A Long-Enduring, Stable Primary Hepatocyte Co-culture Model: Intrinsic Clearance and Metabolite Generation

Delaware Valley Drug Metabolism Discussion Group, 2016

J. Matthew Hutzler, Ph.D., Director, In Vitro Metabolism
In Vitro Metabolism and Scaling

Predicting Metabolic Clearance

- Common methodology to estimate metabolic clearance: incubate test compound (<<K_m) with liver microsomes or hepatocytes and measure rate of substrate depletion

$$Cl_{int} = \frac{0.693}{t_{1/2} \text{ (min)}} \times \frac{25.7 \text{ g liver}}{\text{kg body Wt}} \times \frac{\text{mL inc}}{\text{mg mic protein}} \times \frac{45 \text{ mg mic protein}}{\text{g Liver}} = \text{mL/min/kg}$$

- For low-turnover compounds, traditional incubation systems (e.g. microsomes and hepatocyte suspensions) are inadequate for a reliable clearance projection due to incubation time limitations (~1 hr for microsomes and ~4 hr for hepatocytes in suspension)

Low-Turnover Drug Molecules: A Current Challenge for Drug Metabolism Scientists

J. Matthew Hutzler, Barbara J. Ring, and Shelby R. Anderson

Q² Solutions, a Quintiles Quest Joint Venture, Bioanalytical and ADME Laboratories, Indianapolis, Indiana

Hutzler et al. (2015) Drug Metab Dispos. 43(12), 1917-1928
Cryopreserved Human Hepatocytes in Suspension

Rapid Decrease in Activity With Time

- Effect of time (in humidified incubator at 37° C) on activities in cryopreserved hepatocyte suspensions
- Mean IT_{50} values (time where 50% loss in activity was observed)
  - CYP1A2: 2.69 hrs
  - CYP2C9: 4.47 hrs
  - CYP2D6: 3.03 hrs
  - CYP3A: 1.62 hrs
  - UGT: 1.39 hrs
- Rapid loss of activity that is enzyme-dependent
- Incubation time limit: ~4 hrs
Hepatocytes in Monolayer Culture

Enables Longer Incubation Times, But…

### Pros

- Enzyme activity more stable compared to suspension
- Practical solution – plate cells and dose 4 hrs later
- Some plateable pools now available (5 or 10 donors)

### Cons

- Enzyme activity decreases over first 24 hrs (~50%)
- Not all lots of hepatocytes are plateable
- 24 hrs incubation limit may not be long enough for low CL

![Graphs showing enzyme activity over culture period](image)

* Hepatocytes were plated and allowed to attach for 4 hours prior to initiation of study

- Effects on specific activities in plated cryopreserved hepatocytes (4 preparations)
- $T_{50}$ estimates for CYP1A2 and CYP3A: 21.3 ± 2.1 and 28.8 ± 20.4 hrs

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Smith et al. (2012) *J Pharm Sci.* 101(10), 3989-4002
In Vitro Assay Option for Longer Duration Incubations

HµREL® Hepatic Co-culture Model

HµREL® - a hepatic co-culture platform (with stromal cells)
Robust enzyme activity for days to weeks
Plates arrive ready to use same day
Multiple plate formats available (12, 24, 48, 96 well)
Human, Rat, Dog, Monkey available on same plate for cross-species comparisons
Can use any plateable lot of hepatocytes (single donor or pooled)

Leslie Benet, Presentation at The Boston Society, July 2013

- Q² Solutions has conducted pilot studies with HµREL® co-culture system in effort to potentially utilize for low turnover compounds needing either clearance projection, or metabolite profile information
### Low Clearance In Vitro Assays: A Comparison Study

#### Study Design Summary

<table>
<thead>
<tr>
<th>Condition</th>
<th>Suspension (4 hr)</th>
<th>Plated Monolayer (24 hr)</th>
<th>HµREL® (72 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte Lot</td>
<td>LiverPool™ 5-Donor Mixed Gender Cryoplatable Lot YMD (BioreclamationIVT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thawing Media</td>
<td>CHRM</td>
<td>CHRM</td>
<td>HµREL thaw</td>
</tr>
<tr>
<td>Plating Media</td>
<td>-</td>
<td>Hu Hep Plating Media</td>
<td>HµREL plate</td>
</tr>
<tr>
<td>Incubation Media</td>
<td>HMM</td>
<td>HMM</td>
<td>HµREL Dosing Media</td>
</tr>
<tr>
<td>Seeding density (cells/well)</td>
<td>50,000</td>
<td>45,000</td>
<td>30,000</td>
</tr>
<tr>
<td>Inc Volume</td>
<td>200 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>Substrate Concentration</td>
<td>1 µM</td>
<td>1 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>Plate Design</td>
<td>96 well</td>
<td>96 well</td>
<td>96 well</td>
</tr>
<tr>
<td>Shaker speed</td>
<td>600 rpm</td>
<td>120 rpm</td>
<td>120 rpm</td>
</tr>
<tr>
<td>Time Points (6)</td>
<td>0, 15 min, 0.5, 1, 2, 4 hr</td>
<td>0, 4, 6, 8, 18, 24 hr</td>
<td>0.25, 3, 6, 24, 48, 72 hr</td>
</tr>
<tr>
<td>General procedures</td>
<td>Thaw cells, incubate in suspension for total of 4 hrs</td>
<td>Plate at 8am, dose at noon (t0), sample at 4 pm, 6 pm, 8 pm, 6 am, and noon next day</td>
<td>Receive plates on a Tuesday morning, dose in afternoon, 72 hrs is Friday afternoon</td>
</tr>
</tbody>
</table>

> Stromal cell only plate included as a control for HµREL system study
Low Clearance In Vitro Assays: A Comparison Study

Selected Substrates

- 13 substrates selected based on range of reported clearance values, as well as diversity in cytochrome P450 metabolic clearance mechanisms
- Minimal renal clearance
- Clearance rates estimated by substrate depletion methodology
- Scale-up to intrinsic clearance

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzymes Involved in Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>± Warfarin</td>
<td>CYP2C9, CYP3A, CYP1A2</td>
</tr>
<tr>
<td>Timolol</td>
<td>CYP2D6</td>
</tr>
<tr>
<td>Diazepam</td>
<td>CYP3A, CYP2C19</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>CYP2C9</td>
</tr>
<tr>
<td>Theophylline</td>
<td>CYP1A2</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>CYP2D6</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>CYP2B6</td>
</tr>
<tr>
<td>Verapamil</td>
<td>CYP2D6, CYP3A, UGT</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>CYP2C9, UGT</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>CYP3A</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>CYP2C9</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>CYP2C9</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>CYP3A</td>
</tr>
</tbody>
</table>
Representative Metabolic Stability Figures

NC = no clearance

*internal standard issue
In Vitro Scaling Calculations

1) \( k_{dep} = \text{slope of time (min) vs. Ln\% Remaining; } k_{dep} = \frac{\text{Ln}(2)}{t_{1/2}} \)

2) In Vitro Intrinsic Clearance:
\[
\frac{\text{Ln}(2)}{t_{1/2}} \times \frac{\text{Inc Vol (\muL)}}{\text{Cells} \times 10^6} = \frac{\muL}{\text{min} \times 10^6 \text{ Cells}}
\]

3) Intrinsic Clearance (\( Cl_{\text{int}} \)):
\[
\frac{\text{Ln}(2)}{t_{1/2}} \times \frac{\text{Inc Vol (mL)}}{\text{Cells} \times 10^6} \times \frac{120 \times 10^6 \text{ Hepatocytes}}{\text{g Liver}} \times \frac{25.7 \text{ g Liver}}{\text{Kg BWt}} = \frac{\text{mL}}{\text{min} \times \text{Kg}}
\]

4) \( Cl_{\text{int, in vivo}} \) back-calculated from \( Cl_{\text{in vivo}} \) using the well-stirred model:
\[
Cl_{\text{int, in vivo}} = \frac{Cl_{\text{in vivo}}}{f_{ub} \times \left(1 - \frac{Cl_{\text{in vivo}}}{Q}\right)}
\]
**Intrinsic Clearance Comparison**

(mL/min/kg)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Hepatocyte Suspension</th>
<th>Hepatocyte Monolayer</th>
<th>HμREL Co-Culture</th>
<th>In Vivo Intrinsic Clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(4 Hour Incubation)</td>
<td>(24 Hour Incubation)</td>
<td>(72 Hour Incubation)</td>
<td></td>
</tr>
<tr>
<td>Extrapolation Limits</td>
<td>11.9</td>
<td>1.11</td>
<td>0.560</td>
<td></td>
</tr>
<tr>
<td>(≥20% dep ~3x incubation time)</td>
<td>(t₁/₂ = 720 min)</td>
<td>(t₁/₂ = 4320 min)</td>
<td>(t₁/₂ = 12960 min)</td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>NC</td>
<td>NC</td>
<td>2.53</td>
<td>2.48</td>
</tr>
<tr>
<td>Timolol</td>
<td>NC</td>
<td>NC</td>
<td>2.90</td>
<td>48.9</td>
</tr>
<tr>
<td>Theophylline</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>2.34</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>NC</td>
<td>NP</td>
<td>NC</td>
<td>1.59</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>NC</td>
<td>NP</td>
<td>1.60</td>
<td>9.70</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>14.9</td>
<td>NP</td>
<td>1.79</td>
<td>30.7</td>
</tr>
<tr>
<td>Diazepam</td>
<td>16.5</td>
<td>NC</td>
<td>3.58</td>
<td>18.3</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>22.9</td>
<td>2.22</td>
<td>4.84</td>
<td>1.63</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>38.6</td>
<td>4.04</td>
<td>7.86</td>
<td>46.5</td>
</tr>
<tr>
<td>Verapamil</td>
<td>121</td>
<td>15.9</td>
<td>33.9</td>
<td>310</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>171</td>
<td>NP</td>
<td>20.3</td>
<td>70.3</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>183</td>
<td>NC</td>
<td>5.52</td>
<td>No IV</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>343</td>
<td>6.35</td>
<td>41.9</td>
<td>583</td>
</tr>
</tbody>
</table>

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

- Plated monoculture performs poorly (5/9 non-detects); 24 hour incubation time may not be long enough for a low-turnover compound
- Clearance rate in HμREL co-culture system 2- to 7-fold higher than hepatocyte monoculture format, with improved ability to determine clearance rate (11/13 compounds, or 85%)
In Vitro-In Vivo Correlation Analysis

- Suspended hepatocyte data correlates best overall for moderate/high clearance, but short incubation time limits measuring clearance for low-turnover compounds.

- Clearance estimates from HµREL co-culture trends toward under-prediction for moderate/high clearance compounds, but performance was superior to a monoculture system.

- A long-enduring co-culture system is suited for enabling better assessment of low-turnover drug molecules.

- Additional studies to enrich low-turnover compound data set in progress.
Importance of Metabolite Identification

• Guidance Expectations around Metabolite Profiling (FDA/EMA/Japan):
  ✓ Are the “major” circulating human metabolites produced in the toxicology species? – MIST
  ✓ Are the “major” circulating human metabolites pharmacologically active? – PK/PD
  ✓ Are there “major” circulating human metabolites that may elicit clinically relevant drug interactions? – DDI
  ✓ What are the primary metabolic clearance pathways, including the enzyme(s) responsible? – DDI

  – Prior to Phase I dosing, in vitro systems are relied upon to predict human metabolites
    • For moderate/high clearance drugs, traditional in vitro systems (e.g. microsomes, S9 fraction, suspended hepatocytes) are utilized for these assessments

  – For low turnover drugs and drugs that undergo secondary metabolism, traditional in vitro systems may not be adequate for producing metabolite profiles that resemble in vivo metabolite profiles

  – A longer incubation time is necessary
**Metabolite Identification from HµREL Co-culture**

**Study Design Summary**

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<tr>
<th>Condition</th>
<th>Suspension</th>
<th>HµREL®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte Lot</td>
<td>HSH (5-donor cryoplatable pool)</td>
<td>5-donor cryoplatable pool (Xenotech)</td>
</tr>
<tr>
<td>Thawing Media</td>
<td>CHRM</td>
<td>HµREL thaw</td>
</tr>
<tr>
<td>Plating Media</td>
<td>-</td>
<td>HµREL plate</td>
</tr>
<tr>
<td>Incubation Media</td>
<td>InVitroGRO KHB</td>
<td>HµREL Dosing Media</td>
</tr>
<tr>
<td>Cell density (cells/well)</td>
<td>500,000</td>
<td>188,000</td>
</tr>
<tr>
<td>Inc Volume</td>
<td>500 µL</td>
<td>400 µL</td>
</tr>
<tr>
<td>Substrate Concentration</td>
<td>10 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td>Plate Design</td>
<td>24 well</td>
<td>24 well</td>
</tr>
<tr>
<td>Shaker speed</td>
<td>400 rpm</td>
<td>120 rpm</td>
</tr>
<tr>
<td>Time Points</td>
<td>4 hr</td>
<td>4, 24, 72, 168 hr</td>
</tr>
</tbody>
</table>

- Stromal cell only plate included as a control for HµREL system study

**Mass Spectrometry**

- **System:** Thermo LTQ Orbitrap XL
- **Ionization:** ESI+; **Source:** 5.0 kV; **Capillary:** 350˚C
- **Fragmentation Method:** HCD
- **Collision Energy:** 25 (meloxicam), 50 (timolol), 40 (XK469)
Meloxicam Metabolism

Hepatocyte Suspension - 4 hr

HμREL – 4 hr
- Melox-COOH

HμREL – 24 hr
- Melox + 4H + O
- Melox + 2 H₂O

HμREL – 72 hr

HμREL – 168 hr

Time (min)

10 15 20 25 30 35

Suspended hepatocytes:
- Observed only trace levels of +O (Melox-OH)

HμREL Co-culture:
- Observed progression from +O (Melox-OH) to subsequent acid (Melox-COOH) over time

Comparison to in vivo data:
- Based on ¹⁴C-human:
  - Melox-OH ~9% dose
  - Melox-COOH ~60% dose
  - Turck et. al. 1996

Conclusion:
- Long duration incubations in HμREL system yielded good secondary metabolite and better correlation with in vivo met ID data
Timolol Metabolism

Suspended hepatocytes:
- Observed only trace levels of metabolites, largest +O

HμREL Co-culture:
- Seeing progression from +O to morpholine ring opening (+2O) to loss of ethyl (-C$_2$H$_2$)
- Seeing robust metabolism to yield multiple metabolites

Comparison to in vivo data:
- Based on $^{14}$C-human:
  - Morpholine ring open (+2O) ~22% dose
  - Loss of ethyl bridge from morpholine ~7% dose
- Tocco et. al. 1980

Conclusion:
- Morpholine ring opening with subsequent loss of ethyl bridge readily observed
- Intriguing that +gluc was not observed in suspensions but significant in HμREL
**XK-469 Metabolism**

- **Suspended hepatocytes:**
  - Observed only trace levels metabolites, largest +O

- **HµREL Co-culture:**
  - Seeing +O associated with aldehyde oxidase as largest peak
  - Seeing robust metabolism to an array of non-P450 metabolites

- **Comparison to in vivo data:**
  - Based on human data:
    - AO metabolite accounts for 54% of total urinary excretion, and is most predominant metabolite

- **Conclusion:**
  - Long duration incubations in HµREL system yielded identification of key non-P450 in vivo metabolite for a low-turnover AO substrate (relevant species difference)

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**Diagram:**

- **Hepatocyte Suspension - 4 hr**
  - XOR69

- **HµREL - 4 hr**
  - + Taurine
  - + AcetylCys – 2H
  - + O (AO)
  - + Glycine

- **HµREL - 24 hr**
  - + Cys – 2H

- **HµREL - 72 hr**
  - + Cys – 2H

- **HµREL - 168 hr**

**Time (min)**: 10 15 20 25 30 35

**XOR69**

**AO-mediated hydroxylation**
Conclusions

• Clearance
  – Hepatocytes in suspension performed well for moderate to high-turnover compounds for estimation of intrinsic clearance, but is unsuitable for low-turnover compounds
  – HµREL hepatocyte co-culture system outperformed hepatocytes in monolayer culture for prediction of clearance (85% success), with trend towards under-prediction for moderate to high-turnover compounds
  – Adding to data set with 5 additional low turnover compounds and conducting a lot-to-lot comparison

• Metabolite ID
  – Incubations in HµREL hepatocyte co-culture system over 7 days yielded robust generation of metabolites with time
  – Both secondary metabolites and non-P450 metabolic pathways functional over the duration of incubations (not observed in hepatocyte suspensions)
  – Hepatocyte co-culture model like HµREL with longer incubation times may enable better assessment of possible human metabolites and comparison to preclinical species
Acknowledgements

• Clearance Studies
  – Mark VandenBranden
  – Todd Hieronymus
  – Andy Staton
  – Barbara Ring

• Metabolite ID Studies
  – Taysir Chamem
  – Todd Hieronymus
  – David Heim
  – Rich Burton
  – Shelby Anderson
  – Xiusheng Miao

• HμREL
  – Eric Novik
  – Matt Shipton