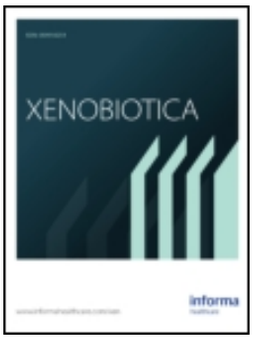

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RESEARCH ARTICLE

A novel matrix for the short-term storage of cells: utility in drug metabolism and drug transporter studies with rat, dog and human hepatocytes

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Abstract

1. The SureTran™ matrix is a novel method facilitating short-term maintenance of fresh primary hepatocyte cellular function and offers the potential use of primary cells "as fresh" for several days post isolation. In the study presented, the maintenance of several key phase I and II drug metabolizing enzyme and drug transporter activities is demonstrated with rat and dog hepatocytes preserved for up to 7 days after cell isolation.
2. Intrinsic clearance values were determined for 60 new chemical entities using rat hepatocytes freshly isolated at AstraZeneca and rat hepatocytes prepared at the facilities of Abcellute Ltd (SureTran™ purveyors), stored and incubated 24 hours after isolation. A very good correspondence in the intrinsic clearance values underlines the utility of the cell maintenance matrix.
3. For human hepatocytes many of the enzyme activities assayed were well maintained for 7 days of storage but some declined to below 50% of initial values between day 4 and 7 of storage. Human OATP1B1 activity was only determined with one batch and declined to 51% of the initial test value by day 4 and further down to 35% by day 7.

Keywords: Fresh Hepatocytes, rat, dog, human, storage, enzyme activity, transporter activity

Introduction

The *in vitro* use of isolated hepatocytes provides the most physiologically relevant model with which to measure qualitative and quantitative aspects of hepatic drug metabolism since they contain the full complement of enzymes a compound is likely to encounter *in vivo*. Additionally, key drug transporters are expressed and can be, under appropriate *in vitro* conditions, correctly orientated in isolated hepatocytes. Accordingly hepatocytes are routinely used to determine metabolic stability, drug transporter dependency and drug-drug interaction potential including the induction of drug metabolizing enzymes (Le Cluyse et al. 2000; Kato et al. 2005; McGinnity et al. 2005; Zhao et al. 2005; Soars et al. 2007; Grime et al. 2008; Kusuhara & Sugiyama 2010) as well as *in vitro* toxicological assessment (Sahi et al. 2010).

Concerns over the availability of freshly isolated human hepatocytes prompted refinement of cryopreservation techniques such that today hepatocytes have effectively become an "off-the-shelf" reagent (Li et al. 1999; Soars et al. 2007). Apart from this convenience, additional advantages include the ability to pool cells obtained from different human donors in a single incubation and the capability to purchase with the drug metabolizing enzymes and inducibility of such enzymes pre-characterized.

Despite all the advances in cryopreservation and the subsequent thawing technique (Li et al. 1999; McGinnity et al. 2004), the process is a somewhat harsh technique typically involving relatively high concentrations of the cytotoxic organic solvent dimethylsulphoxide (DMSO) and rapid changes in temperature. Consequently, despite optimization of the technique and skilful production of

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high quality cryopreserved human hepatocytes (Li 2008), yields of viable hepatocytes are reduced and some key applications such as cell transplant, toxicity testing and assays involving cell culture are perhaps more uncertain (Terry et al. 2010; Sohlenius-Sternbeck & Schmidt 2005; Terry et al. 2007). Advances in the methodology continue to be investigated (Saliem et al. 2012) but it is still a consideration that cell surface proteins such as drug transporters and those involved in cell adhesion may be more at risk of unpredictability in response to the process than intracellular molecules. Variability in the uptake rates of drug transporter substrates certainly indicates that several individual batches of cryopreserved hepatocytes need to be used in this type of study (Shitara et al. 2003; Shitara et al. 2004), although it is not clear to what extent variability in uptake rate is due to donor polymorphisms in transporter expression (Tirona et al. 2001; Michalski et al. 2002; Nozawa et al. 2002; Letschert et al. 2004). Nevertheless, recently it has been observed that OCT1 and OATP1B1/3 activity may not always be well conserved after cryopreservation of human hepatocytes (Badolo et al. 2011). The evidence clearly points to uncertainty in transporter activity retention for cryopreserved hepatocytes. On thawing, cryopreserved hepatocytes often have reduced viability and suffer considerable inter-batch variability in attachment rates and efficiency (Alexandre et al. 2002; Illouz et al. 2008; Saliem et al. 2012) as well as impaired glutathione-conjugation capacity (Sohlenius-Sternbeck & Schmidt 2005). Clearly this is a major issue since cell adhesion and subsequent cell culture is a prerequisite for the study of drug metabolizing enzyme regulation.

SureTran™ is a proprietary medium but it's value in apparently facilitating the successful storage of hepatocytes so that they can be used "as freshly isolated" for several days post isolation without the need for a cryopreservation step, makes it worthy of investigation. In the series of experiments presented here, the maintenance of phase I and II drug metabolizing enzymes as well as drug transporter activity was assessed in rat, dog and human hepatocyte preparations preserved in SureTran™. The aim was to understand these basic properties in order to determine the suitability of the cells for drug metabolism studies.

Materials and methods

Chemicals

S-Mephenytoin, bufuralol and midazolam were purchased from Ultrafine Chemicals (Manchester, UK and Stockholm, Sweden). Acetaminophen, amodiaquine, bupropion, phenacetin, and diclofenac (sodium salt), ethinyl estradiol, gemfibrozil, ketoprofen and NADPH were purchased from Sigma-Aldrich (Dorset, UK and Stockholm, Sweden). Desethylamodiaquine and hydroxybupropion were purchased from TRC (Toronto, Canada). 4-Hydroxydiclofenac was purchased from BD Gentest (Stockholm, Sweden). 1-Hydroxymidazolam, 4-hydroxymephenytoin and 1-hydroxybufuralol were purchased from Ultrafine (Manchester, UK). [^{3}H]-Estrone-3-sulfate (specific activity 2120 GBq/mmol), [^{3}H]-tiotropium and [^{3}H]-taurocholate (4.6 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Williams E, and glutamine were purchased from Sigma-Aldrich (Dorset, UK). HEPES was purchased from Gibco, Life technologies (Paisley, UK). Silicone oil (AR20 and AR200) were purchased from Wacker Chemie GmbH (Munich, Germany) and caesium chloride was purchased from Chempur (Karlsruhe, Germany).

E. coli coexpressing the relevant bacterial membranes containing rat cytochrome P450s coexpressed with rat NADPH-cytochrome P450 reductase and dog cytochrome P450s coexpressed with dog NADPH-cytochrome P450 reductase were purchased from CYPEX (Dundee, UK).

Hepatocytes were purchased from Abcellute Limited (Cardiff, UK). Additionally, rat hepatocytes were isolated at AstraZeneca laboratories in order to compare with the Abcellute rat hepatocytes for key drug metabolizing enzyme and transporter activities and for use in the determination of intrinsic clearance values for 60 AstraZeneca new chemical entities (NCEs). The AstraZeneca prepared hepatocytes were isolated from adult male Sprague-Dawley rats (250–300 g) using a procedure based on the method of Seglen (1976) as described previously (Kenny & Grime 2006). Cryopreserved human hepatocytes were also used for comparison with the Abcellute human hepatocytes. These were prepared from an isolated lobe of human liver (obtained from local hospitals with ethical approval) and cryopreserved as detailed previously (McGinnity et al. 2004). Twenty one batches of cryopreserved human hepatocytes were characterized within 24 hours of isolation and cryopreservation, each batch coming from an individual donor.

Recombinant CYP incubations

Seven human CYP selective probe substrates (phenacetin, 25 μM; diclofenac, 2 μM; S-mephenytoin, 20 μM; bufuralol, 5 μM; midazolam, 2.5 μM; bupropion, 25 μM and amodiaquin, 1 μM) were incubated with rat and dog CYPs to assess probe substrate specificity. The substrates were prepared as methanol stocks and added to incubate such that the final concentration of methanol was 1% v/v. The incubations were performed in phosphate buffer (0.1 M, pH 7.4) containing 1 mM NADPH and 20 pmoles/mL CYP for 10 min. Incubations were terminated by the removal of 50 μL into 100 μL methanol. Samples were placed at –20°C for 2 hours, centrifuged at 2200 g for 15 min and the supernatant (120 μL) was analyzed by LC-MS-MS as described below.

Rat and dog hepatocyte incubations

Hepatocytes in SureTran™ matrix were received from Abcellute Ltd. The cells sit in the gelatinous SureTran™ matrix in petri dishes and these dishes containing the cells were stored in an incubator that maintained a constant 10°C temperature until the day of use. The cells were

then released (on the day 1, 2, 4 and 7) from the matrix according to the Abcellute application notes. Briefly, the plates with cells in matrix were warmed up to 37°C until the matrix was fluid. After centrifugation the cells were washed with Abcellute proprietary media. Following a final cell wash and re-suspension in incubation media (Williams E supplemented with 25 mM HEPES and 2 mM L-glutamine and set to pH 7.4), the hepatocytes (1 million cells/mL, 1 mL incubations) were incubated with test drugs (phenacetin, 25 μM; diclofenac, 2 μM; S-mephenytoin, 20 μM; bufuralol, 5 μM; midazolam, 2.5 μM; bupropion, 25 μM; amodiaquin, 1 μM; ethinyl estradiol, 1 μM; gemfibrozil, 1 μM and ketoprofen, 1 μM) for 60 min. Samples (50 μL) were removed and added to methanol (100 μL) at each of eight time points to terminate. Samples were placed at –20°C for 2 hours and centrifuged at 2200 g for 15 min and the supernatant (120 μL) was analyzed by LC-MS-MS as described below.

[^{3}H]-Estrone–3-sulphate, [^{3}H]-taurocholate and [^{3}H]-tiotropium were used to assess organic anion transporter polypeptide (OATP), sodium dependent taurocholate co-transporting polypeptide (NTCP) and organic cation transporter (OCT) activities. Intrinsic clearance (CLint) values for drug appearance into hepatocytes were determined using a method adapted from the centrifugal filtration technique of Parker & Houston (2008). A vial containing hepatocytes at a concentration of 2 million viable cells/mL was pre-incubated for 5 min in a water bath at 37°C along with a vial containing 500 μL of tritiated substrate in suspension buffer (final concentrations 3 μM estrone sulphate, 5 μM taurocholate or 1 μM tiotropium). Reactions were initiated with the addition of 500 μL of hepatocyte suspension to the substrate.

Aliquots (100 μL) were removed at 10, 20, 30, and 40 s and immediately dispensed into microtubes containing 10 μL of 3 M caesium chloride (lower layer) and 150 μL of silicone oil (density of 1.015 g/mL, upper layer) and were immediately centrifuged at 7000 g for 30 s using a MiniSpin centrifuge (Eppendorf, Cambridge, UK). During this process the hepatocytes pass through the oil into the caesium chloride. After an overnight incubation at –20°C to fully disrupt the hepatocyte cytoplasmic membrane in the caesium chloride, each centrifuge tube was frozen in liquid nitrogen and cut, with collection of the cell pellet in a scintillation vial. After the addition of scintillation cocktail, the amount of radioactivity in the cells was determined using a Packard 2200CA Tri-Carb liquid scintillation counter (PerkinElmer Life and Analytical Sciences, High Wycombe, UK).

For the determination of CLint values for 60 AstraZeneca NCEs for the comparison of Abcellute hepatocytes and freshly prepared rat hepatocytes, hepatocytes (1 million cells/mL, 1 mL incubations) were incubated with NCEs (1 μM in DMSO, 1% v/v) for 60 min. Samples (50 μL) were removed and added to methanol (100 μL) at each of eight time points to terminate. Samples were placed at –20°C for 2 hours and centrifuged at 2200 g for 15 min and the supernatant (120 μL) was analyzed by LC-MS-MS as described below.

Human hepatocyte incubations

Hepatocytes in SureTran™ matrix were received from Abcellute Ltd and released from the matrix according to the Abcellute application notes as described above (rat and dog hepatocyte incubations). Additionally, cryopreserved hepatocytes were thawed as detailed previously (McGinnity et al. 2004) and incubated in the same way as Abcellute cell suspensions as follows: hepatocytes (1 million cells/mL, 50 μL incubations, one incubation for each time point) were incubated with test drugs for 30 min (phenacetin, 25 μM; diclofenac, 2 μM; S-mephenytoin, 20 μM; bufuralol, 5 μM; midazolam, 2.5 μM; bupropion, 25 μM and amodiaquin, 1 μM) or 60 min (ethinyl estradiol, 1 μM; gemfibrozil, 1 μM and ketoprofen, 1 μM). The incubations were stopped by addition of acetonitrile (150 μL) at each of five time points. Samples were placed at –20°C for 30 min and centrifuged at 4000 g for 20 min. The supernatant was diluted with water (1:1) and analyzed by LC-MS-MS as described below.

Atorvastatin and estrone–3-sulphate, were used to assess organic anion transporter polypeptide (OATP) activity. CLint values for drug appearance into hepatocytes were determined using the method of Parker & Houston (2008) described above with the exception that the samples were analyzed using LC-MS-MS.

LC-MS-MS conditions

For the analysis of paracetamol, 4-hydroxy diclofenac, 4-hydroxy S-mephenytoin, 1-hydroxy bufuralol and 1-hydroxy midazolam in samples from rat and dog hepatocyte incubations the LC-MS-MS conditions were as follows: mass spectrometry was performed on a Micromass Quattro Ultima Platinum triple quadrupole mass spectrometer (Waters, Manchester, UK) using multiple reaction monitoring using in positive ion mode with chromatographic separation being performed using a Hewlett Packard 1100 high performance liquid chromatography system (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was achieved with a Devosil C30 column using 30 μL of sample. The mobile phase consisted of water with 0.1% (v/v) formic acid (A) and methanol containing 0.1% (v/v) formic acid (B). The gradient was as follows: 97% A (0–0.3 min), 97% to 5% A (0.3–0.55 min), 5%A (0.55–1.55 min), 5% to 97% A (1.6 min), 97%A to 2.5 min. The flow rate was 1.2 mL/min and the column temperature was 40°C.

For the analysis of the CYP-substrates from incubations with human hepatocytes, except for the analysis of 4-hydroxy S-mephenytoin, a Waters Xevo TQ (Waters, Manchester, UK) in a positive ion mode was used. Chormatographic separation was achieved with a Waters ACQUITY UPLC (Waters, Manchester, UK) with a ACQUITY UPLC BEH C18, 1.7 μm, 2.1 × 30 mm column with an injection volume of 5 μL. The mobile phase consisted of water with 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). The gradient

was as follows: 96% A (0 min), 96% to 5% A (0–0.8 min), 5% A (0.8–1.3 min), 5% to 96% A (1.3–1.4 min), 96% A to 1.8 min. The flow rate was 0.8 mL/min and the column temperature was 40°C. For the analysis of 4-hydroxy S-mephenytoin a TSQ Quantum ultra triple quadropole MS instrument with an APCI ion source was used to carry out the sample analysis in the MRM mode (MS/MS). Chormatographic separation was achieved with Flux HPLC-pump (Thermo scientific, Stockholm, Sweden) with a Phenomenex Synergi 50 × 2 mm, 4 micron column with an injection volume of 10 µL. The mobile phase consisted of water with 0.1% (v/v) formic acid (A) and methanol containing 0.1% (v/v) formic acid (B). The gradient was as follows: 97% A (0–0.25 min), 97% to 0% A (0.25–0.25 min), 0% A (0.25–1.75 min), 5% to 97% A (1.75–1.85 min). The flow rate was 0.65 mL/min and the column temperature was 40°C.

For quantification of the metabolites, standard curves with metabolite standards were performed.

For the analysis of the UGT-substrates (ethinyl estradiol, gemfibrozil and ketoprofen) from incubations with human hepatocytes, an Agilent 6410 triple quadropole (Agilent Technologies Inc., Wilmington, DE, USA) was used. For gemfibrozil and ketoprofen negative electrospray ionization mode were used and for ethinyl estradiol positive electrospray ionization mode was used. Chormatographic separation was achieved with an Agilent 1200 SL LC pump and a Poroshell Fused core C18 50 × 2.1 mm column, 2.7 µm particle size (Agilent Technologies Inc., Wilmington, DE, USA). The sample injection volume was 10 µL. The mobile phase consisted of water with 2% acetonitrile and 0.2% (v/v) formic acid (A) and acetonitrile containing 0.2% (v/v) formic acid (B). The gradient was as follows: 95% to 5% A (0–1.2 min), 5% A (1.2–1.8 min), 5% to 95% A (1.8–1.9 min). The flow rate was 0.7 mL/min and the column temperature was 40°C.

For the determination of intrinsic clearance values for 60 AstraZeneca NCEs, LC-MS-MS was performed as described for the rat and dog experiments with the following modifications: chromatographic separation was achieved using a Hypersil Gold C18 (4.6 × 50 mm, 3 µm) column (Thermo Electron Corporation, Basingstoke, UK) using 10 µL of each sample. The mobile phase consisted of water with 0.1% (v/v) formic acid (A) and methanol containing 0.1% (v/v) formic acid (B). The gradient was as follows: starting at 95% A (0–0.5 min), 95% A to 0% A (0.5–2 min), 0% A (2–3 min), 0 to 95% A (3–4 min). The flow rate was 1.2 mL/min, and the column temperature was 40°C.

Data analysis

Rates of metabolism for the CYP and UGT marker substrates were calculated using data taken only from where metabolite formation was linear with respect to incubation time.

Intrinsic clearance was calculated from product of the linear slope (k) of the natural log concentration–time data and the volume of the incubation (V), such that intrinsic clearance = k. V.

Uptake rates for the drug transporter assays were calculated as described previously (Grime et al. 2008).

Results

CYP substrate selectivity investigation

CYP substrates were selected because they are used as selective probes for the major human drug metabolizing CYPs (Weaver et al. 2003; O'Donnell et al. 2007). To determine the rat and dog CYP specificity, experiments were performed using membranes containing single rat or dog CYPs and CYP reductase. Rat CYP results indicated a similar CYP-selectivity profile to human for phenacetin O-deethylation (metabolized primarily by rat CYP1A2), diclofenac 4-hydroxylation (primarily rat CYP2C11), S-mephenytoin 4-hydroxylation (primarily rat CYP2C6 and CYP2C11), bufuralol hydroxylation (primarily rat CYP2D2), midazolam 1-hydroxylation (primarily rat CYP3A2), bupropion hydroxylation (primarily rat CYP2B1) and amodiaquin N-deethylation (primarily rat CYP2C6) (Figure 1).

For dog CYP studies only two CYPs (CYP2C21 and CYP3A12) were readily commercially available. The results showed that dog CYP2C21 was involved in phenacetin O-deethylation, 4-hydroxylation of S-mephenytoin, 1-hydroxylation of bufuralol and the 1-hydroxylation of midazolam, and that dog CYP3A12 was involved in the 1-hydroxylation of midazolam.

CYP, UGT and drug transporter activities in suspensions of freshly isolated rat hepatocytes

Rat hepatocyte CYP, UGT and drug transporter activities were assayed immediately after isolation of the cells in

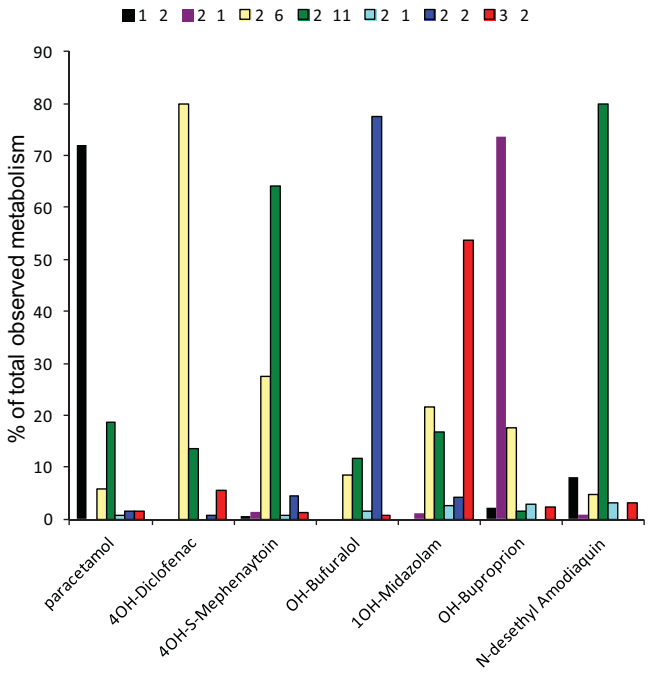

Figure 1. Rat CYP substrate specificity profiles generated using Bactosomes prepared from *E. coli* cells co-expressing recombinant rat CYPs and NADPH-cytochrome P450 reductase.

the laboratories of AstraZeneca, as detailed in materials and methods. The results are shown in Table 1.

Maintenance of rat hepatocyte activities

Good maintenance of rat hepatocyte CYP, UGT and drug transporter activities was demonstrated following assays after 1, 2, 4 and 7 days of hepatocyte storage in SureTran™ matrix in the petri dishes at 10°C (see materials and methods). For rat hepatocytes the mean CYP activities were maintained to at least 80% (of day 1 activity) until day 7 for hydroxy bupropion formation (CYP2B1), 4-hydroxy S-mephenytoin formation (CYP2C6, CYP2C11), 4-hydroxy diclofenac formation (CYP2C11), 1-hydroxy bufuralol formation (CYP2C21, CYP2D15) and 1-hydroxy midazolam formation (CYP3A12, CYP2C21). The phenacetin O-deethylation activity (CYP1A2, CYP2C6) and amodiaquin N-deethylation (CYP2C6) declined from greater than 80% on day four to approximately 60% and 40% respectively on day 7 (Table 2). The three UGT activities investigated, UGT1A1 (ethinyl estradiol as a substrate), UGT2B1 (gemfibrozil) and UGT2B31 (ketoprofen), were maintained at levels greater than 60% of day 1 for 7 days (Table 3).

The oatp activity (estrone-3-sulphate uptake) was maintained above 80% of day 1 activity until day 7. OCT activity (tiotropium uptake) was maintained above 80% of day 1 activity until day 4, but decreased to 60% by day 7. The taurocholate uptake activity was greater than 80% of day 1 for the first 4 days, but declined to 48% by day 7 (Table 4).

Table 1. Mean freshly isolated rat hepatocyte CYP, UGT and drug transporter activities, measured as metabolite formation rate from CYP substrates, intrinsic clearance of UGT substrates and uptake intrinsic clearance of drug transporter substrates (n = 3 incubations with data presented as mean ± standard deviation).

Reaction assayed	Activity
Paracetamol	34 ± 6 pmol/min/million cells
4-hydroxy diclofenac	245 ± 13 pmol/min/million cells
4-hydroxy S-mephenytoin	3 ± 0 pmol/min/million cells
1-hydroxy bufuralol	147 ± 6 pmol/min/million cells
1-hydroxy midzolam	47 ± 3 pmol/min/million cells
1-hydroxy bupropion	74 ± 5 pmol/min/million cells
N-desethyl amodiaquin	16 ± 1 pmol/min/million cells
Ketoprofen CLint	18 ± 1 µL/min/million cells
Ethynyl estradiol CLint	240 ± 17 µL/min/million cells
Gemfibrozil CLint	110 ± 21 µL/min/million cells
Estrone-3-sulphate uptake Clint	215 ± 30 µL/min/million cells

Comparison of CLint values between freshly isolated rat hepatocytes and rat hepatocytes maintained in SureTran™ matrix for 24 hours post isolation

Rat hepatocyte CL_{int} values of 60 AstraZeneca NCEs were shown to be very similar when the compounds were incubated with rat hepatocytes preserved in SureTran™ matrix for 24 hours and rat hepatocytes freshly isolated at AstraZeneca (Figure 2).

Maintenance of dog hepatocyte activities

Good maintenance of dog hepatocyte CYP, UGT and drug transporter activities was demonstrated following assays after 1, 2, 4 and 7 days of hepatocyte storage in SureTran™ matrix. 4-Hydroxy S-mephenytoin formation (CYP2C21) and 1-hydroxy midazolam formation (CYP3A12, CYP2C21) were maintained to at least 80% of day 1 activity until day 7 while 4-hydroxy diclofenac formation (CYP2C21) showed an activity above 90% of the day 1 value at days 2 and 7, but the data showed a drop in activity down to 60% at day 4. For 1-hydroxy bufuralol formation (CYP2C21) the mean activities declined from greater than 80% on day 4 to approximately 60% on day 7. The same was true for hydroxy bupropion formation (Table 5). The UGT activity investigated, UGT2B31 (ketoprofen glucuronidation), showed an excellent maintenance of activity at levels at 100% of day 1 for 7 days (Table 3).

The oatp (estrone-3-sulphate uptake) mean activity was maintained above 80% of day 1 activity until day 4, but then decreased to 60% at day 7. OCT (tiotropium) mean activity was maintained above 80% (of day one activity) until day 7. The mean taurocholate uptake activity was greater than 80% the first 4 days, but declined to approximately 20% day 7 (Table 4).

CYP, UGT and drug transporter activities in suspensions of cryopreserved human hepatocytes

CYP, UGT and OATP activities were assayed in suspensions of thawed cryopreserved human hepatocytes, as detailed in materials and methods. The results are shown in Table 6.

Table 2. Mean rat hepatocyte CYP activities, measured as metabolite formation rate from CYP substrates (n = 3 incubations per day with data presented as mean ± standard deviation).

		Metabolite formation rate (pmol/min/million cells)			
Measured metabolite	Metabolising CYP	Day 1	Day 2	Day 4	Day 7
Paracetamol	CYP1A2, CYP2C6	44 ± 5	42 ± 20	49 ± 27	24 ± 5
4-hydroxy diclofenac	CYP2C11	272 ± 143	259 ± 135	291 ± 199	318 ± 137
4-hydroxy S-mephenytoin	CYP2C6, CYP2C11	3 ± 0	3 ± 1	4 ± 2	2 ± 0
1-hydroxy bufuralol	CYP 2C6, CYP2D2	140 ± 75	130 ± 48	124 ± 70	99 ± 51
1-hydroxy midzolam	CYP3A2, CYP2C11, CYP2C6	30 ± 22	45 ± 12	40 ± 10	49 ± 2
1-hydroxy bupropion	CYP2B1, CYP2C11	78 ± 5	75 ± 14	105 ± 24	75 ± 25
N-desethyl amodiaquin	CYP2C6	14 ± 1	15 ± 5	13 ± 5	8 ± 5

Table 3. Hepatocyte intrinsic clearance of UGT substrates in rat and dog hepatocyte incubations.

	Rat hepatocytes, Clint (μL/min/million cells) (n = 2 incubations per day)				Dog hepatocytes, Clint (μL/min/million cells) (n = 1 incubations per day)			
Substrate	Day 1	Day 2	Day 4	Day 7	Day 1	Day 2	Day 4	Day 7
Ketoprofen	16	18	19	11	11	18	19	100
Ethynyl estradiol	239	251	229	214	ND	ND	ND	ND
Gemfibrozil	100	89	92	94	ND	ND	ND	ND

ND: No data.

Table 4. Hepatocyte uptake intrinsic clearance of drug transporter substrates in rat and dog hepatocyte incubations.

	Rat hepatocytes, Clint (μL/min/million cells) (n = 2 incubations per day)				Dog hepatocytes, Clint (μL/min/million cells) (n = 2 incubations per day)			
Substrate	Day 1	Day 2	Day 4	Day 7	Day 1	Day 2	Day 4	Day 7
Estrone -3- sulphate	263	322	264	201	71	61	68	39
Taurocholate	246	248	214	117	68	61	56	12
Tiotropium	119	136	128	80	8	10	9	9

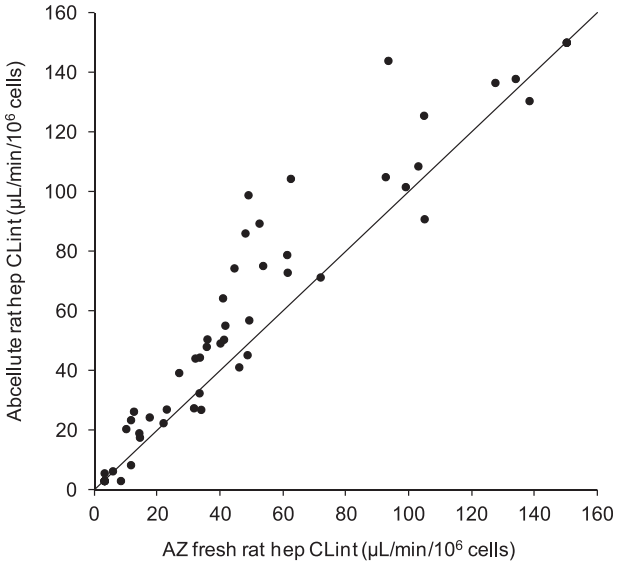

Figure 2. Intrinsic clearance values for 60 NCEs determined using freshly isolated rat hepatocytes and hepatocytes preserved in Abcellute matrix for 24 hours after isolation.

Maintenance of human hepatocyte activities

The human hepatocyte activities were tested on two different occasions with hepatocytes from two separate donors. At the first occasion enzyme activities where determined after 2, 3 and 6 days and on the second occasion after 2, 4 and 7 days of hepatocyte storage in SureTran™ Matrix. For the first batch of human hepatocytes tested, the results showed that CYP activities were maintained to at least 80% (of day 2 activity) until day 6 for CYP2B6 (1-hydroxy bupropion formation), CYP2C8 (amodiaquin N-deethylation) and CYP2D6 (1-hydroxylation of bufuralol). For CYP3A4 (1-hydroxy midazolam formation) mean activity was maintained above 90% (of day two activity) until day 3, but then decreased to 70% at day 6. Additionally, for CYP1A2 (phenacetin O-deethylation) mean activity was maintained above 70% (of day two activity) until day 3, but then decreased to 65% at day 6. For CYP2C19 (4-hydroxy-S-mephenytoin formation) activity was maintained above 50% until day 6 (Table 7).

Table 5. Dog hepatocyte CYP activities, measured as metabolite formation rate from CYP substrates (n = 1 incubations per day).

Measured metabolite	Metabolising CYP	Metabolite formation rate (pmol/min/million cells)			
		Day 1	Day 2	Day 4	Day 7
4-hydroxy diclofenac	CYP2C21	56	55	33	54
4-hydroxy mephenytoin	CYP2C21	1.4	1.8	1.5	1.1
1-hydroxy bufuralol	CYP 2C21	24	24	20	16
1-hydroxy midzolam	CYP3A12, CYP2C21	144	143	125	117
1-hydroxy bupropion		189	188	166	127

For the second batch, the results showed that the CYP3A4 (1-hydroxy midazolam formation) activity was maintained to at least 80% of day 2 activity until day 7. For CYP2C19 (4-hydroxy-S-mephenytoin formation), CYP2D6 (1-hydroxylation of bufuralol), CYP2C8 (amodiaquin N-deethylation) and CYP2B6 (1-hydroxy bupropion formation) mean activities were maintained above 70% of day 2 activity until day 4, but then decreased to 48%, 67%, 78% and 57%, respectively at day 7. Additionally, for CYP1A2 (phenacetin O-deethylation) mean activity was maintained above 50% of day 2 activity until day 4, but then decreased to 38% at day 7 (Table 8).

Interestingly an increase in activity of CYP2C9 (4-hydroxy diclofenac formation) was observed with both batches of human hepatocytes, rising to almost double the observed activity on day 2 (Tables 7 and 8).

With the first batch of human hepatocytes, UGT1A1 (ethinyl estradiol), UGT1A9, UGT2B7 (gemfibrozil and ketoprofen) showed good maintenance from day 2 to day 6, while in the second experiment the activity of UGT1A1 decreased to 44% of the day 2 activity by day 4 and at day 7 only 33% activity was recorded. UGT1A9, UGT2B7 activities remained at 87% of day 2 values until day 4 but then declined to 57% at day 7 (Table 9).

The OATP1B1 activity (atorvastatin uptake) was only determined with the second batch of human hepatocytes

Table 6. Human hepatocyte CYP, UGT and OATP activities from 21 individual batches of cryopreserved human hepatocytes (one donor per batch). Activities were measured as metabolite formation rate (CYP substrates), parent drug intrinsic clearance (UGT substrates) and atorvastatin hepatocytes uptake intrinsic clearance (data presented as mean of three incubations).

	Rate of formation of metabolites (pmol/min/million cells)							CLint (μL/min/million cells)		
Donor	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4	Ethinyl estradiol	Ketoprofen	Atorvastatin
1	172	10	45	36	6	30	44	19	14	108
2	87	13	48	42	5	32	30	31	4	No data
3	16	27	104	46	3	4	10	23	8	No data
4	27	57	149	65	2	5	14	10	10	No data
5	59	10	43	75	29	21	37	15	8	No data
6	102	5	27	58	101	26	26	17	14	No data
7	13	8	21	29	2	4	44	15	4	82
8	11	14	32	29	3	18	17	16	7	40
9	66	12	64	40	1.3	22	23	14	14	24
10	51	11	35	55	6	59	32	17	8	39
11	19	7	41	32	5	9	21	15	9	23
12	15	0.5	20	10	0	13	14	11	11	20
13	14	24	27	19	7	33	26	22	5	48
14	21	14	29	13	20	9	144	3.6	6	39
15	33	16	24	19	3	3	116	18	10	3
16	46	8.5	107	99	21	90	82	22	12	39
17	18	29	39	101	5	110	77	14	9	43
18	32	123	47	99	3	29	209	19	13	51
19	48	17	20	92	1.5	92	82	17	14	27
20	34	11	10	67	4	34	66	13	7	29
21	150	7	19	112	15	61	238	24	10	24
Mean	49	20	45	54	12	34	64	17	9	40
Modal value	16	10	20	19	3	4	14	15	14	39

Table 7. Human hepatocyte CYP activities from donor 1, measured as metabolite formation rate from CYP substrates (n = 3 incubations per day with data presented as mean ± standard deviation).

Measured metabolite	Metabolising CYP	Metabolite formation rate (pmol/min/million cells) Day 2	Day 3	Day 6
Acetaminophen	1A2	144.1 ± 1.3	104.7 ± 6.5	94.9 ± 4.2
4-hydroxy diclofenac	2C9	31.5 ± 2.5	51.6 ± 4.6	58.8 ± 5.0
4-hydroxy mephenytoin	2C19	18.0 ± 2.1	9.8 ± 0.6	10.7 ± 1.3
1-hydroxy bufuralol	2D6	15.1 ± 0.5	13.8 ± 0.6	12.9 ± 1.2
1-hydroxy midazolam	3A4	10.0 ± 0.2	9.0 ± 2.2	7.2 ± 1.1
N-desethyl amodiaquine	2C8	26.5 ± 2.0	33.0 ± 0.9	40.9 ± 4.5
1-hydroxy bupropion	2B6	15.6 ± 0.4	13.5 ± 0.8	14.7 ± 0.7

and declined to 51% of the day 2 value by day 4 and further to 35% by day 7 (Figure 3).

Discussion

In vivo hepatic metabolic clearance can be estimated successfully from *in vitro* data (Grime & Riley 2006). Many laboratories use hepatic microsomes rather than hepatocytes to determine CLint since they have traditionally been viewed as a more flexible system with which to study oxidative biotransformations in terms of ease of preparation from many species, long-term storage and availability. However, hepatocytes provide the most physiologically relevant model with which to measure qualitative and quantitative aspects of hepatic metabolism and hepatic uptake since they contain the full complement of enzymes and uptake transporters a compound is likely to encounter during hepatic metabolism (Soars et al. 2007) and allow a comprehensive identification of metabolites in pre-clinical species and human (Tucker et al. 2001). Furthermore, optimism is emerging that hepatocytes may be used to predict transporter dependent hepatobiliary clearance *in vivo* (Ghibellini et al. 2007; Nakakariya et al. 2012).

Nevertheless, the utility of hepatocytes is counterbalanced by limited flexibility of access to freshly isolated cells. Advances in cryopreservation techniques have addressed this concern (Li et al. 1999) and phase I and phase II enzyme activities are well maintained post cryopreservation (McGinnity et al. 2004; Griffin & Houston 2004). However, non-cryopreserved cells may be attractive for a variety of reasons including the study of drug transporters and enzyme regulation. The fact that the Abcellute® preservation matrix (SureTran™) does not involve either cryoprotectant chemicals such as DMSO or rapid and

Table 8. Human hepatocyte CYP activities from donor 2, measured as metabolite formation rate from CYP substrates (n = 3 incubations per day with data presented as mean ± standard deviation).

		Metabolite formation rate (pmol/min/million cells)		
Measured metabolite	Metabolising CYP	Day 3	Day 6	Day 2
Acetaminophen	1A2	30.3 ± 4.7	24.7 ± 0.6	70.7 ± 15
4-hydroxy diclofenac	2C9	24.0 ± 1.0	30.7 ± 0.6	17.3 ± 0.6
4-hydroxy mephenytoin	2C19	35.0 ± 5.0	21.3 ± 1.5	37.3 ± 12.7
1-hydroxy bufuralol	2D6	7.3 ± 0.6	6.0 ± 0.2	8.7 ± 0.6
1-hydroxy midazolam	3A4	45.0 ± 10.7	44.7 ± 2.1	46.7 ± 11.8
N-desethyl amodiaquine	2C8	30.7 ± 3.1	17.3 ± 0.6	24.0 ± 1.7
1-hydroxy bupropion	2B6	1.4 ± 0.5	0.9 ± 0.2	1.6 ± 0.4

Table 9. Human hepatocyte UGT activities, measured as intrinsic clearance values from UGT substrate metabolism.

	Experiment 1 Clint (μL/min/million cells) (n = 2 incubations per day)			Experiment 2 Clint (μL/min/million cells) (n = 2 incubations per day)		
Substrate	Day 2	Day 3	Day 6	Day 2	Day 4	Day 7
Ketoprofen	8	6	8	No data collected		
Ethynyl estradiol	15	14	17	18	8	6
Gemfibrozil	41	28	35	23	20	13

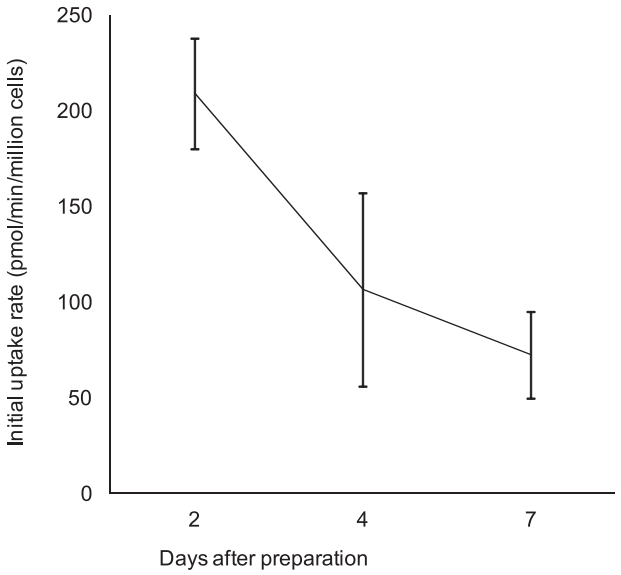

Figure 3. Initial uptake rate for atorvastatin in human hepatocytes (Donor 2). N = 4 separate incubations were performed on each day and data are presented as mean with standard deviation error bars.

significant temperature changes indicates a mechanism for preservation of cellular function that is more likely to facilitate a complete "off the shelf use as fresh" than cryopreserved cells, albeit for only short term maintenance. This work set out to simply determine the general suitability of cells stored in SureTran™ matrix for drug metabolism studies, through understanding the extent to which the major drug metabolizing enzymes and drug uptake transporters are affected by the conditions used.

Human enzyme and drug transporter selective probes were used as the basis for the analysis. An initial experiment was performed in order to ensure that key rat and dog CYP activities were covered by the probe substrates chosen. The results confirmed previously published data (Kobayashi et al. 2002) demonstrating that key rat CYP enzymology was covered (CYPs 1A2, 2B1, 2C6, 2C11, 2D2 and 3A2). For dog CYP studies, the conclusions were limited due to the commercial availability of only CYP2C21 and CYP3A12. However, the results showed that dog CYP2C21 was involved in phenacetin O-deethylation, 4-hydroxylation of S-mephenytoin, 1-hydroxylation of bufuralol and the 1-hydroxylation of midazolam, and that dog CYP3A12 was involved in the 1-hydroxylation of midazolam, concurring with previous reports (Shou et al. 2003; Aidasani et al. 2008). As reported in literature (Soars et al. 2003; Di Marco et al. 2005) ketoprofen, gemfibrozil and ethinylestradiol are suitable probe substrates for UGT metabolism in rat, dog and human hepatocytes.

Hepatocytes start to lose their liver-specific functionality very quickly during the isolation procedure. Disruption of the tissue and isolation of the cells reduces the transcription of most liver-specific genes (Clayton & Darnell 1983; Jefferson et al. 1984), associated with activation of proliferation, increased expression of proto-oncogenes and a decline in the expression of liver-specific transcription factors (DiPersio et al. 1991; Rana et al. 1995). The observed loss of liver specific phenotype that follows is typified by the loss of cytochrome P450. In the experiments described here, the rat and dog drug metabolizing enzymes (CYP and UGT) were very well maintained for the entire period of study. Rat hepatocytes isolated at Abcellute Ltd and then transported to the AstraZeneca laboratory in SureTran™ matrix were also used to define intrinsic clearance values for 60 NCEs. The close correlation between the two CLint datasets underlines the flexibility and utility provided by the novel cell maintenance matrix. This observation suggests that the matrix does preserve activities 'as fresh' and is supported by the fact that baseline levels for rat hepatocyte CYP, UGT and drug transporter activities, determined using rat hepatocytes freshly isolated at AstraZeneca (Table 1), match very well with the activity values determined using rat hepatocytes

isolated in the Abcellute laboratory and transported (in SureTran™ matrix) to AstraZeneca for incubations on day 1, 4 and 7 post isolation (Tables 2–4).

It was not possible to obtain day zero data for any of the cells maintained in SureTran™, (Tables 7–9) since they were prepared in the laboratories of Abcellute. However, due to the enzymic homogeneity of rat hepatocyte preparations, isolating cells at the AstraZeneca laboratory served as an effective surrogate for day 0 for rat. The inter-subject variability in human drug metabolising enzyme expression rendered this approach inadequate for human cell comparison. However, to try and establish typical baseline levels for the various activities, cryopreserved human hepatocyte data from 21 individual donors are shown in Table 6. The inter-individual variability in CYP activity from the different batches is clear and simply confirms that with human hepatocytes there is no substitute for knowing the enzyme activities in the freshly isolated cells (day 0 values) obtained from Abcellute. Nevertheless, the Abcellute human hepatocyte batches 1 and 2 have CYP, UGT and OATP activities that are not out of line with the activities from the twenty one donors. Additionally, the hepatocytes were placed into the SureTran™ matrix immediately after isolation so there is no reason to suspect that the activities would drop between that point and day 1 when activities were first assayed at the AstraZeneca laboratory, particularly given the facts that activities are largely stable to at least day 4 and that the matrix was shown to maintain drug metabolizing competence of rat hepatocyte preparations. It is strongly indicated that the SureTran™ media does preserve baseline activities in rat and human hepatocytes.

The components of the matrix itself remain un-disclosed by Abcellute Ltd but it is gelatinous at 10°C during the cell storage period and it could be considered that the cells are completely quiescent in this phase. However, this is not entirely certain since, despite excellent maintenance of most activities during the period studied, some minor changes in the human hepatocytes over several days of storage were observed. With the second human hepatocyte preparation, CYP2C19 activity declined between day 4 and day 7. Also with the second batch, UGT1A1 activity declined progressively throughout the storage period and UGT1A9 and 2B7 activities fell away after day 4. Interestingly, CYP2C9 activity increased progressively during the storage period in both preparations of human hepatocytes. While only two human hepatocyte preparations were investigated several weeks apart, it is unlikely be the case that the day to day changes were due to technical issues with the assaying, since for each day the cells to be used were pooled, washed and divided out equally from the same central container and assays performed in triplicate. Regulation of gene transcription is of course extremely complex with tight regulation of transcription involving chromosomal and nuclear position, the proximity of activators or repressors and acetylation status of histones and the chromatin structure (Grunstein 1997; Honkakoski & Negishi 2000; Alverez et al. 2003; Chiba et al. 2005; Sasakawa et al. 2005). In the context of the enzyme and transporter activities investigated here a whole range of nuclear receptors and transcription factors with inter-linking cross-regulation are relevant (Schrem et al. 2002; Tirona & Kim 2005) but from the available information it is not obvious why we observed the same pattern in CYP1A2 decline and CYP2C9 elevation compared to the relative stability of other CYPs such as CYP3A4 over the storage period in both human hepatocyte preparations. We therefore do not claim our findings to be anything more than interesting and requiring further detailed study. Moreover, the degree of change observed for CYP1A2 and CYP2C9 do not change the overall conclusion that the SureTran™ matrix rather well maintained all the CYPs studied over the period day 1 to day 4.

All the transporter activities in the rat, dog and human hepatocyte preparations were maintained reasonably well from day 2 to day 4 post cell isolation but declined from day 4 to day 7. The good maintenance of transporter activity over the initial days of storage in SureTran™ suggests a possible advantage over cryopreserved hepatocytes where retention of transporter activity post thawing may be variable (Badolo et al. 2011). In this context is worthy of note that the atorvastatin uptake data from the cryopreserved batches of human hepatocytes (Table 6) are all lower than the day 1 value determined in the Abcellute human hepatocyte batch 1 (Figure 3).

While these studies were only designed to investigate these basic parameters, the results coupled with the excellent cell morphology on day 2 after isolation (on arrival at the AstraZeneca laboratory) suggest that other more involved investigations involving cell culture (CYP induction studies for example) may be successful and in which case this method of short-term cell storage will certainly have an important role to play in facilitating reproducible and effective drug metabolism studies. Further work to investigate the utility of SureTran™ matrix in the study the induction of human drug metabolizing enzymes would of course be valuable but fell outside the scope of this present investigation.

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Declaration of interest

The authors report no conflicts of interest.

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