Research Article

Drug Metabolism & Disposition

Assumptions Underlying Hepatic Clearance Models: Recognizing the Influence of Saturable Protein Binding on Driving Force Concentration and Discrimination Between Models of Hepatic Clearance

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IVIVE  \textit{In-vitro-in-vivo} extrapolation

\(K_D\)  Equilibrium dissociation constant

\(K_m\)  Michaelis-Menten constant

\(K_{nsb}\)  Non-saturable binding constant or binding potential

\(k_{off}\)  Protein binding off rate

\(k_{on}\)  Protein binding on rate

MWSM  Modified well-stirred model

NSB  Non-saturable binding

PTM  Parallel tube model

Q  Perfusion rate

\(Q_H\)  Hepatic flow rate

\(R_{BP}\)  Blood-to-plasma ratio

\(R_N\)  Efficiency number

SB  Saturable binding

SD  Standard deviation

WSM  Well-stirred model
Abstract

One underlying assumption of hepatic clearance models is often underappreciated. Namely, plasma protein binding is assumed to be non-saturable within a given drug concentration range, dependent only on protein concentration and $K_D$. However, *in vitro* hepatic clearance experiments often use low albumin concentrations that may be prone to saturation effects, especially for high-clearance compounds, where the drug concentration changes rapidly. Diazepam isolated perfused rat liver literature datasets collected at varying concentrations of albumin were used to evaluate the predictive utility of four hepatic clearance models (the well-stirred, parallel tube, dispersion, and modified well-stirred model) while both ignoring and accounting for potential impact of saturable protein binding on hepatic clearance model discrimination. In agreement with previous literature findings, analyses without accounting for saturable binding showed poor clearance prediction using all four hepatic clearance models. Here, we show that accounting for saturable albumin binding improves clearance predictions across the four hepatic clearance models. Additionally, the well-stirred model best reconciles the difference between the predicted and observed clearance data, suggesting that the well-stirred model is an appropriate model to describe diazepam hepatic clearance when considering appropriate binding models.

Keywords: Hepatic Clearance, Saturable Plasma Protein Binding, Albumin, Diazepam, In Vitro-In Vivo Extrapolation
Significance Statement:

Hepatic clearance models are vital for understanding clearance. Caveats in model discrimination and plasma protein binding have sparked an ongoing scientific discussion. This study expands our understanding of the underappreciated potential for saturable plasma protein binding. We recognize that $f_u$ must correspond to relevant driving force concentration. These considerations can improve clearance predictions and address hepatic clearance model disconnects. Importantly, even though hepatic clearance models are simple approximations of complex physiological processes, they are valuable tools for clinical clearance predictions.
**Introduction**

"All models are wrong, some are useful." – (Box, 1976)

This paradigm can be applied to all model systems used in drug development. But one area where it becomes abundantly clear is hepatic clearance models. These models are vital for enabling *in-vitro-in-vivo* extrapolation (IVIVE) of hepatic clearance and predicting drug-drug interactions and population differences (e.g., genetic polymorphisms). However, physiological relevance and prediction accuracy vary (Ito and Houston, 2005; Camenisch and Umehara, 2012; Poulin *et al.*, 2012).

Three hepatic clearance models are commonly employed: the well-stirred model (WSM; Eq.1), parallel tube model (PTM; Eq.2), and dispersion model (DM; Eq.3). These three models describe different relationships between hepatic clearance ($CL_H$), hepatic flow rate ($Q_H$), unbound fraction in the experimental system ($f_u$) and unbound intrinsic hepatic clearance ($CL_{int,u}$). $CL_{int,u}$ is the ability of the liver to clear a compound, independent of $Q_H$ and $f_u$ (Jones *et al.*, 1984; Benet *et al.*, 2018; Benet and Sodhi, 2020; Rowland *et al.*, 2021).

\[
WSM: CL_H = \frac{Q_H f_u CL_{int,u,WSM}}{Q_H + f_u CL_{int,u,WSM}} \quad (1)
\]

\[
PTM: CL_H = Q_H \left(1 - e^{-f_u CL_{int,u,PTM}/Q_H}\right) \quad (2)
\]

\[
DM: CL_H = Q_H \left(1 - \frac{-\left((1+\alpha)^2 e^{-\left((1-\alpha)/2DN-\left(1-\alpha/2DN\right)^2\right)}\right)}{(1+\alpha)^2 e^{-\left(1+\alpha/2DN\right)}}\right) \quad (3)
\]

\[a = \sqrt{1 + 4D_N \frac{f_u CL_{int,u,DM}}{Q_H}} \quad (3a)\]

In *vivo*, the $f_u$ can be either the $f_u$ in blood ($f_{u,b}$) or plasma ($f_{u,p}$), which should be corrected for the blood-to-plasma ratio ($R_{BP}$). However, in *in vitro* and *ex vivo* systems, the $f_u$ represents the experimentally measured $f_u$ ($f_{u,exp}$) in the respective system. Additionally, $Q_H$ can refer to the *in vivo* hepatic blood flow or an experimental perfusion rate.
The main differences between the three hepatic models are the applied flow and extent of mixing. The WSM assumes that the system is instantaneously well mixed (Pang and Rowland, 1977a; Rowland et al., 2021). In contrast, the PTM assumes that the liver consists of identical parallel tubes with evenly distributed enzymes without mixing (Pang and Rowland, 1977a; Rowland et al., 2021). Finally, the DM is an intermediate state between WSM and PTM with different degrees of mixing described by the dispersion number \( \Delta N \), estimated with non-eliminated markers (Roberts and Rowland, 1986b). The WSM and PTM are extreme cases of the DM where \( \Delta N \) is infinity or zero, respectively (Roberts and Rowland, 1986a; Diaz-Garcia et al., 1992).

Recently, an additional fourth model of hepatic clearance was introduced, the modified well-stirred model (MWSM; Eq.4; (Hsu et al., 2021)).

\[
\text{MWSM: } \text{CL}_{int,u,MWSM} = f_u \times \text{CL}_{int,u,MWSM}
\]  

(4)

However, the MWSM does not follow the common assumption that \( \text{CL}_{int,u,MWSM} \) is independent of \( Q_H \) (Hsu et al., 2021).

While there are theoretical differences, the hepatic clearance models are built on shared fundamental assumptions. First, the models are derived from Michaelis-Menten kinetics, Fick's diffusion principle, and mass balance considerations (Pang and Rowland, 1977a; Michaelis et al., 2011). Second, the definition of \( \text{CL}_H \) is model-independent and only dependent on \( Q_H \) and the extraction ratio (ER; Eq.5; (Rowland and Pang, 2018, 2022; Korzekwa and Nagar, 2023)).

\[
\text{ER, a measure of elimination efficiency, can be determined from the concentration entering } \left( C_{in} \right) \text{ and leaving the liver } \left( C_{out} \right).
\]

\[
\text{CL}_H = Q_H \times \text{ER} = Q_H \times (1 - F) = Q_H \times \frac{C_{in} - C_{out}}{C_{in}}
\]  

(5)

Third, drugs establish a rapid equilibrium between sinusoidal, extracellular and intracellular spaces (Pang and Rowland, 1977a; Roberts and Rowland, 1986a). Fourth, drugs are fully bioavailable, e.g., after intravenous dosing. Fifth, clearance occurs only in the liver, thus total
body clearance should be corrected for other organ clearances, including renal clearance (Jusko and Li, 2021; Rowland et al., 2021; Rowland and Pang, 2022).

And finally, hepatic clearance is based on the free drug hypothesis. Therefore, only unbound drug is available for elimination (Pang and Rowland, 1977a; Roberts and Rowland, 1986a). Furthermore, plasma protein binding is typically considered linear or non-saturable, characterized by fast on- ($k_{on}$) and off-rates ($k_{off}$), and $f_u$ is assumed to be constant throughout the entire liver (Roberts and Rowland, 1986a; Bteich, 2019).

When comparing the influence of changes in $f_u$ across clearance models, $\text{CL}_{\text{int},u}$ predictions are consistent for low ER drugs (Pang and Rowland, 1977a). However, predictions for high ER compounds can vary significantly depending on the applied model (Pang and Rowland, 1977b; Rowland et al., 1984; Hale et al., 1991; Sodhi et al., 2020). Common explanations for poor clearance predictions and discrepancies among models are lack of physiological relevance, use of incorrect reference concentrations, and theoretical issues with the applied hepatic clearance models (Rowland et al., 1973; Pang and Rowland, 1977b; Roberts and Rowland, 1986a; Diaz-Garcia et al., 1992; Benet and Sodhi, 2020, 2022; Kochak, 2020; Sodhi et al., 2020). However, several underlying assumptions have not been thoroughly tested and could explain the discrepancies.

While further evaluation of the principles of Michaelis-Menten kinetics and non-rate-limiting plasma protein binding may be beneficial, it appears that these assumptions are met in traditional IVIVE approaches (Pang and Rowland, 1977b; Yasumori et al., 1993; Baker and Parton, 2007; Michaelis et al., 2011). Therefore, we focus on the assumption that plasma protein binding is linear, not saturable. If $f_u$ is saturable, it may change throughout the liver and differ between $C_{in}$ and $C_{out}$. Therefore, we hypothesize that the lack of prediction accuracy may be caused by poor understanding of the dynamic binding processes and application of incorrect, linear $f_u$ terms.
To test the effect of saturable plasma protein binding on hepatic clearance model discrimination, we explored a high-quality IPRL dataset comparing diazepam clearance at different human serum albumin (HSA) concentrations (Wang and Benet, 2019; Hsu et al., 2021). While there is data available on many experimental models and drugs, we chose diazepam for our case study as this IPRL dataset provided the richest data source for albumin-related changes in $\text{CL}_H$. This case study serves as a universal reminder to test and carefully consider all underlying assumptions when applying $\text{CL}_H$ models.
Methods

IPRL and equilibrium dialysis data, initially published by Wang and Benet at the University of California San Francisco, USA (Wang and Benet, 2019), and Hsu et al. at the National Defense Medical Center, Taipei, Taiwan (Hsu et al., 2021), were analyzed to evaluate the impact of saturable plasma protein binding on hepatic clearance model discrimination. The original data and key experimental parameters are summarized in Table S1.

Saturable Plasma Protein Binding: The dilution factor (D) was calculated by comparing the HSA concentration in plasma (4%) to that in the perfusate (Eq. 6).

\[
D = \frac{\% \text{HSA in Whole Plasma}}{\% \text{HSA in Perfusate}}
\] (6)

Saturable and non-saturable binding parameters were estimated based on Eq. 7 (Kalvass et al., 2018). The saturable unbound fraction (\(f_{u,sat}\)) is determined by the maximal binding capacity (\(B_{\text{max}}\)), protein binding dissociation constant (\(K_D\)), and non-saturable binding constant or binding potential (\(K_{\text{nsb}}\)). These parameters were estimated from the plasma protein binding data (Wang and Benet, 2019; Hsu et al., 2021) with Eq. 7 using GraphPad Prism 9.1.0 with 1/X^2 weighting (GraphPad Software, San Diego, CA, USA).

\[
f_{u,sat} = \frac{C - \left(\frac{B_{\text{max}}}{D} + K_D \left(\frac{1 + K_{\text{nsb}}}{D}\right)\right)}{\sqrt{\left(C - \left(\frac{B_{\text{max}}}{D} + K_D \left(\frac{1 + K_{\text{nsb}}}{D}\right)\right)\right)^2 + 4K_D \left(\frac{1 + K_{\text{nsb}}}{D}\right)C}}
\] (7)

\[
K_{\text{nsb}} = \frac{B_{\text{max,nsb}}}{K_{D,\text{nsb}}}
\] (7a)

where \(C\) is the total diazepam concentration used for equilibrium dialysis; \(C=1 \, \mu\text{g/ml}=3.512 \, \mu\text{M}\) in both experiments (Wang and Benet, 2019; Hsu et al., 2021).

Eq. 7 includes two binding components, saturable (SB) and non-saturable (NSB), that were considered separately and together, creating three distinct protein binding models. First, plasma protein binding follows the full model, including saturable and non-saturable binding components (SB + NSB). Second, albumin binding is non-saturable (NSB only); therefore, \(B_{\text{max}}\)
is 0 (Bteich, 2019). The third model considers only saturable plasma protein binding while constraining $K_{nas}$ to 0 (SB only). Model fits were compared using the weighted $R^2$ and the sample-size adjusted Akaike's Information Criterion (AICc) determined in GraphPad Prism 9.1.0.

Concentration-dependent changes in diazepam $f_{u,sat}$ were modeled at different HSA concentrations and dilution factors $D$ using the estimated parameters and Eq.7.

**Hepatic Clearance:** Overall hepatic clearance ($CL_H$), extraction ratio (ER), and hepatic availability ($F$) were calculated for each HSA concentration with the model-independent Eq.5 (Rowland *et al.*, 1973; Rowland and Pang, 2018; Wang and Benet, 2019).

Hepatic clearance is driven by different concentrations depending on mixing and dispersion factors applied in the respective models. According to the WSM, the concentration is the same across the entire organ and equal to the concentration $C_{out}$, which drives clearance (Eq.8; Pang and Rowland, 1977a; Rowland *et al.*, 2021)). In the PTM, the drug concentration continuously decreases throughout the liver until it reaches $C_{out}$ at the portal vein. Under these conditions, hepatic clearance is driven by the logarithmic average of $C_{in}$ and $C_{out}$ (Eq.9; Pang and Rowland, 1977a; Rowland *et al.*, 2021)). The driving concentration in the DM is the average hepatic concentration with is determined by the extent of drug distribution in the hepatic blood compartment and the $D_N$ (Eq.10; Roberts and Rowland, 1986a; Diaz-Garcia *et al.*, 1992; Rowland *et al.*, 2021)). On the other hand, the MWSM assumes that hepatic clearance is driven by $C_{in}$ rather than the hepatic concentration (Eq.11; Wang and Benet, 2019; Hsu *et al.*, 2021)).

The driving concentrations, $C_{WSM}$, $C_{PTM}$, $C_{DM}$, and $C_{MWSM}$, were determined using Eq.8-11 (Benet *et al.*, 2021; Hsu *et al.*, 2021). In the following step, the respective $f_{u,sat}$ were calculated using Eq.7 and the estimated binding parameters, $C_{in}$, and $C_{out}$.

$$C_{WSM} = C_{out}$$

$$C_{PTM} = \frac{C_{in}-C_{out}}{\ln C_{in}-\ln C_{out}}$$

$$C_{DM} = \frac{C_{in}^{2}C_{out}}{C_{in}^{2}+C_{out}^{2}}$$

$$C_{MWSM} = \frac{C_{in}^{2}C_{out}}{C_{in}^{2}+C_{out}^{2}}$$
\[ C_{DM} = Q_H \times \frac{C_{in} - C_{out}}{CL_H} \approx C_{in} \]  
(10)

\[ C_{MWSM} = C_{in} \]  
(11)

The relationship between hepatic availability (F) and the HSA concentration in each hepatic clearance model (Eq. 12-15) was fitted to the experimental data in GraphPad Prism 9.1.0 using 1/Y weighting.

\[ F_{WSM} = 1 - \frac{f_u \times CL_{int,u,WSM}}{Q_H + f_u \times CL_{int,u,WSM}} \]  
(12)

\[ F_{PTM} = e^{-f_u \times CL_{int,u,PTM}} / Q_H \]  
(13)

\[ F_{DM} = e^{1 - \alpha / 2D_N} = e \]  
(14)

\[ F_{MWSM} = 1 - \frac{f_u \times CL_{int,u,MWSM}}{Q_H} \]  
(15)

\( D_N \) for these experiments was previously reported (\( D_N = 0.34 \) (Diaz-Garcia et al., 1992)).

The respective \( CL_{int,u} \) values were fitted and used to model ER and \( CL_H \). Model fit was evaluated using the weighted \( R^2 \) and AICc provided by GraphPad Prism 9.1.0.
Results

Saturable Albumin Binding

Diazepam binding to HSA was best described by a single-site saturable binding model ($B_{\text{max}} = 136.8 \mu M$, $K_D = 0.914 \mu M$, $K_{\text{nbs}} = 0 \mu M$; $R^2 = 0.9882$; AICc = -193.7; Fig.1A; Table1).

Additionally, the diazepam concentration-dependent change in $f_{u,\text{sat}}$ at different HSA concentrations was evaluated (Fig.1B; Fig.S1). At the diazepam concentration used in the IPRL experiments (3.512 µM, open circles), protein binding saturation is more pronounced at low (0.025-0.1%; Fig.S1B) than at high HSA concentrations (1-4%; Fig.S1C).

Hepatic Clearance Predictions

The WSM (solid green line; Fig.2A; Table2) best described the observed diazepam F, followed by the DM (dashed purple line) and PTM (dashed red line). Introducing the MWSM did not significantly improve the data fitting when saturable albumin binding was considered (blue dotted line; Fig.2A; Table2). The same model rank order was observed for other clearance measures, such as ER and CL_H (Fig.2B; Fig.S2A-C).

Additionally, $f_{u,\text{sat}}$ for the respective model-dependent driving concentrations (Fig.S2; Table2) better described the observed diazepam clearance data for each of the four hepatic clearance models than the measured $f_{u,\text{exp}}$ at $C_{\text{in}}$ (3.512 µM), across all three clearance parameters (F, ER, and CL_H).

CL_{int,u} is independent of $f_{u,\text{exp}}$ and, therefore, should not vary with the HSA concentration; the observed CL_{int,u} should be similar to the model fitted CL_{int,u} (Fig.3; Table2). The average fold deviation (AFD) was calculated as the ratio of the observed to the fitted CL_{int,u}. The AFD was close to 1 for the WSM (Fig.3; Table2; green circle) and using $f_{u,\text{sat}}$ (solid green circle; AFD=1.2) decreased the variability in the data compared to $f_{u,\text{exp}}$ (open green circle; AFD=1.6). The AFD deviated from unity by more than 3-fold for the DM (Fig.3; Table2; purple diamond), PTM (red square), and MWSM (blue triangle), independent of saturable (closed symbols; AFDs for PTM: 3.8, DM: 3.1, MWSM: 3.1) or linear binding (open symbols; AFDs for PTM: 3.7, DM: 3.3,
MWSM: 3.3). The DM and MWSM deviate most at low HSA concentrations, while the PTM deviates more at high HSA concentrations for both $f_{u,\text{exp}}$ and $f_{u,\text{sat}}$ (Fig.3).
Discussion

As outlined in the introduction, we hypothesize that the lack of clearance prediction accuracy may be caused by poor understanding of the dynamic binding processes and application of incorrect \( f_u \) terms. The static equilibrium \( f_{u,\text{exp}} \) may not accurately reflect the dynamic binding processes in vitro and in vivo.

Here, we show that albumin binding of diazepam is saturable at low HSA concentrations. Therefore, \( f_{u,\text{sat}} \) changes with the drug concentration or plasma protein dilution factor. Saturable albumin binding parameters were determined from the protein binding data (Fig.1; Fig.S1; Table1) and used to model hepatic clearance. Saturable diazepam binding better described the observed \( F \) and \( CL_H \) measurements and predicted \( CL_{\text{int},u} \) across all four hepatic clearance models (Fig.2; Fig.3; Fig.S2&3; Table2; TableS2). Overall, the WSM fits the observed clearance data the best across all HSA concentrations and clearance endpoints (Fig.2; Fig.3; Fig.S2).

Saturable plasma protein binding has been well described for α-1-acid glycoprotein (Smith and Waters, 2018; Bteich, 2019). On the other hand, only a few examples of saturation of albumin binding in plasma have been reported. Cefazolin \( f_{u,p} \) increases with drug concentration in vitro in spiked human plasma and ex vivo patient plasma samples and varied within the same patient at trough and peak concentrations (Vella-Brincat et al., 2007). Other examples of saturable albumin binding include valproic acid, indomethacin, ceftriaxone, and temocillin (Bowdle et al., 1980; Alexandre and Fantin, 2018; Nation et al., 2018). Based on our predictions, saturation of plasma protein binding is unlikely at clinically relevant diazepam concentrations (\( C_{\text{max}} = 0.28-2.21 \) µM (Cortellis, 2022)) in human plasma (4% HSA; Fig.1B; Fig.S1C). However, in vitro assays are often run at lower plasma protein levels. Therefore, in vitro clearance measurements may be more prone to saturation of plasma protein binding (Fig.S1B), which may contribute to IVIVE disconnects. Based on these observations, we recommend performing in vitro assays at physiologically relevant plasma protein and drug concentrations (4% HSA, 100% plasma), as
recently outlined for experimental best practices (Schulz et al., 2023). Alternatively, plasma protein binding parameters can be fitted using Eq.7 and applied to the relevant assay conditions.

Additional experimental caveats should be considered. The determined $B_{\text{max}}$ (136 $\mu$M; Table1) is lower than the albumin plasma concentration (~700 $\mu$M) and the potential binding site concentration ($n=1$, ~700 $\mu$M (Watkins et al., 1994; Krause and Goss, 2018)). This discrepancy may be explained by considering that albumin binding sites can be occupied by other endogenous ligands, such as bilirubin or fatty acids (Koch-Weser and Sellers, 1976; Inoue et al., 1985; Weisiger, 1985; Zucker et al., 1995). Significant differences in binding are reported for native and fatty acid-free albumin (Rowland et al., 1984; Fujino et al., 2018; Li et al., 2020). This competition prevents drug binding, and saturation may occur at lower drug concentrations.

Several groups have explored diazepam IPRL at varying HSA concentrations for hepatic clearance model discrimination (Rowland et al., 1984; Diaz-Garcia et al., 1992; Wang and Benet, 2019; Hsu et al., 2021). The impact of different binding processes on diazepam clearance, including non-specific binding to tubing and other perfusion equipment, was evaluated. While non-specific binding was not observed in the original studies (Rowland et al., 1984; Diaz-Garcia et al., 1992), Wang and Benet reported that $f_{u,\text{exp}}$ did not reach 100%, even in HSA-free buffer, indicating drug loss due to non-specific binding (Wang and Benet, 2019). Additionally, $f_{u,\text{exp}}$ does not depend on the diazepam concentration if albumin is in excess of the drug and most binding sites are available (Rowland et al., 1984). However, this may not be the case with low HSA concentrations. Furthermore, the authors discussed that $f_{u,\text{exp}}$ may change during the liver passage. Yet, the $f_{u,\text{exp}}$ measured with equilibrium dialysis was not significantly different in perfusate and effluent. Therefore, $f_{u,\text{exp}}$ was assumed to be constant for each HSA concentration (Rowland et al., 1984).
However, the lowest albumin concentration in Rowland’s study (1%) does not show saturation based on our modeling results (Figure S1; Rowland et al., 1984) and the changes in $f_{u,exp}$ may not be apparent under these conditions.

In previous diazepam IPRL studies, a significant change in diazepam $F$ was observed when the protein concentration was low ($f_{u,exp} \approx 1$), while clearance was attenuated in the presence of albumin (Rowland et al., 1984; Diaz-Garcia et al., 1992; Wang and Benet, 2019; Hsu et al., 2021). Additionally, model discrimination is only possible at low HSA concentrations when diazepam is rapidly cleared (Diaz-Garcia et al., 1992; Wang and Benet, 2019; Hsu et al., 2021).

As previously shown, clearance of diazepam in IPRL was better described by the WSM or PTM than DM (Rowland et al., 1984; Diaz-Garcia et al., 1992; Wang and Benet, 2019). However, Hsu et al. claimed that the WSM is only superior at high HSA concentrations ($f_{u,exp} < 0.8$). At low HSA concentrations ($f_{u,exp} > 0.8$), the MWSM better predicts diazepam availability (Hsu et al., 2021). The authors propose the MWSM as an extreme case where drugs are highly unbound, and $C_{in}$ drives clearance. However, we were unable to confirm this finding.

In our hands, the MWSM performed poorly, both with $f_{u,exp}$, and when considering saturable protein binding, while the WSM most accurately predicted diazepam $F$ in both cases (Fig. 2; Fig. 3; Fig. S2; Table 2). However, we should note that our analysis is based on the HSA concentration, while previous evaluations have compared changes in $F$ with $f_u$ (Rowland et al., 1984; Diaz-Garcia et al., 1992; Wang and Benet, 2019; Hsu et al., 2021). This change was necessary since $f_{u, sat}$ is different for each model. Therefore, data comparison across hepatic clearance models was only possible when plotting $F$ against HSA concentration. To compare our data with the previously published results, we included the comparison using $f_u$ in the supplemental information (Fig. S3).

For the data analysis, several experimental considerations should be examined. First, in the presence of HSA, $C_{DM}$ is approximately the same as $C_{in}$ throughout the perfusion. Therefore,
there is no significant difference between the $f_{u,\text{sat}}$ for the DM and MWSM. However, the model fit varies significantly.

Second, each perfusion started and ended with the 0% HSA group to ensure that the clearance capacity of the liver did not change throughout the experiment (Wang and Benet, 2019; Sodhi et al., 2020; Hsu et al., 2021). While ER is similar for the repetitions, $F$, $C_{\text{out}}$, and $CL_{\text{int, u}}$ vary drastically. The discrepancy between the two endpoints is likely due to non-specific binding to the apparatus and tissue, which is more pronounced during the first perfusion cycle and likely saturated at later time points. Therefore, we only included the 0% HSA data from the end of the perfusions in our analysis. However, since the 0% HSA data points may be problematic due to non-specific binding and potential loss of enzyme activity, we also analyzed all HSA concentrations (0.025-2%), excluding 0% (Fig.S4; TableS2). While the $CL_{\text{int, u}}$ estimates for each model changed slightly, the overall model discrimination and rank order remained the same. Therefore, inclusion or exclusion of the 0% HSA condition did not affect data interpretation.

Third, the $D_N$ used in this study and the original publications was a literature value ($D_N=0.34$) and not measured in the respective liver preparations (Diaz-Garcia et al., 1992; Wang and Benet, 2019; Hsu et al., 2021). The $D_N$ is generally determined with non-cleared reference markers, such as red blood cells or sucrose. Including these controls in each IPRL experiment could improve the model fit of the DM by correcting for the dispersion processes in the respective liver preparations. The importance of accurate $D_N$ determination is further highlighted by a recent analysis by Sodhi et al. which found that $D_N$ fitted from IPRL experiments with cleared compounds are often large, causing the DM to approach the WSM (Sodhi et al., 2020).

Additionally, several theoretical phenomena must be carefully considered for hepatic model discrimination. First, since clearance is model-independent, all hepatic clearance models have the same value for $F$, $ER$, and $CL_H$ (Jusko and Li, 2021; Rowland et al., 2021; Rowland and Pang, 2022; Korzekwa and Nagar, 2023). However, depending on the degree of mixing and flow patterns, the $CL_{\text{int, u}}$ values vary significantly (Yadav et al., 2021).
Second, any clearance measure can be helpful for a first critical model examination (Pang and Rowland, 1977a; Jones et al., 1984). However, based on previous experiments, ER appears to be a poor model discriminator because it differs least when CL\textsubscript{H} is high, which is “easy” to predict, and is most sensitive to Q\textsubscript{H} (Pang and Rowland, 1977b). Therefore, F appeared to be the most appropriate model discriminator and was used in this study (Rowland et al., 1984). Furthermore, the original analysis of these IPRL data focused on F as a clearance parameter (Wang and Benet, 2019; Hsu et al., 2021) and using the same parameter in our analysis allows direct comparison with the original data interpretation.

Third, hepatic clearance models have distinct physiological relevance. The WSM is considered the least physiological model of hepatic clearance since it assumes instantaneous well-stirred conditions. However, when looking at branching, anastomosis, and mixing within the interconnected system of the liver sinusoid, the WSM may accurately describe physiological processes (Pang and Rowland, 1977b). Similar assumptions also underlie the DM, where local movements against flow direction improve mixing, especially at branch points (Roberts and Rowland, 1986a). On the other hand, the MWSM does not follow the definition of CL\textsubscript{int,H}, which is independent of protein binding and Q\textsubscript{H} (Jones et al., 1984; Benet et al., 2018; Benet and Sodhi, 2020; Rowland et al., 2021). While the MWSM includes f\textsubscript{u}, it does not correct for Q\textsubscript{H} (Benet et al., 2018; Hsu et al., 2021). Therefore, it is biased to highly bound, low ER predictions where the hepatic clearance models cannot be distinguished. The MWSM has no physiological relevance for high ER compounds, such as diazepam, where Q\textsubscript{H} limits clearance. Therefore, the utility of the MWSM may be limited.

Fourth, it is important to distinguish between blood and plasma CL\textsubscript{H} when performing IVIVE, especially when the explored drugs partition into blood cells or bind to blood components other than plasma proteins (Yang et al., 2007; Rowland et al., 2021). However, since IPRLs were performed with Krebs-Ringer bicarbonate buffer containing only diazepam and HSA, with no cells or other blood components, we can assume that the blood to plasma ratio is 1 and
distinction between \( f_{u,b} \) and \( f_{u,p} \) is not necessary. Therefore, the relevant measured \( f_{u,exp} \) and modeled \( f_{u,sat} \) values were used for the modeling exercises.

Lastly, while the diazepam saturable binding data presented here provides a promising strategy for improving \( CL_H \) predictions, additional compounds and assay formats should be explored to evaluate the potential impact of saturable plasma protein binding. Other IPRL datasets were explored for assessment of saturable binding, but did not provide sufficient information for conclusive analysis, due to the lack of data at very low HSA concentrations (Pang and Rowland, 1977b; Jones et al., 1984; Roberts and Rowland, 1986b). Nevertheless, this analysis provides an important first step to understanding underlying misconceptions and disconnects concerning plasma protein binding in \( CL_H \) predictions.

As an aside, saturable plasma protein binding may partially explain observed plasma protein-mediated uptake effects (PMUE). As outlined in our recent review paper on the topic (Schulz et al., 2023), we believe that other alternative explanations, such as saturable plasma protein binding, can consolidate the observed PMUE under the free drug hypothesis (Schulz et al., 2023). If plasma protein binding is saturated, the \( f_{u,sat} \) available for clearance is larger than expected based on linear binding assumptions. Therefore, \( CL_{int,u} \) appears larger, which has been attributed to PMUE (Schulz et al., 2023). In the present analysis, \( CL_{int,u} \) increases with increasing HSA, when considering linear \( f_{u,exp} \), which could be interpreted as PMUE. However, \( CL_{int,u} \) is constant across the HSA concentrations when correcting for saturable plasma protein binding in the WSM. Therefore, PMUE may be an artifact of applying inaccurate \( f_u \) values and violating underlying model assumptions. However, further exploring the contribution of saturable binding to PMUE goes beyond the scope of this manuscript.

Finally, in concordance with George Box (Box, 1976), we demonstrate that “all models are wrong, but some are useful.” Careful testing of all underlying assumptions is vital for proper model application. To this end, we evaluated the linear binding assumption underlying hepatic clearance models.
The intent of this work was to evaluate the assumptions underlying hepatic clearance models applicable to *in vitro*, *ex vivo* and *in vivo* models. Rather than predicting a distinct physiologically relevant *in vivo* CL\textsubscript{Hi}, we evaluated the predicted CL\textsubscript{Hi} as a function of changing experimental conditions. To this end, consideration of saturable plasma protein binding improved hepatic clearance predictions for diazepam. High-clearance compounds, where the concentration changes rapidly and significantly throughout the experiment, may be prone to this phenomenon. Most importantly, saturable binding can reconcile the observed clearance data under the WSM, which described the observed clearance processes well, showing that while clearance models may not be physiologically sound, they can be useful for clearance predictions. Introducing new hepatic clearance models does not improve clearance predictions when f\textsubscript{u,sat} values are employed. Further research should focus on enhancing f\textsubscript{u} measurements and predictions, as a better understanding of binding processes will improve our understanding of the mechanisms underlying IVIVE disconnects. In addition to saturable protein binding, other potential causes of poor f\textsubscript{u} predictions should be evaluated, including rate-limiting dissociation.
Acknowledgements:

We thank Dr. Leslie Z. Benet (UCSF) and Dr. Jasleen Sodhi (Septerna) for their critical review of the manuscript and their scientific comments and discussions.

Data Availability Statement:

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contributions:

Participated in research design: Schulz Pauly, Phipps, Kalvass

Performed data analysis: Schulz Pauly, Wang

Wrote or contributed to the writing of the manuscript: Schulz Pauly, Wang, Phipps, Kalvass
References:


Cortellis (2022) Drug Discovery Intelligence (CDDI), Clarivate.


Sodhi JK, Wang H-J, and Benet LZ (2020) Are there any experimental perfusion data that preferentially support the dispersion and parallel tube models over the well-stirred model of organ elimination? *Drug Metab Dispos* **48**:dmd.120.090530.


Footnotes

This manuscript was sponsored and funded by AbbVie.

Conflict of Interest Statement:

All authors are employees of AbbVie and may own AbbVie stock. AbbVie contributed to the design; participated in the collection, analysis, and interpretation of data, and in writing, reviewing, and approval of the final publication. The manuscript contains no proprietary AbbVie data.
Figure Legends

Figure 1. Saturable albumin binding of diazepam. A) The measured unbound fraction of diazepam (1 µg/ml=3.512 µM; (Mean ± standard deviation (SD))) \( f_{u,exp} \) varies with the dilution factor \( D \) of HSA compared to plasma. Non-saturable binding (solid grey line, NSB only) poorly describes the observed binding phenomena. The full saturable binding model accurately represents diazepam binding; however, non-saturable binding is negligible (black dotted line). Saturable binding with no non-saturable binding accurately represents diazepam binding (solid red line, SB only). B) The concentration dependence of \( f_{u,sat} \) varies with the HSA concentration (0.025%-4%). Diazepam albumin binding is more saturable at low HSA concentrations than at high HSA concentrations (Fig.S1). The open circles represent the measured \( f_{u,exp} \) at 3.512 µM diazepam, while the lines reflect the modeled \( f_{u,sat} \) values at different HSA concentrations (0.025% - blue dashed line, 0.075% - green dashed line, 0.5% light blue dashed line, 2% - light green dashed line, 0.04% solid purple line, 0.1% - orange dotted line, 1% - light purple dotted line, 4% - solid red line).

Figure 2. Hepatic Availability and Clearance Predictions. Considering saturable plasma protein binding, the WSM (solid green line) described the hepatic availability (\( F(A) \)) and hepatic clearance (\( CL_H (B) \)) data (black circles) best, compared to PTM (red dashed line) and DM (purple dashed line). Introducing new hepatic clearance models, like the MWSM (blue dotted line), does not further improve clearance predictions when appropriate \( f_{u,sat} \) values are employed (Fitting parameters in Table2 (Mean ± SD)).

Figure 3. Hepatic Clearance Predictions. The observed \( CL_{int,u} \) at different HSA concentrations were compared with the modeled \( CL_{int,u} \) for the respective models. \( CL_{int,u} \) for \( f_{u,sat} \) (solid symbols) are more consistent across HSA concentrations than \( CL_{int,u} \) for \( f_{u,exp} \) (open symbols) across all four hepatic clearance models. Saturable binding improves \( CL_{int,u} \) prediction accuracy across all four hepatic clearance models. Additionally, the WSM \( CL_{int,u} \) (green circles) best describes the
observed data (Mean ± SD), while the PTM (red squares), DM (purple diamond) and MWSM (blue triangle) deviate significantly.
Tables

Table 1. Saturable Protein Binding Parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SB + NSB</th>
<th>SB only</th>
<th>NSB only</th>
</tr>
</thead>
<tbody>
<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt; [µM]</td>
<td>136.8</td>
<td>136.8</td>
<td>0</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt; [µM]</td>
<td>0.9140</td>
<td>0.9140</td>
<td>-</td>
</tr>
<tr>
<td>K&lt;sub&gt;nsb&lt;/sub&gt;</td>
<td>~4.930*10&lt;sup&gt;-32&lt;/sup&gt;</td>
<td>0</td>
<td>110</td>
</tr>
<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt; (weighted)</td>
<td>0.9882</td>
<td>0.9882</td>
<td>0.8601</td>
</tr>
<tr>
<td>AICc</td>
<td>-189.7</td>
<td>-193.7</td>
<td>-162.4</td>
</tr>
</tbody>
</table>

Best fit values of the saturable albumin binding parameters were derived from the IPRL datasets (Wang and Benet, 2019; Hsu et al., 2021) in GraphPad Prism 9.1.0. with 1/X<sup>2</sup> weighting.
### Table 2. Unbound Intrinsic Clearance Prediction Accuracy.

<table>
<thead>
<tr>
<th>Model</th>
<th>WSM</th>
<th>PTM</th>
<th>DM</th>
<th>MWSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fitted CL\textsubscript{int,u} [ml/min]</td>
<td>145.4</td>
<td>29.03</td>
<td>56.27</td>
<td>14.43</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>131.7-157.6</td>
<td>24.5-31.8</td>
<td>50.6-61.3</td>
<td>12.9-14.8</td>
</tr>
<tr>
<td>(R^2) (weighted)</td>
<td>0.9870</td>
<td>0.8953</td>
<td>0.9637</td>
<td>0.5463</td>
</tr>
<tr>
<td>AICc</td>
<td>-80.77</td>
<td>-51.89</td>
<td>-67.68</td>
<td>-32.16</td>
</tr>
<tr>
<td>Average Fold Deviation (AFD)</td>
<td>Observed/Modeled</td>
<td>1.2±0.2</td>
<td>3.8±0.9</td>
<td>3.1±0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model</th>
<th>WSM</th>
<th>PTM</th>
<th>DM</th>
<th>MWSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fitted CL\textsubscript{int,u} [ml/min]</td>
<td>103.9</td>
<td>38.74</td>
<td>56.14</td>
<td>15.57</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>84.4-119.1</td>
<td>38.4-42.2</td>
<td>49.2-61.8</td>
<td>14.3-16.0</td>
</tr>
<tr>
<td>(R^2) (weighted)</td>
<td>0.9497</td>
<td>0.9275</td>
<td>0.9474</td>
<td>0.6921</td>
</tr>
<tr>
<td>AICc</td>
<td>-63.23</td>
<td>-58.42</td>
<td>-62.87</td>
<td>-37.90</td>
</tr>
<tr>
<td>Average Fold Deviation (AFD)</td>
<td>Observed/Modeled</td>
<td>1.6±0.3</td>
<td>3.7±0.9</td>
<td>3.3±0.3</td>
</tr>
</tbody>
</table>

CL\textsubscript{int,u} was determined by fitting the hepatic availability (F) data for each model. Consistency of CL\textsubscript{int,u} across HSA concentrations was compared for the four hepatic clearance models, \(f_{u,exp}\), and \(f_{u,sat}\) in GraphPad Prism 9.1.0. with \(1/Y\) weighting. The average fold deviation (AFD) between the observed and model fitted CL\textsubscript{int,u} was reported as Mean ± SD.
Figure 1

A

Measured $f_{u, exp}$ [%]

Dilation Factor D

% HSA

B

Fitted $f_{u, sat}$ [%]

Diazepam Concentration [µM]

$0.025\%$ $0.075\%$ $0.5\%$ $2\%

$0.04\%$ $0.1\%$ $1\%$ $4\%$ $3.512 \, \mu M$