Using the Dynamic Well-Stirred Model to Extrapolate Hepatic Clearance of OATP Substrates without Assuming Albumin-Mediated Hepatic Drug Uptake

Zhengyin Yan*, Li Ma, Nicky Hwang, Julie Huang, Jane R. Kenny, Cornelis E.C.A Hop

Department of Drug Metabolism and Pharmacokinetics
Genentech Inc., South San Francisco, CA, 94080

*Send Correspondence to:
Zhengyin Yan, Ph.D.
Department of Drug Metabolism and Pharmacokinetics
Genentech, Inc
1 DNA Way
South San Francisco, CA 94080
Telephone No: (610)-639-9876
Email Address: lexington1528@gmail.com
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**List of Abbreviations:** $f_D$, dynamic free fraction; $\text{PS}_{\text{int},u}$, unbound intrinsic uptake clearance; $\text{PS}_{\text{int}}$, intrinsic uptake clearance; WSM, well-stirred clearance model; dWSM, dynamic well-stirred model; $f_u$, fraction of unbound; LC-HRMS liquid chromatography high resolution mass spectrometry.
ABSTRACT

Extrapolating in vivo hepatic clearance from in vitro uptake data is a known challenge, especially for OATP substrates, and the well-stirred model (WSM) commonly yields systematic under-predictions for those anionic drugs hypothetically due to “albumin-mediated hepatic drug uptake”. In the present study, we demonstrate that the WSM incorporating the dynamic free fraction ($f_D$), a measure of drug protein binding affinity, performs reasonably well in predicting hepatic clearance of OATP substrates. For a selection of anionic drugs including atorvastatin, fluvastatin, pravastatin, rosuvastatin, pitavastatin, cerivastatin, and repaglinide, this dynamic well-stirred model (dWSM) correctly predicts hepatic plasma clearance within 2-fold error for six out of seven OATP substrates examined. The geometric mean of clearance ratios between the predicted and the observed values falls in the range of 1.21-1.38. As expected, the WSM with unbound fraction ($f_u$) systematically under-predicts hepatic clearance with greater than 2-fold error for five out of seven drugs, and the geometric mean of clearance ratios between the predicted and the observed values is in the range of 0.20-0.29. The results suggest that, despite its simplicity, the dWSM operates well for transporter-mediated uptake clearance, and that clearance under-prediction of OATP substrates may not necessarily be associated with the chemical class of the anionic drugs, nor is it a result of albumin-mediated hepatic drug uptake as currently hypothesized. Instead, the superior prediction power of the dWSM confirms the utility of the dynamic free fraction in clearance prediction and the importance of drug plasma binding kinetics in hepatic uptake clearance.
Significance Statement

The traditional well-stirred model (WSM) consistently under predicts OATP-mediated hepatic uptake clearance, hypothetically due to the albumin-mediated hepatic drug uptake. In this manuscript, we apply the dynamic well-stirred model (dWSM) to extrapolate hepatic clearance of the OATP substrates, and our results show significant improvements in clearance prediction without assuming albumin-mediated hepatic drug uptake.
INTRODUCTION

Human organic anion-transporting polypeptide transporters (OATPs) consist of a family of influx transporters expressed in various tissues including liver, brain, and intestine (Hilgendorf et al., 2007). Among all OATP transporters, OATP1B1, 1B3 and 2B1 are highly expressed on the sinusoidal membrane of hepatocytes, where they uptake endogenous substrates as well as xenobiotics from blood and transport into hepatocytes for disposition (Hagenbuch et al., 2008). Over the past decades, there has been an increasing body of evidence that some OATPs, particularly OATP1B1 and 1B3, play a critical role in the disposition of anionic drugs such as commonly prescribed statins (Rocha et al., 2018). Additionally, a number of studies have shown that OATPs are involved in clinical drug-drug interaction (DDI) of some marketed drugs (Balasubramanian et al., 2021). Therefore, it is of importance to predict hepatic clearance of OATP substrates accurately in order to properly project human clinical doses and assess the DDI risk in drug discovery and early development.

For low permeability OATP substrates such as statins, passive diffusion is negligible and the transporter-mediated uptake is the rate-limiting step in hepatic elimination (Maeda et al., 2011). Thus, the extended well-stirred model (Sirianni et al., 1997) is abridged to eq. 1 for extrapolating hepatic blood clearance from in vitro intrinsic uptake clearance (IVIVE).

\[
CL_H = \frac{Q_H \cdot f_{u,b} \cdot PS_{int,u}}{Q_H + f_{u,b} \cdot PS_{int,u}}
\]

Where \(CL_H\) is hepatic blood clearance, \(Q_H\) is the liver blood flow, \(f_{u,b}\) is the fraction of unbound drug in blood, and \(PS_{int,u}\) represents the in vivo intrinsic uptake clearance determined in hepatocytes in buffer.

It is widely recognized that this IVIVE approach consistently yields prominent under-predictions especially for highly bound OATP substrates such as pitavastatin, atorvastatin,
fluvastatin, and cerivastatin (Izumi et al., 2017; Nozaki et al., 2020; Fujino et al., 2018). In other words, the hepatic clearance \( (CL_H) \) calculated from eq. 1 is lower compared to the observed values, implying that these drugs seem to be eliminated by the liver more efficiently than expected from the unbound drug level estimated with \( f_u \). To rationalize this discrepancy, some researchers have postulated “protein-mediated uptake” (PMU) as a likely mechanism responsible for hepatic clearance under-prediction (Li et al., 2020; Miyauchi et al., 2018; Bowman et al., 2019; Kim et al., 2019). Briefly, the PMU postulates that the interaction between the drug-albumin complex and hepatocytes somehow facilitates the dissociation of the drug-protein complex, resulting in an elevated level of unbound drug on the surface of hepatocytes. Thus, the \( \textit{in vivo} \) PS\( _u \) of the OATP substrate is higher than the value predicted from the \( \textit{in vitro} \) drug uptake data after accounting for the effect of plasma binding on the intrinsic uptake clearance with \( f_u,b \) (Li et al., 2020; Bowman et al., 2019; Kim et al., 2019; Miyauchi et al., 2022).

Given that \( f_u \) is a measure of the drug binding extent in plasma at equilibrium, we have introduced the “dynamic free fraction” \( (f_D) \) as a new binding parameter describing drug binding kinetics or drug binding affinity to plasma protein (Yan et al., 2023). For a drug molecule “i” in a given drug-protein mixture, it stays either in the bound (ON) or free (OFF) state at any given time period \((dT)\). Statistically, \( f_D \) is as follows:

\[
f_D = \frac{\sum dT_{i,\text{off}}}{\sum dT_{i,\text{total}}} = \frac{\Delta T_{\text{off}}}{\Delta T_{\text{total}}} \tag{2}
\]

Where \( dT_{i,\text{off}} \) represents the time that the drug molecule “i” stays in the “OFF” state during the entire given time interval \( (dT_{i,\text{total}}) \). Basically, \( f_D \) is the ratio of the total time all drug molecules spend in the “OFF” state \( (\Delta T_{\text{off}}) \) relative to the total time interval \( (\Delta T_{\text{total}}) \).
Conceptually, if a drug binds to the protein with high affinity, it stays longer time in the “ON” state, resulting in a low \( f_o \) because of its slow dissociation rate.

Recently, we have demonstrated that the effect of protein binding on metabolic intrinsic clearance (\( \text{CL}_{\text{int}} \)) should be accounted for by using \( f_{D,b} \) measured in blood (\( \text{CL}_{\text{int}} = f_{D,b} \times \text{CL}_{\text{int},u} \)), which results in more accurate predictions of hepatic metabolic clearance from liver microsomal data (Yan et al., 2024). Extending this concept to OATP-mediated drug uptake, we rationalize that \( f_{D,b} \) should be applied to account for the effect of blood protein binding on the intrinsic drug uptake clearance (Supplemental SM1):

\[
\text{PS}_{\text{int}} = f_{D,b} \times \text{PS}_{\text{int},u}
\]

A dynamic version of the well-stirred model (dWSM) for hepatic blood uptake clearance is expressed below.

\[
\text{CL}_H = \frac{Q_H \times f_{D,b} \times \text{PS}_{\text{int},u}}{Q_H + f_{D,b} \times \text{PS}_{\text{int},u}} \tag{4}
\]

The current study aims to (i) evaluate the performance of the dWSM (eq. 4) in extrapolating in vivo clearance of OATP substrates from in vitro uptake data; and (ii) conceptualize the physiological relevance of drug binding kinetics to hepatic uptake clearance.

**Materials and Methods**

**Material.** Human plasma pooled from three individual donors was obtained from Bioreclamation IVT (NY, USA). Human recombinant cytochrome P450 3A4 (Supersomes rCYP3A4) was purchased from BD Biosciences (San Jose, CA). OATP substrates, including atorvastatin, fluvastatin, pravastatin, rosuvastatin, pitavastatin, cerivastatin and repaglinide, were
obtained directly from the Genentech Compound Management, and the drugs were previously purchased from Sellek Chemicals (Houston, TX) and Toronto Research Chemicals (North York, Canada). Reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) was from Sigma-Aldrich (St. Louis, MO).

**Determination of the Dynamic Free Fraction in Plasma.** Dynamic free fraction in human plasma \( (f_{DP}) \) was determined as described previously (Yan et al., 2023) with the minor customizations. Briefly, individual drugs – atorvastatin, fluvastatin, pravastatin, rosuvastatin, pitavastatin, cerivastatin, and repaglinide – were diluted in phosphate buffer (pH 7.4) or human plasma (pH 7.4) premixed with the reporter enzyme (rCYP3A4). The resulting mixtures were warmed for 15 min at 37°C, and the enzyme reaction was then initiated by adding NADPH to the reaction mixtures. Both buffer and plasma reaction mixtures each contained 10 µM drug, 100 pmol/mL reporter enzyme, and 0.25 mM NADPH in a final volume of 100 µL. All incubations were performed in triplicate in a water-bath at 37°C for 20 min. The reaction was terminated by adding 400 µL of acetonitrile containing 100 nM propranolol as the internal standard (IS), which was then followed by the addition 100 µL of plasma to the buffer samples and 100 µL of buffer to plasma samples respectively to neutralize the matrix effect. After brief vortexing, the samples were centrifuged for 15 min at 2000 g to remove protein precipitates. The supernatant (200 µL) was dried and reconstituted with 150 µL 10% acetonitrile in water, and then analyzed for primary enzyme products using a Shimadzu Nexera liquid chromatography interfaced with the Thermo Orbitrap Exploris 240 mass spectrometry (LC-HRMS). Reporter enzyme binding assay conditions and MS detection information are summarized in Supplemental Table ST1.
The dynamic free fraction in plasma \( f_{D,P} \) was calculated using the peak area ratios of enzyme product to IS in buffer and plasma wells, obtained using LC–HRMS, by following the equation below (Yan et al., 2023):

\[
f_{D,P} = \frac{\text{product peak area, protein}}{\text{IS peak area, protein}} \div \frac{\text{product peak area, buffer}}{\text{IS peak area, buffer}}
\]

It is important to point out that the reporter enzyme method works only at initial reaction condition (Yan et al., 2023). Too much enzyme or extended incubation time can lead to the violation of the initial reaction conditions, whereas too little enzyme or insufficient incubation may not produce enough product for LC-MS analysis. Because of low turnover of statins by the reporter enzyme, assay conditions were optimized by increasing the enzyme concentration (100 pmol/mL) and incubation time (20 min) to ensure sufficient product conversion for analytical detection (LC-MS) and maintain the enzymatic reaction at the initial reaction condition. The \( f_{D,P} \) value does not change with substrate and protein concentrations as long as the reaction occurs under initial reaction condition.

**Source of Pharmacokinetic Datasets.** Except for \( f_{D,P} \), the majority of pharmacokinetic data of the OATP substrates were obtained from a very recent paper published by Kim et al. (2019), which include blood-to-plasma partition ratio \( R_b \), observed hepatic clearance (Supplemental Table ST2), unbound fraction \( f_{u,P} \) in human plasma, and one set of *in vitro* uptake clearance data measured in suspended human hepatocytes (Supplemental Table ST3). Given donor variability in the expression of OATPs (Badée et al., 2015) and data variability in the drug uptake assay, a literature search was conducted to collect additional *in vitro* uptake clearance data measured in suspended human hepatocytes from different donor pools using the oil-spin method. The literature search covered the past 20 years, and literature-compiled *in vitro* uptake data were grouped into two separate datasets for IVIVE analysis: the singly measured dataset.
published by Kim et al. (2019) which represents the performance of the most recent hepatic uptake assay, and a pooled dataset averaged (geometric mean) from all literature values which represents different donors and data variability in the assay performed by various researchers (Supplemental Table ST3).

**Extrapolation of Hepatic Clearance from the In Vitro Uptake Clearance.** The *in vitro* hepatic uptake clearance (µl/min/million cells) compiled from literature was scaled up to *in vivo* clearance (PS_{int,u} ml/min/kg) by applying the following physiological scaling factors: 120 million cells/g liver and 25.7 g liver/kg body weight (Davies et al., 1993). The scaled intrinsic uptake clearance (PS_{int,u}) was used in the dynamic WSM with the effect of drug partition (R_B) in blood (eq. 5) to calculate hepatic plasma clearance (CL_{H,P}) (Yang et al., 2007).

\[
CL_{H,P} = \frac{Q_H \times f_{D,P} \times PS_{int,u}}{Q_H + f_{D,P} \times PS_{int,u}/R_B}
\]  

(eq. 5)

For each individual drug, the ratio of predicted to observed plasma clearance value was calculated, and a ratio in the range of 0.5-2.0 was considered a successful prediction. Additionally, the geometric mean of the clearance ratios was calculated.

Similarly, this scaled intrinsic uptake clearance was also used in the traditional well-stirred model with f_{u,P} (eq. 6) to calculate hepatic plasma clearance for comparison.

\[
CL_{H,P} = \frac{Q_H \times f_{u,P} \times PS_{int,u}}{Q_H + f_{u,P} \times PS_{int,u}/R_B}
\]  

(eq. 6)

**RESULTS**
We initially attempted to determine the dynamic free fraction in human plasma for all OATP substrates examined by Kim et al. (2019), but failed to measure $f_{D,P}$ for valsartan, nateglinide, and glibenclamide due to low detection sensitivity and poor metabolic turnover by the reporter enzyme in the drug binding assay, which is a recognized limitation of the method (Yan, et al. 2023). The $f_{D,P}$ values measured for atorvastatin, fluvastatin, pravastatin, rosuvastatin, pitavastatin, cerivastatin, and repaglinide are summarized in Table 1. It appears that the OATP substrates bind to albumin in plasma with different binding affinity with a rank order from high to low affinity: cerivastatin ($f_{D,P} 0.066$) > fluvastatin ($f_{D,P} 0.1536$) > atorvastatin ($f_{D,P} 0.4320$) > pitavastatin ($f_{D,P} 0.5983$) > pravastatin ($f_{D,P} 0.7384$) ≈ repaglinide ($f_{D,P} 0.7469$) > rosuvastatin ($f_{D,P} 0.9156$). As a whole, all seven of the OATP substrates tested had an $f_{D,P}$ value higher than their corresponding $f_u$ values (Kim et al. 2019), and this observation ($f_{D,P} > f_u$) is in agreement with our previous dataset generated in human plasma for a diverse group of drugs (Yan et al., 2024).

The most dramatic difference between measured $f_{D,P}$ and $f_u$ was observed for pitavastatin and repaglinide with ratios greater than 110, which is followed by fluvastatin (35.9), atorvastatin (14.0), cerivastatin (9.0), and rosuvastatin (6.8). The smallest difference between $f_{D,P}$ and $f_u$ was seen for pravastatin with a ratio of 1.3. Given that $f_{D,P}$ is a measure of drug binding affinity in plasma (eq. 2) or the dissociation rate of drug-protein complex (Yan et al., 2023), the differences between $f_{D,P}$ and $f_u$ values suggest that the anionic drugs show distinct binding profiles in human plasma. Pravastatin exhibits the lowest binding extent (highest $f_u$, 0.563), and it binds to a low affinity site of albumin in plasma ($f_{D,P} 0.7384$). Both cerivastatin and fluvastatin are highly bound in human plasma ($f_u < 0.01$), and presumably the former binds to a high affinity site of albumin ($f_{D,P}, 0.0660$), whereas the latter binds to albumin with more moderate affinity ($f_{D,P}, 0.1536$). Atorvastatin, pitavastatin, repaglinide, and rosuvastatin bind to plasma extensively ($f_u < 0.05$),
but their binding affinity is low ($f_{D,P} > 0.4$) due to their high OFF rates of the drug-protein complex. This difference between the two binding parameters in plasma is explainable, considering that acidic drugs predominately bind to albumin and the molar ratio of albumin to the drug is high (>60) under the binding assay conditions. If one given drug binds extensively to plasma with low affinity, the drug can have a high portion of molecules in the OFF state (high $f_{D,P}$), which are available to interact with their biological target even though the binding extent of the drug is high (low $f_{u,P}$). This is simply because drug protein binding is reversible and the drug-protein complex has a high dissociation rate ($k_{off}$). Theoretically, this can be the case in the in vivo situation where albumin concentration in plasma is very high (625-650 µM) (Peters, 1996) compared to most drugs (~0.1-10 µM).

Hepatic plasma uptake clearance was calculated using the measured $f_{D,P}$ and in vitro uptake clearance as described in the Methods. As shown in Table 2, when the singly measured in vitro uptake clearance dataset (Kim et al., 2019) was used, the dWSM (eq. 5) correctly predicted hepatic plasma uptake clearance for six out of seven OATP substrates (atorvastatin, fluvastatin, pravastatin, rosuvastatin, pitavastatin, and repaglinide) with a clearance ratio of predicted to observed values within 2-fold. Cerivastatin is the only exception, exhibiting a low degree of over-prediction with a clearance ratio higher than 2-fold (predicted/observed, 2.35) but less than 3-fold (Table 2). Overall, the geometric mean of hepatic clearance ratios is 1.21 for the singly measured dataset. For a more representative assessment, the pooled in vitro uptake clearance dataset was used to extrapolate hepatic plasma clearance using the dWSM (eq. 5). Similar to that of the single dataset, the same six OATP substrates – atorvastatin, fluvastatin, pravastatin, pitavastatin, rosuvastatin, and repaglinide – showed a clearance ratio of predicted to observed values within 2-fold; the dWSM also yielded a moderate degree of hepatic clearance over-
prediction for cerivastatin with a clearance ratio of 2.47 (predicted/observed). For the entire group, the geometric mean of hepatic clearance ratios is 1.38, which is slightly higher than that obtained from the single dataset, and no significant difference was observed in predicted hepatic plasma clearance between the pooled and the single dataset. Overall, no clear under-prediction was observed for both datasets.

To further assess the impact of *in vitro* data variability on the prediction, the spread of predicted uptake clearance was analyzed for the pooled dataset. As shown in Table 2, the range of predicted plasma clearance values is generally tight, considering that the pooled *in vitro* uptake data were generated from different donor pools by different researchers over a period of two decades. The ratio of predicted vs observed plasma clearance ranges from 0.42 for pravastatin to 3.19 for cerivastatin, and most of the *in vitro* uptake data points yielded hepatic plasma clearance within two-fold.

For direct comparison, hepatic plasma uptake clearance was also calculated using $f_{u,r}$ in the traditional well-stirred model (eq. 6). As shown in Table 3, although the WSM correctly predicted hepatic plasma clearance within 2-fold for cerivastatin and pravastatin using the single and the pooled datasets, atorvastatin, fluvastatin, rosuvastatin, pitavastatin, and repaglinide showed multiple-fold under-prediction regardless of whether the single uptake dataset or the pooled uptake datasets were examined. The ratios of predicted to observed plasma clearance values are in the range of 0.08-0.67, and the geometric means of clearance ratios are 0.20 for the single dataset and 0.29 for the pooled dataset. The spread of predicted uptake plasma clearance also indicated consistently systematic under-predictions of hepatic clearance for the OATP substrates.
DISCUSSION

The tenet of IVIVE is the proportionality between \textit{in vitro} intrinsic clearance and \textit{in vivo} intrinsic clearance for both liver enzymes and transporters (Rane et al., 1977; Houston, 1994). OATP-mediated drug uptake takes place in the contact between blood and the sinusoidal membrane of hepatocytes. Conceptually, it is reasonable to assume that the drug uptake process follows the “well-stirred condition” more favorably compared to enzyme-mediated drug elimination since it does not involve passive diffusion across the cell membrane, intracellular protein binding, and potential drug partition into organelles of hepatocytes. However, when the traditional WSM (eq. 1) is applied to predicting transporter-mediated hepatic plasma clearance, some OATP substrates such as fluvastatin, rosuvastatin, pitavastatin, and repaglinide are among the drugs exhibiting the worst under-prediction (Bowman et al., 2019). Such prominent \textit{in vitro}-\textit{in vivo} disconnect in OATP-mediated drug uptake has been frequently cited as the most appealing evidence supporting different versions of the PMU (Weisiger, 1981; Li et al., 2020; Bowman et al., 2019; Kim et al. 2019; Miyauchi et al., 2022).

In the present study, we demonstrate that the dynamic WSM model (eq. 5) incorporating $f_{D,p}$ is able to predict hepatic plasma uptake clearance within 2-fold error for six out of the seven OATP substrates examined (atorvastatin, fluvastatin, rosuvastatin, pitavastatin, pravastatin, and repaglinide), suggesting that there is an alternative way to predict uptake clearance without the PMU. There were no dramatic differences in the prediction accuracy between the single and pooled datasets (Table 2), indicating that the performance of the dWSM is consistent and not dataset specific, despite inherent data variability of the drug uptake assay. Among the seven OATP substrates, cerivastatin was the only outlier with greater than 2-fold but less than 3-fold error. Mechanistically, it remains unclear why cerivastatin exhibited a low level of over-
prediction, assuming both *in vitro* uptake clearance and observed hepatic plasma clearance are accurate. The success rate (85%) of the dWSM in uptake clearance prediction appears comparable to the prediction of hepatic metabolic clearance from liver microsomal data (Yan et al., 2024). Overall, the results together with our theoretical analysis (Supplemental Material SM1) confirm the utility of the dynamic WSM in hepatic clearance prediction and the critical role of drug binding kinetics in plasma in drug elimination (Baker et al., 2007; Weisiger, 1985).

Conceptually, physiological relevance of drug plasma binding kinetics to hepatic drug uptake clearance can be elaborated using the dynamic free fraction in time terms. If a drug binds to plasma protein with low affinity, it exhibits a high $f_{D,P}$ value and spends longer time in the “OFF” state in blood compared to drugs with high binding affinity (eq. 2) (Yan et al., 2023). As depicted in Fig. 1, assuming that the blood stream carries both repaglinide (low affinity, $f_{D,P}$ 0.7469) and cerivastatin (high affinity, $f_{D,P}$ 0.066) simultaneously through the liver, repaglinide statistically would spend 10-times longer time in the “OFF” state than cerivastatin during the transit in the liver. As a result, repaglinide would be far more likely to encounter the OATP transporter than cerivastatin as both drugs move through the liver, given that the estimated transit time of blood in the liver is approximately in the range of 10-35s (Albrecht et al., 1999) while the dissociation of the drug-albumin complex likely occurs within milliseconds in most cases (Berezhkovski, 2014). The difference in $f_{D,P}$ conceptually explains why repaglinide exhibited higher hepatic clearance and extraction ratio ($CL_{H,P}$ 7.18 ml/min/kg, ER 0.56) in the clinic compared to cerivastatin ($CL_{H,P}$ 2.9 ml/min/kg, ER 0.24) (Table ST2), despite having nearly identical $f_{u,P}$ (0.00676 vs 0.00734) (Table 1) and cerivastatin having higher intrinsic uptake clearance ($PS_{int,u}$ range ~46-197 µL/min/million vs ~39-114 µL/min/million) (Table 2). Alternatively, even though both drugs are highly bound to albumin in plasma (>99%), the
binding affinity of repaglinide in plasma ($f_{D,p} 0.7469$) is significantly lower than cerivastatin ($f_{D,p} 0.0660$), and thus it is conceivable that cerivastatin is much harder than repaglinide to be extracted out of the blood by the OATP transporter in the liver. For the same reason, atorvastatin, fluvastatin, and pitavastatin show higher ER ratios (ER 0.65-0.47) than cerivastatin (ER 0.24) (Supplemental ST2), the drug with the highest binding affinity to albumin in the group (Table 2), even though the drugs bind to plasma extensively ($f_{u,p} 0.004-0.03$). The results are consistent with our previous study (Yan et al. 2024), suggesting that drug binding affinity in plasma determines hepatic drug uptake clearance, not binding extent. This explains why some drugs yielded more accurate clearance prediction without incorporating protein binding ($f_{u,p} 1.0$) in the WSM (Obach, 1999), since the drugs can dynamically be nearly totally free in plasma with a $f_{D,p}$ value close to 1, as seen for pitavastatin, rouvuvastatin, and repaglinide (Table 1), even though they bind extensively in plasma.

Historically, $f_{u,p}$ has commonly been used to calculate the surrogate of true free drug concentration in plasma and pharmacokinetic parameters such as unbound intrinsic metabolic clearance and drug uptake rates. Such practice has frequently resulted in “unexpected datasets” for many highly bound compounds, which are difficult to interpret in the context of the free drug theory (Schulz et al., 2023). As a measure of binding extent, $f_{u,p}$ does not capture the drug binding kinetics or the dissociation of drug-protein complex in plasma. For instance, pitavastatin has a $f_u$ value (0.00541) significantly lower than its $f_{D,p}$ (0.5983) (Table 1), suggesting that the true free drug level in plasma is more than 100-fold higher than the surrogate presumably due to fast dissociation of the drug-albumin complex. When $f_{u,p}$ is used to back calculate the unbound intrinsic uptake clearance ($PS_{int,u,cal.}$) from the apparent value ($PS_{int}$) measured in the presence of plasma, the calculated value ($PS_{int,u,cal.}=PS_{int}/f_{u,p}$) is significantly greater than the actual unbound
intrinsic uptake clearance ($P_{\text{int,u}}$) measured in buffer. This extra value ($P_{\text{int,u,cal.}}-P_{\text{int,u}}$) is often interpreted as the result of “protein mediated uptake” by some researchers (Li et al., 2020; Bowman et al., 2019; Kim et al. 2019; Miyauchi et al., 2022). We argue that it is truly a mathematical artifact, which appears consistent with two recent studies suggesting that the PMU is likely an artifact compounded by biochemical events other than drug transporter activity (Yin et al., 2022; Yin et al., 2023). For highly free pravastatin, the dissociation of the drug-albumin complex does not dramatically affect the true free drug level in plasma ($f_{u,p} 0.5630$ vs $f_{D,p} 0.7384$) (Table 1). As a result, pravastatin does not exhibit marked PMU, and both traditional WSM and the dWSM are able to predict hepatic plasma uptake clearance within 2-fold error (Table 2 &3).

It is important to recognize that $f_{u,p}$ and $f_{D,p}$ are two different binding parameters. The former is a measure of binding extent, and the latter is a measure of drug binding affinity in plasma. The divergence between $f_{u,p}$ and $f_{D,p}$ for highly bound drugs is a unique drug binding phenomenon in plasma due to the high molar ratio (~100) of albumin relative to the drug, which can help explain some poorly understood observations. For instance, highly bound drugs are more likely to exhibit a more dramatic divergence in $f_{u,p}/f_{D,p}$ leading to more prominent under predictions are observed more frequently for these drugs (Francis et al., 2020). Also, most of acidic drugs bind to albumin extensively (Peters, 1996), and they appear to represent a distinct chemical class with more prevalent under-prediction with $f_{u,p}$ compared to neutral and basic drugs (Tess et al., 2023; Poulin et al., 2021; Tess et al., 2020).

Although the PMU seems plausible for explaining the in vitro to in vivo disconnect associated with OATP transporters, some key mechanistic details have not been fully elaborated in various versions of PMU (Bowman et al., 2019; Kim et al., 2019; Poulin et al., 2021). For instance, the hypothetical binding partner of albumin still remains elusive despite its critical role.
in the PMU. Additionally, under normal physiological conditions, there are approximately 100-fold more free albumin molecules (620-630 µM) in plasma (Peters, 1996) compared to drug-bound albumin (assuming total drug level ≈ 1-5 µM), and it remains unclear how the drug-albumin complex can out-compete the unbound albumin for the binding partner on the surface of hepatocytes. Otherwise, the drug-albumin complex would need to have 100-fold higher selectivity toward its binding partner compared to unbound albumin in order to carry the drug molecules preferentially to the proximity of the OATP transporter without much competition from its unbound counterpart. Furthermore, given that the albumin concentration is significantly higher than the drug in plasma, it is likely that the drug-albumin complex maintains a stoichiometry of 1 between the drug and albumin. In other words, one can anticipate that each drug-albumin complex molecule likely only carries one drug molecule to the transporter because the total drug molecules available in plasma are very limited (1%) relative to albumin. Even if the interaction between the drug-albumin complex and its binding partner can facilitate the dissociation of drug from the protein-drug complex, it is difficult to anticipate a dramatic increase in the free drug level around the OATP transporter, given that each drug-albumin complex likely has only one drug molecule to release. Since the drug-albumin complex must compete for the binding partner with its more abundant counterpart (unbound albumin), it is difficult to rationalize how a 100-fold increase in the local drug concentration is achieved for OATP substrates such as pitavastatin and repaglinide, unless the stoichiometry of the drug-albumin complexes is much greater. In contrast, our results provide a theoretical explanation for the IVIVC disconnect of drug uptake clearance, which is in agreement with literature suggesting that the PMU is likely an artifact (Yin et al., 2022; Yin et al., 2023).

CONCLUSIONS
Hepatic clearance under-prediction has been a known challenge especially for OATP substrates, and a pragmatic strategy is to incorporate compound-specific empirical scalers into the traditional WSM to mathematically correct (Miyauchi et al., 2018; Bowman et al., 2019; Kim et al., 2019). In the present study, we demonstrate that the dynamic WSM performed well in predicting OATP-mediated hepatic clearance without clear systematic under-prediction. This was achieved without using empirical scalers or assuming “albumin-facilitated drug uptake”, confirming that the dWSM functions well for transporter-mediated hepatic drug eliminations. Contrary to the current assumption (Tess et al., 2023; Poulin et al., 2021; Francis et al., 2020; Tess et al., 2020), our results indicate that hepatic clearance of acidic drugs can be predicted with comparable accuracy to neutral and basic drugs, given that three chemical classes are not different mechanistically in regards to protein binding and disposition. However, prediction challenges can rise for some drug candidates including acidic compounds if the compounds exhibit low conversion rates by the reporter enzyme (rCYP3A4) and inadequate sensitivity in the ESI-HRMS detection (Yan et al., 2023). Also, a violation of initial reaction condition or nonlinearity of kinetics can potentially lead to inaccurate $f_{D,p}$ values and poor prediction outcomes. Although the number of drugs examined is limited, this study confirms the utility of dynamic free fraction in hepatic clearance prediction and provides compelling evidence that drug plasma binding kinetics is a key determinant in hepatic clearance.
Authorship Contributions

Participated in research design: Yan, Ma.

Conducted experiments: Ma, Huang

Performed data analysis: Yan, Ma, Hwang

Wrote or contributed to the writing of the manuscript: Yan, Ma, Hwang, Kenny, Hop.

Address correspondence to:

Dr. Zhengyin Yan, Department of Drug Metabolism and Pharmacokinetics, Genentech, 1 DNA Way, South San Francisco, CA 94080. Email: lexington1528@gmail.com

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The Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the paper.

Declaration of Competing Interest

No author has an actual or perceived conflict of interest with the contents of this article.
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REFERENCE


Yin M, Storelli F, Unadkat JD (2022) Is the protein-mediated uptake of drugs by organic anion transporting polypeptides a real phenomenon or an artifact? Drug Metab Dispos. 50:1132-1141.

**Figure Legend.**

**Figure 1.** A schematic of OATP-mediated hepatic uptake comparing two drugs with different binding affinity. R, repaglinide, a drug with a longer “OFF” time ($dT_{off,R}$); C, cerivastatin, a drug with a shorter “OFF” time ($dT_{off,C}$). Blue and red (triangle and cycle) represent drug molecules in the “OFF” and “ON” states, respectively.
Table 1. Comparison of the dynamic free fraction ($f_{D,P}$) and the fraction of unbound ($f_{u,P}$) of OATP substrates in plasma. High $f_{D,P}$ value suggests low binding affinity.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$f_{D,P}$</th>
<th>$f_{u,P}$</th>
<th>Ratio ($f_{D,P}/f_{u,P}$)</th>
<th>Binding profile in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>0.4320</td>
<td>0.03080</td>
<td>14.0</td>
<td>High binder with low binding affinity</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.1536</td>
<td>0.00428</td>
<td>35.9</td>
<td>High binder with moderate binding affinity</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.7384</td>
<td>0.56300</td>
<td>1.3</td>
<td>Low binder with low binding affinity</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.9156</td>
<td>0.13400</td>
<td>6.8</td>
<td>Moderate-to-high binder with low binding affinity</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.5983</td>
<td>0.00541</td>
<td>110.6</td>
<td>High binder with low binding affinity</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>0.0660</td>
<td>0.00734</td>
<td>9.0</td>
<td>High binder with high binding affinity</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.7469</td>
<td>0.00676</td>
<td>110.5</td>
<td>High binder with low binding affinity</td>
</tr>
</tbody>
</table>
Table 2. A summary of hepatic uptake clearance predicted using the dWSM incorporating with the dynamic free fraction ($f_{D,P}$).

<table>
<thead>
<tr>
<th>Drug</th>
<th>In Vitro PS$_{u,inf}$ (µl/min/million, cell)</th>
<th>Observed CL$_{H,P}$ (mL/min/kg)</th>
<th>CL$_{H,P}$ predicted by dWSM (mL/min/kg)</th>
<th>CL$_{H,P}$ Ratio (dWSM) (predicted/observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single dataset (Kim et al. 2019)</td>
<td>Pooled dataset (SM2*)</td>
<td>Single dataset</td>
<td>Pooled dataset</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>Range</td>
<td>Average</td>
<td>Range</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>24.2</td>
<td>62.13</td>
<td>24.2 - 100.0</td>
<td>8.93</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>62.1</td>
<td>101.80</td>
<td>62.1 - 120.8</td>
<td>8.7</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>3.55</td>
<td>3.30</td>
<td>1.8 - 4.79</td>
<td>7.2</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>4.01</td>
<td>9.54</td>
<td>4.01 - 12.6</td>
<td>8.36</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>36.2</td>
<td>70.73</td>
<td>26.7 - 154.2</td>
<td>5.59</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>77.5</td>
<td>100.85</td>
<td>46.4 - 197.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>39.2</td>
<td>81.55</td>
<td>39.2 - 114.0</td>
<td>7.18</td>
</tr>
</tbody>
</table>

* SM2, Supplemental Table SM2.
Table 3. A summary of hepatic uptake clearance predicted using the WSM incorporating with the dynamic free fraction ($f_{u,P}$).

<table>
<thead>
<tr>
<th>Drug</th>
<th>In Vitro PS$_{u,inf}$ (µL/min/million, cell)</th>
<th>Observed CL$_{H,P}$ (mL/min/kg)</th>
<th>CL$_{H,P}$ predicted by WSM (mL/min/kg)</th>
<th>CL$_{H,P}$ Ratio (WSM)</th>
<th>CL$_{H,P}$ Ratio (predicted/observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single dataset (Kim et al. 2019)</td>
<td>Pooled dataset (SM*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>24.2</td>
<td>62.13</td>
<td>8.93</td>
<td>4.12</td>
<td>0.22</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>62.1</td>
<td>101.80</td>
<td>8.7</td>
<td>1.22</td>
<td>0.09</td>
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<tr>
<td>Pravastatin</td>
<td>3.55</td>
<td>3.30</td>
<td>7.2</td>
<td>3.83</td>
<td>0.56</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>4.01</td>
<td>9.54</td>
<td>8.36</td>
<td>1.48</td>
<td>0.18</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>36.2</td>
<td>70.73</td>
<td>5.59</td>
<td>1.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>77.5</td>
<td>100.85</td>
<td>2.9</td>
<td>1.92</td>
<td>0.53</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>39.2</td>
<td>81.55</td>
<td>7.18</td>
<td>1.50</td>
<td>0.11</td>
</tr>
<tr>
<td>Geomean</td>
<td>0.20</td>
<td>0.32</td>
<td>0.15 - 0.45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SM2: Supplemental Table SM2.
Figure 1.