Clearance Studies Using Cryopreserved Pooled Human (LiverPool™) Hepatocytes: Reproducibility Within One Lot And Between Lots

Anders Eneroth[(1)], Lotta Vrang[(1)], Leif Astner[(1)], Britt-Louise Sahlberg[(1)] and Nicola J. Hewitt[(2)].
1-Medivir AB, Lunastigen 7, 14144 Huddinge, Sweden; 2 – In Vitro Technologies Inc., 1450 South Rolling Road, Baltimore, MD21227, USA.

Abstract
Cryopreserved hepatocytes are a good model for metabolism and clearance assays because they retain both phase 1 and 2 enzyme activities (Hallifax D, Rawden HC, Hakooz N and Houston JB. Prediction of metabolic clearance using cryopreserved human hepatocytes: kinetic characteristics for five benzodiazepines. Drug Metab Dispos. 2005;33(12):1852-8.). We used pooled human cryopreserved hepatocytes (LiverPool™ Hepatocytes) from 5 and 10 donors to determine the intrinsic clearance (CL_{int}) of 6 substrates (reflecting both phase 1 and 2 mediated metabolic clearance). The aim of our studies was to determine the reproducibility within one Lot and between four different Lots. LiverPool™ hepatocytes were thawed and incubated in suspension with 2μM midazolam (MID (CYP3A4)), 2μM phenacetin (PHEN (CY1A2)), 2μM burfuralol (BURCYP2D6)), 2μM diazepam (DIAZ (CYP2C19)), 20μM 4-methylumbelliferone (4MU (glucronosyltransferases)), 20μM umbelliferone (UM (glucronosyltransferases and sulfurtransferases) in HBSS buffer for up to 2h. MID, PHEN, BUR, DIAZ and UM were also incubated at 2μM as a cocktail incubation. The CL_{int} of MID in 9 different incubations using Lot 1 was 14.6±1.6 μl/min/million cells, with a range of 12 to 17μl/min/million cells. The average CL_{int} of MID in all 4 Lots was 14.9±5.1(range 10-22)μl/min/million cells. 4MU clearance was also reproducible within in one Lot (20.3±2.6(range 16-24)μl/min/million cells in Lot 1 (n=7)) and between 4 different Lots (19.0±1.0(range 18-20)μl/min/million cells). There was a good correlation between the CL_{int} of each compound incubated either as a single substrate or as a cocktail (R^2=0.85), despite the concentration of UM and 4MU being 10-fold higher in the single compound incubation. Using average data from both single and cocktail incubations and all four LiverPool™ Lots, the rank order of CL_{int} was UM (24.3±5.8μl/min/million cells) > 4MU (20.3±3.6μl/min/million cells) > MID (17.3±5.2μl/min/million cells) > PHEN (12.0±2.8μl/min/million cells) > BUR (2.2±2.3μl/min/million cells) > DIAZ (0.8±1.1μl/min/million cells). In conclusion, Liverpool™ hepatocytes correctly ranked the CL_{int} of each substrate, whether incubated as a single compound or as a cocktail. There was little difference in the CL_{int} between 5-donor and 10-donor LiverPool Lots. LiverPool hepatocytes can be used for use in clearance incubations. Their advantage is that they represent the 'average donor' and Lot-to-Lot variation is minimized, unlike single donor Lots, which do show variation in enzyme activities.

Introduction
It is estimated that about 60% of marketed compounds are cleared by hepatic CYP mediated metabolism (McGinnity et al., 2004) making the liver the main focus for clearance studies. The pharmacokinetics of drugs are important to establish in the early phases of development and therefore, there has been an increase in the use of in vitro techniques to increase the throughput of these assays. Cryopreserved hepatocytes offer a good alternative to fresh cells for suspension assays and as such are now more frequently used in clearance prediction assays (Blanchard et al., 2005; Soars et al., 2002; Bachmann et al., 2003; Shibata et al., 2002). Moreover, human hepatocytes are suggested to be more predictive of human hepatic clearance then animal hepatocytes or allometric scaling (Zuegge et al., 2001). Blanchard et al. (2006) compared the clearance of six compounds in cryopreserved hepatocytes from three different donors. The CL_{int} was donor-dependent and was linked to differences in the levels of the enzymes involved in the metabolism of the compounds. Single donor incubations allow for calculations for extremes of characteristics and can be used for assays later on in the study of a lead compound. However, for earlier and higher throughput assays, a pool of hepatocytes from different donors may give a better estimation of clearance in an 'average donor'. Cryopreserved hepatocytes pooled from several donors are now commercially available (LiverPool™). These reflect the average donor in the same way as pooled microsomes but their advantage is that they are an intact whole cell system. Previously three compounds with low, medium and high clearances were compared in fresh and pooled (LiverPool™) hepatocytes (Koganti et al., poster ISSX 2005). The clearance was predicted equally well in both fresh and LiverPool hepatocytes with equivalent CL_{int} values being determined by both models. Here, we report the reproducibility of the enzyme activities and clearance of phase 1 and 2 substrates between different batches of LiverPool hepatocytes. The compounds were midazolam (CYP3A4 substrate), phenacetin (CYP1A2 substrate), burfuralol (CYP2D6 substrate), diazepam (CYP2C19 substrate) 4-methylumbelliferone (4MU UGT1A6) and umbelliferone (UGT1A6 and SULT1A1 substrate).

Table 1. Enzyme characteristics of LiverPool™ Lots

Lot	Viability	CYP2D6	CYP2C19	CYP3A4	CYP1A2	UGT1A6	SULT1A1
YJD – 5 donors	72%	30	1	165	27	470	41
MOO – 5 donors	77%	25	2	318	15	144	32
HOD – 10 donors	84%	24	3	107	28	391	62
DWY – 10 donors	73%	24	2	169	21	287	38
Donors on IVT web site:							
Mean of 58 donors	79%	23	16	115	29	230	33
Range		1-96	0-175	6-675	1-125	10-540	5-110

Enzyme activities (supplied by IVT) are expressed as **pmol/min/million cells**. Substrates were dextromethorphan (CYP2D6), S-mephenytoin (CYP2C19), testosterone (CYP3A4), phenacetin (CYP1A2) and umbelliferone (7-hydroxycoumarin, UGT1A6 and SULT1A1).

Methods
LiverPool hepatocytes (Lots: YDJ, MOO, HOD, DWY) were obtained from In Vitro Technologies Inc. (IVT), Baltimore, USA. The relevant Lot enzyme characteristics issued by IVT are shown in Table 1. Hepatocytes were rapidly thawed in a 37°C water bath and transferred to 48ml of warmed HBSS pH7.4 thawing medium. After centrifugation at 60g, the cells were resuspended in HBSS, pH7.4 and the cell number and viability assessed by Trypan blue exclusion. Cells were diluted to 1.5million cells/ml and 1ml was incubated with test compounds (See Table 2 for concentrations) and aliquots were taken for up to 2h. Cocktail incubations were based on the method reported by Floby et al., 2004. Parent compound disappearance was determined by LC-MS/MS. The CL_{int} was determined using the following equation: CL_{int} (μl/min/million cells) = (ml/million cells) x 0.693/$t_{1/2}$.

Table 2. Compounds used and concentrations in single and cocktail incubations

Substrate	Enzyme(s) involved in metabolism	Conc. in single substrate incubation (μM)	Conc. in cocktail incubation (μM)	IVT substrate and concentration for same enzymes
Midazolam	CYP3A4	2	2	50μM Testosterone
Phenacetin	CYP1A2	2	2	15μM Phenacetin
Burfurolol	CYP2D6/CYP2C19	2	2	8μM Dextromethorphan
Diazepam	CYP2C19	2	2	20μM S-mephenytoin
Umbelliferone	UGT1A6	20	2	30μM Umbelliferone
4-MU	UGT1A6	2	n.d.	30μM Umbelliferone

n.d. = not determined

Table 3. Intrinsic clearance values in LiverPool™ hepatocytes incubated with substrates either as a cocktail or in single substrate incubations.

Substrate	CL_{int} in Lot YJD only	Average CL_{int} from up to 4 lots, single and cocktail incubations	Reported CL_{int} from literature	Reference
Midazolam	14.9± 1.6	17.3 ± 5.2 (n=4)	14.0 ± 8.0	McGinnity et al.
4MU	20.3 ± 2.6	19.0 ± 1.0 (n=4)	No reference	
Umbelliferone	17	24.3 ± 5.8 (n=4)	No reference	
Burfurolol	0	2.2 ± 2.3 (n=4)	No reference	
Phenacetin	n.d.	12.0 ± 2.8 (n=2)	14.72 ± 0.29	Shibata et al.
Diazepam	n.d.	0.8 ± 1.1 (n=4)	1.4	Benet et al.
			0.05 ± 0.03	Shibata et al.

n.d. = not determined, 'n' refers to number of Lots tested (one may have both single and cocktail data)
Values are expressed as μl/min/million cells

Results
The enzyme activities of the four Lots of LiverPool hepatocytes were similar, especially when compared to the range of enzyme activities in single donor Lots (Table 2).

The CL_{int} values for midazolam, umbelliferone, burfuralol and diazepam were comparable in single substrate or cocktail incubations (Figure 1).

There was a good inter-assay reproducibility of the CL_{int} of midazolam and 4MU when incubated as single substrates in hepatocytes from one Lot, YJD (Table 3). Incubations were repeated 9 times with midazolam and 7 times with 4MU.

The CL_{int} of midazolam incubated as a single substrate in four different Lots ranged between 12 and 17μl/min/million cells, with an average of 14.9 ± 1.6μl/min/million cells. This value also correlated that found by others (14.0 ± 8.0μl/min/million cells by McGinnity et al., 2003). When cocktail incubation values were also included, the mean CL_{int} was marginally higher (Table 3) but was not statistically different from the single substrate value.

There was a link between the CL_{int} of the five compounds and the level of the enzyme activities involved in their metabolism (Figure 2). UGT-mediated metabolism was the highest activity and correlated to a high CL_{int} (of umbelliferone) and CYP2C19 and CYP2D6 were the lowest activities, correlating to low CL_{int} (of diazepam and bufuralol). The clearance of midazolam was more clearly linked to specfic Lot characteristics than the other compounds.

Figure 1. A comparison of CL_{int} values determined from single substrate or cocktail incubations.

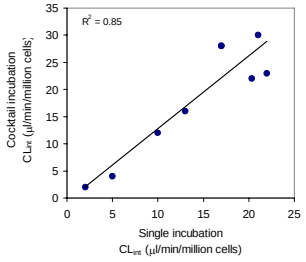
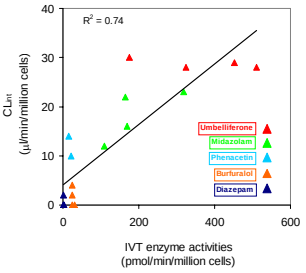


Figure 2. A comparison of substrate CL_{int} values the enzyme activities involved in their metabolism.



Conclusions
For the compounds we tested, single substrate or cocktail incubations can be used to determine the CL_{int}. Although our cocktail incubations were successful, they may be more problematic for other compounds that interfere with each others detection.

LiverPool™ hepatocytes are useful in determining the CL_{int} of diagnostic and lead compounds, since they represent the 'average donor'.

There was a very good reproducibility of CL_{int} values from one Lot to another, suggesting that pooled hepatocytes Lots are consistent in their enyzme characteristics.

The major advantages of LiverPool™ hepatocytes is that Lots can be used interchangeably without concern about Lot-to-Lot variation and that they are cryopreserved and thus can be used whenever experimental needs arise.

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