A Novel Relay Method for Determining Low-Clearance Values

Li Di, Patrick Trapa, R. Scott Obach, Karen Atkinson, Yi-An Bi, Angela C. Wolford,
Beijing Tan, Thomas S. McDonald, Yurong Lai, Larry M. Tremaine

Pharmacokinetics, Dynamics and Metabolism, Pfizer Inc., Groton, CT 06340, USA: LD,
PT, RSO, KA, YAB, ACW, BT, TSM, YL, LMT
Corresponding Author:
Li Di
Pharmacokinetics, Dynamics and Metabolism
Pfizer Inc.
Eastern Point Road
Groton, CT 06340
LI.DI@PFIZER.COM
860-715-6172

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Abbreviations
ADME = Absorption, Distribution, Metabolism, and Excretion
CYPs = Cytochrome P450
DMPK = Drug Metabolism and Pharmacokinetics
DMSO = Dimethyl Sulfoxide
F_u,inc = Fraction Unbound under Incubation Condition
IVIVC = In Vitro-In Vivo Correlation
LC/MS = Liquid Chromatography / Mass Spectrometry
MAO = Monoamine Oxidase
MeOH = Methanol
Abstract

A novel relay method has been developed using cryopreserved human hepatocytes to measure intrinsic clearance of low-clearance compounds. The relay method involved transferring the supernatant from hepatocyte incubations to freshly thawed hepatocytes at the end of the 4-hour incubation to prolong the exposure time to active enzymes in hepatocytes. An accumulative incubation time of 20 hours or longer in hepatocytes can be achieved in the method. The relay method was validated using seven commercial drugs (diazepam, disopyramide, theophylline, timolol, tolbutamide, S-warfarin and zolmitriptan) metabolized by various CYPs with low human in vivo intrinsic clearance around 2-15 mL/min/Kg. The results showed that the relay method produced excellent predictions of human in vivo clearance. The difference between in vitro and in vivo intrinsic clearance was within two fold for most compounds, which is similar to the standard prediction accuracy for moderate to high clearance compounds using hepatocytes. The relay method is a straightforward, relatively low cost and easy to implement new tool to address the challenges of low clearance in drug discovery and development.
**Introduction**

Prediction of in vivo hepatic clearance with in vitro metabolism assays is important in drug discovery and development since hepatic clearance impacts half-life, oral bioavailability, dose and dosing regimens (Obach, 2011). In vitro methods to measure CYP450 mediated metabolism have been well-developed and scaling factors are well-established to predict in vivo clearance (Hosea et al., 2009). However, for non-CYP mediated metabolism and transporter mediated elimination, prediction of in vivo clearance is much more challenging. Developing predictive tools and improving prediction accuracy is a very active research area (Kilford et al., 2009; Zientek et al., 2010; Maeda et al., 2011).

Some drugs and experimental compounds have low clearance. They are slowly metabolized and do not have significant turnover in liver microsome and hepatocyte assays. Low-clearance compounds in drug discovery are increasingly more prevalent due to a number of reasons. Firstly, high throughput metabolic stability assays in conjunction with metabolite identification enable rapid development of structure-metabolic stability relationships (Di and Kerns, 2003; Di et al., 2008; Hop et al., 2008). Effective design strategies have been put into place to overcome metabolic liabilities through structural modifications (Kerns and Di, 2008; Di et al., 2009). More and more metabolically stable compounds are being generated. It has been estimated, based on our in house in vitro data, that approximately 30% of drug candidates have intrinsic clearance values of less than 10 ml/min/kg (i.e., no significant turnover of parent in microsomal or hepatocyte stability assays). For some projects, the entire series have low clearance and
it is difficult to differentiate among the compounds based on intrinsic clearance. Secondly, the recent approach of utilizing uptake transporters for liver targeting (Oballa et al., 2011; Pfefferkorn et al., 2012), leading to a shift of chemical space toward high polarity and low permeability, further contributes to low-clearance compounds. Liver targeting compounds tend to be metabolically stable and their elimination pathways typically involve transporter-mediated biliary and/or renal clearance.

Though reducing metabolic clearance might be the goal of drug discovery teams, low-clearance compounds present great challenges for DMPK scientists to predict human clearance and half-life, and to differentiate among stable compounds. The typically employed tools established for IVIVC cannot be applied to these cases due to low or insignificant turnover of the parent compounds. When compounds are stable in human liver microsomes and human hepatocytes, no in vitro clearance prediction can be obtained. Single species or allometric scaling will have to be used to estimate human clearance. In such cases, species differences in metabolism may lead to large uncertainty in dose prediction and half-life estimation. For example, several low-clearance drug candidates required to test in humans in order to obtain reliable PK parameters. Many of these compounds ultimately failed due to the half-life being too long. Because of the lack of adequate tools, low-clearance compounds may progress to human studies to verify the actual PK profiles. These compounds could otherwise be terminated earlier using in vitro predictions. For these reasons, low-clearance compounds often fail in the clinical trials due to undesirable PK profiles (Obach, 2011) (too long a half-life, complex dosing regimen, toxicity due to accumulation and concerns on difficulty to “wash off” when needed). When there is no measurable turnover in liver microsomes and hepatocytes, it is
also difficult for project teams to differentiate compounds based on metabolic stability during lead optimization and candidate selection.

Currently available tools for low clearance are quite limited and imperfect. The most common approach is to monitor metabolite formation instead of parent deletion after in vitro microsomal or hepatocyte incubations (Stringer et al., 2009). Monitoring metabolite formation can be a valuable tool for low-clearance compounds when the importance of a particular metabolic pathway in the overall metabolic clearance of the compounds is known. However, metabolite standards or radio-labeled parents are required for this approach (Carlile et al., 1998; Shaffer and Langer, 2007). This method is appropriate for late stage development candidates, but not for studying a large number of compounds in early drug discovery in a higher throughput setting. Another approach to address low-clearance compounds is the HepatoPac® platform developed by Hepregen Corporation (http://www.hepregen.com/). Early investigations of the system showed promising results in metabolite identification (Wang et al., 2010) and clearance prediction (Chan et al., 2011; Quinn et al., 2011); however, more validation is needed to demonstrate the reliability and reproducibility in predicting human clearance for low-clearance compounds.

In this paper, we explore a new methodology for low-clearance that can be applied in early drug discovery with reasonable throughput and cost. The development of a novel relay method determining intrinsic clearance of low-clearance compounds using cryopreserved human hepatocytes has proven to be straightforward and can be established easily in any DMPK laboratory.
Material and Methods

Materials

Test compounds were obtained from Pfizer Global Material Management (Groton, CT) or purchased from Sigma-Aldrich (St. Louis, MO). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless specified. Polypropylene plates were from Thermo (Pittsburgh, PA) for 96-wells and from Costar (Sigma-Aldrich, St. Louis, MO) for 24-wells.

Relay Method Using Human Hepatocytes

Pooled cryopreserved human hepatocytes of 10 donors were purchased from Celsis IVT (Baltimore, MD). This lot of pooled hepatocytes was used for all the studies. When selecting new lots of hepatocytes, enzyme activities need to be verified using marker compounds. Upon thawing, the hepatocytes were re-suspended in Williams E medium (WEM GIBCO-BRL, cat#C1984, custom formula # 91-5233EC) supplemented with HEPES and Na₂CO₃. The cells were counted using the Trypan Blue exclusion method, and the 24-well hepatocyte plates containing 0.5 million cells/mL were spiked with a compound at a final concentration of 1 μM (final DMSO of 0.025% and MeOH of 0.125%), in a final incubation volume of 0.50 mL. The plates were covered with Breathe-Easy™ gas permeable membranes (Diversified Biotech, Dedham, MA) and incubated at 37°C with 95% O₂/ 5% CO₂, 75% relative humidity for 4 hours at 150 rpm in a humidified incubator. At time 0 and 4.0 hr, 25 μL of hepatocyte suspension was removed from the incubation and added to 50 μL of cold acetonitrile containing internal standard to quench the reaction. The samples were centrifuged ((Eppendorf, Hauppauge,
NY) at 3000 rpm for 10 minutes at 4°C and 50 µL of supernatant was transferred to a clean plate, dried completely and reconstituted prior to LC-MS/MS analysis. The remaining hepatocyte suspensions in the incubation plate were centrifuged (3000 rpm, 10 minutes, 4°C). The supernatant of 300 µL was transferred to a clean 24-well plate and stored at -80°C until the next relay experiment. For the 2nd relay experiment, the supernatant plates were warmed to 37°C for 20 min and hepatocytes were added to the samples to give a final cell density 0.5 million cells/mL. The plates were incubated at 37°C for 4 hr, sampled and processed as described above. Five relays were performed to give a total incubation time of 20 hr. The supernatant from the last relay was saved in case more relays were needed for compounds with remarkably low clearance. Standard curves were prepared under the same conditions.

**LC-MS/MS Quantification**

The LC mobile phases were: (A) HPLC grade water containing 0.1% formic acid; and (B) acetonitrile containing 0.1% formic acid. A solvent gradient from 5% (B) to 95% (B) over 2.0 minutes at the flow rate of 0.4 ml/min was used to elute the compounds from the column (Kinetex C18, 30x2 mm, 2.6μm, Phenomenex, Torrance, CA). The cycle-time was 3 minutes / injection. An aliquot of 5 µL sample was injected for analysis using a CTC PAL autosampler (Leap Technology, Carrboro, NC). For timolol, tolbutamide, diazepam and warfarin, the analysis was conducted with Shimadzu HPLC AD20 pumps (Columbia, MD) connected to an AB Sciex (Foster City, California) API 5500 triple quadrupole mass spectrometer equipped with a TurboIonSpray source using MRM mode. Analyst™ 1.5.2 software (Applied Biosystems, Foster City, CA) was applied to data
collection, processing and analysis. For theophylline and zolmitriptan, the analysis was performed on Thermo (Waltham, MA) ultra-pressure liquid chromatography (UPLC) on a Kinetex column (C18, 30x2 mm, 2.6 μm, Phenomenex, Torrance, CA) with an Accela 1250 pump (West Palm Beach, FL), which was connected to a Q-Exactive orbitrap high resolution mass spectrometer equipped with TurboIonSpray source (Thermo, Waltham, MA). A full scan mode from m/z 150 to 600 was applied to detect each compound. LCquan software (version 2.5) from Thermo (Waltham, MA) was utilized for data collection, processing and analysis. Terfenadine was used as an internal standard for LC–MS/MS quantification in positive ion MRM mode. All the test compounds had good linearity with R² > 0.99 and the limit of quantitation was 1 nM for all the compounds.

Calculations

The relay assay requires transfer of the supernatant between wells for the different incubations. Drug is lost due to hepatocyte uptake, nonspecific binding or other sources, and dilution occurs as new hepatocytes and medium are added post transfer. These losses necessitate a correction before comparison back to time zero concentrations. The correction equation follows (Eq. 1):

\[
C_{\text{corrected}} = C_{n,\text{total}} \times (\text{recovery})^n \times \left(\frac{\text{well volume}}{\text{transfer volume}}\right)^n \times \prod_{i=1}^{n} \left(\frac{C_{i-1,\text{total}}}{C_{i-1,\text{supernatant}}}\right) \quad (\text{Eq. 1})
\]

Here, \(C_{\text{corrected}}\) is the total concentration of the well after transfer corrected for loss and dilution. Recovery accounts for the fraction of the compound irretrievable due to nonspecific binding to the well or other sources, and should be set to unity unless it is known that drug is lost after each transfer at a location not captured in the total-concentration measurement. Recovery can be estimated experimentally by measuring the...
change in concentration of a representative solution from before and after addition to a well in absence of cells (buffer control); however, low-clearance compounds are often polar and therefore would not be expected to experience high levels of nonspecific binding. N is the number of transfers. The ratio of the well volume to the supernatant-transfer volume corrects for the dilution which occurs after each transfer. The above equation assumes that the ratio is fixed, i.e. the total and transfer volume remain constant throughout the relay assay. Deviations can be handled by the equation listed below. The final term, the product of the ratios of the total and supernatant concentrations in the steps previous to the transfer corrects for loss in the hepatocyte precipitate.

Take, for example, the following data:

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Supernatant Concentration (µM)</th>
<th>Total Concentration (µM)</th>
<th>Total Corrected Concentration (µM)</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.24</td>
<td>1.67</td>
<td>1.670</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.560</td>
<td>0.867</td>
<td>0.867</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.0838</td>
<td>0.159</td>
<td>0.409</td>
<td>after 1st transfer</td>
</tr>
<tr>
<td>12</td>
<td>0.0132</td>
<td>0.0257</td>
<td>0.209</td>
<td>after 2nd transfer</td>
</tr>
</tbody>
</table>

For the first two points (t = 0 and 4 hours), the total concentration is equal to the corrected concentration because no transfer has occurred. For the third time point:

\[
C_{corrected} = 0.159 \times (1)^1 \times \left(\frac{500}{300}\right)^1 \times \left(\frac{0.867}{0.56}\right) = 0.409
\]

For the fourth point:
These corrected values can now be compared directly to values obtained at early time points prior to any transfer. Note: if the transfer amount or volume of the well changes with transfers, the general equation becomes (Eq. 2):

\[
C_{\text{corrected}} = C_{n,\text{total}} \times (\text{recovery})^n \times \prod_{i=1}^{n} \left( \frac{\text{well volume}}{\text{transfer volume}} \right) \times \prod_{i=1}^{n} \left( \frac{C_{i-1,\text{total}}}{C_{i-1,\text{supernatant}}} \right)
\]  (Eq. 2)

Half-life and intrinsic clearance were calculated using Eq. 3 and Eq. 4 with the corrected concentration \(C_{\text{corrected}}\) discussed above.

\[
T_{1/2} = \ln 2 / -(\text{slope of the ln % remaining of drug vs. time plot}) = \text{minutes} \quad (\text{Eq. 3})
\]

\[
CL_{\text{at}} = \ln 2 \times \frac{1}{T_{1/2} \text{ (min)}} \times \frac{\text{mL incubation}}{0.5 \text{ M cells}} \times \frac{120 \text{ M cells}}{\text{g liver}} \times \frac{21 \text{ g liver}}{\text{kg}} = \text{ml/min/kg} \quad (\text{Eq. 4})
\]

Human in vivo intrinsic clearance was calculated using the well-stirred model (Eq. 5) (Brown et al., 2007) based on human IV clearance data from the literature, where \(Q_H\) is hepatic blood flow (20.7 ml/min/kg); \(CL_b\) is hepatic blood clearance; \(f_u_p\) is fraction unbound in plasma; \(R_B\) is blood to plasma concentration ratio.

\[
CL_{\text{at}} = \frac{f_u_p \times CL_b}{R_B \times (1 - \frac{CL_b}{Q_H})} \quad (\text{Eq. 5})
\]
Results and Discussion

Human liver microsomes and hepatocytes are the most commonly used systems to predict human PK parameters since they are derived from human material and have historically been demonstrated to have high prediction accuracy for metabolically cleared compounds. The challenge of using human liver microsomal and hepatocyte assays for low-clearance compounds is that enzyme activities in these systems decrease with time and they are only suitable for metabolic rate determination within certain limits of incubation time (e.g., human liver microsomes ~ 1 hr, human hepatocytes ~ 4 hr). Incubating beyond these time limitations leads to too much loss of enzyme activity to generate reliable kinetic information. If compounds do not show significant turnover (<20%) during the optimal incubation times, it will be difficult to determine the metabolic rate using these in vitro systems, and therefore difficult to predict human in vivo clearance.

To overcome the impediment due to the loss of enzyme activity during incubation, a novel relay method for low-clearance measurement has been developed. In the method, the supernatant is transferred from hepatocyte incubations to freshly thawed hepatocytes at the end of the 4-hour incubation to prolong the exposure time to active enzymes in hepatocytes (Figure 1). The relay assay conditions are summarized in Table 1 and are quite similar to a regular human hepatocyte assay. The only difference is the addition of the relay steps to prolong the exposure time. Through this method, drugs can be continuously metabolized beyond 4 hr by replacing with freshly thawed hepatocytes every 4 hours. Since the assay is essentially an extension of the regular hepatocyte assay
that has proven to be effective, the relay method is likely to have similar performance (pros and cons) as the standard hepatocyte stability assay.

Seven commercial drugs covering various CYPs and various degrees of low intrinsic clearance were selected for method development. The structures of the compounds are shown in Figure 2 and all compounds have reported human IV clearance data. The intrinsic clearance values were calculated based on the well-stirred model (Eq. 5) and the results are summarized in Table 2 along with the in vitro intrinsic clearance measured using the relay method. Correction for $f_{u, \text{inc}}$ (fraction unbound in the incubation) was not applied due to the high free fraction of the test compounds in the hepatocyte incubation ($f_{u, \text{inc}}$ near 1). The kinetic plots are shown in Figure 3 and the correlation coefficients ($R^2$) are greater than 0.90 for all the compounds suggesting good linearity for the metabolic kinetic measurement. The slope from 0-hour to the 1st time point (4 hr incubation) is the maximum decay in a regular hepatocyte assay. It is clear that the slope is very low with minimal change in concentration for low-clearance compounds, which makes it difficult to determine intrinsic clearance in the regular hepatocyte assay. The relay method gave very good prediction of human intrinsic clearance with most compounds within 2 fold of the in vivo human data (Table 2). The results are consistent with hepatocyte prediction accuracy of moderate/high clearance compounds (Hallifax et al., 2010). The exceptions are timolol (2.5 fold) and zolmitriptan (3.3 fold) which could potentially be due to the effect of 2D6 polymorphism on timolol and the extrahepatic contribution of MAO for zolmitriptan. Hepatocyte also under-predicts sumatriptan (metabolized by MAO) human intrinsic clearance in vivo(Obach et al., 2008), supporting
extrahepatic contribution of MAO in metabolizing their substrates. The relay method is a very forgiving assay because even with many transferring in several days with a number of analysts, the results are quite reproducible as shown by the tight standard errors. The relay method variability is quite similar to the regular suspension hepatocyte stability assay even though it involves multiple steps.

The number of relays for each study can be flexible depending on the intrinsic clearance of the compounds. For screening purposes, five relays have been shown to be successful to achieve a total incubation time of 20 hr in active hepatocytes. The supernatant of the last relay should be frozen and saved in case additional relays are needed for a particular compound with extremely low clearance. The maximum number of relays is dependent on LC-MS detection limit since there is a dilution in each relay when freshly thawed hepatocytes are added to the supernatant. For compounds with typical LC-MS sensitivity with 0.1 – 1 nM detection limit, 10-15 relays can be achieved. Dilution of freshly thawed hepatocytes with fresh buffer during each relay is important in maintaining a healthy environment for the cells, since the fresh media provides additional nutrients to the cells and dilutes the waste generated by the cells from the previous incubations.
Conclusions

The relay method shows excellent correlation with in vivo human clearance data for low clearance compounds. It offers a straightforward and reliable measurement for low clearance, which can be easily adapted in any DMPK laboratory without additional investment for new instrumentation. The assay is relatively low cost (~$3000 / plate of 24-wells). Miniaturization of the assay to a higher density format (e.g., 96-well format) will further reduce the use of hepatocytes and the cost of the assay. The method can also be applied to reaction phenotyping and metabolite identification of low-clearance compounds. The novel relay method presents a new opportunity to address low-clearance issues in drug discovery and development.
Acknowledgements

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Authorship Contributions

Participated in research design: Li Di, Patrick Trapa, R. Scott Obach, Karen Atkinson, Yi-An Bi, Angela C. Wolford, Beijing Tan, Thomas S. McDonald, Yurong Lai, Larry M. Tremaine

Conducted experiments: Karen Atkinson, Yi-An Bi, Angela C. Wolford, Beijing Tan, Thomas S. McDonald

Performed data analysis: Li Di, Patrick Trapa, Karen Atkinson, Yi-An Bi, Angela C. Wolford, Beijing Tan, Thomas S. McDonald

Wrote or contributed to the writing of the manuscript: Li Di, Patrick Trapa, R. Scott Obach, Karen Atkinson, Beijing Tan, Larry M. Tremaine
References


http://www.hepregen.com/.


Figure Legends

Figure 1. Relay Method for Low Clearance

Figure 2. Structures of the Low Clearance Test Compounds

Figure 3. Kinetic Plot of Low Clearance Compounds Using the Relay Method. Error bar represents standard error.
Table 1. Relay Assay Conditions

<table>
<thead>
<tr>
<th>Assay Parameters</th>
<th>Assay Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Compound Concentration</td>
<td>1 µM</td>
</tr>
<tr>
<td>Hepatocyte Cell Density</td>
<td>0.5 Million Cells/mL</td>
</tr>
<tr>
<td>Organic Solvent Content</td>
<td>DMSO of 0.025% &amp; MeOH of 0.125%</td>
</tr>
<tr>
<td>Incubation Volume</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Supernatant Transfer Volume</td>
<td>0.3 mL</td>
</tr>
<tr>
<td>Plate Format</td>
<td>24 Well</td>
</tr>
<tr>
<td>Incubation Temperature</td>
<td>37º C</td>
</tr>
<tr>
<td>Incubation Conditions</td>
<td>95% O₂ / 5% CO₂ / 75% RH</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>4 hr / relay</td>
</tr>
<tr>
<td>Standard Number of Relays</td>
<td>5 relays</td>
</tr>
</tbody>
</table>
Table 2. Comparison of *In Vivo* Human Intrinsic Clearance with *In Vitro* Intrinsic Clearance Using the Relay Method

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Metabolizing Enzymes</th>
<th>Human In Vivo Intrinsic Clearance (mL/min/Kg)</th>
<th>Relay Method Intrinsic Clearance (mL/min/Kg) ± SE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold Difference between In Vitro and In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>CYP3A, 2C19</td>
<td>15 (Hallifax et al., 2010)</td>
<td>15 ± 0.85</td>
<td>1.0</td>
</tr>
<tr>
<td>Disopyramide</td>
<td>CYP3A</td>
<td>5.9 (Obach et al., 2008)</td>
<td>4.8 ± 0.55</td>
<td>1.2</td>
</tr>
<tr>
<td>Theophylline</td>
<td>CYP1A2</td>
<td>2.6 (Hallifax et al., 2010)</td>
<td>2.8 ± 0.60</td>
<td>1.1</td>
</tr>
<tr>
<td>Timolol</td>
<td>CYP2D6</td>
<td>36- 49 (Obach et al., 2008; Hallifax et al., 2010)</td>
<td>14 ± 2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>CYP2C9</td>
<td>4.9 (Brown et al., 2007)</td>
<td>7.4 ± 0.40</td>
<td>1.5</td>
</tr>
<tr>
<td>S-Warfarin</td>
<td>CYP3A, 2C9</td>
<td>4.5 (Hallifax et al., 2010)</td>
<td>4.2 ± 0.65</td>
<td>1.1</td>
</tr>
<tr>
<td>Zolmitriptan</td>
<td>CYP1A2, MAO</td>
<td>13 (Obach et al., 2008)</td>
<td>3.5 ± 0.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>n= 4 for all the compounds with the exception of theophylline (n=2) and zolmitriptan (n=3).

<sup>b</sup>Under-prediction of in vivo clearance could potentially be due to extrahepatic contribution of MAO.
Figure 2

- Diazepam
- Disopyramide
- Theophylline
- Timolol
- Tolbutamide
- S-Warfarin
- Zolmitriptan
Figure 3-2

Disopyramide

%Remaining

$R^2 = 0.95$

Time (hour)
Figure 3-4

Timolol

% Remaining

R² = 0.93

Time (Hour)