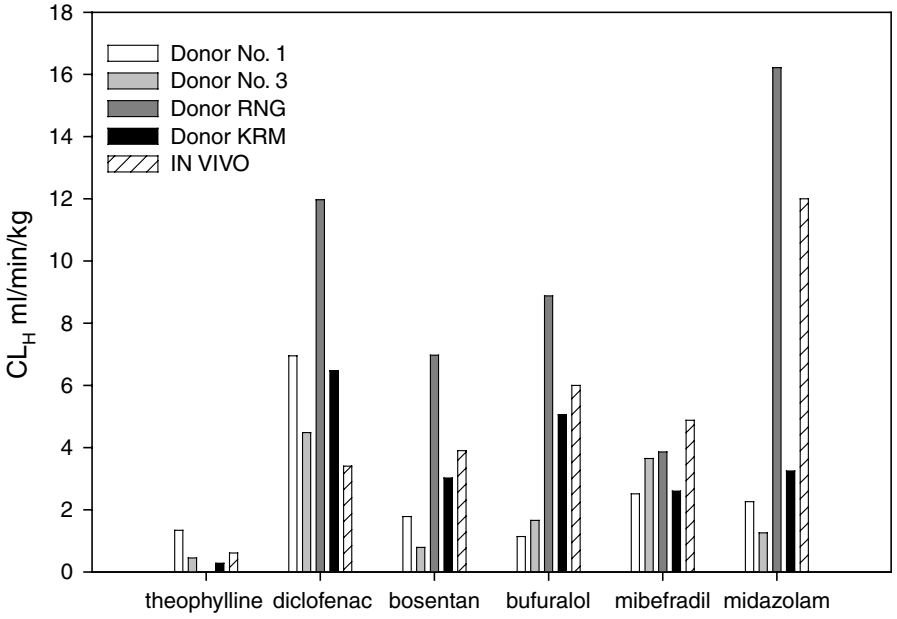

Fig. 3. Values of hepatic clearance (CL_H) obtained for theophylline, diclofenac, bosentan, bufuralol, mibefradil and midazolam using cryopreserved human hepatocytes from donors No. 1, No. 3, RNG and KRM versus of values of hepatic clearance observed in vivo.

Table 3
Values of intrinsic clearance and hepatic clearance (CL_H) obtained for theophylline, diclofenac, bosentan, bufuralol, mibefradil and midazolam using rat hepatocytes used either in primary culture or in suspension compared to values observed in vivo

Compounds (10 μM)	Donor	Prediction of CL_H (ml/min/kg) using				CL_H (ml/min/kg) observed in vivo
		CPC CL_H (ml/min/kg)	FSH CL_H (ml/min/kg)	CPSH CL_H (ml/min/kg)	CSH CL_H (ml/min/kg)	
Theophylline CYP 1A2, 2E1, 3A4	No. 1	0.02	0.36	1.8	1.3	0.61
	No. 2	0.1	1.64	0.35	ND	
Diclofenac CYP 2C9	No. 1	5.9	5.76	8.35	6.95	3.4
	No. 2	3.9	7.45	7.94	ND	
Bosentan CYP 3A4, 2C9	No. 1	1.6	0.77	3.2	1.78	3.9
	No. 2	0.825	1.12	1.23	ND	
BUFURALOL CYP 2D6	No. 1	3.5	0.51	2	1.14	6
	No. 2	6.12	7.21	3.80	ND	
Mibefradil CYP 3A4, UGT	No. 1	3	1.76	3.2	2.5	4.88
	No. 2	1.06	3.04	2.80	ND	
Midazolam CYP 3A4	No. 1	4.1	3.08	3.5	2.3	12.0
	No. 2	1.77	3.97	5.39	ND	

ND, no values of clearance determined (cryopreserved hepatocytes were not available for this donor).

or over-predicted using FSH, CPSH or CSH (Table 3). For example, for theophylline (in vivo observed CL_H = 0.61 ml/min/kg), the predicted clearance was 0.36 (FSH), 1.8 (CPSH) and 1.3 (CSH) ml/min/kg for donor No. 1, respectively, and 1.64 (FSH) and 0.35 (CPSH) ml/min/kg for donor No. 2, respectively, while predicted CL_H from CPC values were underestimated: 0.02 (donor No. 1) and 0.1 (donor No. 2) ml/min/kg, respectively.

For the four other compounds, namely bufuralol, mibefradil, midazolam (medium clearance compounds) and bosentan (a low clearance compound), the predicted CL_H values were underestimated by 2- to 5-fold compared to the observed in vivo values, whatever the experimental conditions used (FSH, CPSH or CSH). For example, predictions of in vivo CL_H obtained for midazolam using CPC, FSH, CPSH or CSH were 4.1, 3.08, 3.5 and 2.3 ml/min/kg, respectively, for donor No. 1, and 1.77, 3.97 and 5.39 ml/min/kg, respectively, for donor No. 2 (Table 3), clearly under-predicting the in vivo observed CL_H(12.0 ml/min/kg).

3.3. Predicted in vivo CL_H using cryopreserved human hepatocytes isolated from 4 donors (No. 1, No. 3, RNG and KRM)

Four sets of cryopreserved human hepatocytes, isolated from four different donors (donors No. 1, No. 3: obtained in house; RNG and KRM: commercially available), were used in suspension. The in vivo predicted CL_H were determined for the six test compounds in these hepatocytes preparations and were compared to the in vivo observed CL_H.

As expected, and related to genetic polymorphism of the main CYPs involved in the metabolism of these reference compounds (Table 3), the predicted clearances obtained from the four donors of hepatocytes were donor and compound dependent, reflecting interindividuals' variabilities.

For example, the predicted CL_H for diclofenac, bosentan and bufuralol using cryopreserved hepatocytes from donor KRM were close to the in vivo observed CL_H (less than twofold under/overpredicted), whereas predicted CL_H for midazolam, mibefradil and theophylline were 2- to 4-fold underestimated compared to the in vivo observed clearance when using this hepatocyte preparation. The CL_H predicted for diclofenac, mibefradil and theophylline using cryopreserved hepatocytes from donor No. 3 were close to the observed in vivo CL_H, whereas CL_H predicted for midazolam, bosentan and bufuralol were 3- to 6-fold underestimated with this hepatocyte preparation.

4. Discussion

Because of the increasing number of compounds synthesised weekly, the pharmaceutical industry needs to develop systems to speed up screening and ranking, in order to test new chemical entities with a higher throughput and a high degree of accuracy. Traditionally, human hepatocytes were used in primary cultures but were limited by both scientific issues such as their tendency to under-predict CL_H and technical issues such as the erratic and unpredictable availability of human liver samples for hepatocyte isolation. During the last few years, suspensions of rat hepatocytes were shown [1,7] to improve the prediction of in vivo hepatic metabolic clearance, in 24- as well as in 48-well plates, and similar results have been reported with suspensions of human hepatocytes [8–10,27] (Blanchard et al., submitted[4]). This opened up the possibility of using cryopreserved human hepatocytes that usually do not attach to collagen-coated plates.

The main objective of this study was to evaluate hepatocytes in suspension in a miniaturised format, e.g., 96-well plates, in order to increase throughput for compound testing via automation and to decrease cell consumption for the assay, which is of great interest in the context of screening and ranking of compounds at the early phase of drug discovery. The first step was an optimisation of this system using rat hepatocytes, and the second step was the evaluation of this model under the predetermined conditions (obtained with rat hepatocytes) with different sources and preparations of human hepatocytes, namely fresh or cold/cryopreserved human hepatocytes.

The preevaluation study, performed using rat hepatocytes, showed that hepatocytes in suspension or in primary culture both predicted the in vivo plasma clearances of diclofenac, bosentan, bufuralol and theophylline rather well, whereas the in vivo clearances of midazolam and mibefradil were under-predicted. The reasons for this tendency to under-predict, such as extra-hepatic (intestinal?) metabolism that obviously cannot be discussed "covered" with pure hepatic models, involvement of active transport and so on, have been extensively in a previous paper [1].

A "proof of concept" study was then carried out with human hepatocytes. In the present study, human hepatocytes used either immediately after isolation (FSH) or 20 h after cold-storage (CPSH) were isolated from liver resections obtained from three human donors (donor Nos.1, 2 and 3) under conditions that have been reported to be optimal [28].

For low clearance compounds, such as theophylline, predicted clearances obtained in suspensions (respectively, 0.36 and 1.8 ml/min/kg in FSH and CPSH for donor No. 1 and 1.64 and 0.35 ml/min/kg in FSH and CPSH for donor No. 2) were globally higher than those obtained in cultures (0.02 and 0.1 ml/min/kg, respectively). Results obtained for theophylline confirmed that the 96-well format can also be run for low clearance compounds. In fact, clearance values obtained for this compound, if not improved, were at least as good as those obtained with CPC. For these compounds, the difficulty lies more in the sensitivity of the analytical method than in the CYPs activities/cell accessibilities [1,10]. Further to cold preservation, and in agreement with the range of viability obtained after such cold-storage [10,29], the viability of hepatocytes isolated from donor No. 2 was low (close to 30%). However, no Percoll centrifugation was performed before use because of the high number of cells that could be lost during this step. Cell density used for incubation was based on viable-cells, and 3 million viable hepatocytes were incubated in each preparation. It is noteworthy that this low viability (and, consequently, the presence of dead cells) did not affect the prediction, even for lipophilic compounds such as mibefradil. These results confirm that hepatocytes could be used in suspension for up to 20 h after isolation and cold-storage in U.W., the time required for shipment and setting up of the experiment.

However, the most convenient storage condition for human hepatocytes, enabling a full, free handling, is cryopreservation. Results achieved in the present work with hepatocytes from donor No. 1 confirmed results obtained in other publications, showing that values of clearance obtained with fresh and cryopreserved hepatocytes are similar [1,27,30–32].

Four lots of cryopreserved human hepatocytes, obtained from two different sources, were evaluated. Donor Nos.1 and 3 were provided in house, and donors RNG and

KRM were purchased commercially. Results obtained confirm, that when using 150,000 cells per well in 96-well plates, the capacity of human hepatocytes to predict hepatic clearance of very low as well as of medium/high compounds is not altered by cryopreservation, whatever the source of supply. The viability of cryopreserved human hepatocytes obtained for these four donors, varying from 41% to 90%, did not affect their capacity to predict clearance.

Based upon the four batches of human hepatocytes that we investigated, variabilities observed in the predicted CL_H were both donor and compound dependent. Values of CL_H were underestimated for midazolam, bosentan and bufuralol in donor No. 2, whereas CL_H were close to in vivo observed values for theophylline, diclofenac and bosentan. Considering donor RNG for example, values of predicted CL_H were difficult to measure (0.02 ml/min/kg) for theophylline, whereas clearances were over-predicted for diclofenac, and close to in vivo observed values for midazolam, mibefradil and bosentan. These variations, depending on the donor and on the compound, in the capacity to successfully predict CL_H reflected, at least partly, the interindividual variability. This confirmed that several human donors must be used to determine clearance, to ensure covering interindividual variability of the population, particularly for polymorphic cytochromes. Indeed, some authors [33–35] have shown that the expressions of most of the CYP 450 (1A2, 2A6, 2B6, 2C9, 2D6, 2E1 and 3A4) were polymorphic, with 200- to 20,000-fold variations of the expression levels among the population. It is described in the literature that, using preparations from different subjects, a 3- to 5-fold variation was found in the in vitro metabolic clearance for some standard compounds [8].

In conclusion, this miniaturised assay of human hepatocytes in suspension in a 96-well format gives encouraging results for the screening of new compounds. This model enables, primarily, a decrease in the number of hepatocytes used, which is of great value for human hepatocytes, but also allows an increase in the number of compounds that can be screened, with a much higher speed, but still with a certain degree of accuracy. Moreover, hepatocytes can be used in suspension, either for up to 24 h of cold storage after isolation, a time required for shipment and setting up of the experiment, or after cryopreservation. This latter condition presents the huge advantage to enable their use whenever needed and to avoid the dependency upon an erratic and unpredictable supply of fresh human cells. Results achieved can be considered very satisfactory for screening and ranking of compounds at the early phase of drug discovery.

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