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A Novel Unified Approach to Predict Human Hepatic Clearance for Both Enzyme- and Transporter-Mediated Mechanisms Using Suspended Human Hepatocytes

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Running Title

IVIVE of Human Hepatic Clearance

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Abbreviations

AFE, average fold error; BSA, bovine serum albumin; CL_H, systemic plasma clearance mediated by hepatic elimination; $CL_{H,int}$, hepatic intrinsic clearance; $CL_{H,int,metabolism}$, intrinsic hepatic metabolic clearances; $CL_{H,int,biliary}$, intrinsic biliary clearance; $CL_{H,int,passive}$, intrinsic hepatic passive permeability; $CL_{H,int,uptake}$, intrinsic hepatic uptake clearance; $CL_{HHEP,int}$, intrinsic hepatocyte metabolic clearance; $CL_{HHEP,app}$, apparent hepatocyte metabolic clearance; CL_R, renal clearance; CL_P, total plasma clearance; CYP, cytochrome P450; ECCS, extended clearance classification system; $f_{u,p}$, the unbound fraction in plasma; $f_{u,intracellular}$, unbound fraction in hepatocyte intracellular water; $f_{u,buffer}$, unbound fraction in buffer without BSA; $f_{u,buffer,BSA}$, unbound fraction in buffer with BSA; $f_{u,HHEP}$, unbound fraction in suspended hepatocytes; $f_{u,HLM}$, unbound fraction in liver microsomes; *in vitro* to *in vivo* extrapolation; *Kp*, hepatocyte to buffer partitioning ratio; $Kp_{HHEP,BSA}$, hepatocyte to buffer partitioning ratio with BSA; $Kp_{uu,liver/liver,plasma}$, unbound liver tissue to liver plasma partitioning ratio; LC, liquid chromatography; LC-MS/MS, liquid chromatography– tandem mass spectrometry; NTCP, sodium-taurocholate co-transporting polypeptide; NHP, non-human primate; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; *PSF,* physiological scaling factor; Q_H, hepatic blood flow; $R_{B/P}$, the blood to plasma ratio; UGT, UDP-glucuronosyltransferases; UPLC, ultra-performance liquid chromatography.

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Abstract

The accurate prediction of human pharmacokinetics is critically important in modern drug discovery since it drives both pharmacological and toxicological effects. Although significant progress has been made to predict drug disposition by hepatic drug metabolizing enzymes, predicting transporter-mediated clearance is still highly uncertain. Furthermore, different approaches are often used to predict clearance with and without transporter involvement, hence the major clearance pathway for a compound must first be determined to know which approach to use. Due to these challenges, a novel unified method has been developed to predict human hepatic clearance for both enzyme- and transporter-mediated mechanisms using cryopreserved suspended human hepatocytes. This method hypothesizes that, once *in vitro* metabolic stability is scaled by partition coefficients between hepatocytes and buffer with 4% BSA, it can better predict *in vivo* clearance. With this method, good *in vitro* - *in vivo* correlation of human hepatic clearance has been obtained for a set of thirty-two structurally diverse compounds, including transporters such as organic anion-transporting polypeptides substrates. The clearance predictions for most compounds are within 3-fold of observed values. This is the first time that multiple compounds result in good *in vitro* - *in vivo* extrapolation using an entirely "bottom-up" approach without any empirical scaling factor when transporter-mediated clearance is involved. Potential exceptions are compounds with significant biliary and/or extra-hepatic clearance. The method offers an alternative approach to more accurately predict human hepatic clearance when multiple complex mechanisms are involved.

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Key Words

Clearance, IVIVE, *Kp*, Metabolism, Transporters, OATP

Introduction

Pharmacology and toxicology effects are driven by free drug exposure at the site of action (e.g., blood, liver, brain, and muscle) (Smith et al., 2010). Therefore, the accurate prediction of human pharmacokinetics (PK) and tissue exposure is critically important in modern drug discovery. PK information not only enables medicinal chemistry design to achieve optimal dosing regimen, but also helps to plan clinical studies during early drug developmental phases. Over the past decade, significant progress has been made to predict hepatic metabolic clearance mediated by drug metabolizing enzymes, such as cytochrome P450 (CYP) and UDP-glucuronosyltransferases (UGT) (Kilford et al., 2009; Hallifax et al., 2010; Di et al., 2012b; Di et al., 2013). These advances, in conjunction with high throughput screening of metabolic stability and early metabolite identification, enable project teams to successfully reduce metabolic clearance so significantly that low clearance assays are often needed to measure intrinsic clearance of many drug discovery compounds (Di and Obach, 2015). Although metabolism is the major elimination pathway for most drugs, the role of transporters has been increasingly recognized in drug clearance (Kalliokoski and Niemi, 2009). For example, organic-anion-transporting polypeptide (OATP) 1B1 and 1B3 play essential roles in hepatic clearance of statins, sartans, and glinides. Compared to enzyme-mediated processes, predicting transporter-mediated clearance is highly uncertain. Human translation for transporter-mediated clearance is challenging since transporter activity is different between *in vitro* systems and *in vivo*, so empirical scaling factors are required to correct rates derived from *in vitro* assays. This phenomenon has been reported in multiple studies and was summarized in a previously published review article (Li et al., 2014a). The use of empirical scaling factors substantially decreases our ability to predict human PK because the values can be assay- and compound-dependent (Li et al., 2014a). For novel

compounds in drug discovery, it becomes difficult to determine which scaling factors should be used to predict human PK. This problem was partially resolved when global scaling factors were identified as being applicable to multiple OATP substrates (Li et al., 2014b). However, for compounds with hepatic uptake clearance that are not mediated by OATPs but by other transporters (e.g., sodium-taurocholate co-transporting polypeptide (NTCP) or organic anion transporters (OATs)), it is not clear if OATPs and other transporters share the same scaling factors. Another limitation is that the estimated scaling factors for active uptake of OATP substrates are usually quite high (e.g., 10 to 150 (Li et al., 2014a)), indicating the *in vitro* transporter activities are much lower than *in vivo*. Even weak signals from the *in vitro* systems can be translated to major uptake clearance *in vivo*. As such, *in vitro* systems often fail to identify OATP substrates that have weak signals due to poor sensitivity, especially for highly lipophilic compounds with high passive permeability, despite the fact that transporters may actually play a significant role in their *in vivo* clearance. This makes it difficult to develop structure-transporter relationships and build confidence in *in vivo* human translation from *in vitro* transporter assays. Furthermore, with the current approaches, the major clearance pathway for a compound must be determined to know which method of PK prediction to utilize. For example, suspended human hepatocyte stability or human liver microsomal stability assays are typically used to predict human hepatic clearance if metabolism is the dominant clearance pathway, while plated human hepatocyte uptake assays may be used if transporter uptake is the rate-determining step for clearance. Determination of major clearance pathways is challenging, especially when *in vitro* to *in vivo* translation is disposition-, pathway- and compound-dependent.

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Facing these challenges, we have developed a novel method to predict human *in vivo* hepatic clearance for compounds that are potential substrates for drug metabolizing enzymes and/or transporters. This new approach eliminates the use of empirical scaling factors for *in vitro* to *in vivo* extrapolation (IVIVE) by using the extended clearance concept. Compared to the current available methods in the literature, this approach improves the precision and accuracy of prediction. More importantly, it uses a unified method to predict human hepatic clearance independent of disposition pathways of enzyme-mediated metabolism and/or transporter-mediated clearance. This significantly reduces the ambiguity and complexity, leading to increased confidence in human PK prediction regardless of major clearance mechanism.

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Materials and Methods

Materials

Test compounds were obtained from Pfizer Compound Management and Distribution (Groton, CT) or Sigma-Aldrich (St. Louis, MO). Cryopreserved human hepatocytes (Lot DCM, custom-pooled, 10 donors, male and female) were from BioreclamationIVT, LLC (Hicksville, NY). The individual donors were genotyped for the major drug metabolizing enzymes and transporters, and are mostly wild type. Human plasma was from Sera Laboratories International Ltd (a division of BioreclamationIVT, West Sussex, UK) and human blood was obtained from Clinical Trials Laboratory Services Ltd (London, UK). Williams' medium E (WEM Gibco-BRL, catalog #C1984, custom formula number 91-5233EC) contained 26 mM sodium bicarbonate and 50 mM hepes. InVitroGRO HI media and MPER buffer were obtained from Thermo Fisher Scientific (Waltham, MA). The equilibrium dialysis device (96-well format) and cellulose membranes (MWCO 12-14K) were from HTDialysis, LLC (Gales Ferry, CT). Breathe Easy™ sealing membranes, BSA (free of fatty acid, catalog # A4612) and other reagents were purchased from Sigma-Aldrich unless specified.

Measurement of Metabolic Stability in Human Hepatocytes

Metabolic stability of compounds was determined using cryopreserved suspended human hepatocytes in the standard 4-hour incubation assay for high to moderate clearance compounds (Di et al., 2012a) or 20-hour hepatocyte relay assay for low clearance compounds (Di et al., 2012b). The detailed relay protocols have been reported previously (Di et al., 2012a; Di et al., 2012b). Briefly, test compounds were added to suspended human hepatocytes at 0.5 or 2 million

cells/mL in WEM buffer and incubated at 37°C in a humidified CO_2 incubator (75% relative humidity, 5% CO_2/air) for 4 hours on an orbital shaker (150 rpm). For compounds that do not have significant turnover, five days of 4-hour incubations were used to give an accumulative relay incubation time of 20 hours. At various time points, an aliquot of the sample was collected and quenched with cold acetonitrile containing internal standard(s) (CP-628374 or a cocktail of terfenadine, indomethacin, and metoprolol). Quenched solutions were centrifuged, and the supernatants were analyzed by LC-MS/MS. The apparent metabolic intrinsic clearance ($CL_{HHEP,app}$) was determined based on the degradation half-life estimated from area ratio-time profile data.

Determination of Fraction Unbound

Human plasma protein binding and human liver microsomal assays were measured at Unilabs York Bioanalytical Solutions (York, UK). Binding in InVitroGRO HI media containing 4% BSA (abbreviated as BSA media here after) was performed at Pfizer Groton labs. Equilibrium dialysis method was used for fraction unbound (f_u) measurements as described previously (Riccardi et al., 2017). Briefly, the dialysis membranes and dialysis device were assembled following the manufacturer instructions (HTDialysis, LLC, Gales Ferry, CT). Human plasma, BSA media, or human liver microsomes (0.7-0.8 mg/mL) containing 1 or 2 μM test compounds with 1% DMSO were dialyzed against phosphate buffered saline (PBS) for 6 hours in a humidified incubator (75% relative humidity, 5% CO_2/95% air) at 37°C and shaking at 200 RPM for 6 hours. Quadruplicates of binding were measured for each compound. Samples were matrix matched and quenched with cold acetonitrile containing internal standard(s). The

solutions were centrifuged and the supernatants were analyzed with LC-MS/MS. Pre-saturation method was also used for highly bound compounds (Riccardi et al., 2015). Calculation of fraction unbound has been discussed previously (Riccardi et al., 2015; Riccardi et al., 2016; Riccardi et al., 2017).

Determination of Blood-to-Plasma Ratio

Human blood-to-plasma ratio was measured by Unilabs York Bioanalytical Solutions (York, UK). Test compounds were incubated with fresh human blood at 1 μM in quadruplicate in a humidified incubator (95% relative humidity, 5% CO_2/95% air) at 37°C and shaking at 450 RPM for 1 and 3 hours. At the end of incubations, plasma samples were obtained by centrifuging blood samples at 3000 ×g for 7 minutes. Both plasma and blood samples were matrix-matched with each other and quenched with acetonitrile containing internal standard. The solutions were centrifuged, and the supernatants were analyzed by LC-MS/MS. Peak area ratios were used to calculate blood-to-plasma ratio.

In Vitro Kp Measurement

The method for hepatocyte to buffer partitioning ratio (Kp_{HHEP}) measurement has been discussed previously (Riccardi et al., 2017). Briefly, test compounds (1 μM) were added to suspended human hepatocytes (0.5 million cells/mL) in either WEM buffer or InVitroGRO HI media containing 4% BSA and incubated on an orbital shaker (150 ×g) in a 37°C humidified incubator (75% relative humidity, 5% CO_2/95% air) for 1-4 hours in duplicate. At the end of the incubation, the suspended cells were centrifuged (500 ×g, 3 minutes) and supernatants were

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sampled. The remaining medium was removed and cells were washed twice with cold PBS and lysed with MPER buffer. Samples were quenched with cold acetonitrile containing internal standard and centrifuged. The supernatants were transferred for LC-MS/MS analysis using standard curves from both cells and media. *Kp* is the total cell concentration divided by the total buffer concentration.

Physiochemical Property Measurements

Physiochemical properties including $logD_{7.4}$, *pKa*, and permeability (P_{app}) measurements were conducted using methods reported previously (Shalaeva et al., 2008; Di et al., 2011; Li et al., 2014c).

LC-MS/MS Quantification

A generic LC-MS/MS method is presented here and similar methods were used based on compound properties. LC mobile phases were: (A) 0.1% formic acid in water, (B) 0.1% formic acid in acetonitrile. Solvent gradient was run from 5% (B) to 95% (B) over 1.1 minutes at a flow rate of 0.5 mL/min. UPLC column (BEH C18, 1.7 µm, 50x2.1 mm, Waters, Milford, MA) was used and the injection volume was 10 µL. A CTC PAL autosampler (LEAP Technologies, Carrboro, NC), an Agilent 1290 binary pump (Santa Clara, CA) and an AB Sciex (Foster City, CA) API 6500 triple quadrupole mass spectrometer with a TurboIonSpray source in MRM mode were used for sample analysis. Analyst™ 1.6.1 software (Applied Biosystems, Foster City, CA) was used for data collection and analysis.

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Mathematical Model

The extended clearance concept defines hepatic intrinsic clearance ($CL_{H,int}$) as a function of metabolic ($CL_{H,int,metabolism}$), biliary efflux ($CL_{H,int,biliary}$), passive permeability ($CL_{H,int,passive}$), and hepatic uptake ($CL_{H,int,uptake}$) intrinsic clearance.

$$CL_{H,int} = \frac{\left(CL_{H,int,metabolism} + CL_{H,int,biliary}\right)\left(CL_{H,int,uptake} + CL_{H,int,passive}\right)}{\left(CL_{H,int,metabolism} + CL_{H,int,biliary} + CL_{H,int,passive}\right)} \quad (1)$$

Our method (i.e. Method 1) assumes that (1) *in vitro* $CL_{H,int,uptake}$ values with BSA are equivalent to the *in vivo* $CL_{H,int,uptake}$ values, and (2) *in vitro* $CL_{H,int,metabolism}$ and $CL_{H,int,passive}$ values with (or without) BSA are equivalent to the *in vivo* values. Alternatively speaking, we assume that (1) BSA is only required for the hepatic active uptake *in vitro* - *in vivo* ratio to be one (without BSA, the *in vitro* assay under-predicts *in vivo* active uptake); and (2) *in vitro* metabolism (and passive diffusion) values with and without BSA are the same, hence the *in vitro* - *in vivo* ratios are one regardless of using BSA in the assay. The traditional method (i.e. Method 2) assumes that *in vitro* $CL_{H,int,metabolism}$, $CL_{H,int,passive}$, and $CL_{H,int,uptake}$ clearance (all without BSA) rates are equivalent to the *in vivo* clearance rates. Since we do not have an *in vitro* tool to estimate $CL_{H,int,biliary}$, it is assumed to be zero for both methods.

All equations are derived assuming (pseudo-) steady state, well-stirred conditions. Assuming that metabolism is the only elimination pathway in the liver, the amount of compound in the liver plasma eliminated by $CL_{H,int}$ is equivalent to the amount of liver intracellular compound eliminated through $CL_{H,int,metabolism}$.

$$CL_{H,int} \cdot C_{liver,plasma} \cdot f_{u,p} = CL_{H,int,metabolism} \cdot C_{liver,intracellular} \cdot f_{u,intracellular} \quad (2)$$

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$$CL_{H,int} = \frac{CL_{H,int,metabolism} \cdot C_{liver,intracellular} \cdot f_{u,intracellular}}{C_{liver,plasma} \cdot f_{u,p}} = CL_{H,int,metabolism} \cdot Kp_{uu,liver/liver,plasma} \quad (3)$$

$f_{u,p}$ and $f_{u,intracellular}$ are the unbound plasma fraction and unbound hepatocyte intracellular fraction. $Kp_{uu,liver/liver,plasma}$ is the unbound liver tissue to liver plasma partitioning ratio. Please note that this parameter is <u>not</u> equivalent to the unbound liver tissue to systemic plasma partitioning ratio ($Kp_{uu,liver}$). $CL_{H,int,metabolism}$ is predicted with the intrinsic hepatocyte metabolic clearance without BSA ($CL_{HHEP,int}$) and a physical scaling factor (*PSF*). The value of *PSF* is calculated as the product of human liver weight per body weight (i.e., 21 g / kg) and human hepatocytes per liver weight (i.e., 120 million hepatocytes /g).

$$CL_{H,int,metabolism} = CL_{HHEP,int} \cdot PSF \quad (4)$$

$CL_{HHEP,int}$ is estimated using apparent metabolic intrinsic clearance estimated from suspended human hepatocyte without BSA ($CL_{HHEP,app}$) after accounting for the potential asymmetrical distribution due to residual transporter activity in the suspended hepatocyte assay.

$$CL_{HHEP,int} = \frac{CL_{HHEP,app} \cdot \left(V_{inc} - V_{cell} \cdot \left(1 - Kp_{HHEP}\right)\right)}{Kp_{HHEP} \cdot f_{u,intracellular} \cdot V_{inc}} \quad (5)$$

V_{inc} and V_{cell} are the total incubation (0.9987 mL) and intracellular (0.0013 mL) volumes of the suspended hepatocyte assay. The hepatocyte to buffer partitioning ratio (Kp_{HHEP}) is determined as the ratio of intracellular hepatocyte to buffer concentrations.

$$Kp_{HHEP} = C_{HHEP,intracellular} / C_{buffer} \quad (6)$$

With above equations, $CL_{H,int}$ is calculated as following.

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$$\begin{aligned} CL_{H,int} &= CL_{H,int,metabolism} \cdot Kp_{uu,liver/liver,plasma} \\ &= CL_{HHEP,int} \cdot PSF \cdot Kp_{uu,liver/liver,plasma} \\ &= \frac{CL_{HHEP,app} \cdot \left(V_{inc} - V_{cell} \cdot \left(1 - Kp_{HHEP}\right)\right)}{Kp_{HHEP} \cdot f_{u,intracellular} \cdot V_{inc}} \cdot PSF \cdot Kp_{uu,liver/liver,plasma} \end{aligned} \quad (7)$$

In this study, $Kp_{uu,liver/liver,plasma}$ is estimated as the hepatocyte to buffer partitioning ratio with BSA ($Kp_{HHEP,BSA}$) as following.

$$\begin{aligned} Kp_{uu,liver/liver,plasma} &= \left(C_{HHEP,BSA,intracellular} \cdot f_{u,intracellular}\right) \Big/ \left(C_{buffer,BSA} \cdot f_{u,buffer,BSA}\right) \\ &= Kp_{HHEP,BSA} \cdot f_{u,intracellular} \Big/ f_{u,buffer,BSA} \end{aligned} \quad (8)$$

The parameters $f_{u,buffer}$ and $f_{u,buffer,BSA}$ present the buffer unbound fraction with and without 4% BSA. $C_{HHEP,BSA,intracellular}$ and $C_{buffer,BSA}$ are the hepatocyte intracellular and buffer concentrations in the presence of 4% BSA. The dialysis assay is used to determine $f_{u,buffer,BSA}$, while $f_{u,buffer}$ is assumed to be 1. Finally, $CL_{H,int}$ is calculated with the equation below.

$$\begin{aligned} CL_{H,int} &= \frac{CL_{HHEP,app} \cdot \left(V_{inc} - V_{cell} \cdot \left(1 - Kp_{HHEP}\right)\right)}{Kp_{HHEP} \cdot f_{u,intracellular} \cdot V_{inc}} \cdot PSF \cdot Kp_{uu,liver/liver,plasma} \\ &= CL_{HHEP,app} \cdot \frac{Kp_{HHEP,BSA} \cdot \left(V_{inc} - V_{cell} \cdot \left(1 - Kp_{HHEP}\right)\right)}{Kp_{HHEP} \cdot f_{u,buffer,BSA} \cdot V_{inc}} \cdot PSF \end{aligned} \quad (9)$$

It is worth noting that $CL_{H,int}$ is the hepatic intrinsic clearance referring to compound elimination from the unbound liver plasma. The following equations are used to predict systemic plasma clearance mediated by hepatic elimination (CL_H), i.e. clearance referring to the compound elimination from unbound systemic plasma. The equation essentially calculates the extraction from the hepatic plasma flow assuming a well-stirred condition, where Q_H represents hepatic blood flow (20 mL/min/kg) and $R_{B/P}$ represents the blood to plasma ratio.

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$$CL_H = Q_H \cdot \frac{CL_{H,int} \cdot f_{u,p}}{CL_{H,int} \cdot f_{u,p} / R_{B/P} + Q_H} \quad (10)$$

The observed CL_H values are from previously published clinical studies, after subtraction of the renal clearance (CL_R) from the total plasma clearance (CL_P).

For comparison, $CL_{H,int}$ (Method 2) is also calculated by a traditional approach without using hepatocyte *Kp* data (Hallifax et al., 2010).

$$CL_{H,int} = \frac{CL_{HHEP,app}}{f_{u,HHEP}} \cdot PSF \quad (11)$$

Here $f_{u,HHEP}$ represents the unbound fraction in a suspended hepatocyte study. $f_{u,HHEP}$ is assumed to be equivalent with the unbound fraction in liver microsomes ($f_{u,HLM}$) under similar assay conditions ($f_{u,HLM}$ at 1 mg/mL is similar to $f_{u,HHEP}$ at 1 million cells/mL) (Austin et al., 2005).

Assessing Prediction Accuracy and Precision

Prediction precision and accuracy was assessed with average fold error (AFE) Eq. 11, and bias Eq. 12, where AFE approximates variance from unity, and bias is degree away from unity.

$$\text{AFE} = 10^{\frac{1}{N} \cdot \sum \left| \log_{10} \frac{\text{Predicted}}{\text{Observed}} \right|} \quad (12)$$

$$\text{bias} = 10^{\frac{1}{N} \cdot \sum \log_{10} \frac{\text{Predicted}}{\text{Observed}}} \quad (13)$$

N is the number of observations.

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Results

A novel *in vitro* method has been developed, hereafter referred to as Method 1, using suspended human hepatocytes with and without 4% BSA to measure metabolic stability and partition coefficients. Thirty-two structurally diverse compounds are included in the current study, covering a wide range of physicochemical properties and five of the six ECCS (extended clearance classification system; 1a, 1b, 2, 3b, and 4) classes (Varma et al., 2015). The physicochemical and *in vitro* data are summarized in Table 1 for compounds less likely to have biliary excretion (i.e. ECCS class 1a, 1b and 2) and in Table 2 for compounds with the potential for significant biliary excretion (i.e. ECCS classes 3b, and 4). The comparison between the predicted and observed hepatic clearance values are shown in Tables 3 and 4 for those two groups of compounds. For compounds without significant biliary excretion (n=19), good IVIVE is observed for hepatic clearance using this novel extended clearance method (Method 1), where the compounds are centered along the unity line (Figure 1A). Greater than 80% of the compounds (16 out of 19) have predictions within 3-fold of observed values. The outliers are pitavastatin, telmisartan, and theophylline. Conversely, when the traditional method (Method 2, which only includes metabolic clearance) is used, most compounds appear on the right-hand bottom of the graph (Figure 1B), indicating a systemic under-prediction of hepatic clearance. Only 26% (5 out of 19 compounds) of the predicted hepatic clearance values are within 3-fold of the observed values, suggesting that transporter-mediated clearance plays a significant role in addition to metabolic clearance. Similar trends are observed for the prediction of hepatic intrinsic clearance using Methods 1 and 2 (Figures 1C and 1D). When applying Methods 1 and 2 to the compounds in Table 1, the AFE values for hepatic clearance (CL_H) are 1.9 and 4.1, and the bias values are 0.84 and 0.30, respectively (Table 3). The smaller AFE value and the bias value

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closer to one both indicate that Method 1 is more accurate and precise, consistent with what has been observed in Figure 1. Similarly, with Methods 1 and 2, the AFE values for intrinsic hepatic clearance ($CL_{H,int}$) are 2.5 and 5.8, and the bias values are 0.71 and 0.21, respectively. This indicates that Method 1 also performs much better than Method 2 in predicting intrinsic clearance.

For compounds with potentially significant biliary excretion (i.e., ECCS 3a, 3b, and 4 compounds in Table 2, n=13), 38% (5 out of 13 compounds) of the predicted hepatic clearance values are within 3-fold (Figure 2A) with Method 1. When Method 2 is applied, 23% (3 out of 13 compounds) of the predicted values are within 3-fold (Figure 2B). Intrinsic clearance values are also under-predicted (Figures 2C and 2D). Consistent with data shown in the plots, large AFE values and bias values deviating from one are observed for this group of compounds with biliary clearance (Table 4).

Discussion

Significant advances have been made over the years to predict human metabolic clearance. However, prediction of human hepatic clearance for complex mechanisms of enzyme-transporter interplay is still a major challenge in drug discovery and development. Although several new approaches have been developed, practical applications of the methods are limited in providing guidance for medicinal chemistry design and accurate predictions of human PK. The restrictions of these approaches, as highlighted in the introduction, are due to the limitation of compound- and transporter-dependent scaling factors, inability to detect transporter activity for highly lipophilic permeable compounds, and the need to know the rate-determining step prior to clearance prediction. We have developed a novel experimental approach to predict human clearance for enzyme- and transporter-mediated mechanisms. This method was developed based on liver-to-plasma Kp_{uu} work previously published (Riccardi et al., 2016; Riccardi et al., 2017). Based on the hypotheses that (1) the metabolic, passive, and uptake intrinsic clearance rates in the *in vitro* hepatocyte assay, in the presence of 4% BSA, are equivalent to *in vivo* rates, and (2) the metabolic intrinsic clearance rates in the *in vitro* hepatocyte assay, in the absence of 4% BSA, are equivalent to *in vivo* rates, we decided to expand this method to predict human clearance mediated by both drug metabolizing enzymes and transporters. A large set of structurally diverse compounds with different clearance mechanisms and transporter involvement (OATPs, NTCP, OATs) and have human IV clearance data were used for the method validation. This is the first study with a large set of compounds demonstrating strong human IVIVE for clearance involving transporters using a bottom up approach without any empirical scaling factors. This novel method incorporating the extended clearance concept with

transporter effects provides a much better prediction of human clearance than using the traditional metabolic clearance alone.

The data obtained in this study are different from those using plated hepatocytes without BSA, where large scaling factors are required for uptake transporters in order to predict human PK (Jones et al., 2012; Li et al., 2014b). This indicates that the suspended hepatocytes in this novel *in vitro* system (InVitroGRO HI media supplemented with 4% BSA) are able to function similarly to *in vivo* under physiological conditions, leading to a possible translation from *in vitro* to *in vivo* without any scaling factors. The 4% BSA was used to mimic the physiological amount of albumin and can be applied to various species. A similar phenomenon has been reported recently using plated human hepatocytes in human plasma, but the approach was only verified with pravastatin (Mao et al., 2018). *In vitro* hepatocyte systems supplemented with proteins appear to more closely mimic *in vivo* disposition, although the underlining mechanisms are not entirely clear. One explanation is based on the "facilitated-dissociation" model in which interaction of the albumin-drug complex with the cell surface enhances dissociation of the complex to provide more unbound drug molecules to be transported (Tsao et al., 1988; Miyauchi et al., 2018). This is an area to further investigate in the future.

Accurate measurement and prediction of human biliary clearance and enterohepatic recirculation continues to be particularly challenging. Since there is currently no reliable *in vitro* method to predict biliary excretion, we have assigned the biliary excretion to be zero for all compounds in this study. As expected, this assumption leads to under-prediction of clearance for some compounds with significant biliary clearance (Table 4, Figure 2). The magnitude of under-prediction is dependent upon the extent of biliary excretion that contributes to the total hepatic intrinsic clearance. These compounds are still included in the current analysis to test the extent of

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misprediction if the biliary clearance is arbitrarily fixed at zero due to the lack of the method accurately predicting biliary excretion. The sandwich culture hepatocyte assay has been previously developed as a method to estimate biliary excretion. However, mechanistically translating the *in vitro* biliary clearance estimate in sandwich culture assay to *in vivo* biliary clearance is still challenging since compound-dependent scaling factors may be required, and high uncertainty is also associated with the estimated scaling factors (Li et al., 2014b). Furthermore, estimating *in vivo* biliary clearance from clinical pharmacokinetic data is usually confounded by the enterohepatic recirculation. As such, it is difficult to use *in vivo* biliary clearance to validate a prediction made by *in vitro* tools (such as sandwich cultured human hepatocyte). This lack of good *in vivo* validation data can be a key missing piece in developing *in vitro* assays and using them in drug discovery. Hence, it will be helpful in the future if new modeling tools can be developed to estimate clinical biliary excretion and enterohepatic recirculation from existing systemic pharmacokinetic data, or if new experimental technology can be developed to directly measure these two processes. Clearance of two compounds (pitavastatan and telmisartan) mainly cleared by UGTs are under-predicted (>3-fold) by the current approach. This could be, in part, due to extrahepatic metabolism contributed by some of the UGTs. Future understanding of extrahepatic metabolism will be required to increase our confidence in clearance prediction of these groups of compounds.

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Authorship Contributions

Participated in research design: Riccardi, Tess, Di, and Li.

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Contributed new reagents or analytic tools: Riccardi and Tess.

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Figure Captions

Figure 1. Observed and predicted clearance for compounds without the potential for biliary excretion as a major clearance pathway. (A) predicted systemic plasma clearance mediated by hepatic elimination (CL_H) with the novel extended clearance method. (B) predicted CL_H with the traditional metabolic clearance method. (C) predicted hepatic intrinsic clearance ($CL_{H,int}$) with the novel extended clearance method. (D) predicted $CL_{H,int}$ with the traditional metabolic clearance method. The black dashed, blue dotted, and red solid lines represent unity, 3-fold from unity, and method bias. Yellow triangles, green circles, and blue squares represent ECCS class 1a, 1b, and 2 compounds.

Figure 2. Observed and predicted clearances for compounds with the potential for biliary excretion as a major clearance pathway. (A) predicted systemic plasma clearance mediated by hepatic elimination (CL_H) with Method 1. (B) predicted CL_H with Method 2. (C) predicted hepatic intrinsic clearance ($CL_{H,int}$) with Method 1. (D) predicted $CL_{H,int}$ with Method 2. The black dashed and blue dotted lines represent unity and 3-fold from unity. Purple diamonds and orange circles represent ECCS class 3b and 4 compounds.

Table 1. Physicochemical properties and *in vitro* inputs for compounds without the potential for biliary excretion as the major clearance pathway. (ECCS represents extended clearance classification system; A and B represent acidic and basic *pKa* values.)

	MW (g/mol)	$logD_{7.4}$	*pKa*	P_{app} (10^{-6} cm/s)	ECCS	$R_{B/P}$	$f_{u,p}$	$f_{u,buffer,BSA}$	$CL_{HHEP,app}$ (µL/min/10^6 cells)	Kp_{HHEP}	$Kp_{HHEP,BSA}$	$f_{u,HLM}$
Diclofenac	296.2	1.2	4.4 (A) [a]	23	1a	0.66	0.0038	0.0026	67	18	0.95	0.85
Ibuprofen	206.3	0.9 [a]	4.4 (A) [a]	31	1a	0.55	0.012	0.018	24	8.5	0.21	0.95
Meloxicam	351.4	0.5	4.0 (A)	35	1a	0.57	0.0082	0.020	2.1	22	0.92	0.92
Nateglinide	317.4	1.1	3.8 (A) [a]	11	1a	0.6 [b]	0.011	0.0043	11	8.4	0.20	0.89 [a]
Tolbutamide	270.4	0.4	5.1 (A) [a]	26	1a	0.56	0.030	0.058	1.8	0.69	0.14	0.91
Atorvastatin	558.6	1.3	4.4 (A) [a]	1.9	1b [c]	0.57	0.048	0.043	9.4	88	31	0.46
Bosentan	551.6	1.1	4.7 (A)	7.5	1b	0.67	0.028	0.034	5.6	44	8.2	0.63
Cerivastatin	459.6	1.8	4.6 (A) 5.5 (B)	14	1b	0.56	0.018	0.019	13	121	12	0.47
Fluvastatin	411.5	1.4	4.4 (A)	11	1b	0.69	0.0092	0.025	33	32	6.2	0.70 [a]
Glipizide	445.5	0.4	4. 9 (A)	8.8	1b	0.60	0.040	0.063	2.0	3.2	0.55	0.95
Glyburide	494.0	2.1	5.0 (A)	14	1b	0.57	0.0024	0.0070	23	45	1.3	0.65
GSK269984A	406.2	2.1	2.3 (A) [a] 5.9 (B) [a]	14	1b	0.65	0.0024	0.00081	37	150	2.4	0.24
Irbesartan	428.5	1.4	4.3 (A) [a] 7.0 (B) [a]	9.3	1b	0.60	0.016	0.046	14	50	12	0.76
Montelukast	586.2	5.1	4.2 (A) [a] 5.3 (B) [a]	< 0.1	1b [c]	0.67	0.000092	0.00019	9.6	1.9	0.17	0.00067
Pitavastatin	421.5	1.2	4.2 (A) [a] 5.0 (B) [a]	5.7	1b	0.55	0.0080	0.055	11	22	3.0	0.67
Repaglinide	452.6	2.2	4.3 (A) 5.9 (B)	20	1b	0.86	0.012	0.0086	33	64	1.5	0.49
Telmisartan	514.6	2.4	4.1 (A) 6.1 (B)	18	1b	0.61	0.0060	0.014	13	440	45	0.57
Theophylline	180.2	0.1	8.6 (B)	27	2	0.71	0.70	0.76	1.2	0.13	0.27	1.0
Timolol	316.4	0.6 [a]	8.6 (B) [a]	20	2	0.82	0.73	0.81	2.6	6.1	7.1	0.83

[a] *in silico*; [b] assumed; [c] based on *in vivo* PK properties rather than RRCK P_{app}

Table 2. Physicochemical properties and *in vitro* inputs for compounds with the potential for biliary excretion as the major clearance pathway. (ECCS represents extended clearance classification system; A and B represent acidic and basic *pKa* values.)

	MW (g/mol)	$logD_{7.4}$	*pKa*	P_{app} (10^{-6} cm/s)	ECCS	$R_{B/P}$	$f_{u,p}$	$f_{u,buffer,BSA}$	$CL_{HHEP,app}$ (μL/min/10^6 cells)	Kp_{HHEP}	$Kp_{HHEP,BSA}$	$f_{u,HLM}$
Lesinurad	404.3	0.5	3.1 (A)	1.0	3b	0.60	0.010	0.0040	2.9	53	0.82	0.68 [a]
Losartan	422.9	1.7 [a]	4.5 (A) [a]	1.3	3b	0.62	0.029	0.017	9.3	56	7.8	0.79
PF-05089771	500.45	2.3	6.0 (A)	3.8	3b	0.60	0.0015	0.0059	11	34	2.8	0.41
PF-05150122	541.0	1.1	6.5 (A) 7.4 (B)	3.2	3b	0.64	0.065	0.18	5.8	27	6.3	0.85
PF-05186462	531.9	0.8	4.1 (A)	0.81	3b	0.66	0.0016	0.015	2.4	24	4.1	0.67
PF-05241328	436.9	2.0	3.6 (A)	3.9	3b	0.56	0.0020	0.0084	10	300	2.8	0.38
Pravastatin	424.5	−0.4	4.3 (A)	0.31	3b	0.55	0.63	0.49	0.29	1.7	9.4	0.80
Rosuvastatin	481.5	0.1	4.3 (A)	0.75	3b	0.57	0.16	0.19	0.32	14	24	0.77
Valsartan	435.5	−0.8	3.4 (A) 4.7 (A)	0.32	3b	0.70	0.0041	0.0060	0.58	14	0.88	0.79
Disopyramide	339.5	0.6 [a]	9.4 (B) [a]	1.3	4	0.70	0.37	0.88	1.2	3.0	4.6	0.94
Maraviroc	513.7	12.0	9.4 (B)	1.8	4	0.68	0.51	0.33	1.9	4.6	3.3	0.64
Ranitidine	314.4	−0.7	8.5 (B)	0.57	4	1.0	0.84	1.0	1.7	2.1	1.3	0.95 [a]
Zolmitriptan	287.4	0.4 [a]	8.9 (B) [a]	< 0.1	4	1 [b]	0.75	0.84	0.26	3.0	4.1	0.84

[a] *in silico*; [b] assumed

Table 3. Predicted human and observed human hepatic clearance for compounds without the potential for biliary excretion as the major clearance pathway. (ECCS represents extended clearance classification system.)

Compound	Observed CL_H (mL/min/kg)	Predicted CL_H Method 1 (mL/min/kg)	Predicted CL_H Method 2 (mL/min/kg)	Observed $CL_{H,int}$ (mL/min/kg)	Predicted $CL_{H,int}$ Method 1 (mL/min/kg)	Predicted $CL_{H,int}$ Method 2 (mL/min/kg)	Reference for observed values
Diclofenac	4.1	6.5	0.67	1500	3400	190	(Willis et al., 1979; NDA-207238, 2017)
Ibuprofen	0.89	0.93	0.71	79	83	62	(Bushra and Aslam, 2010; Pavliv et al., 2011)
Meloxicam	0.16	0.093	0.045	20	11	5.5	(Schmid et al., 1995)
Nateglinide	1.3	1.5	0.35	120	150	30	(Weaver et al., 2001)
Tolbutamide	0.18	0.46	0.15	6.1	16	5.0	(Scott and Poffenbarger, 1979; Flesch et al., 1997)
Atorvastatin	8.9	5.4	1.7	890	220	41	(Lennernas, 2003)
Bosentan	2.2	2.0	0.52	92	84	19	(Weber et al., 1996)
Cerivastatin	2.7	2.6	0.89	200	190	55	(Muck et al., 1997; Muck, 2000)
Fluvastatin	7.3	4.2	0.91	1700	660	110	(Tse et al., 1992; Lindahl et al., 1996)
Glipizide	0.53	0.55	0.21	14	14	5.3	(Pentikainen et al., 1983)
Glyburide	1.2	0.60	0.18	580	260	78	(Neugebauer et al., 1985; Karma et al., 2009)
GSK269984A	2.0	3.8	0.63	980	2200	280	(Ostenfeld et al., 2012)
Irbesartan	2.1	2.4	0.64	160	200	43	(Vachharajani et al., 1998)
Montelukast	0.57	0.99	1.8	6400	12000	23000	(Cheng et al., 1996; Balani et al., 1997)
Pitavastatin	5.6	0.53	0.28	1400	70	36	(NDA-022363, 2009)
Repaglinide	7.3	2.6	1.5	1000	250	140	(Hatorp et al., 1998; NDA-020741, 2017)
Telmisartan	10	1.8	0.27	10000	360	47	(Stangier et al., 2000a; Stangier et al., 2000b)
Theophylline	0.87	4.0	1.8	1.3	8.0	2.9	(Conrad and Nyman, 1980; Caldwell et al., 1986; Vestal et al., 1986; Lombardi et al., 1987; NDA-019211, 2009)
Timolol	6.4	4.8	4.0	14	9.3	7.3	(Wilson et al., 1982; McGourty et al., 1985)
N		19	19		19	19	
AFE		1.9	4.1		2.5	5.8	
bias		0.84	0.30		0.71	0.21	

Table 4. Predicted human and observed human hepatic clearance for compounds with the potential for biliary excretion as the major clearance pathway. (ECCS represents extended clearance classification system.)

Compound	Observed CL_H (mL/min/kg)	Predicted CL_H Method 1 (mL/min/kg)	Predicted CL_H Method 2 (mL/min/kg)	Observed $CL_{H,int}$ (mL/min/kg)	Predicted $CL_{H,int}$ Method 1 (mL/min/kg)	Predicted $CL_{H,int}$ Method 2 (mL/min/kg)	Reference for observed values
Lesinurad	0.93	0.29	0.092	100	30	9.3	(Shen et al., 2015)
Losartan	7.5	4.1	0.75	630	210	27	(Lo et al., 1995)
PF-05089771	0.17	0.59	0.079	120	410	53	(Jones et al., 2016)
PF-05150122	3.2	1.2	0.98	64	20	16	(Jones et al., 2016)
PF-05186462	1.3	0.12	0.013	890	74	8.0	(Jones et al., 2016)
PF-05241328	5.7	0.077	0.10	5900	39	51	(Jones et al., 2016)
Pravastatin	7.3	3.5	0.50	34	7.9	0.83	(Singhvi et al., 1990)
Rosuvastatin	6.8	1.1	0.15	110	7.5	0.95	(Martin et al., 2003)
Valsartan	0.35	0.066	0.0071	85	16	1.7	(Flesch et al., 1997)
Disopyramide	0.30	1.6	1.0	0.84	5.0	3.0	(Lima et al., 1984)
Maraviroc	7.2	3.9	2.7	30	11	6.6	(Abel et al., 2008)
Ranitidine	2.6	2.1	3.2	3.6	2.8	4.4	(Chau et al., 1982; van Hecken et al., 1982)
Zolmitriptan	8.7	0.78	0.54	21	1.1	0.73	(Dixon et al., 1997; Seaber et al., 1998)
N		13	13		13	13	
AFE		4.6	11		6.4	16	
bias		0.34	0.12		0.25	0.080	

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Figure 1.

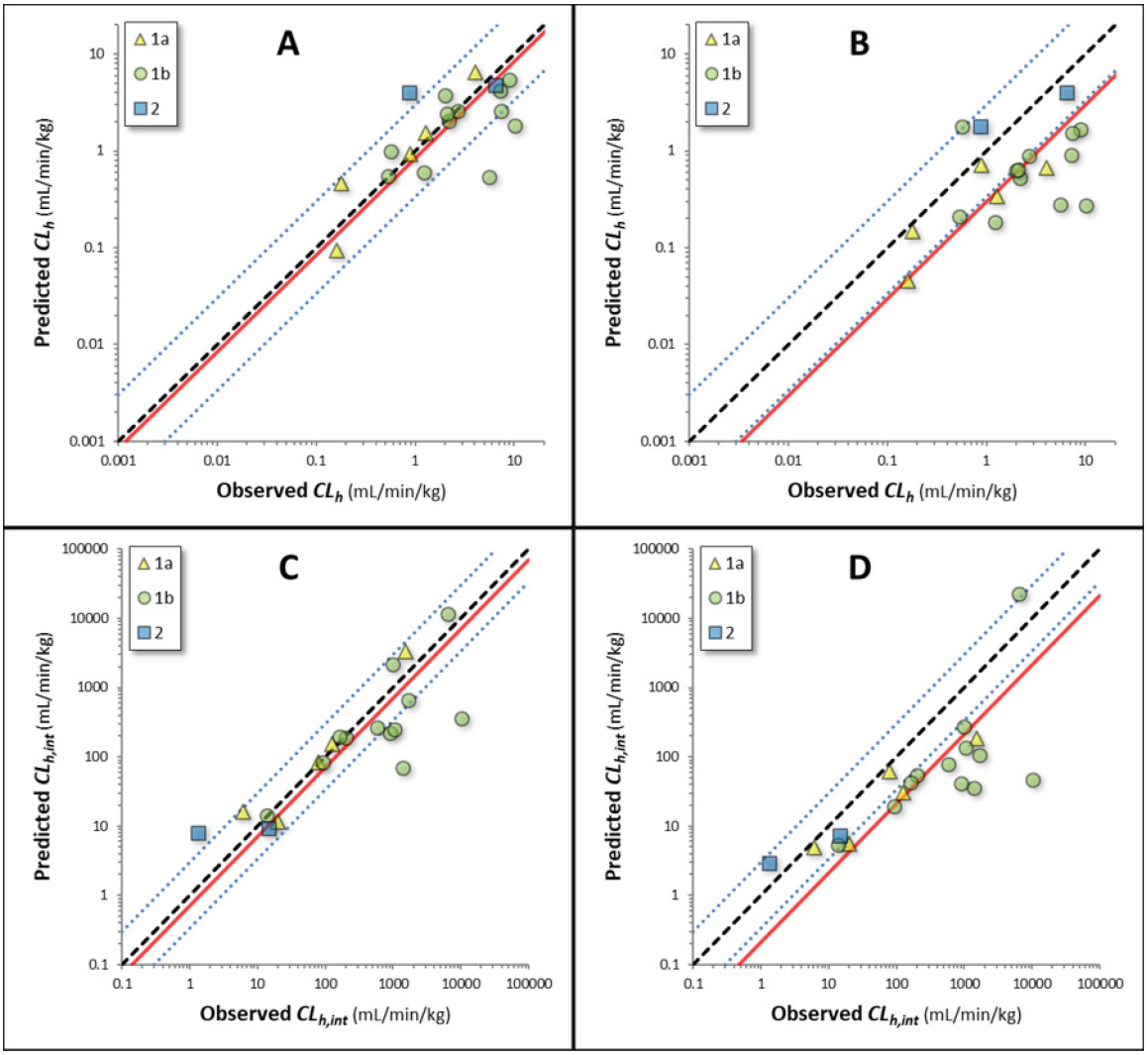

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Figure 2.