A Comparison of Suspension, Plated Monoculture, and HμREL® Co-Culture Hepatocyte Models for Estimating Intrinsic Clearance of Low-Turnover Drugs

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Abstract

To directly compare three in vitro human hepatocyte models for calculating intrinsic clearance of low-turnover drugs, 13 drugs with diverse metabolic pathways and a range of intrinsic clearances (±warfarin, timolol, theophylline, alprazolam, prednisolone, meloxicam, diazepam, tolbutamide, metoprolol, verapamil, glimepiride, efavirenz, and diclofenac) were selected for incubation using the same lot of cryopreserved hepatocytes, a comparison that has yet to be performed. Incubations were conducted with pooled cryoplateable human hepatocytes (5 donors) in a 96-well format, with cell densities of 50,000 (suspension), 45,000 (monoculture), and 30,000 cells/well (HμREL co-culture). HμREL co-culture plates were prepared at HμREL Corporation, and shipped for immediate use 6 days later. Maximum incubation times for each hepatocyte system were 4 hours (suspension), 24 hours (monoculture), and 72 hours (co-culture). Depletion rates were calculated and converted to an in vitro intrinsic clearance for comparison between hepatocyte systems and to the back-calculated in vivo intrinsic clearance values for correlation analysis.

Intrinsic clearance lower limits were 11.9 mL/min/kg for suspended hepatocytes, 1.1 mL/min/kg for monoculture, and 0.56 mL/min/kg for the HμREL co-culture model. Using these limits, intrinsic clearance for 8 of the 13 compounds tested in suspended hepatocytes was able to be calculated. Compounds with no observable clearance included low-turnover drugs ±warfarin, timolol, theophylline, alprazolam, and prednisolone, indicating an insufficient incubation time. Following a 24-hour incubation using a monoculture hepatocyte model, only 4 of 9 compounds incubated had enough depletion to enable intrinsic clearance calculations. Meanwhile, intrinsic clearance for 11 of 13 compounds was able to be calculated using the HμREL co-culture system following a 72-hour incubation, with the exceptions being theophylline and alprazolam.

Regarding scaling to reported in vivo clearance values, when utilizing suspended hepatocytes, intrinsic clearance for moderate/high-turnover compounds was predicted within 2- to 3-fold of the in vivo intrinsic clearance. Thus, suspended hepatocytes provided the most accurate estimation of clearance. However, for low-turnover compounds, a model providing longer incubation times is required. The 24-hour plated hepatocyte incubations did not provide added benefit for low-turnover compounds, with markedly less activity compared to suspension incubations. Using the HμREL co-culture system, intrinsic clearance for most of the low-turnover compounds was able to be measured, and with activity 2- to 7-fold higher than simply plating hepatocytes in monoculture, although under-prediction of in vivo intrinsic clearance was observed for higher turnover compounds. However, the HμREL co-culture system, with the ability to incubate at least 72 hours, would be a preferable model for estimating intrinsic clearance of low-turnover compounds such as ±warfarin, tolbutamide, and prednisolone.

Introduction

- Developing drug candidates with favorable pharmacokinetic parameters is a goal for the pharmaceutical industry. In vitro metabolism assays enable the rapid identification of optimized drug candidates with low clearance.
- Drugs that are slowly metabolized present a challenge for scientists because typical in vitro models (liver microsomes and hepatocytes) have become unreliable for predicting clearance for drugs with this profile.
- Traditional in vitro models are limited by incubation time (typically 60 minutes for microsomal incubations and 4 hours for suspended hepatocyte incubations), making it difficult to determine and differentiate the intrinsic clearance of compounds with low turnover.
- Various in vitro approaches are available for extending incubation times to enable the prediction of intrinsic clearance. Two of these methods were directly compared in this study to standard suspensions using the same lot of cryopreserved plateable pooled hepatocytes: 1) monolayer culture of cryopreserved plateable pooled hepatocytes, and 2) the HμREL hepatic co-culture system (a co-culture of cryopreserved plateable pooled hepatocytes and non-parenchymal, stromal cells).
- A set of commercially available drugs with diverse reported clearance rates was incubated in three hepatocyte systems. To our knowledge, this is the first direct comparison of multiple hepatocyte systems using the same lot of cryopreserved hepatocytes.

A Diverse Set of Substrates Was Selected for Hepatocyte Incubations

Drug	Enzymes Involved in Metabolism
±Warfarin	CYP2C9, CYP3A, CYP1A2
Timolol	CYP2D6
Diazepam	CYP3A, CYP2C19
Tolbutamide	CYP2C9
Theophylline	CYP1A2
Metoprolol	CYP2D6
Efavirenz	CYP2B6
Verapamil	CYP2D6, CYP3A, UGT
Diclofenac	CYP2C9, UGT
Alprazolam	CYP3A
Glimepiride	CYP2C9
Meloxicam	CYP2C9
Prednisolone	CYP3A

Substrates were selected based on range of reported clearance values, as well as for diversity in metabolic clearance mechanisms.

Materials

- Cryoplateable pooled hepatocytes (n = 5 donors), Lot YMD (Bioreclamation/IVT, Baltimore, MD)
- Suspension
  - Cryopreserved hepatocyte recovery media (CHRM) thawing media (APSciences, Inc., Columbia, MD)
  - Hepatocyte maintenance media (HMM; Lonza, Walkersville, MD)
- Monolayer
  - CHRM thawing media (APSciences, Inc.)
  - InVitroGRO CP plating media (Bioreclamation/IVT)
  - InVitroGRO HI incubation media (Bioreclamation/IVT)
- HμREL Co-culture
  - PlatinumHeps maintenance media (HμREL Corporation, North Brunswick, NJ)
  - PlatinumHeps dosing media (HμREL Corporation)

References:
Di L and Obach RS (2014). Addressing the Challenges of Low Clearance in Drug Research. *AAPS J*. **17(2)**: 352-357.
Smith CM, et al. (2012). A Comprehensive Evaluation of Metabolic Activity and Intrinsic Clearance in Suspensions and Monolayer Cultures of Cryopreserved Primary Human Hepatocytes. *J. Pharm. Sci.* **101(10)**: 3989-4002.
Hallifax D, Foster JA, and Houston JB (2010). Prediction of Human Metabolic Clearance from In Vitro Systems: Retrospective Analysis and Prospective View. *Pharm. Res.* **27**: 2150-2161.

Methods

Comparison of Incubation Condition for Hepatocyte Models

Condition	Suspension	Monolayer	HμREL Co-Culture*
Thawing media	CHRM	CHRM	Thawed by HμREL
Plating media	–	InVitroGRO CP plating media	Plated by HμREL
Incubation media	HMM	InVitroGRO HI incubation media	HμREL dosing media
Max. incubation time	4 hour	24 hour	72 hour
Seeding density	50,000 cells/well	45,000 cells/well	30,000 cells/well
Incubation volume	200 µL	100 µL	100 µL
Substrate conc.	1 µM	1 µM	1 µM
Plate design	96 well	96 well	96 well
Shaker speed	600 rpm	120 rpm	120 rpm
Time points (6)	0, 0.25, 0.5, 1, 2, 4 hr	0, 4, 6, 8, 18, 24 hr	0.25, 3, 6, 24, 48, 72 hr

*Incubations with the HμREL co-culture system also included a plate with the stromal cells only.

LC/MS/MS Bioanalysis

MS System:	AB SCIEX API 4000 with turbo ion spray interface at 550 °C
Analytical Column:	MAC-MOD Analytical ACE HALO Phenyl-Hexyl (2.1 x 30 mm, 2.7 µm particle size)
Guard Column:	Phenomenex SafetyGuard C18 Cartridge (2.0 mm)
Mobile Phase Buffer:	2.5 mM Ammonium formate, 1 mM formic acid
Mobile Phase A:	2.5:97.5 Mobile phase buffer/water
Mobile Phase B:	2.5:97.5 Mobile phase buffer/acetonitrile
Flow Rate:	0.6 mL/min (gradient elution)

Data Analysis

- Each time point was analyzed for the concentration of substrate remaining and expressed as the percent remaining as compared to the zero-minute concentration. The natural log of the percent remaining substrate (LN%) at each incubation time point was used to calculate a rate of depletion [slope of the natural log of the concentration of remaining compound vs. time (minutes)]. Substrate depletion slopes (k_{dep}) were estimated using GraphPad PRISM.
- Equations:

1) k_{dep} = slope of time (min) vs. LN% Remaining

2) In Vitro Half-Life: $t_{1/2} = \frac{LN(2)}{k_{dep}}$

3) In Vitro Intrinsic Clearance: $\frac{LN(2)}{t_{1/2}} \times \frac{\text{Inc Vol}\,(\mu L)}{\text{Cells} \times 10^6} = \frac{\mu L}{\text{min} \times 10^6\,\text{Cells}}$

4) Intrinsic Clearance (CL_{int}): $\frac{LN(2)}{t_{1/2}} \times \frac{\text{Inc Vol}\,(mL)}{\text{Cells} \times 10^6} \times \frac{120 \times 10^6\ \text{Hepatocytes}}{\text{g Liver}} \times \frac{25.7\ \text{g Liver}}{\text{kg BWt}} = \frac{mL}{\text{min} \times \text{kg}}$

5) In Vivo Intrinsic Clearance (CL_{int}): $\frac{CL}{f_{uB} \times \left(1 - \frac{CL}{Q_H}\right)}$

Where $t_{1/2}$ = half-life; Inc Vol = incubation volume; BWt = body weight; f_{uB} = free fraction of blood; Q_H = hepatic blood flow in human (21 mL/min/kg)

Results

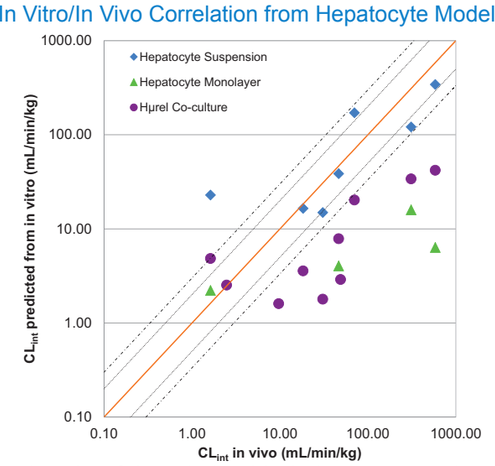
Comparison of Scaled Intrinsic Clearance (CL_{int}) Values (mL/min/kg)

Drug	Hepatocyte Suspension (4 Hour Incubation)	Hepatocyte Monolayer (24 Hour Incubation)	HμREL Co-Culture (72 Hour Incubation)	In Vivo Intrinsic Clearance
Extrapolation Limits (≥20% dep ~3x incubation time)	11.9 ($t_{1/2}$ = 720 min)	1.11 ($t_{1/2}$ = 4320 min)	0.560 ($t_{1/2}$ = 12960 min)	
±Warfarin	NC	NC	2.53	2.48
Timolol	NC	NC	2.90	48.9
Theophylline	NC	NC	NC	2.34
Alprazolam	NC	NP	NC	1.59
Prednisolone	NC	NP	1.60	9.70
Meloxicam	14.9	NP	1.79	30.7
Diazepam	16.5	NC	3.58	18.3
Tolbutamide	22.9	2.22	4.84	1.63
Metoprolol	38.6	4.04	7.86	46.5
Verapamil	121	15.9	33.9	310
Glimepiride	171	NP	20.3	70.3
Efavirenz	183	NC	5.52	No IV
Diclofenac	343	6.35	41.9	583

NC = No clearance observed (below cut-off value)
NP = Assay was not performed

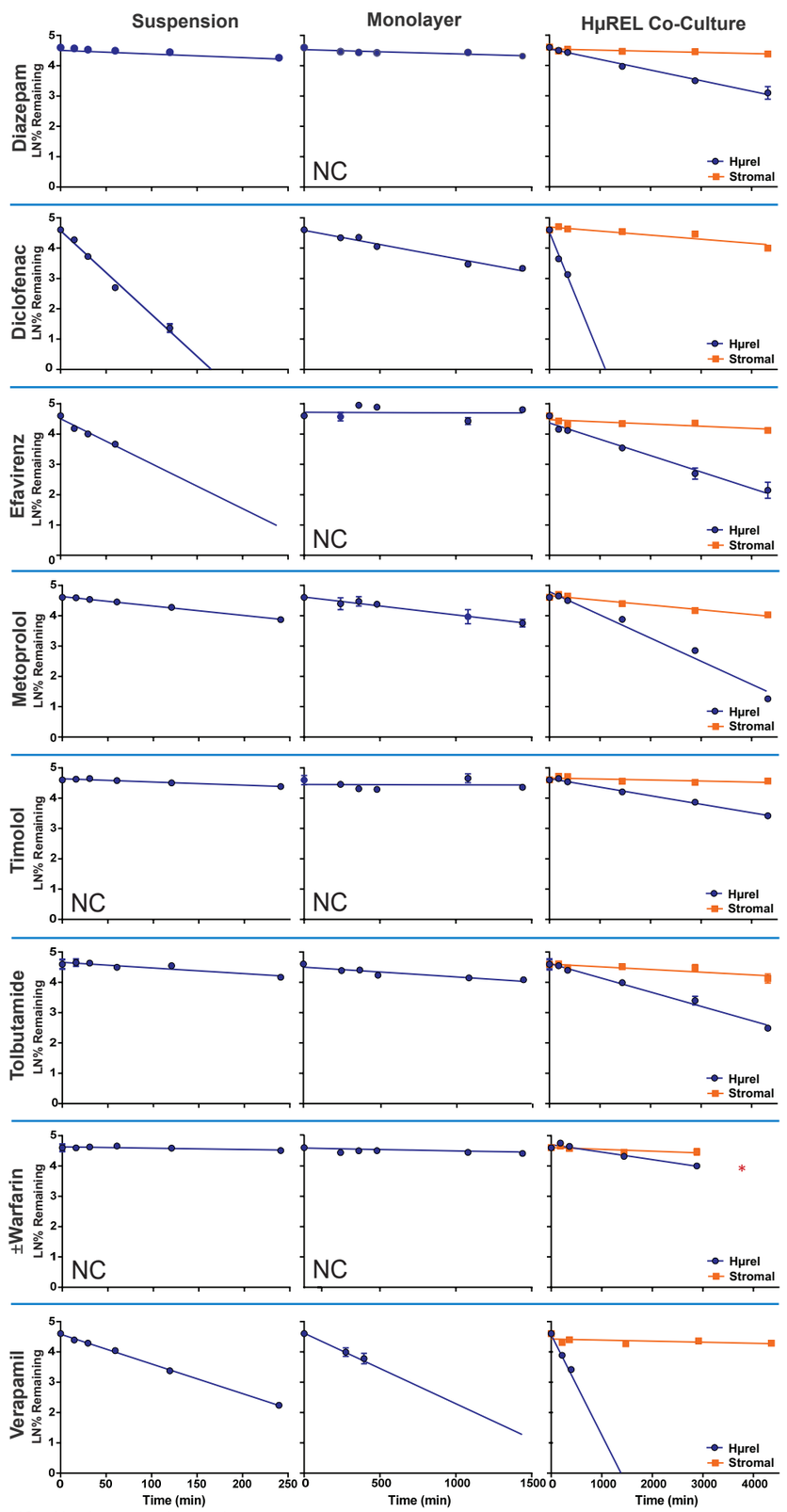
The table contains the scaled intrinsic clearance values for each hepatocyte system calculated from the depletion rate of each substrate using equation 4. The extrapolation limits for each system (~3x the incubation time) are included in the table. In vivo CL_{int} values were back-calculated using reported in vivo clearance values and the rearranged well-stirred model (equation 5).

In Vitro/In Vivo Correlation from Hepatocyte Models



The graph shows a log plot of the calculated intrinsic clearance values from our study as compared to the reported in vivo values. The solid line represents the line of unity while the dashed lines represent 2- and 3-fold deviations.

Representative Graphs of Metabolic Stability of Substrates in Hepatocyte Models



NC = No clearance observed
*Internal standard issue with the terminal time point for ±warfarin. These data were excluded from analysis.

Conclusions

- Under our assay conditions for suspended hepatocytes, moderate- to high-turnover compounds yielded results that compared favorably to the reported in vivo results. However, for low-turnover compounds, a model providing longer incubation times was required.
- The plated hepatocyte system did not provide any added benefit for these low-turnover compounds and under-predicted metabolic clearance for the high-turnover compounds.
- Using the HμREL co-culture system, we were able to measure metabolic clearance for 11 of 13 (85%) compounds.
- With a trend towards under-prediction for moderate- to high-clearance compounds, the HμREL co-culture system was the most appropriate for low-turnover drugs.
- If a measurable clearance cannot be determined and longer incubation times are required, the HμREL co-culture system would provide a preferred hepatocyte model to monoculture for measuring metabolic clearance of low-turnover compounds.

Acknowledgements:
- HμREL Corporation for this collaboration and scientific discussions.
- Suzanne Swift-Spencer for poster creation.

HμREL Corporation