A Novel Relay Method for Determining Low-Clearance Values

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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-h incubation to prolong the exposure time to active enzymes in hepatocytes. An accumulative incubation time of 20 h or longer in hepatocytes can be achieved using the method. The relay method was validated using seven commercial drugs (diazepam, disopyramide, theophylline, timolol, tolbutamide, S-warfarin, and zolmitriptan) that were metabolized by various cytochrome P450s with low human in vivo intrinsic clearance at approximately 2 to 15 ml·min⁻¹·kg⁻¹. 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 2-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-use 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 because hepatic clearance affects half-life, oral bioavailability, and dose and dosing regimens (Obach, 2011). In vitro methods to measure cytochrome P450 (P450)-mediated metabolism have been well developed, and scaling factors are well established to predict in vivo clearance (Hosea et al., 2009). However, for non-P450-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 for a number of reasons. First, 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⁻¹·kg⁻¹ (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. Second, a recent approach of using 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.

Although reducing metabolic clearance might be the goal of drug discovery teams, low-clearance compounds present great challenges for drug metabolism and pharmacokinetics (DMPK) scientists to predict human clearance and half-life, and to differentiate among stable compounds. The tools typically used to establish in vitro-in vivo correlation cannot be applied in these cases because of 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 are required to test in humans to obtain reliable pharmacokinetics (PK) parameters. Many of these compounds...
ultimately failed because the half-life was 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 in vivo using in vitro predictions. For these reasons, low-clearance compounds often fail in the clinical trials because of 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 depletion 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 radiolabeled 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 article, 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.

Materials 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 unless specified. Polypropylene plates were from Thermo Fisher Scientific (Waltham, MA) for 96-wells and from Costar (Sigma-Aldrich) 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 selecting new lots of hepatocytes, enzyme activities need to be verified during lead optimization and candidate selection.

LC-MS/MS Quantification. The LC mobile phases were as follows: (A) HPLC grade water containing 0.1% formic acid, and (B) acetonitrile containing 0.1% formic acid. A solvent gradient from 5% (A) to 95% (B) over 2.0 min at the flow rate of 0.4 ml/min was used to elute the compounds from the column (Kinex C18, 30 × 2 mm, 2.6 μm; Phenomenex, Torrance, CA). The cycle-time was 3 min/injection. A 5-μl aliquot of the 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 (Shimadzu, Columbia, MD) connected to an AB Sciex (Foster City, CA) API 5500 triple quadrupole mass spectrometer equipped with a TurboIonSpray source using multiple reaction monitoring 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 Fisher Scientific (Waltham, MA) ultrapressure liquid chromatography on a Kinex column (C18, 30 × 2 mm, 2.6 μm; Phenomenex) with an Acella 1250 pump (Thermo Scientific, West Palm Beach, FL), which was connected to a Q-Exacte orbitrap high-resolution mass spectrometer equipped with TurboIonSpray source (Thermo Fisher Scientific). A full-scan mode from m/z 150 to 600 was applied to detect each compound. LCQquand software (version 2.5; Thermo Fisher Scientific) was used for data collection, processing, and analysis. Terfenadine was used as an internal standard for LC-MS/MS quantification in positive ion multiple reaction monitoring mode. All of the test compounds had good linearity with R² > 0.99, and the limit of quantitation was 1 nM for all of 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 posttransfer. These losses necessitate a correction before comparison back to time 0 concentrations. The correction equation follows (eq. 1):

\[ C_{\text{corrected}} = C_{\text{total}} \times (\text{recov}) \times \left( \frac{\text{well volume}}{\text{transfer volume}} \right)^n \times \frac{1}{1 + \left( \frac{C_{\text{supernatant}} - 1}{C_{\text{total}} - 1} \right)} \]

Here, C_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 it 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

Transfer Supernatant

FIG. 1. Relay method for low clearance.
supernatant-transfer volume corrects for the dilution that 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.

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

Table 2 illustrates example data. For the first two points \((t = 0\) and \(4\ h))$, the
total concentration is equal to the corrected concentration because no transfer
has occurred. For the third time point:

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

For the fourth point:

\[
C_{\text{corrected}} = 0.0257 \times \left( \frac{500}{300} \right)^{\frac{1}{2}} \times \left( \frac{0.159}{0.0838} \right) \times \left( \frac{0.867}{0.56} \right) = 0.209
\]

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

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

**Fig. 2.** Structures of the low-clearance test compounds.
from the literature, where model (eq. 5) (Brown et al., 2007) based on human intravenous clearance data. If compounds do not show significant turnover (leads to too much loss of enzyme activity to generate reliable kinetic measurement) during incubation, a novel relay method for low-clearance measurement can be used 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 3). The results are consistent with hepatocyte prediction accuracy, with most compounds within 2-fold of the in vivo human data (Table 3). The results are consistent with hepatocyte prediction accuracy, with most compounds within 2-fold of the in vivo human data (Table 3).

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

$$T_{1/2} = \ln 2 / (\text{slope of the ln % remaining of drug vs. time plot}) = \text{minutes}$$

$$CL_{\text{int}} = \ln 2 \times \frac{1}{T_{1/2} \text{(min)}} \times \frac{\text{ml incubation}}{0.5 \text{ M cells}} \times \frac{120 \text{ Mcells}}{\text{g liver}} \times \frac{21 \text{ g liver}}{\text{kg}}$$

$$= \text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$$

Human in vivo intrinsic clearance was calculated using the well stirred model (eq. 5) (Brown et al., 2007) based on human intravenous clearance data from the literature, where $Q_H$ is hepatic blood flow (20.7 ml · min$^{-1}$ · kg$^{-1}$); $CL_{\text{int}}$ is hepatic blood clearance; $f_u$ is fraction unbound in plasma; and $R_b$ is blood-to-plasma concentration ratio.

$$CL_{\text{int}} = \frac{f_u}{R_b} \times \frac{CL_b}{1 - \frac{CL_b}{Q_H}}$$

### Results and Discussion

Human liver microsomes and hepatocytes are the most commonly used systems to predict human PK parameters because 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 $\sim$1 h, human hepatocytes $\sim$4 h). Incubating beyond these time limitations leads to too much loss of enzyme activity to generate reliable kinetic information. If compounds do not show significant turnover ($\sim$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-h incubation to prolong the exposure time to active enzymes in hepatocytes (Fig. 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 h by replacing with freshly thawed hepatocytes every 4 h. Because 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 P450s and various degrees of low intrinsic clearance were selected for method development. The structures of the compounds are shown in Fig. 2, and all compounds have reported human intravenous clearance data. The intrinsic clearance values were calculated based on the well stirred model (eq. 5), and the results are summarized in Table 3 along with the in vitro intrinsic clearance measured using the relay method. Correction for fraction unbound in the incubation ($f_{u,\text{inc}}$) 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 Fig. 3, and the correlation coefficients ($R^2$) are greater than 0.90 for all the compounds, which suggests good linearity for the metabolic kinetic measurement. The slope from 0 h to the 1st time point (4 h incubation) is the maximal delay 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 3). The results are consistent with hepatocyte prediction accuracy, with most compounds within 2-fold of the in vivo human data (Table 3). The results are consistent with hepatocyte prediction accuracy, with most compounds within 2-fold of the in vivo human data (Table 3). The results are consistent with hepatocyte prediction accuracy, with most compounds within 2-fold of the in vivo human data (Table 3). The results are consistent with hepatocyte prediction accuracy, with most compounds within 2-fold of the in vivo human data (Table 3).

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
FIG. 3. Kinetic plot of low-clearance compounds using the relay method. Error bar represents S.E.
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References


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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.

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Authorship Contributions

Participated in research design: Di, Trapa, Obach, Atkinson, Bi, Wolford, Tan, McDonald, Lai, and Tremaine.

Conducted experiments: Atkinson, Bi, Wolford, Tan, and McDonald.

Performed data analysis: Di, Trapa, Atkinson, Bi, Wolford, Tan, and McDonald.

Wrote or contributed to the writing of the manuscript: Di, Trapa, Obach, Atkinson, Tan, and Tremaine.