Novel Method to Predict In Vivo Liver-to-Plasma $K_{puu}$ for OATP Substrates Using Suspension Hepatocytes

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Abbreviations

BSA, bovine serum albumin; CI, confidence interval; DDI, drug-drug interactions; DHP, dehydropravastatin; HPLC, high performance liquid chromatography; IC_{50}, half maximal inhibitory concentration; IS, internal standard; I.V., intravenous; f_{u}, fraction unbound; f_{u,cell}, fraction unbound of cells; f_{u,liver}, fraction unbound of liver; f_{u,media}, fraction unbound of media; f_{u,p}, fraction unbound of plasma; K_{p}, partition coefficient; K_{puu}, unbound partition coefficient; NASH, non-alcoholic steatohepatitis; IVIVE, In vitro – in vivo extrapolation/correlation; LC, liquid chromatography; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDCK-LE, Madin-Darby Canine Kidney-low efflux cell line; MWCO, molecular weight cutoff; MVA, mevalonic acid; NHP, non-human primate; OATP, organic anion-transporting polypeptide; P_{app}, apparent permeability; PD, pharmacodynamics; PET, positron emission tomography; PK, pharmacokinetics; PK/PD, pharmacokinetics/pharmacodynamics; PBS, phosphate buffered saline; RH, relative humidity; RPM, revolutions per minute; RT, room temperature.
Abstract

The ability to predict human liver-to-plasma unbound partition coefficient ($K_{puu}$) is of great importance to estimate unbound liver concentration, develop PK/PD relationships, predict efficacy and toxicity in the liver, and model drug-drug interaction (DDI) potential for drugs that are asymmetrically distributed into the liver. A novel in vitro method has been developed to predict in vivo $K_{puu}$ with good accuracy using cryopreserved suspension hepatocytes in InVitroGRO HI media with 4% BSA. Validation was performed using six OATP substrates with rat in vivo $K_{puu}$ data from IV infusion studies where steady state was achieved. Good in vitro-in vivo correlation (IVIVE) was observed as the in vitro $K_{puu}$ values were mostly within two fold of in vivo $K_{puu}$. Good $K_{puu}$ IVIVE in human was also observed with in vivo $K_{puu}$ data of dehydropravastatin from positron emission tomography and in vivo $K_{puu}$ data from PK/PD modeling for pravastatin and rosvastatin. Under the specific $K_{puu}$ assay conditions, the drug metabolizing enzymes and influx/efflux transporters appear to function at physiological levels. No scaling factors are necessary to predict in vivo $K_{puu}$ from in vitro data. The novel in vitro $K_{puu}$ method provides a useful tool in drug discovery to project in vivo $K_{puu}$. 
Introduction

Liver is an important organ for many disease targets, such as dyslipidemia, diabetes, obesity, and NASH. It is critical to understand the unbound drug concentration in the liver, as it impacts pharmacological activity, metabolic and biliary clearance, and DDI (Smith et al., 2010). For compounds that are not actively transported and are not influenced by membrane potential or pH gradient (Scott et al., 2016), the unbound drug concentration in the liver will be the same as that in the plasma. In this case, the unbound partition coefficient ($K_{puu}$) between liver and plasma is close to 1. When compounds are uptake transporter substrates (e.g., OATPs), $K_{puu}$ values can be greater than 1 due to active influx. $K_{puu}$ represents the distribution of unbound drugs between liver and plasma in vivo or between hepatocytes and media in vitro when multiple processes, including metabolism, uptake, efflux, and passive diffusion, have achieved steady state. $K_{puu}$ can be described using the extended clearance equation incorporating the multiple mechanisms (Shitara et al., 2006; Watanabe et al., 2010; Yabe et al., 2011). It is important to be able to estimate in vivo $K_{puu}$, since it is the link between unbound plasma concentration and unbound liver concentration. Because it is challenging to measure unbound liver concentration directly in higher species (e.g., NHP) and humans, the ability to predict $K_{puu}$ will enable direct estimation of unbound liver concentrations from unbound plasma concentrations.

Currently, several in vitro methods (Riccardi et al., 2016) are available to estimate $K_{puu}$, including the binding method (Mateus et al., 2013), the kinetic method (Yabe et al., 2011) and the temperature method (Shitara et al., 2013). However, validation of these
methods with \textit{in vivo} exposure/pharmacology or \textit{in vitro} activity data is fairly limited (Shitara et al., 2013; Riccardi et al., 2016). IVIVE for $K_{puu}$ using hepatocyte systems for OATP substrates has not been established. Several studies have shown internalization or down-regulation of transporters in the hepatocyte systems (Roelofsen et al., 1995; Bow et al., 2008; Kimoto et al., 2012; Kunze et al., 2014; Bridget et al., 2015; Vildhede et al., 2015), although others have shown no significant difference in transporter abundance between hepatocytes and liver tissues (Prasad et al., 2014; Badee et al., 2015). It is uncertain whether a direct translation is possible without scaling factors from \textit{in vitro} hepatocytes to \textit{in vivo} $K_{puu}$. In this study, we explored the IVIVE of $K_{puu}$ using cryopreserved suspension rat and human hepatocytes for OATP substrates. The ability to predict \textit{in vivo} liver-to-plasma $K_{puu}$ from \textit{in vitro} systems will provide a useful tool in drug discovery to predict unbound liver concentration as well as clearance and dose, design drugs for liver targeting, develop PK/PD (pharmacokinetic/pharmacodynamics) relationships for disease targets residing in the liver, and model DDI due to inhibition /induction of liver enzymes when transporters are involved in the distribution processes.
Materials and Methods

Materials

Test compounds were obtained from Sigma-Aldrich (St. Louis, MO) or Pfizer (Groton, CT). PF-04991532 (Compound 19 in the reference) (Pfefferkorn et al., 2012) and PF-05187965 (Compound 7 in the reference) (Stevens et al., 2013) were synthesized according to the methods reported in the referenced publications. Rat (14 male and 14 female, pooled) and human (6 male, pooled) plasma, cryopreserved human hepatocytes (Lot DCM, custom-pooled of both male and female, 10 donors) and Wistar-Han rat hepatocytes (Lot VSU, 35 male pooled donors) were purchased from BioreclamationIVT, LLC (Hicksville, NY). Human liver (1 male donor) was from Analytical Biological Services Inc. (Wilmington, DE). Wistar-Han rat liver (4 male donors) was obtained internally at Pfizer Global Research and Development (Groton, CT). 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 purchased from Thermo Fisher Scientific (Waltham, MA). BSA (free of fatty acid, catalog # A4612) and other reagents were from Sigma-Aldrich unless specified. The equilibrium dialysis device (96-well format) and cellulose membranes (MWCO 12-14K) were obtained from HTDialysis, LLC (Gales Ferry, CT). Breathe Easy™ sealing membranes were obtained from Sigma-Aldrich.

Determination of Fraction Unbound

In preparation of in vitro $f_u$ measurement, rat and human liver tissues were homogenized in phosphate buffered saline (1:5 tissue : PBS dilution) at RT with an Omni TH tissue
homogenizer (Omni International, Kennesaw, GA). A probe (7 mm x 110 mm) was used for 30 second pulses at high speed. InVitroGRO HI media containing 4% BSA and plasma were used directly without any dilution for binding determination. Before an experiment, the dialysis membranes were soaked in water for 15 minutes, 30% ethanol / water for 15 minutes, and PBS for 15 minutes or overnight. The dialysis device was put together following the instructions from the manufacturer (http://htdialysis.com/page/1puq4/Operating_Instructions.html). Compound stock solutions were prepared at 200 µM in DMSO, added to matrices (1:100 dilution), and mixed well with a multichannel pipettor (Eppendorf®, VWR, Radnor, PA). The final test compound concentration for equilibrium dialysis was 2 µM with 1% DMSO. A aliquot (150 µL) of matrix (plasma, liver homogenate or assay media) containing 2 µM test compound was added to the donor side of the membrane and PBS (150 µL) was added to the receiver side of the membrane. The dialysis device was sealed with Breathe Easy™ membranes. Quadruplicates were used for each compound in binding experiments. The dialysis device was incubated in a humidified incubator (75% relative humidity (RH), 5% CO₂/95% air) at 37°C for 6 hours at 200 RPM with an orbital shaker (VWR, Radnor, PA). Alternative binding methods (pre-saturation or dilution) were also used for highly bound compounds to ensure equilibrium had been achieved (Riccardi et al., 2015). At the incubation was completed, matrix samples (15 µL) from the donor wells were added to 45 µL of PBS in a 96-well plate. Dialyzed PBS (45 µL) from the receiver wells were added to the blank matrix (15 µL). Matrix material (15 µL) containing with 2 µM compound from both before and after incubation was taken and added to 45 µL PBS in a 96-well plate. They were used assess recovery and stability of the samples. Cold
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acetonitrile (200 µL) containing internal standard (IS, a cocktail of 5 ng/mL terfenadine and 0.5 ng/mL tolbutamide) was added to all the samples for protein precipitation. The samples were vortexed for 3 minutes (VWR, Radnor, PA) and centrifuged at 3000 RPM for 5 minutes (Allegra 6R, Beckman Coulter, Fullerton, CA) at RT. The supernatant was transferred, dried down, reconstituted with solvents and injected to LC-MS/MS for analysis. Sertraline was used on every incubation plate for quality control. Calculation of fraction unbound (fu) has been discussed previously (Riccardi et al., 2015; Riccardi et al., 2016).

**In Vitro Kpu Measurement**

The cryopreserved hepatocytes were thawed and re-suspended in WEM. The number of cells and viability were determined using the Trypan Blue exclusion method. Cell suspensions were centrifuged (Allegra 6R, Beckman Coulter, Fullerton, CA) at 50 g, RT for 3 minutes. Media were removed and cells were re-suspended in InVitroGRO HI media supplemented with 4% BSA. Test compounds (1 mM) were prepared in DMSO and 1 µL was added to the suspended hepatocytes at 0.5 million cells/mL in 1 mL. The final compound concentration is 1 µM with 0.1% DMSO. Two to four replicates were used for each compound. The suspension was incubated at 37 °C in a humidified incubator (75% RH, 5% CO₂/95% air) with for 4 hours to ensure steady state was reached. At the end of the incubation, the cell suspension was centrifuged for 3 minutes at 500 RPM and supernatant was sampled to determine the medium concentration. The remaining medium was removed from the hepatocytes. The cells were washed with cold PBS 3 times (1 mL each time) and lysed with 75 µL MPER buffer. The solution of the
lysed cells was sampled for analysis. Cold acetonitrile with IS was added to both the supernatant and the cell lysed solutions and mixed. The solution was centrifuged at 3000 RPM for 5 min at RT and the supernatants were transferred for LC-MS/MS analysis using standard curves from both media and cells. Calculation of \textit{in vitro} $K_{puu}$ has been discussed previously (Riccardi et al., 2016). Here, $f_u_{cell}$ is replaced with $f_u_{liver}$ since the two values are quite comparable as liver comprised of 80% hepatocytes by volume (Bayliss and Skett, 1996).

\textit{In Vivo Rat Liver-to-Plasma $K_{puu}$ Determination}

The intravenous (I.V.) infusion experiments in rats were conducted at BioDuro (Shanghai, China). Wistar-Han rats (male, n=3, fed) were infused I.V. through a jugular vein cannula (JVC) at a rate of 4 - 9 $\mu$L/min with test compounds using a programmable pump (Harvard 2000, Harvard Apparatus, Holliston, MA). The doses were selected based on the I.V. bolus data and the detection limit. Infusion time was determined using a duration greater than 5 times the terminal half-life that was predetermined from IV bolus data. Under this condition, $K_{puu}$ should be close to steady state, because greater than 97% steady state is achieved at 5 times half-life (Ito, 2011; Hedaya, 2012). Dose and formulation of the compounds are summarized in Table 1. At the end of infusion, blood samples were obtained from the carotid artery catheter and livers were also collected. Liver samples were rinsed with saline and patted dry with a paper towel and the blood vessels attached were also removed all to minimize the potential contamination from blood and bile. Since the total volume of the biliary tree is quite small compared to the liver for both rat (0.5%) and human (0.3%) (Casali et al., 1994; Masyuk et al., 2001),
the impact of bile contamination on liver concentration is likely to be small. Concentrations were determined using LC-MS/MS. Both pravastatin and its isomer (van Haandel et al., 2016), 3’α-hydroxy-pravastatin, were included in the calculation of $K_{puu}$. Free concentrations were calculated by multiplying the total plasma or total liver concentration by the $f_u$ values of plasma or liver. The \textit{in vivo} unbound liver-to-plasma ratio, $K_{puu}$, was obtained by dividing steady state unbound liver concentration by unbound plasma concentration.

\textit{LC-MS/MS Quantification}

A generic LC-MS/MS method is discussed here and equivalent methods were also used based on compound properties. Two LC mobile phases were used: (A) 95% 2 mM ammonium acetate in water and 5% 50/50 methanol/acetonitrile, (B) 90% 50/50 methanol/acetonitrile and 10% 2 mM ammonium acetate in water, or (A) water with 0.1% formic acid, (B) acetonitrile with 0.1% formic acid. A flow rate of 0.5 mL/min was used with solvent gradient from 5% (B) to 95% (B) over 1.1 minutes to elute the compounds from the UPLC column (BEH C18, 1.7 μm, 50x2.1 mm, Waters, Milford, MA). The injection volume was 10 μL and the cycle time was 2.5 minutes / injection. 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. Data collection processing and analysis were conducted with Analyst\textsuperscript{TM} 1.6.1 software (Applied Biosystems, Foster City, CA).
Human in Vivo K<sub>p uu</sub> Estimation Based on PK/PD Modeling

The in vivo human K<sub>p uu</sub> of rosuvastatin and paravastatin were calculated as the ratio between in vitro and in vivo IC<sub>50</sub> values for HMG-CoA reductase inhibition. The in vitro IC<sub>50</sub> values were obtained from the literature (McTaggart et al., 2001; Holdgate et al., 2003; Gazzerro et al., 2012). The in vivo IC<sub>50</sub> was estimated using PK/PD modeling (See supplementary material: Equation S1). The rosuvastatin model published previously has adapted to estimate the in vivo IC<sub>50</sub> in humans (Aoyama et al., 2010). This is a two compartment PK model developed with an indirect PD response incorporating circadian rhythm of mevalonic acid (MVA) production (Aoyama et al., 2010). Pravastatin plasma PK (Pan et al., 1990; van Luin et al., 2010) and plasma MVA concentrations in response to pravastatin (Nozaki et al., 1996) were used in the PK/PD modeling with the same method as rosuvastatin. All modeling was performed in NONMEM 7.2 (ICON Plc, Dublin, Ireland).

Calculation Methods

F<sub>u</sub> was calculated with Equation (1) based on compound concentrations or area ratios between test compound and IS. For samples with diluted matrices, f<sub>u</sub> was obtained with Equations (2) and (3), where D is the dilution factor. The calculations of recovery and stability are shown in Equation (4) and Equation (5), respectively. In vitro K<sub>p uu</sub>, unbound cell concentration and unbound medium concentration were obtained using Equations (6) – (8).

\[
f_u = \frac{\text{Receiver Concentration}}{\text{Donor Concentration}} \quad \text{Eq (1)}
\]

\[
\text{Diluted } f_u' = \frac{\text{Receiver Concentration}}{\text{Donor Concentration}} \quad \text{Eq (2)}
\]
Undiluted $f_i^* = \frac{1/D}{((1/f_u^{ad})-1) + 1/D}$  \hspace{1cm} \text{Eq (3)}

% Recovery = \frac{\text{Donor Concentration + Receiver Concentration}}{\text{Donor Concentration at Time Zero}} \times 100\% \hspace{1cm} \text{Eq (4)}

\text{Stability as % Remaining} = \frac{\text{Concentration at Last Time Point}}{\text{Concentration at Zero Time Point}} \times 100\% \hspace{1cm} \text{Eq (5)}

K_{\text{prx}} = \frac{C_{u,\text{cell}}}{C_{u,\text{medium}}} \hspace{1cm} \text{Eq (6)}

C_{u,\text{cell}} = C_{\text{total,cell}} \times f_{u,\text{cell}} \hspace{1cm} \text{Eq (7)}

C_{u,\text{medium}} = C_{\text{total,medium}} \times f_{u,\text{medium}} \hspace{1cm} \text{Eq (8)}
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Results

Development of IVIVE requires high quality \textit{in vivo} $K_{\text{puu}}$ data in order to verify the \textit{in vitro} results. As \textit{in vivo} rat $K_{\text{puu}}$ data can be obtained relatively easily by using IV infusion to ensure steady state has been achieved, $K_{\text{puu}}$ IVIVE was first developed using the rat animal model. The method was then extended to humans, where quality \textit{in vivo} $K_{\text{puu}}$ data are quite limited. Four statins (cerivastatin, fluvastatin, rosvastatin and pravastatin) and two Pfizer internal liver-targeting compounds (PF-04991532 (Pfefferkorn et al., 2012) and PF-05187965 (Stevens et al., 2013)) were used for the study of rat IVIVE. All six compounds are OATP substrates consisting of a carboxylic acid functional group. Cerivastatin and pravastatin are OATP1B1 substrates, rosvastatin and fluvastatin are substrates of OATP1B1, 1B3 and 2B1, and the two Pfizer compounds (PF-04991532 and PF-05187965) are substrates of OATP1B1 and OATP1B3 (University of Washington Drug-Drug Interaction Database, UCSF-FDA TransPortal, (Pfefferkorn et al., 2012; Stevens et al., 2013)) The plasma and liver concentrations, measured at steady state, from \textit{in vivo} rat IV infusion studies are shown in Table 2. $K_{\text{puu}}$ was calculated based on \textit{in vivo} liver-to-plasma $K_p$ and \textit{in vitro} $f_{\text{up}}$ and $f_{\text{u,liver}}$ from the equilibrium dialysis assay. The \textit{in vivo} $K_{\text{puu}}$ values of the compounds range from 2.2 to 57 covering a wide range of liver distribution properties. The \textit{in vitro} $K_{\text{puu}}$ data using cryopreserved suspension rat hepatocytes are summarized in Table 3. \textit{In vitro} $K_{\text{puu}}$ was calculated by multiplying \textit{in vitro} $K_p$ with $f_{\text{u,cell}}$ and dividing by $f_{\text{u,media}}$. Since $f_{\text{u,cell}}$ is similar to $f_{\text{u,liver}}$, as 80\% of liver cells are hepatocytes (Bayliss and Skett, 1996), rat $f_{\text{u,liver}}$ data from Table 2 were used as $f_{\text{u,cell}}$ for the \textit{in vitro} $K_{\text{puu}}$ calculation. From this and previous studies (Riccardi et al., 2016), a 4-hour incubation is a conservative time point to achieved steady
state in cells for most compounds. For example, the $K_{puu}$ values for cerivastatin in rat hepatocytes were $39 \pm 2.0$, $34 \pm 3.2$, $24 \pm 1.4$, $31 \pm 0.42$ and $23 \pm 2.3$ at 1, 2, 3, 4 and 5 hours with 0.1 $\mu$M incubation, suggesting steady state has been achieved even at a 1 hour incubation. The comparison between \textit{in vitro} $K_{puu}$ from suspension rat hepatocytes and \textit{in vivo} rat $K_{puu}$ is shown in Table 4. A good correlation between \textit{in vitro} and \textit{in vivo} has been observed and the $K_{puu}$ values are mostly within two-fold of each other. The lower $K_{puu}$ value of pravastatin compared to the other three statins could potentially be due to higher biliary clearance, higher basolateral efflux, and lower active uptake. It appears that the drug metabolizing enzymes and influx/efflux transporters are functioning at the physiological levels in the suspension rat hepatocytes under the specific assay conditions. No scaling factors are necessary to predict \textit{in vivo} $K_{puu}$ from \textit{in vitro} data.

High quality \textit{in vivo} $K_{puu}$ data from human are very scarce. Liver biopsy is invasive and positron emission tomography (PET) imaging has a number of limitations (e.g., interference from metabolites, nonspecific binding to tissues at low doses). Nevertheless, a few \textit{in vivo} human $K_{puu}$ values are available to evaluate the \textit{in vitro} $K_{puu}$ method. Human liver PET data has been reported for $[^{11}C]$dehydropravastatin (DHP) (Shingaki et al., 2014). The $K_{puu}$ was estimated to be 2.0 using terminal phase (post 15 minutes) DHP data and in-house measured pravastatin human $f_{up}$ (0.64) and $f_{u,liver}$ (0.17) values. It has been shown that the transporter and dispositional properties of DHP and pravastatin are very similar using \textit{in vitro} sandwich cultured human hepatocyte assay and \textit{in vivo} rat studies (personal communication with Prof. Yuichi Sugiyama). Therefore, the $K_{puu}$ value of DHP can be used as a surrogate for $K_{puu}$ of pravastatin.
Because high quality directly measured human \textit{in vivo} $K_{puu}$ data are fairly limited, human $K_{puu}$ values of rosuvastatin and pravastatin were obtained indirectly using PK/PD modeling. Other statins were not included in the modeling due to interference of active metabolites or insufficient literature data. The human \textit{in vivo} liver-to-plasma $K_{puu}$ of rosuvastatin was estimated to be 10. The average of literature reported value for \textit{in vitro} IC$_{50}$ was 7 nM, ranging from 2 to 10 (McTaggart et al., 2001; Holdgate et al., 2003; Gazzerro et al., 2012). The \textit{in vivo} IC$_{50}$ of rosuvastatin was fitted to be 2.1 ng/mL with a 95\% confidence interval (CI) of 1.87 – 2.32 ng/mL. The corresponding unbound IC$_{50}$ was 0.7 nM (95\% CI 0.62 – 0.78 nM) using in house measured $f_{up}$ of 0.16. The PK/PD model fits the data well (supplementary material, Figure S1) and the parameter estimates agree with those published previously (supplementary material, Table S1) (Aoyama et al., 2010). The human \textit{in vivo} liver-to-plasma $K_{puu}$ of pravastatin was estimated to be 5.3. The average of the reported \textit{in vitro} IC$_{50}$ values was 48 nM ranging from 29 to 70 nM (Gazzerro et al., 2012). The \textit{in vivo} IC$_{50}$ of pravastatin was modeled to be 6.0 ng/mL with a 95\% CI of 3.6 – 12.7 ng/mL, converting to unbound \textit{in vivo} IC$_{50}$ of 9.0 nM (95\% CI of 5.4 – 19.3 nM) based on in-house measured $f_{up}$ of 0.64. The modeling results are shown in the supplementary material, Table S2 and Figure S2.

The comparison of human \textit{in vivo} $K_{puu}$ from PET or PK/PD modeling and \textit{in vitro} $K_{puu}$ from suspension human hepatocytes is shown in Table 5. The \textit{in vitro} assay predicted \textit{in vivo} DHP $K_{puu}$ from PET data well (2.3 vs. 2.0). The $K_{puu}$ data from PK/PD modeling carry some uncertainties as it is an indirect \textit{in vivo} measure of $K_{puu}$ from many \textit{in vitro} and \textit{in vivo} parameters. Certain assumptions were made to obtain the $K_{puu}$ values from
PK/PD modeling. It is assumed that the \textit{in vitro} assays fully capture the \textit{in vivo} conditions, and that the measured \textit{in vitro} IC\textsubscript{50} can be directly used to quantitatively explain the PD (e.g., MVA) response to unbound liver drug concentrations. In addition, there are only eight and five (respectively) time point measurements of plasma concentrations of MVA in each group of the rosuvastatin and paravastatin studies, and only one PD study of each statin was available to be included in the analysis. Giving the uncertainty of the \textit{in vivo} $K_{\text{puu}}$ values from PK/PD modeling, it is reasonable that the \textit{in vitro} $K_{\text{puu}}$ data are within 2- and 4-fold of the \textit{in vivo} $K_{\text{puu}}$ estimates from PK/PD. The human $K_{\text{puu}}$ IVIVE is similar to that observed in rat based on limited data, suggesting the \textit{in vitro} $K_{\text{puu}}$ method with suspension hepatocytes is a suitable \textit{in vitro} tool to predict \textit{in vivo} $K_{\text{puu}}$ under specific assay conditions. As more human \textit{in vivo} $K_{\text{puu}}$ data become available, the performance of \textit{in vitro} $K_{\text{puu}}$ method will continue to be verified.
Discussion

Determination of unbound liver concentration is critical to understand pharmacology of disease targets in the liver, develop PK/PD relationships, predict DDI potentials, and anticipate liver toxicity. The ability to predict in vivo liver-to-plasma $K_{puu}$ from in vitro assays is highly desirable as there is no easy way to measure human unbound liver concentration. With $K_{puu}$ values, unbound liver concentrations can be estimated from unbound plasma concentrations, which can be readily measured. As reported in the literature, a direct translation of in vitro $K_{puu}$ to in vivo $K_{puu}$ can be challenging that transporter protein levels and functions in the in vitro systems are quite different than in vivo (Roelofsen et al., 1995; Bow et al., 2008; Kimoto et al., 2012; Kunze et al., 2014; Bridget et al., 2015; Vildhede et al., 2015). Typically, empirical scaling factors are needed to predict in vivo drug disposition from in vitro data (Jones et al., 2012; Li et al., 2014). The scaling factors are system dependent and can vary with assay conditions, such as cell culture time/media, plated vs. suspension cells, BSA vs. no proteins, cell types (transfected cells vs. hepatocytes), and medium composition. However, under this specific assay condition with cryopreserved hepatocyte suspension in InVitroGRO HI media with 4% BSA, the transporters and enzymes appear to be functioning at the physiological level. Good $K_{puu}$ IVIVE has been observed in both rat and human without any scaling factors. Both the specific assay media and the physiological amount of BSA (4%) in the assay are important to generate good $K_{puu}$ IVIVE. This is the first time that an in vitro assay shows good prediction of in vivo $K_{puu}$ for OATP substrates. Based on the extended clearance concept, $K_{puu}$ is affected by intrinsic clearance of passive diffusion, active hepatic uptake, sinusoidal efflux, biliary excretion, as well as
metabolism. Based on some literature data, cryopreserved suspended hepatocytes did not retain proper functional activity of efflux transporters. This was likely due to the internalization or down-regulation of some transporters (Bow et al., 2008). However, the information is controversial in the literature, as efflux activity has been reported in suspension hepatocytes of multiple species including rat, human, dog and monkey (Li et al., 2008). The mechanistic understand of why this particular assay condition seems to perform better others requires further investigation.

The *in vivo* $K_{puu}$ measures the unbound drug concentration between liver and arterial blood rather than liver blood. The liver blood has lower drug concentrations than arterial blood for high extraction drugs. Theoretically, for compounds with high liver extraction, the *in vivo* measured $K_{puu}$ will be lower than the *in vitro* experimental $K_{puu}$. However, in practice, these differences have not been observed. The *in vitro* $K_{puu}$ values are shown to be both higher or lower than the *in vivo* $K_{puu}$. This could potentially be due to experimental variability from both *in vitro* and *in vivo* assays, making it difficult to detect the differences. This theoretical difference between *in vivo* and *in vitro* $K_{puu}$ appears to be inconsequential for $K_{puu}$ IVIVE.

In this study, we observed good correlation between *in vivo* $K_{puu}$ from PK/PD modeling and *in vitro* $K_{puu}$. The *in vivo* $K_{puu}$ was calculated as the IC$_{50}$ ratio of HMG-CoA inhibition between *in vitro* and *in vivo*. The *in vitro* IC$_{50}$ was measured using human liver microsomes. Assuming *in vitro* IC$_{50}$ fully captures *in vivo* conditions (e.g. enzyme activity, substrate concentration, pH, and temperature), has no impact of transporters and
represents the intrinsic inhibitory activity, this measured in vitro IC₅₀ should explain the in vivo effect directly if intracellular unbound drug concentration in the liver is known. However, in the absence of liver drug concentration, the human in vivo IC₅₀ was estimated using PK/PD modeling based on unbound plasma concentration. The unbound statin concentration in the liver can be higher than unbound plasma concentrations due to OATP active uptake. Therefore, the IC₅₀ ratio between in vitro and in vivo reflects the unbound drug concentration difference between liver and plasma and can be used as a surrogate for Kₚᵤᵤ. This novel in vitro Kₚᵤᵤ method provides a new tool to assess in vivo Kₚᵤᵤ in drug discovery. The information is useful to estimate human unbound liver drug concentrations, predict efficacy and model DDI risks for drugs that are active influx/efflux in the liver by transporters.
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Authorship Contributions

Participated in research design: Riccardi, Lin, Li, Niosi, Ryu, Hua, Atkinson, Kosa, Litchfield, Di.

Conducted experiments: Riccardi, Ryu, Hua.

Performed data analysis: Riccardi, Lin, Li, Niosi, Ryu, Hua, Atkinson, Kosa, Litchfield, Di.

Wrote or contributed to the writing of the manuscript: Li, Atkinson, Kosa, Di.
References


Focus on Proteins Determining Drug Exposure. *Journal of Proteome Research* **14:**3305-3314.


## Table 1. Rat IV Infusion Experimental Conditions

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (mg/kg/h)</th>
<th>Dosing Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerivastatin</td>
<td>0.10</td>
<td>Saline</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.10</td>
<td>12% (w/v) SBECD (sulfobutylether-β-cyclodextrin) in water</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.10</td>
<td>12% (w/v) SBECD in water</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.10</td>
<td>12% (w/v) SBECD in water</td>
</tr>
<tr>
<td>PF-04991532</td>
<td>0.12</td>
<td>Phosphate buffered saline (PBS), pH 7.4</td>
</tr>
<tr>
<td>PF-05187965</td>
<td>1.2</td>
<td>12% (w/v) SBECD in water with 3 molar equivalent 1N HCl</td>
</tr>
</tbody>
</table>
### Table 2. *In Vivo* Rat IV Infusion Study for Determination of Liver-to-Plasma $K_{p_{uu}}$

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Plasma Concentration at Terminal Time Point (ng/mL)</th>
<th>Liver Concentration at Terminal Time Point (ng/mL)</th>
<th>Total Liver-to-Plasma Ratio ($K_p$)</th>
<th>Rat Plasma $f_{u,p}$</th>
<th>Rat Liver $f_{u,liver}$</th>
<th>In Vivo Rat Liver-to-Plasma $K_{p_{uu}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerivastatin</td>
<td>261 ± 70</td>
<td>6717 ± 960</td>
<td>27 ± 8.0</td>
<td>0.016 ± 0.001</td>
<td>0.017 ± 0.001</td>
<td>29 ± 8.5</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>245 ± 34</td>
<td>8990 ± 1490</td>
<td>37 ± 7.4</td>
<td>0.011 ± 0.001</td>
<td>0.013 ± 0.001</td>
<td>44 ± 8.7</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>14 ± 3.7</td>
<td>178 ± 50</td>
<td>13 ± 2.2</td>
<td>0.044 ± 0.009</td>
<td>0.19 ± 0.02</td>
<td>57 ± 9.5</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>33 ± 19</td>
<td>171 ± 15</td>
<td>6.7 ± 4.5</td>
<td>0.54 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>2.2 ± 1.5</td>
</tr>
<tr>
<td>PF-04991532</td>
<td>172 ± 67</td>
<td>1170 ± 364</td>
<td>7.1 ± 2.0</td>
<td>0.12 ± 0.01</td>
<td>0.096 ± 0.02</td>
<td>5.7 ± 1.6</td>
</tr>
<tr>
<td>PF-05187965</td>
<td>76 ± 21</td>
<td>323 ± 13</td>
<td>4.4 ± 1.0</td>
<td>0.27 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>2.4 ± 0.57</td>
</tr>
</tbody>
</table>
Table 3. *In Vitro* $K_p$ and $K_{puu}$ between Cells and Media in Rat Suspension

**Hepatocytes**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$f_{u,\text{media}}$</th>
<th>$f_{u,\text{cell}}^*$</th>
<th>$K_p$</th>
<th>$K_{puu}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerivastatin</td>
<td>0.022 ± 0.003</td>
<td>0.017 ± 0.001</td>
<td>27 ± 2.6</td>
<td>21 ± 2.0</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.016 ± 0.001</td>
<td>0.013 ± 0.001</td>
<td>27 ± 1.8</td>
<td>22 ± 1.5</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.19 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>35 ± 0.6</td>
<td>35 ± 0.6</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.49 ± 0.06</td>
<td>0.18 ± 0.02</td>
<td>8.3 ± 0.8</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>PF-04991532</td>
<td>0.12 ± 0.01</td>
<td>0.096 ± 0.02</td>
<td>8.9 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>PF-05187965</td>
<td>0.36 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>10 ± 0.4</td>
<td>4.2 ± 0.2</td>
</tr>
</tbody>
</table>

* $f_{u,\text{cell}}$ is assumed to be the same as $f_{u,\text{liver}}$ in Table 2, as 80% of the liver is made of hepatocytes by volume (Bayliss and Skett, 1996).
Table 4. Correlation between In Vivo Rat Liver-to-Plasma $K_{puu}$ and In Vitro Suspension Rat Hepatocyte $K_{puu}$

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$P_{app}$ MDCK-LE (10$^{-6}$ cm/s) *</th>
<th>In Vivo Rat Liver-to-Plasma $K_{puu}$</th>
<th>In Vitro Suspension Rat Hepatocyte $K_{puu}$</th>
<th>Fold Difference in Vivo $K_{puu}$/ In Vitro $K_{puu}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerivastatin</td>
<td>10.3</td>
<td>29 ± 8.5</td>
<td>21 ± 2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>7.8</td>
<td>44 ± 8.7</td>
<td>21 ± 1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.9</td>
<td>57 ± 9.5</td>
<td>35 ± 0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.4</td>
<td>2.2 ± 1.5</td>
<td>2.9 ± 0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>PF-04991532</td>
<td>1.0</td>
<td>5.7 ± 1.6</td>
<td>7.1 ± 0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>PF-05187965</td>
<td>0.5</td>
<td>2.4 ± 0.6</td>
<td>4.2 ± 0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* $P_{app}$ was measured using MDCK-LE method (Di et al., 2011) and the values were obtained from the following references (Pfefferkorn et al., 2012; Stevens et al., 2013; Varma et al., 2015).
Table 5. Correlation between *in Vitro* and *in Vivo* $K_{puu}$ for Humans

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>In Vivo</em> Liver-to-Plasma $K_{puu}$</th>
<th><em>In Vitro</em> Suspension Hepatocyte $K_{puu}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pravastatin</td>
<td>2.0 (DHP, PET), 5.3 (PK/PD)</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>10 (PK/PD)</td>
<td>39 ± 5.0</td>
</tr>
</tbody>
</table>

Pravastatin human $f_u = 0.64 ± 0.16$, $f_{u,liver} = 0.17 ± 0.02$, $f_{u,media} = 0.49 ± 0.06$

Rosuvastatin human $f_u = 0.16 ± 0.05$, $f_{u,liver} = 0.31 ± 0.02$, $f_{u,media} = 0.19 ± 0.02$
Supplementary Material

Novel Method to Predict *In Vivo* Liver-to-Plasma $K_{puu}$ for OATP Substrates Using Suspension Hepatocytes

Keith Riccardi, Jian Lin, Zhenhong Li, Mark Niosi, Sangwoo Ryu, Wenyi Hua, Karen Atkinson, Rachel E. Kosa, John Litchfield, Li Di

Drug Metabolism and Disposition

Figure Legend

Figure S1. Plasma mevalonic acid (MVA) profiles in response to rosuvastatin. (A) plasma MVA profiles in healthy control subjects of morning dosing group; (B) plasma MVA profiles in healthy control subjects of evening dosing group; (C) plasma MVA profiles in healthy subjects with rosuvastatin morning dose; (D) plasma MVA profiles in healthy subjects with rosuvastatin evening dose. LB: 95% lower prediction band; UB: 95% upper prediction band.

Figure S2. Plasma mevalonic acid (MVA) profiles in response to pravastatin. (A) plasma MVA profiles in healthy control subjects of morning dosing group; (B) plasma MVA profiles in healthy control subjects of evening dosing group; (C) plasma MVA profiles in healthy subjects with pravastatin morning dose; (D) plasma MVA profiles in healthy subjects with pravastatin evening dose. LB: 95% lower prediction band; UB: 95% upper prediction band.

Table Legend

Table S1. Model Parameters of Rosuvastatin PK/PD modeling
Table S2. Model Parameters of Pravastatin PK/PD modeling
Table S1. Model Parameters of Rosuvastatin PK/PD modeling

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Estimates</th>
<th>SE%</th>
<th>Annotations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>ml/min/kg</td>
<td>11.3</td>
<td>2.20</td>
<td>Plasma clearance</td>
</tr>
<tr>
<td>V1</td>
<td>L/kg</td>
<td>3.11</td>
<td>8.87</td>
<td>Volume of distribution in central compartment</td>
</tr>
<tr>
<td>Q12</td>
<td>ml/min/kg</td>
<td>6.81</td>
<td>26.0</td>
<td>Inter-compartment clearance</td>
</tr>
<tr>
<td>V2</td>
<td>L/kg</td>
<td>3.55</td>
<td>24</td>
<td>Volume of distribution in peripheral compartment</td>
</tr>
<tr>
<td>MAT</td>
<td>hour</td>
<td>2.48</td>
<td>9.19</td>
<td>Mean absorption time</td>
</tr>
<tr>
<td>Fa</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>Bioavailability, Fixed (Rosenson, 2003)</td>
</tr>
<tr>
<td>IC</td>
<td>ng/ml</td>
<td>4.75</td>
<td>2.01</td>
<td>Initial plasma concentration of MVA</td>
</tr>
<tr>
<td>Kamp</td>
<td>ng/ml/h</td>
<td>1.04</td>
<td>3.65</td>
<td>Amplitude of MVA production rate</td>
</tr>
<tr>
<td>tz</td>
<td>hour</td>
<td>16.4</td>
<td>0.787</td>
<td>Acrophase time of MVA synthesis rate</td>
</tr>
<tr>
<td>kout</td>
<td>1/hour</td>
<td>0.773</td>
<td>0.176</td>
<td>Elimination rate of MVA</td>
</tr>
<tr>
<td>IC50</td>
<td>ng/ml</td>
<td>2.09</td>
<td>3.57</td>
<td>IC50 of rosuvastatin based on its plasma concentration</td>
</tr>
<tr>
<td>γ</td>
<td>-</td>
<td>1.66</td>
<td>9.34</td>
<td>Hill coefficient</td>
</tr>
</tbody>
</table>

Table S2. Model Parameters of Pravastatin PK/PD modeling

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Estimates</th>
<th>SE%</th>
<th>Annotations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>ml/min/kg</td>
<td>10.3</td>
<td>5.94</td>
<td>Plasma clearance</td>
</tr>
<tr>
<td>V1</td>
<td>L/kg</td>
<td>0.125</td>
<td>16.1</td>
<td>Volume of distribution in central compartment</td>
</tr>
<tr>
<td>Q12</td>
<td>ml/min/kg</td>
<td>6.63</td>
<td>15.5</td>
<td>Inter-compartment clearance</td>
</tr>
<tr>
<td>V2</td>
<td>L/kg</td>
<td>0.413</td>
<td>8.31</td>
<td>Volume of distribution in peripheral compartment</td>
</tr>
<tr>
<td>MAT</td>
<td>hour</td>
<td>1.79</td>
<td>2.21</td>
<td>Mean absorption time</td>
</tr>
<tr>
<td>Fa</td>
<td>-</td>
<td>0.18</td>
<td>-</td>
<td>Bioavailability, Fixed (Rosenson, 2003)</td>
</tr>
<tr>
<td>IC</td>
<td>pmol/mL</td>
<td>76.9</td>
<td>2.95</td>
<td>Initial plasma concentration of MVA</td>
</tr>
<tr>
<td>Kamp</td>
<td>pmol/ml/h</td>
<td>5.29</td>
<td>23.3</td>
<td>Amplitude of MVA production rate</td>
</tr>
<tr>
<td>tz</td>
<td>hour</td>
<td>14.1</td>
<td>2.05</td>
<td>Acrophase time of MVA synthesis rate</td>
</tr>
<tr>
<td>kout</td>
<td>1/hour</td>
<td>0.272</td>
<td>23.7</td>
<td>Elimination rate of MVA</td>
</tr>
<tr>
<td>IC50</td>
<td>ng/mL</td>
<td>5.95</td>
<td>5.88</td>
<td>IC50 of rosuvastatin based on its plasma</td>
</tr>
<tr>
<td>γ</td>
<td>-</td>
<td>1.35</td>
<td>34.2</td>
<td>Hill coefficient</td>
</tr>
</tbody>
</table>
Equation S1.

(Please refer to Tables S1 and S2 for symbol annotations)

PK equations:

\[ K_{10} = \frac{CL \times 60}{V_1 \times 1000} \]
elimination rate constant from central compartment

\[ K_{12} = \frac{Q_{12} \times 60}{V_1 \times 1000} \]
intercompartment clearance from central

\[ K_{21} = \frac{Q_{12} \times 60}{V_2 \times 1000} \]
intercompartment clearance from peripheral

\[ K_a = \frac{1}{MAT} \]
absorption rate constant

\[ \frac{d(Q_p)}{dt} = K_a \times Q_a - K_{10} \times Q_p - K_{12} \times Q_p + K_{21} \times Q_d \]
derivative of plasma drug amount

\[ \frac{d(Q_d)}{dt} = K_{12} \times Q_p - K_{21} \times Q_d \]
derivative of peripheral drug amount

\[ \frac{d(Q_a)}{dt} = -K_a \times Q_a \]
derivative of drug amount for absorption

\[ C_p = \frac{Q_p}{V_1} \]
plasma drug concentration

PD equations:

\[ K_m = K_{out} \times IC - \frac{K_{amp} \times K_{out}^2 \times \left( \cos \left( \frac{2\pi \times tz}{24} \right) - \frac{2\pi}{24 \times K_{out}} \times \sin \left( \frac{2\pi \times tz}{24} \right) \right)}{K_{out}^2 + \left( \frac{2\pi}{24} \right)^2} \]
mean MVA synthesis rate

\[ K_{in} = K_m + K_{amp} \times \cos \left( \frac{2\pi \times (t - tz)}{24} \right) \]
MVA synthesis rate

\[ \frac{d(MVA)}{dt} = K_{in} \times \left( 1 - \frac{C_p}{IC_{20} + C_p} \right) - K_{out} \times MVA \]
derivative of plasma MVA
**Figure S1.** Plasma mevalonic acid (MVA) profiles in response to rosuvastatin. (A) plasma MVA profiles in healthy control subjects of morning dosing group; (B) plasma MVA profiles in healthy control subjects of evening dosing group; (C) plasma MVA profiles in healthy subjects with rosuvastatin morning dose; (D) plasma MVA profiles in healthy subjects with rosuvastatin evening dose. LB: 95% lower prediction band; UB: 95% upper prediction band.
Figure S2. Plasma mevalonic acid (MVA) profiles in response to pravastatin. (A) plasma MVA profiles in healthy control subjects of morning dosing group; (B) plasma MVA profiles in healthy control subjects of evening dosing group; (C) plasma MVA profiles in healthy subjects with pravastatin morning dose; (D) plasma MVA profiles in healthy subjects with pravastatin evening dose. LB: 95% lower prediction band; UB: 95% upper prediction band.