METABOLIC STABILITY AND CLEARANCE OF PHARMACEUTICAL CHEMICALS IN PRE-POOLED CRYOPRESERVED HEPATOCYTES

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Abstract

Cryopreserved hepatocytes express both phase I and phase II enzymes and facilitate early evaluation of the metabolic stability of pharmaceuticals. Availability of pre-pooled cryopreserved hepatocytes further enhances the utility of this model by reducing donor to donor variability. In this study, the metabolic stability of twenty nine pharmaceutical compounds was evaluated in pre-pooled cryopreserved human hepatocytes (10 donor pool). All compounds were evaluated at a single concentration of 5 µM and at six time points ranging from 0 to 4 hours. Clearance of these chemicals by hepatocytes was calculated by the AUC method using the trapezoidal rule. The obtained *in vitro* clearance values were used to categorize the chemicals as low (<1.0 µL/min/million cells), moderate (≥1.0 and ≤5.0 µL/min/million cells), and high (>5.0 µL/min/million cells). This classification system was previously reported in the literature. *In vivo* clearance values were obtained from the literature and were also used to categorize the chemicals as low (< 5.0 mL/min/kg), moderate (≥5.0 and ≤20.0 mL/min/kg), and high (>20 mL/min/kg) clearance compounds. The *in vitro* clearance values provided correct category predictions for 65%, under predictions for 14%, and over predictions for 21% of the evaluated compounds. The predicted *in vivo* hepatic clearance values correlated with the actual *in vivo* clearance values with an $r^2 = 0.501$. The data demonstrated the potential utility of pre-pooled cryopreserved human hepatocytes in determining metabolic stability and in classifying pharmaceuticals as low, moderate, or high clearance compounds.

Introduction

***In vitro* drug metabolism data have been increasingly utilized in understanding as well as predicting pharmacokinetics *in vivo*. Several investigators have demonstrated the utility of human hepatocytes in predicting *in vivo* hepatic clearance (Zuegge, et. al., 2001; Shibata, et.al, 2002; Bachmann, et.al, 2003; Naritomi, et.al., 2003; McGinnity, et.al., 2004; Blanchard, et.al., 2005).**

Freshly isolated hepatocytes are not readily available and using hepatocytes from individual donors may result in significant donor to donor variability in the metabolic clearance of pharmaceuticals. The availability of individual cryopreserved hepatocytes that can be pooled prior to experimentation or pre-pooled cryopreserved hepatocytes from commercial sources address these issues. Studies have shown that individual cryopreserved hepatocytes are comparable to freshly isolated hepatocytes in determining intrinsic clearance. In the current study, the potential of pre-pooled cryopreserved hepatocytes (10 donor pool) in predicting clearance of several pharmaceutical chemicals was evaluated.

Several methods are reported in the literature for determining intrinsic clearance *in vitro* and for the scaling of intrinsic clearance to predict *in vivo* hepatic clearance. In the current study, substrate depletion approach at low concentrations was used to determine intrinsic clearance. Two different models, Well Stirred model and Parallel Tube model, were used to calculate predicted *in vivo* hepatic clearance.

Materials and Methods

Hepatocyte Suspensions. **Fresh Human Hepatocyte Suspensions (FHHS), Individual Cryopreserved Human Hepatocytes (ICHH; 5 donors), and Pre-Pooled Cryopreserved Human Hepatocytes (PPCHH; LiverPool™; 10 donor pool) were obtained from In Vitro Technologies.**

Preparation of Cryopreserved Human Hepatocyte Suspensions. **Vials were thawed in a 37°C water bath, and cells were diluted in *InVitro*GRO™ CP medium. ICHH from 5 donors were pooled at the time of thawing. Cells were pelleted and resuspended in supplemented Krebs-Henseleit buffer. Cell counts and viability were determined by Trypan blue exclusion. The cell suspensions were then diluted to 2 million viable cells per ml with supplemented Krebs-Henseleit buffer.**

Incubations. **The chemicals were prepared in water, methanol, or acetonitrile at 0.5 mM concentrations and diluted with supplemented Krebs-Henseleit buffer to dosing concentrations of 10 µM. Aliquots (150 µL) of the 10 µM chemical solutions were transferred to uncoated 48-well plates. Hepatocyte suspensions (150 µL) were added to the wells and the mixtures were incubated in a 37°C, 5% CO_2, humidified incubator, on an orbital shaker for 0, 0.5, 1.0, 2.0, 3.0, and 4.0 hours. The final concentration of the chemicals in the incubation mixtures was 5 µM, in an incubation volume of 300 µL containing 0.3 million viable hepatocytes. At each time point, a 250 µL aliquot of incubation mixture was harvested and mixed with an equal volume of methanol. The samples were stored at –70°C until analysis.**

Sample Analysis. **The samples were analyzed by LC/MS to quantify the amount of parent chemical remaining in the incubation mixtures at each time point.**

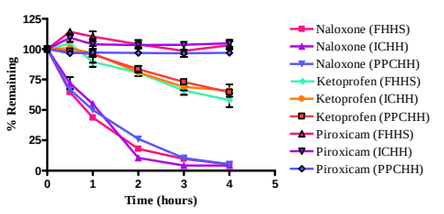


Figure 1. Metabolic stability of model pharmaceuticals in human hepatocytes.

Well Stirred Model

Compound	$CL_{int,\ in\ vitro}$ (µL/min/million cells) FHHS	ICHH	PPCHH	Predicted $CL_{H,\ in\ vivo}$ (mL/min/kg) FHHS	ICHH	PPCHH	Actual $CL_{in\ vivo}$ (mL/min/kg)
Naloxone	13.1	14.5	11.7	13.7	14.1	13.2	22
ketoprofen	2.22	1.73	1.81	5.1	4.2	4.4	1.2
Piroxicam	0	0	0.133	0.0	0.0	0.4	0.036

Liver blood flow (LBF)= 20.7 mL/min/kg
Liver weight (LW) = 25.7 g/kg body weight
Hepatocellularity (SF_{liv}) = 120 million cells/g of liver
Predicted $CL_{H,\ in\ vivo}$ = (LBF * $CL_{int,\ in\ vitro}$ * SF_{liv} * LW/1000) / (LBF + ($CL_{int,\ in\ vitro}$ * SF_{liv} * LW/1000))

Parallel Tube Model

Compound	$CL_{int,\ in\ vitro}$ (µL/min/million cells) FHHS	ICHH	PPCHH	Predicted $CL_{H,\ in\ vivo}$ (mL/min/kg) FHHS	ICHH	PPCHH	Actual $CL_{in\ vivo}$ (mL/min/kg)
Naloxone	13.1	14.5	11.7	17.8	18.3	17.1	22
ketoprofen	2.22	1.73	1.81	5.8	4.7	4.9	1.2
Piroxicam	0	0	0.133	0.0	0.0	0.4	0.036

Liver blood flow (LBF)= 20.7 mL/min/kg
Liver weight (LW) = 25.7 g/kg body weight
Hepatocellularity (SF_{liv}) = 120 million cells/g of liver
Predicted $CL_{H,\ in\ vivo}$ = LBF * (1 - e(-($CL_{int,\ in\ vitro}$ * SF_{liv} * LW) / (LBF * 1000)))

Table 2. Calculation of predicted *in vivo* hepatic clearance ($CL_{H,\ in\ vivo}$) by the Well Stirred and Parallel Tube models.

1. Enter concentrations

Time (hr)	Conc. (µM)
0	2.350
0.5	1.570
1	1.180
2	0.615
3	0.245
4	0.123

2. Time at end of linear phase (t$_2$): 4.0 hours

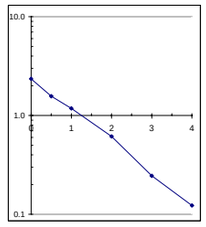
3. Conc. at end of linear phase (C$_2$): 0.1 µM

Area block	Area (nmol-hr/mL)
A0-0.5	0.980
A0.5-1	0.688
A1-2	0.898
A2-3	0.430
A3-4	0.184
A4-∞	0.167

4. Value of β: 0.73763 /hr

5. AUC$_{0-\infty}$ = 3.346 nmol-hr/mL **

6. Clearance = **11.70639** µL/min/million cells



** If the 4 *hr* time point is dropped, AUC3-4 will not be summed, and AUC3-∞ will be calculated and summed instead of AUC4-∞.

Calculations:

- AUC$_{4-\infty}$=[(tn-tx)*(Cx+Cn)]/2
- AUC$_{2-\infty}$=C$_2$/β
- AUC$_{x-\infty}$=AUC$_{4-\infty}$+AUC$_{3-4}$+...AUC$_{0-1}$
- β=-2.303(logC$_{2}$-logC$_{3}$)/(t$_{3}$-t$_{0}$)
- Cl=D0/AUC$_{0-\infty}$/0.3/60 (Note: 0.3 million hepatocytes per incubation and 60 min per hour)

Table 1. Calculation of *in vitro* intrinsic clearance ($Cl_{int,\ in\ vitro}$) by the trapezoidal rule.

Substrate	$CL_{int,\ in\ vitro}$ (µL/min/million cells)	Predicted $CL_{H,\ in\ vivo}$ (mL/min/kg)	Actual $CL_{in\ vivo}$ (mL/min/kg)	F_{metab} (%)
Naloxone (B)	11.7	13.2	22	>90
Ketoprofen (A)	1.81	4.40	1.2 +/- 0.3	>90
Piroxicam (A)	0.133	0.40	0.036 +/- 0.008	>90
Verapamil (B)	6.09	9.85	15 +/- 6	>90
Propranolol (B)	4.45	8.25	16 +/- 5	>90
Imipramine (B)	3.16	6.63	15 +/- 4	>90
Diltiazem (B)	1.33	3.42	12 +/- 4	>90
Ranitidine (B)	0.103	0.31	10.4 +/- 1.1	<20
Terfenadine (B)	14.2	14.06	8.8 +/- 2.0	>90
Promethazine (B)	4.5	8.31	8.5 +/- 3.5 (4)	>90
Erythromycin (B)	0.673	1.89	9.1 +/- 4.1	>90
Cimetidine (N)	0.0773	0.24	8.3 +/- 2	30-40
Nortryptiline (B)	-0.378	-1.24	7.2 +/- 1.8	>90
Atenolol (B)	0.397	1.16	2.0 +/- 0.2	<10
Enalapril (A)	0.144	0.43	4.9 +/- 1.5	<20
Lidocaine (B)	1.87	4.51	9.2 +/- 2.4	>90
Etoposide (B)	-0.0336	-0.10	0.68 +/- 0.23	~50
Tolbutamide (A)	-0.81	-2.84	0.24 +/- 0.04	>90
Dexamethasone (B)	1.22	3.18	3.7 +/- 0.9	>90
Warfarin (A)	-0.474	-1.57	0.045 +/- 0.024	>90
Chlorzoxazone (N)	1.37	3.51	3.02 +/- 1.33 (5)	>90
Phenacetin (N)	5.14	8.98	19.6 (2)	
Dextromethorphan (B)	3.07	6.50	6 (3)	>70
Testosterone (N)	21.9	15.84	12.4 +/- 5.1 (6)	>90
S-Mephenytoin (B)	0.755	2.09	0.45 (7)	
Caffeine (N)	-0.0914	-0.29	1.4 +/- 0.5	>90
Carbamazepine (N)	-0.575	-1.94	1.7 +/- 0.3	>90
Terbutaline (B)	0.406	1.18	3.4 +/-0.6	
Disopyramide (B)	0.0689	0.21	1.2 +/- 0.4	30-40

Table 3. Summary of the pharmaceuticals evaluated, their *in vitro* intrinsic clearance ($CL_{int,\ in\ vitro}$), predicted *in vivo* hepatic clearance ($CL_{H,\ in\ vivo}$) using the Well Stirred model, and the actual *in vivo* clearance ($CL_{in\ vivo}$).

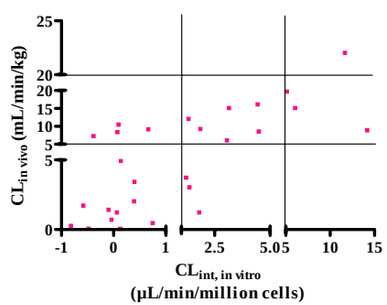


Figure 2. Classification of pharmaceuticals based on their *in vitro* intrinsic clearance.

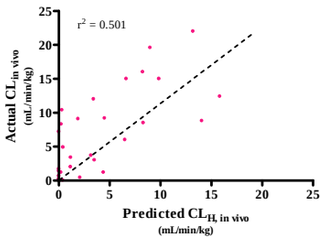


Figure 3. Correlation of predicted *in vivo* hepatic clearance obtained using Well Stirred model and actual *in vivo* clearance.

Results

- **No significant differences were observed with freshly isolated human hepatocytes, individual cryopreserved hepatocytes, or pre-pooled cryopreserved hepatocytes in the metabolism of high, moderate and low cleared compounds (naloxone, ketoprofen, and piroxicam).**
- **Calculation of predicted *in vivo* hepatic clearance by the Well Stirred or the Parallel Tube models resulted in similar correlation. The predicted hepatic clearance values were within 2-fold of the actual clearance values for 43% of the compounds and within 3-fold for 61% of the compounds.**
- **Inclusion of fraction unbound in plasma values in the calculation of predicted clearance calculations resulted in a poorer correlation (data not shown).**
- **Separation of the compounds into basic (B), acidic (A), or neutral (N) categories did not increase the correlation of predicted to actual clearance values (data not shown).**

Conclusions

- **Pre-pooled cryopreserved hepatocytes were comparable to freshly isolated hepatocytes in determining intrinsic clearance.**
- ***In vitro* intrinsic clearance was useful in the classification of 65% chemicals into low, moderate, and highly cleared groups with 14% underpredictions and 21% overpredictions.**
- **Accurate prediction of actual *in vivo* clearance values was not achieved. However, incubations in presence of serum, as shown recently (Bachmann, et.al., 2003; Blanchard, et.al., 2005) may improve the predictive capability of this model.**