Optimization of a Plated Cryopreserved Human Hepatocyte Model for Assessment of the Metabolic Stability Of Low Clearance Compounds using Dextromethorphan, Midazolam, Tolbutamide, and Theophylline

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INTRODUCTION

The in vitro metabolic stability of compounds is routinely used to estimate hepatic clearance by scaling the measured in vitro half-life (Obach, 1999; Obach, 2001). Historically, the use of liver microsomes for the prediction of hepatic clearance has been limited to compounds that are metabolized by cytochromes P450 (Fisher et al., 2000). Recently published data has highlighted the importance of non-P450 mediated clearance mechanisms (Hutzler et al., 2012) warranting the routine use of hepatocytes for metabolic stability assessments. However, hepatocyte suspension models have limitations due to the loss of activity and viability over time. This poses a significant challenge for predicting the hepatic clearance of compounds with lower metabolic turnover and has led to the use of plated hepatocyte models to extend the duration of incubations. The use of a plated cryopreserved hepatocyte model is supported by a recent report demonstrating that culture of hepatocytes can extend the suspension-level P450 half-lives from ~2-3 hrs to ~20-30 hrs in culture (Smith et al., 2012). However, the use of plated hepatocytes inherently introduces a number of unique experimental variables and the effect of each of these variables on clearance predictions is not well understood. The current study investigates cell seeding density and the effects of orbital shaker speed, delay of study initiation following cell plating, and overlay vs. non-overlay effects on the measured half-life for dextromethorphan, midazolam, tolbutamide, and theophylline using plated cryopreserved human hepatocytes. These representative compounds were chosen to assess the predictability of the model for low, moderate, and high clearance compounds.

METHODS AND MATERIALS

Materials:

Cryopreserved hepatocyte recovery media (CHRM®--CM7000 (GIBCO®)); plating media (William's E Medium (WEM) and hepatocyte plating supplement pack (serum-containing) CM3000 (GIBCO®); overlay media (WEM, no Phenol Red), supplement pack CM4000, and Geltrex™ LDEV-free reduced growth factor basement membrane matrix (GIBCO®); culture media (WEM, n Phenol Red (GIBCO®)) and 15mM HEPES; Collagen I-coated plates (CM1024) were obtained from Life Technologies (Carlsbad, CA). Dextromethorphan (DEX), Midazolam (MDZ), Tolbutamide (TOL), theophylline (THE), formic acid, acetonitrile, methanol, and HPLC grade water were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were obtained from standard commercial sources.

Experimental Methods:

Primary Human Hepatocytes

Primary human hepatocytes were isolated by a modification of the 2-step collagenase perfusion method described previously by (LeCluyse *et al.*, 2005) and cryopreserved using proprietary procedures. Final cell viability prior to plating was determined by Trypan Blue™ exclusion and was ≥ 70%. Cryopreserved primary human hepatocytes were thawed in a 37ºC water bath and transferred into CHRM® Medium and centrifuged at 100xg for 10 minutes. Supernatants were removed and cells were resuspended in approximately1 mL of Plating Medium per $1x10^6$ total cells. Final cell densities were adjusted to 0.8 – 1.0 $x10^6$ cells/mL prior to seeding. Collagen I-coated plates were pre-wetted with 60 µL of plating media. Primary hepatocytes were cultured at 37 °C in a humidified culture chamber with 95% relative humidity/5% air/CO_2 for approximately 4 hrs post seeding prior to initiating incubations. When appropriate, cultures were overlayed with Geltrex™ at a concentration of 0.35mg/mL.

Metabolic Stability Assay

Chemical stock solutions (1 mM in DMSO) were added to pre-warmed (37˚C) incubation medium (William's E Medium containing 15 mM HEPES) in polypropylene tubes to achieve a 1 µM final concentrations in incubations solutions. Approximately 4 hours after initial seeding, spent culture media was removed and incubation solutions were added. The plates were returned to 5% CO_2 and 37˚C incubator for the duration of the experiment. At appropriate time points, supernatants were removed from the plates and transferred to polypropylene matrix tubes, and flash frozen in a dry ice bath. Cell monolayers were crashed with 100 µL of solution containing 92:5:3 Water:ACN:Formic Acid with 100 nM Tolbutamide-d9, sealed, and frozen at -80˚C.

Cell Health Assessments

Cell morphology assessments were determined by observation using a Zeiss Axiovert inverted research microscope equipped with phase-contrast optics, a 3 CCD camera, and imaging computer with image analysis software. Morphology markers used for determination of cellular stress included cell shape, nucleus integrity/size/shape, cytosolic clarity, cell membrane integrity, organelle size, lipid droplets/content, cell-cell contacts (e.g. canaliculi), cell debris, and cell excretion products. ATP depletion and LDH leakage assays were performed using the Cell-titer Glow assay system (Promega, Madison, WI) and CytoToxOne™ (Promega, Madison, WI) LDH assay kit according to manufacturer's instructions.

LC/MS/MS Analysis. Quantitation was performed using an AB Sciex API-5000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface in positive or negative ion mode and connected in-line to a Waters Acquity UPLC system. Separation was performed using a LUNA C18 5µm (3.0 x 30mm) column held at 40°C. Mobile phase was flowed at 0.5 mL/minute. Initial conditions were 98% mobile phase A (5 mM amonium formate with 0.05% formic acid in water) and 2% mobile phase B (95:5 acetonitrile/methanol with 0.05% formic acid). Initial conditions were held for 0.5 minutes, followed by a 2.5 min linear gradient to 98% mobile phase B (hold for 0.25 minutes), followed by re-equilibration to initial conditions.

Calculations.

The half life is calculated using the slope of the linear regression from natural log [percent compound remaining] vs. time.

$$T_{1/2} = \frac{\ln 2}{\text{Slope}}$$

Scaling *to* Intrinsic Clearance*: Cl_{int} (ml/min/kg)*

$$Cl_{int} = \frac{0.693}{T_{1/2\ (min)}} * \frac{\text{g liver weight}}{\text{kg of body wt}} * \frac{\text{ml incubation}}{\text{number of cells}} * \frac{\text{Number of hepatocytes}}{\text{1 g of liver weight}}$$

Scaling to Hepatic Clearance*: Cl_{iH} (ml/min/kg)*

$$Cl_H = \frac{Q_h * fu * Cl_{int}}{Q_h + (fu * Cl_{int})}$$

RESULTS

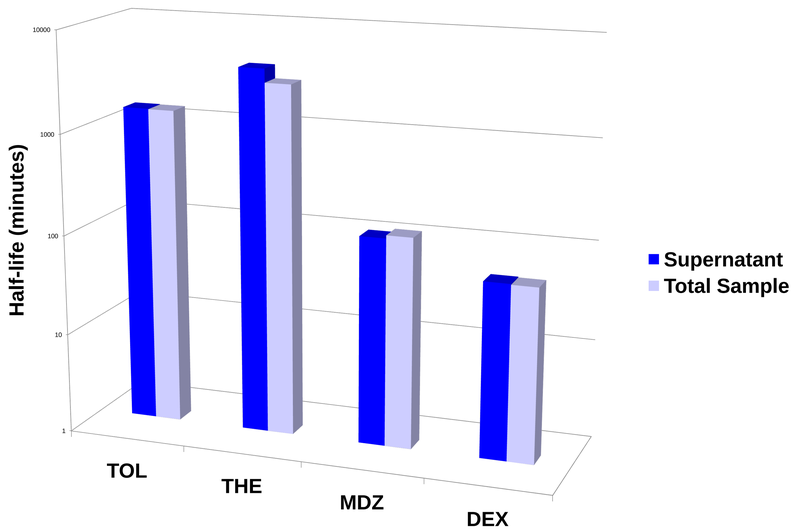


Figure 1. Effect of supernatant and total sample analysis on the observed half-life of dextromethorphan, midazolam, tolbutamide, and theophylline in human hepatocytes at a seeding density of 50k cells/well shaken at 200 rpm.

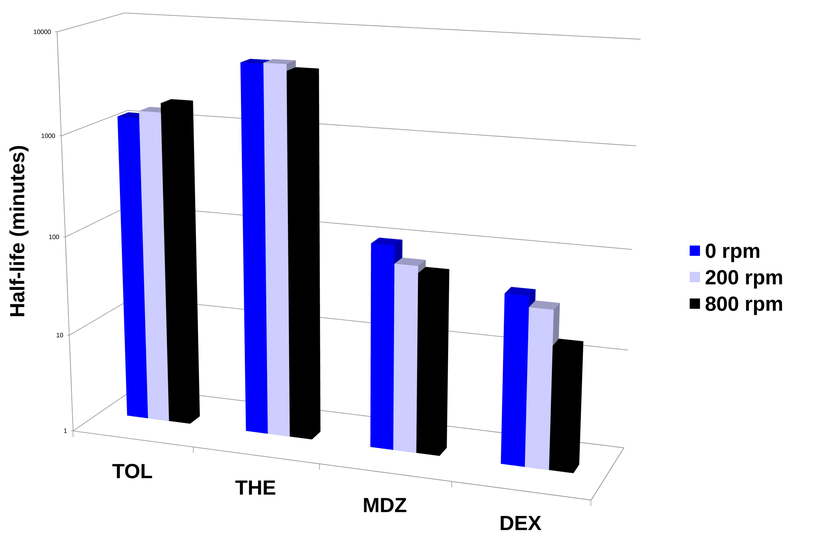
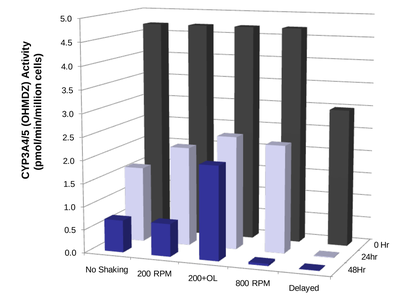
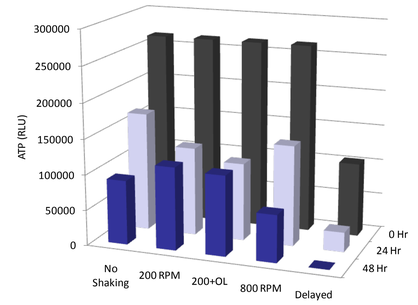
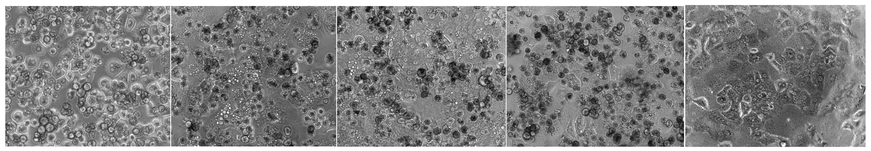


Figure 2. Effect of shaker speed on the half-life of dextromethorphan, midazolam, tolbutamide, and theophylline in human hepatocytes at a seeding density of 50k cells/well.







Initial Attachment | 48 hrs, 200 RPM | 48 hrs, 200 RPM, Overlay | 48 hrs, No Shaking | 48 hrs, 800 RPM

Figure 3. Temporal effect of shaker speed and culture conditions on CYP3A4/5 metabolic activity and cell health at 50,000 cells/well (96-well, Hu8125, no daily feeding).

Compound	Class	In vivo $Cl_{non\text{-}renal,\ obs}$	% Q_h	In vitro CL_h (mL/min/kg)
S-Warfarin	Acidic	0.1	0.5	0.7
Tolbutamide	Acidic	0.4	2	0.8
S-Mephenytoin	Basic	0.5	2	1.5
Alprazolam	Neutral	0.8	4	0.4
Theophylline	Neutral	1.1	5	2.1
Clozapine	Basic	2.9	14	3.4
Triazolam	Neutral	4.7	23	1.7
Prednisolone	Neutral	4.9	24	3.6
Zolpidem	Neutral	5.7	28	2.1
Accuracy	% within 2-fold			56
	% within 3-fold			89

Table 1. Prediction of hepatic clearance for nine low clearance compounds using the well-stirred model.

DISCUSSION and CONCLUSIONS

➢Seeding density titration experiments were conducted with density ranging from 10,000 to 80,000 cells/well. The optimal seeding density was determined to be 50,000 cells/well.

➢Higher rpm shaker speeds caused increased clearance of high clearance compounds and decreased clearance of low clearance compounds. These results may be due to increased sheer forces on cell membranes that have a deleterious effect on cell membrane integrity, consistent with observed cell health data. The optimal shaker speed was determined to be 200 rpm.

➢The presence of an overlay caused increased metabolic capacity and clearance of high clearance compounds and decreased clearance of low clearance compounds with a more predominant effect on high clearance compounds.

➢The model predicted the observed hepatic clearance within 2-fold for 56 percent of the compounds and within 3-fold for 89 percent of the compounds.

REFERENCES

1.Obach RS (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab Dispos* **27:**1350-1359.

2.Obach RS (2001) The prediction of human clearance from hepatic microsomal metabolism data. *Curr Opin Drug Discov Devel* **4:**36-44.

3.Fisher MB, Campanale K, Ackermann BL, VandenBranden M, and Wrighton SA (2000) In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. *Drug Metab Dispos* **28:**560-566.

4.Hutzler JM, Yang YS, Albaugh D, Fullenwider CL, Schmenk J, and Fisher MB (2012) Characterization of aldehyde oxidase enzyme activity in cryopreserved human hepatocytes. *Drug Metab Dispos* **40:**267-275.

5.Smith CM, Nolan CK, Edwards MA, Hatfield JB, Stewart TW, Ferguson SS, Lecluyse EL, and Sahi J (2012) A comprehensive evaluation of metabolic activity and intrinsic clearance in suspensions and monolayer cultures of cryopreserved primary human hepatocytes. *J Pharm Sci* **101:**3989-4002.

6.LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, and Richert L (2005) Isolation and culture of primary human hepatocytes. *Methods Mol Biol* **290:**207-229.