

Protein Binding and Hepatic Clearance: Re-Examining the Discrimination between Models of Hepatic Clearance with Diazepam in the Isolated Perfused Rat Liver Preparation

Hong-Jaan Wang and Leslie Z. Benet

School of Pharmacy, National Defense Medical Center, Taipei, Taiwan (H.-J.W.), and Department of Bioengineering and Therapeutic Sciences, Schools of Pharmacy and Medicine, University of California San Francisco, San Francisco, California (L.Z.B.)

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ABSTRACT

This study re-examined the hepatic extraction for diazepam, the only drug for which isolated perfused rat liver (IPRL) studies have been reported not to be consistent with the well stirred model of organ elimination when only entering and exiting liver concentration measurements are available. First, the time dependency of diazepam equilibrium fraction unbound measurements from 4 to 24 hours was tested, reporting the continuing increases with time. The results showed that the time dependency of equilibrium protein-binding measurements for very highly bound drugs may be an issue that is not readily overcome. When examining C_{out}/C_{in} (F_{obs}) measurements for diazepam when no protein is added to the incubation media, IPRL outcomes were consistent with previous reports showing marked underpredictability of in vivo clearance from in vitro measures of elimination in the absence of protein for very highly bound drugs, which is markedly diminished in the presence of albumin. F_{obs} for diazepam at additional low concentrations of protein that would allow discrimination of the models of hepatic elimination produced results that were not consistent with the dispersion and

parallel-tube models. Therefore, although the outcomes of this study were similar to those reported by Rowland and co-workers, when no protein is added to the perfusion media, these IPRL results for diazepam cannot be reasonably interpreted as proving that hepatic organ elimination is model-independent or as supporting the dispersion and parallel-tube models of organ elimination.

SIGNIFICANCE STATEMENT

The only drug experiments for which isolated perfusion rat liver studies do not support hepatic clearance being best described by the well stirred model have been carried out with diazepam at zero protein concentration. This study repeated those studies, confirming the previous results at zero protein concentration, but the addition of low protein-binding conditions capable of differentiating the various models of hepatic elimination are more consistent with the well stirred model of hepatic elimination. These experimental studies do not support the preference for alternate models of hepatic elimination or the proposal that hepatic organ clearance is model-independent.

Introduction

In 2018, Benet et al. (2018) proposed that calculating organ clearance (CL) as the product of the extraction ratio (ER) and the blood flow to the organ (Q) as given in eq. 1, as first proposed by Rowland (1972), was consistent with the well stirred model in pharmacokinetics. They maintain that this is true since the driving force concentration for elimination is assumed to be the concentration entering the organ, C_{in} , rather than the concentration within the organ: $Rate\ Out = Q \cdot (C_{in} - C_{out}) = CL \cdot C_{in}$. That is, there is no incremental clearance within the organ, and all elimination is driven only by the entering concentration:

$$CL = Q \cdot ER = Q \cdot \frac{C_{in} - C_{out}}{C_{in}} \quad (1)$$

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Rowland and Pang (2018) questioned this concept and have argued that eq. 1 “simply express[es] [a] proportionality between the observed rate of elimination and a reference concentration” and is not model-dependent. That is, clearance as calculated by eq. 1 is the organ clearance for all models of organ elimination, whether drug elimination in the organ follows the parallel-tube model, various axial dispersion models, or the well stirred model.

It is universally agreed that the various models of organ clearance can be differentiated only for high-clearance (ER) substrates, and in only three published studies is the difference in models for high ER drugs in rat isolated perfused organs directly tested. All three of those studies, including two from the Rowland laboratory, conclude that the data are consistent with the well stirred model, not alternate hepatic clearance models. Pang and Rowland (1977) evaluated the effect of changing organ blood flow on the ER of lidocaine; the Rowland laboratory also evaluated the effect of changing blood flow on the ER of meperidine (Ahmad et al., 1983); and Jones et al. (1984) evaluated the effect of changing protein binding on the extraction ratio of propranolol. Yet, these experimental results are generally ignored.

ABBREVIATIONS: C_{in} , entering concentration; CL , clearance; CL_{int} , intrinsic clearance; C_{out} , exiting concentration; DZP, diazepam; ER , extraction ratio; F_{est} , estimated availability; F_{obs} , observed availability; $f_{u,B}$, fraction unbound in blood; HSA, human serum albumin; IPRL, isolated perfusate rat liver; LC-MS/MS, liquid chromatography-tandem mass; Q , organ blood flow; RED, rapid equilibrium dialysis.

In each of those studies for high-clearance (ER) drugs, the investigators concluded that the outcomes were consistent with the well stirred model as given in eq. 1, as represented by eq. 2, as opposed to the parallel tube model given in eq. 3:

$$CL_H = \frac{Q_H \cdot f_{u,B} \cdot CL_{int,u}}{Q_H + f_{u,B} \cdot CL_{int,u}} \quad (2)$$

$$CL_H = Q_H \left(1 - e^{-\frac{f_{u,B} \cdot CL_{int,u}}{Q_H}} \right), \quad (3)$$

where the hepatic clearance, CL_H , is calculated from the hepatic blood flow, Q_H , the fraction unbound in blood, $f_{u,B}$, and the unbound intrinsic clearance, $CL_{int,u}$.

Two experimental organ clearance studies with the low hepatic clearance drug, diazepam, in which diazepam has been manipulated to be high clearance in the absence of plasma proteins, were reported by the Rowland laboratory (Rowland et al., 1984; Díaz-García et al., 1992) to demonstrate preference for the parallel-tube and axial dispersion models versus the well stirred model. Yet, the only experimental data values detailed in these two articles showing preference for the alternate models were carried out when no protein was present in the perfusion media, and the outcomes C_{out}/C_{in} measures differed by about 4-fold: 0.042 (CV = 76%) as reported by Rowland et al. (1984) using a gas liquid chromatographic analytical method and 0.011 (CV = 45%) and reported by Díaz-García et al. (1992) using radiolabel measurements. Although the authors reported that “diazepam did not bind to the perfusion apparatus” (Díaz-García et al., 1992), we and other drug metabolism scientists are quite wary of carrying out studies in the absence of any binding protein in the drug containing media, especially since diazepam in human plasma is so highly protein bound (98.5%; Allen and Greenblatt, 1980). There is also marked uncertainty as to how protein-binding measurements should be interpreted and used in metabolism/transport studies as we and others have reviewed (Poulin et al., 2016; Bowman and Benet, 2018; Bteich et al., 2019). In vitro measures in the absence of protein (albumin) markedly underpredict in vivo clearance; this difference is diminished in the presence of albumin (Poulin and Haddad, 2018; Kim et al., 2019). Therefore, in the present work, we have re-examined the rat liver perfusion clearance of diazepam in the absence of protein, but also at additional protein concentrations that discriminate, using mass spectrometric assay methods, which hepatic disposition model describes the hepatic clearance of diazepam.

Materials and Methods

Chemicals. With approval to use a controlled substance from the University of California San Francisco (UCSF), diazepam (DZP) and its internal standard diazepam-d5 were obtained from Spectrum (Gardena, CA) and Cerilliant (Round Rock, TX), respectively. The buffer reagents used in the IPRC experiments, including taurocholate, sodium bicarbonate, and Krebs-Ringer powder, were all purchased from Sigma-Aldrich (St. Louis, MO). Fatty acid-free human serum albumin (HSA) was supplied commercially from SeraCare (Milford, MA). Rapid equilibrium dialysis (RED) device inserts were purchased from Thermo Scientific (Rockford, IL). All other chemicals and solvents for the analysis were of analytical or liquid chromatography-mass spectrometry (LC-MS) grade.

Diazepam Protein Binding. Equilibrium dialysis experiments with DZP were carried out using RED device inserts (Waters et al., 2008). DZP was prepared at 1 $\mu\text{g}/\text{ml}$ in Krebs' buffer containing varied HSA concentrations (w/v) of 0%, 0.025%, 0.05%, 0.1%, 0.5%, 1%, 2%, and 4%. A 500- μl volume of DZP testing solution was spiked into the sample chamber of the insert, followed by the addition of 750 μl of blank buffer in the adjacent chamber. The loaded inserts were assembled in the Teflon-based plate, and the plate was sealed with a self-adhesive lid, swayed at 20 strokes per minute under 37°C on an incubator shaker (Feasterville, PA) for the required experimental times of 4, 8, 12, or 24 hours.

Surgery and Perfusion of Livers. Male Sprague-Dawley rats (350–400 g; Charles River Laboratory, Hollister, CA) were housed in the UCSF animal care facility with a 12-hour light/dark cycle and allowed free access to water and food. Approval for their use in experiments was obtained from the Committee on Animal Research of UCSF. Anesthesia was achieved by intraperitoneal injection with a 1 ml/kg dose of ketamine/xylazine (91 mg, 9.1 mg/ml) before surgery. Livers were isolated for perfusion ex situ, as described previously with slight modifications (Wu and Benet, 2003; Lau et al., 2004). Oxygenated Krebs-Ringer bicarbonate buffer (pH 7.4), supplemented with different concentrations of HSA (ranging from 0% to 2%), sodium bicarbonate (15 mM), and sodium taurocholate (10 μM), was pumped through the liver at a flow rate of 15 ml/min via a catheter inserted in the portal vein. Perfusion was performed in eight rats at 37°C in a single-pass manner from a reservoir containing 500 ml of medium, through a 1- μm pore-size glass fiber filter, oxygenator, and bubble trap placed before the liver. The perfusate was oxygenated by carbogen (95% O_2 /5% CO_2) as it passed through the semipermeable tube before entering the liver. Liver viability was judged on the basis of its appearance (uniformly pink to brown), portal vein pressure (8–10 mm Hg), and the pH of perfusate (in the range of 7.35–7.45), as well as metabolic capability. After a 20-minute stabilization period, single-pass perfusions were started sequentially, with the perfusates containing 1 $\mu\text{g}/\text{ml}$ DZP and different HSA concentrations (0%, 0.025%, 0.05%, 0.1%, 0.5%, and 2.0%). The order of the perfusate concentrations randomly followed one of the two sequences depicted in Fig. 1. During each rat perfusion experiment, 2-ml samples from the inferior vena cava (C_{out}) were taken at 16, 17, 18, 19, and 20 minutes. At the end of each perfusion period, a 2-ml influx sample (C_{in}) was obtained. Because of fluctuating bile flow rate from the cannulated bile duct after liver isolation, no attempt was made to quantitate DZP in the bile.

Sample Preparation and Analysis. A simple, quick method of protein precipitation was used in sample preparation. Each 200- μl perfusate sample was combined with 40 μl of internal standard solution (500 ng/ml in acetonitrile), as well as 560 μl of cold acetonitrile. The final sample/internal standard/organic solvent mixtures were then mixed briefly, followed by centrifugation at 13,000g for 10 minutes. Then, 200 μl of the supernatant was transferred into high-performance liquid chromatography screw-cap vials. Preliminary studies indicated no matrix effects as a function of different HSA concentrations; therefore, calibration curves for DZP were constructed over the range of 1–1000 ng/ml in perfusate with 2% HSA.

DZP and its internal standard DZP-d5 were analyzed using a Shimadzu LC-20AD high-performance liquid chromatography (Kyoto, Japan) coupled to a Biosystems-Sciex API 4000 series triple-quadrupole mass spectrometer (Foster City, CA) with an electrospray ionization interface. Chromatographic separation was carried out on a Thermo BDS Hypersil C18 column (4.6 \times 100 mm, 5 μm ; Thermo Fisher, Waltham, MA) using a mobile phase composed of solvent A (2 mM ammonium acetate and 0.1% formic acid in water) and solvent B (2 mM ammonium acetate and 0.1% formic acid in methanol) under an isocratic program with a 3-minute run time and a 0.7 ml/min flow rate. The autosampler was conditioned at 10°C, and the injection volume was set at 10 μl . Acquisition for

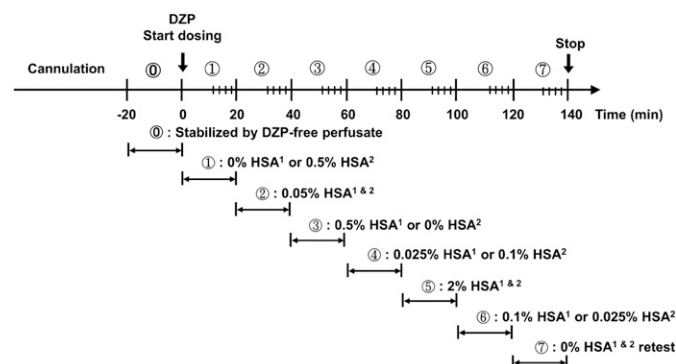


Fig. 1. Drug-loading design of diazepam (1 $\mu\text{g}/\text{ml}$) combined with varied HSA concentrations in IPRC studies. Two sequences of HSA concentrations were used, and individual rats were randomly assigned to a sequence. The Arabic number superscripts indicate where the individual HSA concentrations were in the testing sequence (i.e., in sequence 1, 0.1% HSA was studied between 100 and 120 minutes, and in sequence 2, this HSA concentration was studied between 60 and 80 minutes).

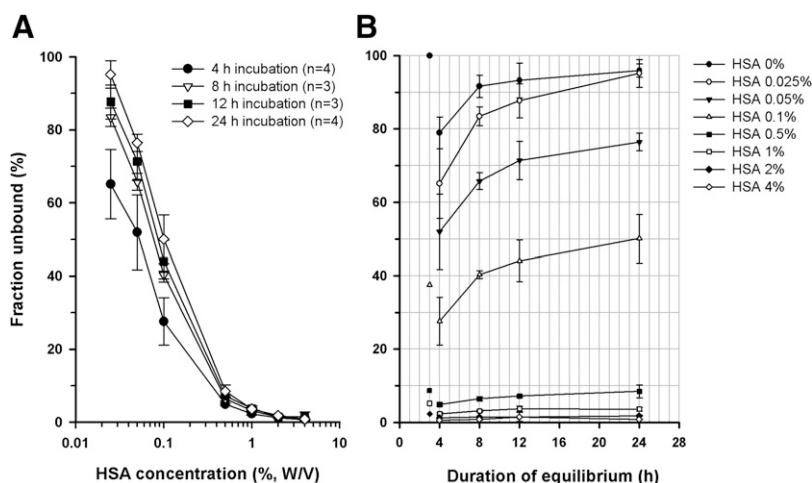


Fig. 2. (A) Plot of the free fraction of diazepam in the perfusate as a function of logarithmic HSA concentration for incubation times ranging from 4 to 24 hours. (B) Solid lines connect the fraction unbound as a function of equilibration time reported here. Data points at 3 hours represent the fraction unbound reported by Díaz-García et al. (1992).

tandem mass spectrometry was performed in positive ionization mode, and the multiple reaction monitoring mode was selected for quantification of the analytes. The precursors to production ion transitions in the analytes were 285.2→154.1 and 285.2→193.0 for DZP, as well as 290.2→154.1 for DZP-d5 (Marin et al., 2012). The ion-source temperature was maintained at 550°C, and the ion spray voltage was 5.0 kV. Analyst 1.4.2 software (Applied Biosystems-Sciex, Foster City, CA) was used to collect and process the data.

Models of Hepatic Drug Clearance. Three models of hepatic elimination are considered in the figures comparing the outcomes by Díaz-García et al. (1992): the well-stirred model, the parallel-tube model, and the axial dispersion model with a dispersion number of 0.34. The theoretical figures comparing the various hepatic models with changing protein binding or changing flow rate are based on the assumption that eq. 1 is model independent. Although we disagree with that assumption, and that only the well-stirred model simulation is relevant, we

calculated the theoretical curves here, following the procedure described in Díaz-García et al. (1992), so that a comparison with previous publications can be made. This was done by using the mean observed f_u and C_{out}/C_{in} (F_{obs}) values for all protein concentrations and estimating the $CL_{int,u}$ for each of the models. This value was then used to determine the estimated C_{out}/C_{in} (F_{est}) values at each value of f_u , from which the hypothetical curve representing each of the models could be drawn.

Results

Equilibrium Dialysis. Variations of diazepam protein binding under different HSA concentrations are shown in Fig. 2 and Table 1, reflecting the time-dependent changes in diazepam protein binding as a function of equilibration times between 4 and 24 hours. The time dependency of

TABLE 1
Diazepam fraction unbound (%) as a function of incubation time and human serum albumin (HSA) concentration

Dialysis Time	HSA (% w/v)	Mean	SD	CV%	Ratio 24 h/4 h
4 h (n = 4)	0	78.9	4.3	5.5	
	0.025	65.1	9.5	14.6	
	0.05	52.0	10.2	19.6	
	0.1	27.6	6.5	23.5	
	0.5	4.91	0.29	8.0	
	1	2.34	0.34	14.7	
	2	1.25	0.11	8.8	
	4	0.646	0.042	6.5	
8 h (n = 3)	0	91.6	3.0	3.3	
	0.025	83.4	2.5	3.0	
	0.05	65.8	2.3	3.5	
	0.1	40.3	1.1	2.6	
	0.5	6.44	0.63	9.8	
	1	3.17	0.26	8.3	
	2	1.46	0.05	3.4	
	4	0.826	0.201	24.3	
12 h (n = 3)	0	93.2	4.8	5.1	
	0.025	87.7	4.7	5.3	
	0.05	71.4	5.2	7.3	
	0.1	44.0	5.7	7.3	
	0.5	7.18	0.39	5.5	
	1	3.75	0.76	20.2	
	2	1.52	0.08	5.1	
	4	0.818	0.137	16.7	
24 h (n = 4)	0	96.0	1.8	1.9	1.22
	0.025	95.2	3.8	4.0	1.46
	0.05	76.4	2.4	3.1	1.47
	0.1	50.1	6.6	13.2	1.81
	0.5	8.48	1.79	21.2	1.73
	1	3.62	0.48	13.3	1.55
	2	1.81	0.17	9.6	1.51
	4	0.837	0.106	12.7	1.30

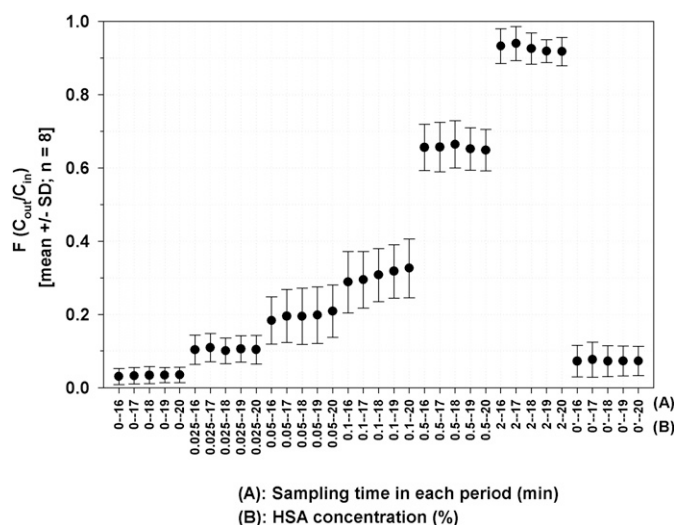


Fig. 3. The availabilities of diazepam in IPRL studies under varied HSA concentrations in a total of seven periods with constant perfusate flow rate (15 ml/min) and drug concentration (1 μ g/ml). Flow-out samples were collected every minute from 16 to 20 minutes in each period, including the flow-in perfusate at the last sampling time.

equilibrium protein-binding measurements is well recognized. For example, Obach (1997) demonstrated it to be a potential error source for in vitro-in vivo extrapolation predictions for warfarin, imipramine, and propranolol. Table 1 presents the mean values of percent fraction unbound for the four different dialysis times at the eight different HSA concentrations. The table also presents the standard deviations at each HSA concentration and the coefficient of variation of these measures. Although there are differences in variability (coefficient of variation) at different dialysis times and different HSA concentrations, no pattern is discernable. That is, the coefficients of variation appear to be randomly distributed across the 32 different mean values. Particularly relevant is comparison of the ratio of mean protein-binding values at each of the HSA concentrations for the 24-hour measurements compared with the 4-hour measurements. The change in the extent of binding for zero HSA concentration, where the fraction unbound percentages increase from 78.9% to 96.0% over the 18-hour time period (ratio of 1.22), was very similar to the proportional change in the extent of protein binding at 4% HSA, where the percent unbound increased from 0.65% to 0.84% (ratio of 1.30). The protein-binding change for all HSA concentrations changed similarly, ranging from 1.22 to 1.82. Thus, although it may appear in Fig. 2A that significant changes in protein binding with respect to incubation time occur only at low HSA concentration, the actual data as presented in Table 1 demonstrate that the relative change in the extent of binding from 4 to 24 hours is approximately the same for all HSA concentrations.

IPRL Experiments. We conducted IPRL using eight male rat livers using two different perfusion sequences and varying HSA concentrations (Fig. 1). Figure 3 depicts the observed C_{out}/C_{in} ratios for all eight rats at the six different HSA concentrations, each with five measurements taken at minutes 16–20, as indicated. Two perfusion sequences were used; therefore, C_{out}/C_{in} ratios were averaged for the two sequences. The final 0% HSA measurement in each rat (depicted as 0) is shown separately from the average of the earlier 0% HSA perfusions. Compared with the first period of HSA-free perfusion (0–20 or 40–60 minutes), the availability of DZP at 120–140 minutes was increased approximately 2-fold ($0.033\% \pm 0.022\%$ vs. $0.073\% \pm 0.043\%$, $P < 0.05$). The individual results for the eight rats in the IPRL studies are depicted in Fig. 4, where the 0% HSA results are for the initial

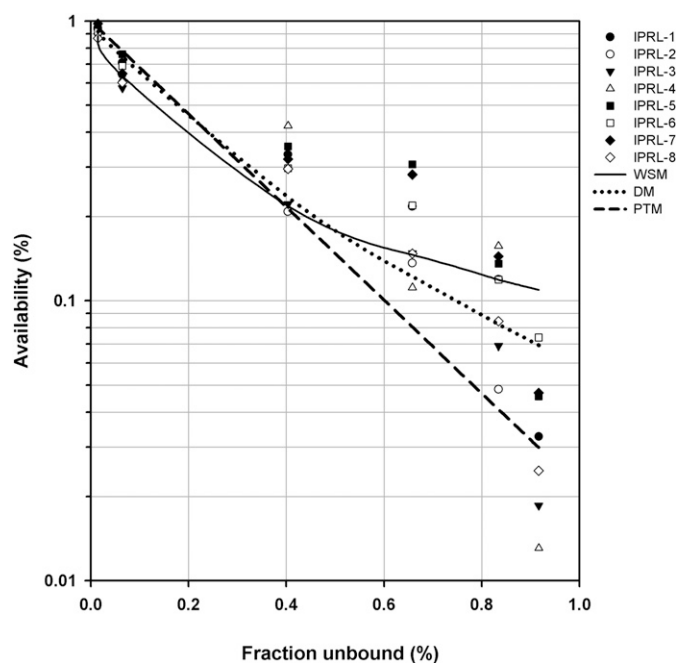


Fig. 4. Estimated results of well stirred (WSM), parallel-tube (PTM), and dispersion (DM) models for the relationship between availability and fraction unbound of diazepam. Symbols represent the individual results in each of the IPRL studies. Values for the zero protein added to the incubation are for the initial measurements.

measurements only. The average F_{obs} values are presented in Table 2, together with the calculated F_{est} for each model using the average 8-hour equilibrium fraction unbound measurements for each HSA concentration. (Although F_{est} values would differ slightly for the different “equilibrium times” chosen, the differentiation between models would be similar.) The mean F_{obs} values were then used for each model to generate the theoretical curves, as depicted in Figs. 4 and 5. Figure 4 displays the individual data, and Fig. 5 depicts the average of the experimental F_{obs} values for each HSA concentration compared with fraction unbound and the theoretical curves for the three models of hepatic elimination. The last three columns of Table 2 give the estimated F values for each model for each HSA concentration.

Discussion

The motivation for the present study resulted from our interest in re-examining the data from apparently the only isolated rat liver perfusion drug studies that provided results that were not best explained by the well stirred model of organ clearance. Those studies (Rowland et al., 1984; Díaz-García et al., 1992) examined diazepam clearance and protein binding at HSA concentrations of 0%, 0.1%, 0.5%, 1%, and 2%. The authors reported that “Preliminary experiments showed that equilibrium was reached within 3 hours, that volume shifts were negligible, and that diazepam did not bind to the experimental system.” The mean experimental percent fraction unbound values from these studies are included in Fig. 2B and shown at 3 hours. Our study using LC-MS/MS measurements in the RED device yielded lower results.

The variation of diazepam protein binding under different HSA concentrations is shown in Fig. 2. The fraction unbound of diazepam in the HSA-free medium was 78.9% after 4-hour incubation, not reaching the theoretical value of 100% even after a 24-hour incubation, as opposed to the 100% free fraction reported by Díaz-García et al. (1992) after a 3-hour incubation. As shown in Table 1, the fractions unbound at 0.1% HSA ranged from 27.6% after 4-hour incubation to 50.1% after 24-hour incubation compared with the reported 37.6% after a 3-hour

TABLE 2

Observed (F_{obs}) and estimated availability (F_{est}) of diazepam in the human serum albumin under varying protein (HSA) binding conditions and measured 8-hour fraction unbound for three different hepatic drug clearance models: WSM, PTM, and DM.

HSA (%)	$f_{u,8h}$ (%)	F_{obs} (%)	Estimated Availability; F_{est} (%)		
			WSM	PTM	DM
0	0.92	3.3	10.7	3.0	6.9
0.025	0.83	10.9	11.7	4.1	8.1
0.05	0.66	19.6	14.3	8.1	12.1
0.1	0.40	30.7	21.4	21.3	23.3
0.5	0.06	65.6	63.1	78.1	74.4
2	0.01	92.7	88.3	94.6	93.2

DM, dispersion model; PTM, parallel-tube model; WSM, well stirred model.

incubation by Díaz-García et al. (1992). None of the protein binding measurements at various HSA concentrations reached equilibrium at 24 hours, all of which exhibit a similar degree of nonequilibrium, independent of protein concentration. Further, the variability of the measurement and the difference of the measurement between 4 and 24 hours appear to be independent of protein concentration. We conclude that the time dependency of equilibrium protein-binding measurements for very highly bound drugs may be an issue that is not readily overcome.

For the isolated perfused diazepam liver studies, we showed results similar to those of Rowland and co-workers for the initial measurements in the absence of added protein. That is, the mean data for this point (0% HSA; 91.6% average fraction unbound; $F_{obs} = 0.033$) appear to be consistent with the parallel-tube model (see Figs. 4 and 5). As depicted in Fig. 4, there is great variability in the eight individual measurements at zero protein addition ($F_{obs} = 0.033$; CV = 67%), with this high variability ($F_{obs} = 0.042$; CV = 76%) also reported by Rowland et al. (1984) using a gas liquid chromatographic analytical method and ($F_{obs} = 0.011$; CV = 45%) reported by Díaz-García et al. (1992) using radiolabel measurements. Our second measures at zero protein

addition of F_{obs} at 120–140 minutes ($F_{obs} = 0.073\%$; CV = 59%) were statistically higher than the earlier first measurements, probably reflecting the decrease in liver function with time, as this parameter is much more sensitive than traditional measures of liver viability. When our second measure at zero protein was plotted in Fig. 5, this mean value appears to be more consistent with the dispersion model (0% HSA; 91.6% average fraction unbound; $F_{obs} = 0.073$). The 2-fold difference here in our measurements should be considered in light of the 4-fold difference reported in the two studies by Rowland and co-worker.

The investigations of Rowland and co-workers did not study any other protein concentrations that would readily allow comparison of the different models. Thus, we investigated 0.025% and 0.05% protein concentrations in our IPRL studies. As seen in Figs. 4 and 5, these studies appear to be more consistent with the well stirred model. Table 2 shows the estimated availability for each of the models (under the assumption that clearance was model-independent). At lower protein concentrations, where the diazepam clearance (ER) is high, differentiation between the models is obvious. This differentiation dissipates as protein binding increases and clearance (ER) decreases. Comparing the least-square differences between the three models as presented in Table 2 and shown in Fig. 5, the initial average F_{obs} measurement when no protein is added to the perfusion media is consistent with the theoretical parallel-tube curve and the later 120- to 140-minute measurement is consistent with the dispersion model theoretical curve. Thus, it appears that we confirm the experimental results observed by Rowland et al. (1984) and Diaz-Garcia et al. (1992), respectively, when no protein is added to the perfusion media; however, when we studied low concentrations of protein that could differentiate the various theoretical models, our results appear to be consistent with the well stirred model. As noted, currently there is increasing attention being paid to how fraction unbound is interpreted in predicting metabolic interactions and the effect that albumin can have on these protein-binding and metabolism measurement as measured in the absence in protein markedly underpredict in vivo clearance as recently reviewed (Bowman and Benet, 2018; Breich et al., 2019). Recent studies with hepatocytes report markedly better in vitro-in vivo extrapolation predictability in the presence of albumin than in its absence (Poulin and Haddad, 2018; Kim et al., 2019). Thus, although we can confirm similar outcomes to that reported by Rowland and co-workers when no protein is added to perfusion media, those results cannot be reasonably interpreted as proving that eq. 1 is model-independent.

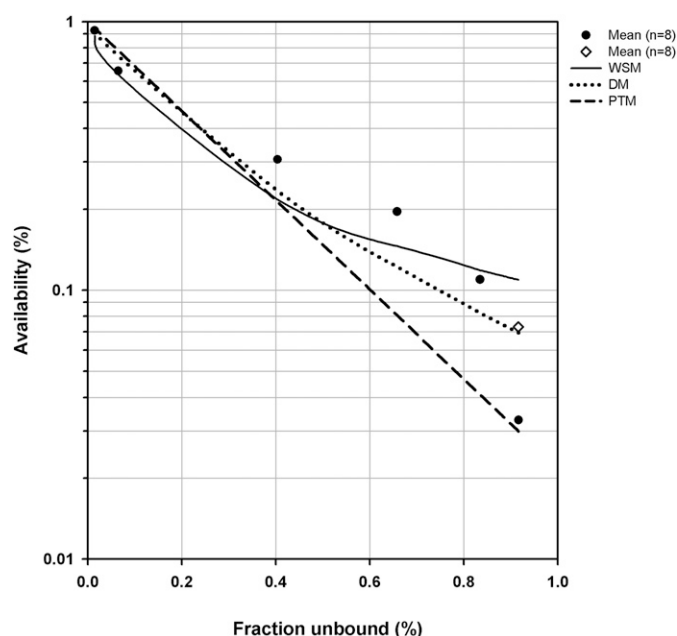


Fig. 5. Estimated results of well stirred (WSM), parallel-tube (PTM), and dispersion (DM) models for the relationship between availability and fraction unbound of diazepam. Symbols represent the mean results ($n = 8$) in all of the IPRL studies. For zero protein additions, ●Initial measurements between 0 and 20 minutes and 40–60 minutes; ◊Second measurements in each perfusion at 120–140 minutes.

Authorship Contributions

Participated in research design: Wang, Benet.

Conducted experiments: Wang.

Performed data analysis: Wang, Benet.

Wrote or contributed to the writing of the manuscript: Wang, Benet.

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Address correspondence to: Dr. Leslie Z. Benet, Department of Bioengineering and Therapeutic Sciences, Schools of Pharmacy and Medicine, University of California San Francisco, 533 Parnassus Avenue, Room U-68, San Francisco, CA 94143-0912. E-mail: leslie.benet@ucsf.edu
