Does Addition of Protein to Hepatocyte or Microsomal In Vitro Incubations Provide a Useful Improvement in In Vitro-In Vivo Extrapolation Predictability?[S]

Tsubasa Kameyama,[1] Jasleen K. Sodhi,[2] and Leslie Z. Benet

Department of Bioengineering and Therapeutic Sciences, Schools of Pharmacy and Medicine, University of California San Francisco, San Francisco, California

ABSTRACT

Accurate prediction of in vivo hepatic clearance is an essential part of successful and efficient drug development; however, many investigators have recognized that there are significant limitations in the predictability of clearance with a tendency for underprediction for primarily metabolized drugs. Here, we examine the impact of adding serum or albumin into hepatocyte and microsomal incubations on the predictability of in vivo hepatic clearance. The addition of protein into hepatocyte incubations has been reported to improve the predictability for high clearance (extraction ratio) drugs and highly protein-bound drugs. Analyzing published data for 60 different drugs and 97 experimental comparisons (with 17 drugs being investigated from two to seven) we confirmed the marked underprediction of clearance. However, we could not validate any relevant improved predictability within twofold by the addition of serum to hepatocyte incubations or albumin to microsomal incubations. This was the case when investigating all measurements, or when subdividing analyses by extraction ratio, degree of protein binding, Biopharmaceutics Drug Disposition Classification System class, examining Extended Clearance Classification System class 1B drugs only, or drug charge. Manipulating characteristics of small data sets of like compounds and adding scaling factors can appear to yield good predictability, but the carryover of these methods to alternate drug classes and different laboratories is not evident. Improvement in predictability of poorly soluble compounds is greater than that for soluble compounds, but not to a meaningful extent. Overall, we cannot confirm that protein addition improves in vitro-in vivo extrapolation predictability to any clinically meaningful degree when considering all drugs and different subsets.

SIGNIFICANCE STATEMENT

The addition of protein into microsomal or hepatocyte incubations has been widely proposed to improve hepatic clearance predictions. To date, studies examining this phenomenon have not included appropriate negative controls where predictability is achieved without protein addition and have been conducted with small data sets of similar compounds that don't apply to alternate drug classes. Here, an extensive analysis of published data for 60 drugs and 97 experimental comparisons couldn't validate any relevant clinically improved clearance predictability with protein addition.

Introduction

Drug discovery and development is an extremely expensive, time-consuming process with a poor success rate. It is well recognized that an accurate prediction of a new molecular entity's pharmacokinetic properties prior to any in vivo studies could improve the efficiency of drug development. Clearance is one of the most important pharmacokinetic parameters, as clearance determines drug exposure, which drives drug efficacy and potential toxicity, and directly contributes to projection of safe and efficacious new molecular entity dosing regimens.

Metabolic stability studies are routinely conducted to predict in vivo drug clearance for metabolized drugs in the early drug discovery stage, where various approaches to predict in vivo clearance from in vitro systems, such as utilization of microsomes and hepatocytes, have been established as recently reviewed (Sodhi and Benet, 2021). Attempting to predict in vivo hepatic clearance using the in vitro-in vivo extrapolation (IVIVE) method involves measuring an intrinsic clearance (CL_{int}), defined as the intrinsic ability of the liver to remove the drug in the absence of protein binding or flow limitations in microsomes or hepatocytes, and applying biologic scaling factors and a liver model to scale the in vitro measures to predict the in vivo hepatic clearance. However, a tendency for significant underprediction from the IVIVE method for primarily metabolized drugs and for drugs where clearance is rate limited by hepatic uptake has been documented (Bowman and Benet, 2016; Wood et al., 2017; Kim et al., 2019). Recently, we examined the theoretical basis of IVIVE predictions from first principles, suggesting a number of future pathways that should be investigated toward improved IVIVE success (Benet and Sodhi, 2020).

Here, we focus on the effect of adding serum or protein, such as albumin, to hepatocyte and microsomal incubations. It has been reported that hepatocyte incubations with 100% serum more accurately predict in vivo hepatic clearance in rats (Blanchard et al., 2004) and

DRUG METABOLISM & DISPOSITION

humans (Blanchard et al., 2005, 2006). Additionally, it has been reported that albumin could improve predictions of in vivo hepatic clearance from microsomal incubations. Furthermore, based on human hepatocyte studies, considerable underprediction of in vivo hepatic clearance (CL_H), especially of highly protein-bound organic anion transporter protein 1B1 (OATP 1B1) substrate drugs, has been widely reported (Soars et al., 2007; Zou et al., 2013). These CL_H predictions are improved (but still result in underpredictions) by inclusion of plasma or plasma proteins (e.g., albumin) in human hepatocyte transport studies. This has been attributed to the observed higher intrinsic uptake clearance (passive and active) in the presence of plasma proteins (Kim et al., 2019; Bowman et al., 2020). These data have led to the hypothesis of a protein-mediated transport effect (Miyauchi et al., 2018). We recently reviewed the evolution of the fraction unbound terms included when implementing IVIVE, the concept of protein-facilitated uptake, and the mechanisms that have been proposed to account for facilitated uptake (Bowman and Benet, 2018). Although protein-facilitated uptake has been mainly reported as the mechanism for the serum albumin effect in hepatocyte incubations (Bowman and Benet, 2018; Bowman et al., 2019; Bteich et al., 2019; Kim et al., 2019), the mechanism is not clearly understood. Protein-mediated increased predictability is also observed in microsomal preparations, and we maintain that this may be due to the lack of detailed testing of multiple negative control substrates for which the albumin-effect is not expected. We propose the possibility that for any poorly soluble compound, the addition of protein in in vitro incubations may enhance assay conditions by preventing nonspecific binding and potentially boosting drug concentrations available for metabolism.

Here, to examine the effect of adding protein to metabolic stability incubations, we compiled measured CL_{int} values from hepatocyte and microsomal incubations with and without protein addition and compared the accuracy of in vivo CL_H predictions from these data using the IVIVE method. Since plasma, serum, or human serum albumin (HSA) is typically added to metabolic stability incubations rather than blood, and due to the potential inaccuracies in determination of fraction unbound in blood ($f_{u,b}$) for transporter substrates as we have recently noted (Benet and Sodhi, 2020), we evaluated IVIVE accuracy in terms of plasma clearance in addition to the typically reported blood clearance-based IVIVE assessment. We also examined the protein effect on IVIVE success with respect to drug properties, such as the extraction ratio (ER), the extent of protein binding, the Biopharmaceutics Drug Disposition Classification System (BDDCS) (Wu and Benet, 2005) (to examine the impact of IVIVE success on low versus high solubility drugs), charge class, and for the drug subset where clearance is rate limited by hepatic OATP uptake.

Methods

Data Collection. In vitro CL_{int} values derived from incubations with protein ($CL_{int, in vitro, with protein}$) were collected from seven studies for human hepatocytes for 52 drugs, with 73 individual drug measurements (values presented in Supplemental Table 1) (Shibata et al., 2002; Bachmann et al., 2003; Blanchard et al., 2006; Chao et al., 2009; Mao et al., 2012; Kim et al., 2019; Nishimuta et al., 2019) and 12 studies for

human microsomes for 15 drugs with 24 individual measurements (values presented in Supplemental Table 2) (Ludden et al., 1997; Carlile et al., 1999; Baba et al., 2002; Tang et al., 2002; Rowland et al., 2007, 2008a,b; Wattanachai et al., 2011, 2015; Gill et al., 2012; Walsky et al., 2012; Palacharla et al., 2017). A total of 60 different drugs were evaluated here. In these investigations, the authors incubated human hepatocytes with 100% human serum or 1%–5% HSA, and human microsomes with 2%–4% HSA or bovine serum albumin. From the numbers above, often the same drug was included in multiple investigations with the addition of serum. To avoid the detrimental impact of variability between laboratories, we scaled up each measurement separately rather than averaging in vitro CL_{int} values from multiple investigations. In vitro CL_{int} values derived from human hepatocyte and microsomal incubations without serum ($CL_{int, in vitro, without protein}$) were collected from the above-mentioned publications in priority; however, the Nishimuta et al. (2019) and Shibata et al. (2002) studies did not evaluate incubations without protein (26 drugs, 28 measurements). To maintain comparisons with and without protein addition $CL_{int, in vitro, without protein}$ values from the Wood et al. (2017) database were incorporated (see Supplemental Tables 1 and 2). A number of drugs were excluded from this analysis because either corresponding $CL_{int, in vitro, without protein}$ values were unavailable or there was not a reliable estimate of in vivo clearance listed in either the Wood et al. (2017) database or the Lombardo et al. (2018) database of intravenous clearance of 1352 drugs. For human hepatocytes, these drugs were: atomoxetine, buspirone, cisapride, disopyramide, felodipine, fentanyl, fluconazole, fluoxetine, ketoconazole, lansoprazole, mebendazole, moferatone, mycophenolic acid, nicardipine, nimodipine, oxybutynin, paclitaxel, paroxetine, rivastigmine, telmisartan, tolcapone, ziprasidone, and zoniporide. For human microsomes, these drugs were: amodiaquine, bupropion, chlorzoxazone, dextromethorphan, ezetimibe, mephenytoin, terfenadine, trifluoperazine, and 5-hydroxytryptophol. Additionally, tolbutamide from the Carlile et al. (1999) study was excluded as concentration-dependent binding was observed and unbound CL_{int} could thus not be calculated from reported data.

In Vitro-In Vivo Extrapolation Approach. In vitro intrinsic clearance derived from incubations with protein ($CL_{int, in vitro, with protein}$) and in vitro intrinsic clearance derived from incubations without protein ($CL_{int, in vitro, without protein}$) were scaled to whole liver in vivo total and in vivo unbound values using eq. 1.

$$Predicted\ CL_{int, in vivo with or without protein} = \frac{CL_{int, in vitro, with or without protein} \cdot PBSF \cdot LW}{f_{u, hep}\ or\ f_{u, mic}}$$

(1)

To ensure that consistent scaling factors were employed for all investigations, we used the physiologic scaling factors for hepatocellularity and a microsomal recovery factor of 120×10^6 hepatocytes/g liver and 40 mg microsomal protein/g liver. Liver weight was assumed to be 21.4 g/kg body weight. Fraction unbound in hepatocyte incubation ($f_{u, hep}$) and fraction unbound in microsomal incubations ($f_{u, mic}$) were taken from the original publications; however, if $f_{u, hep}$ with and without 100% human serum was not reported, we used fraction unbound in plasma ($f_{u, p}$) and $f_{u, hep}$ from the published databases mentioned above (Wood et al., 2017; Lombardo et al., 2018). For procainamide, bufuralol, and drugs studied in the Kim et al. (2019) investigation, $f_{u, hep}$ was

estimated from an empirical relationship as indicated in Supplemental Table 1. Predicted in vivo hepatic blood clearance ($CL_{H,b}$) was scaled using the well-stirred model (eq. 2).

$$Predicted\ CL_{H,b} = \frac{Q_{H,b} \cdot f_{u,b} \cdot Predicted\ CL_{int,\,in\,vivo\,with\,or\,without\,protein}}{Q_{H,b} + f_{u,b} \cdot Predicted\ CL_{int,\,in\,vivo\,with\,or\,without\,protein}}$$

(2)

Liver blood flow ($Q_{H,b}$) was assumed to be 20.7 mL/min/kg, and $f_{u,b}$ was referenced from the published data set of Wood et al. (2017) and for the OATP rate limited drugs from Kim et al. (2019). For the drugs that are not listed in the Wood et al. (2017) compilation, $f_{u,b}$ values were derived from the quotient of $f_{u,p}$ and the reported blood-to-plasma partitioning ratio (R_{BP}). For the drugs that did not have reported R_{BP} values, we assumed the value to be equal to 1 for a basic or neutral compound and 0.55 (1-hematocrit) for an acidic compound.

When calculating predicted $CL_{H,b}$, the value of $f_{u,b}$ is required to scale up predicted in vivo intrinsic clearance ($CL_{int,\,in\,vivo}$) values. However, our laboratory has recently recognized that determinations of $f_{u,b}$ may be inaccurate for transporter substrates (Benet and Sodhi, 2020). This is because calculations of $f_{u,b}$ from measurements of $f_{u,p}$ and the R_{BP} ratio relies on the free drug theory, under the theoretical assumption that free unbound concentrations in the plasma are equal to free drug concentrations within the red blood cell—a scenario that is not valid for substrates of transporters expressed in the erythrocyte membrane. Therefore, we also evaluated IVIVE-error associated with in vivo hepatic plasma clearance ($CL_{H,p}$) to avoid using $f_{u,b}$. In calculations of predicted $CL_{H,p}$, utilization of the liver plasma flow ($Q_{H,p}$) is required. $Q_{H,p}$ was calculated with eq. 3, where the liver blood flow was 20.7 mL/min/kg and hematocrit (Hct) was 0.45.

$$Q_{H,p} = Q_{H,b} \cdot (1 - Hct) = Q_{H,b} \cdot 0.55 = 11.4\ mL/min/kg \quad (3)$$

Predicted $CL_{H,p}$ was determined from predicted $CL_{int,\,in\,vivo,\,with\,or\,without\,protein}$ using the well-stirred model (eq. 4).

$$Predicted\ CL_{H,p} = \frac{Q_{H,p} \cdot f_{u,p} \cdot Predicted\ CL_{int,\,in\,vivo\,with\,or\,without\,protein}}{Q_{H,p} + f_{u,p} \cdot Predicted\ CL_{int,\,in\,vivo\,with\,or\,without\,protein}}$$

(4)

To investigate the effect of serum or HSA addition for hepatic clearance prediction, we collected observed $CL_{H,b}$ (Wood et al., 2017) and $CL_{H,p}$ (Lombardo et al., 2018) values and compared IVIVE-error (predicted $CL_{H,b}$/observed $CL_{H,b}$ or predicted $CL_{H,p}$/observed $CL_{H,p}$) with and without serum. For the drugs that are not listed in the Wood et al. (2017) compilation, $CL_{H,b}$ values were derived from the quotient of $CL_{H,p}$ and R_{BP}. Plasma clearance values reported in Lombardo et al. (2018) were total clearance values; however, since the drugs evaluated in this data set are primarily metabolized BDDCS class 1 and 2 drugs (with minor contribution of renal and biliary elimination), total plasma clearance was assumed to reflect only hepatic clearance. We used ER values of ≤0.3 and ≥0.7 as the cutoffs for low and high ER, respectively, to investigate any potential clearance-dependent trends in IVIVE success. The drugs with ER between 0.3 and 0.7 were defined as moderate ER. For the 11 drugs where clearance is believed to be rate limited by OATP hepatic uptake, the CL_{int} values are, in fact, the intrinsic hepatic uptake clearances. As we have reviewed previously (Benet et al., 2018), when intrinsic hepatic basolateral efflux is negligible or much smaller than the sum of intrinsic hepatic metabolic and biliary clearance, the well-stirred model relationship substituting intrinsic hepatic uptake clearance for intrinsic hepatic elimination clearance is valid. Additionally, we investigated the trends related to BDDCS (Wu and Benet, 2005; Benet et al., 2011; Hosey et al., 2016), charge class, clearance category, and protein binding in terms of prediction accuracy of in vivo hepatic clearance.

Results

Initially, we made a comparison of clearance-dependent IVIVE-error with and without protein addition for both blood and plasma clearance (Fig. 1), visually confirming the clearance-dependent underprediction error recognized by the field for hepatocyte and microsomal incubations (Hallifax et al., 2010; Bowman and Benet, 2016; Bowman et al., 2019). Tables 1 and 2 report the predictability of hepatocyte and microsome data, respectively, within twofold of total clearance for all drugs, then divided based on ER (low, moderate, and high). For the hepatocyte results of $CL_{H,b}$ without serum, predictability accuracy within twofold for all, low, moderate, and high ER drugs was 32.4%, 31.3%, 34.5%, and 30.0%, respectively, suggesting that there was no discrimination based on extraction ratio. In the results with serum addition, predictability within twofold for all, low, moderate, and high ER drugs was 41.1%, 46.9%, 32.3%, and 50.0%, respectively, again demonstrating a similar IVIVE prediction success for all clearance categories in the presence of protein and a slight overall increase as compared with the predictions without serum. A similar trend was observed for hepatocyte predictions of $CL_{H,p}$, with all, low, moderate, and high ER drugs without serum providing IVIVE accuracies within twofold of 17.9%, 22.7%, 26.7%, and 10.0% and in the presence of protein were 27.5%, 50.0%, 31.3%, and 9.7%. A similar outcome is observed in Table 2 for addition of protein into microsomal incubations. In the absence of albumin, IVIVE accuracies within twofold for $CL_{H,b}$ for all, low, moderate, and high ER drugs were 20.8%, 25.0%, 28.6%, and 11.1% and in the presence of albumin were 37.5%, 25.0%, 42.9%, and 44.4%. Additionally, in the absence of albumin, IVIVE microsomal based accuracies within twofold for $CL_{H,p}$ for all, low, moderate, and high ER drugs were 20.8%, 25.0%, 60.0%, and 0.0% and in the presence of albumin were 33.3%, 37.5%, 100.0%, and 0.0% (Table 2).

To examine the relationship between IVIVE predictability and protein binding, we examined the potential for $f_{u,b}$- (or $f_{u,p}$)-dependent IVIVE error with and without serum (Fig. 2). For the hepatocyte results of $CL_{H,b}$ without serum, 14.7% accurate prediction within twofold was obtained for highly bound drugs with $f_{u,b}$ less than 0.1, whereas 48.6% was obtained for drugs with $f_{u,b}$ 0.1 or more (Table 3). The addition of serum yielded 40.0% accurate prediction within twofold for drugs with $f_{u,b}$ less than 0.1, compared with 42.1% for drugs with $f_{u,b}$ 0.1 or more. A similar trend is observed for hepatocyte IVIVE predictions of $CL_{H,p}$, with the percent within twofold for highly bound drugs ($f_{u,p}$ less than 0.1) increasing from 8.8% to 37.1% with the addition of serum and for low-binding drugs ($f_{u,p}$ greater than 0.1) decreasing from 27.3% to 17.6% within twofold in the presence of serum. These results indicate that f_u-dependent IVIVE error is apparent for the hepatocytes incubations without serum (Fig. 2, A and C; red dots), with larger underprediction errors for those drugs with $f_{u,b}$ or $f_{u,p}$ values less than 0.1, and the addition of serum into the hepatocytes incubation (Fig. 2, A and C; green dots) improves IVIVE for highly bound drugs with f_u values less than 0.1, whereas minimal or decreasing changes are observed for low-binding drugs. A different outcome is observed (Table 4) for addition of albumin into microsomal incubations, with respect to IVIVE accuracy for poorly versus highly bound drugs. For highly bound drugs, IVIVE accuracies within twofold for $CL_{H,b}$ were 28.6% and did not change in the presence of albumin (28.6%), but for low binders, predictability within twofold improved from 10.0% to 50.0%. For $CL_{H,p}$, success within twofold for highly bound drugs without albumin was 35.7%, and this value decreased to 28.6% in the presence of albumin. For poorly bound drugs, a marked increase in IVIVE success was observed with the addition of albumin, with values increasing from 0.0% to 30.0% for $CL_{H,p}$. These results indicate that the addition of albumin into microsomal incubations only improves IVIVE for poorly
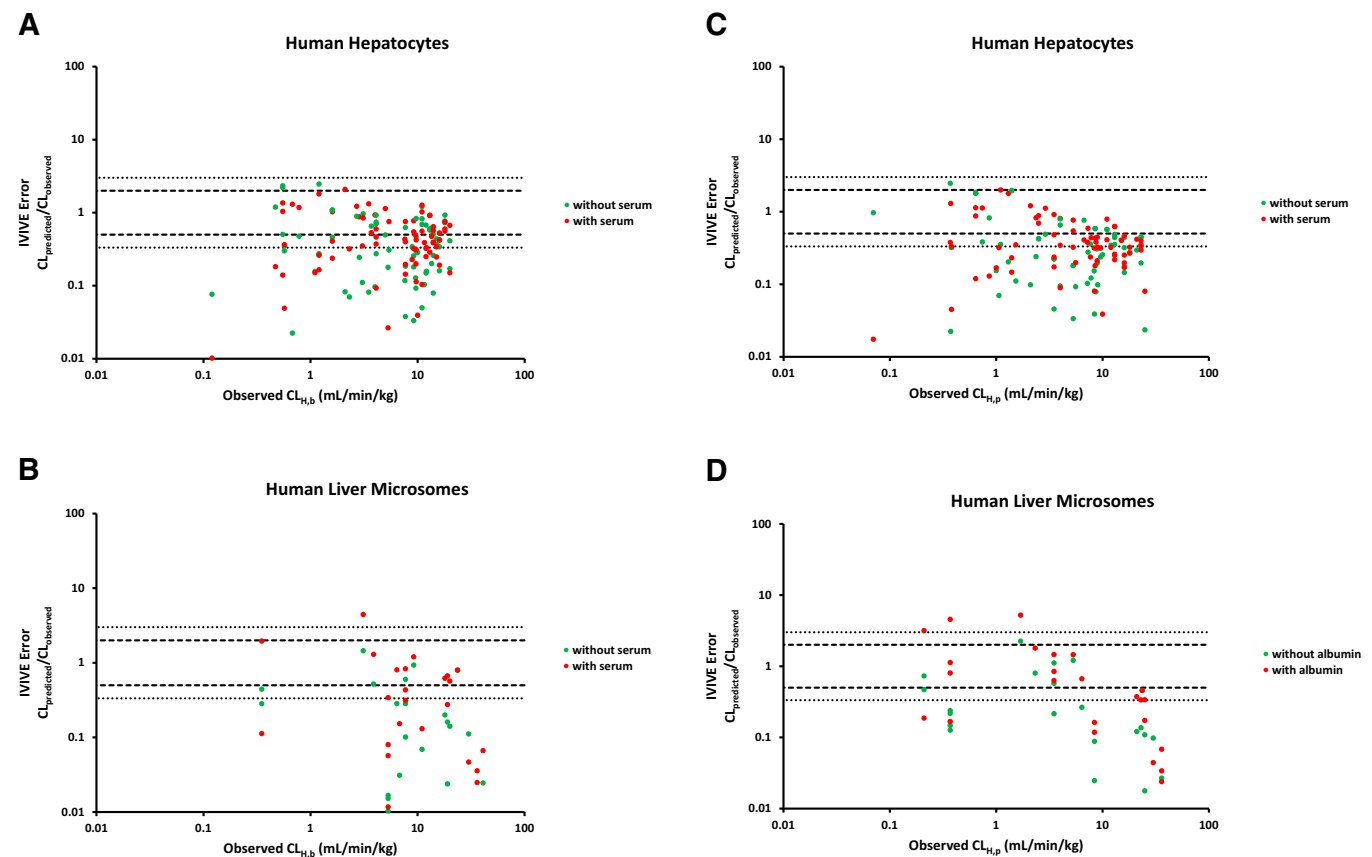
Fig. 1. Comparison of IVIVE error (predicted in vivo clearance/observed in vivo clearance) from the metabolic stability incubations with and without serum/albumin addition versus observed in vivo clearance using blood in vivo clearance from the Wood et al. (2017) data set for (A) hepatocytes and (B) microsomes and plasma in vivo clearance from the Lombardo et al. (2018) data set for (C) hepatocytes and (D) microsomes. Dashed lines indicate within twofold, and dotted lines indicate within threefold predictability. Green dots are incubations without serum/albumin addition, whereas red dots are incubations to which serum/albumin has been added.

bound drugs with f_u values 0.1 or more, the opposite result to the general belief that addition of protein increases the prediction preferentially for highly bound drugs in hepatocyte incubations.

We further investigated the association of BDDCS class with IVIVE-error with and without serum addition, comparing highly soluble class 1 and 3 drugs versus poorly soluble class 2 and 4 drugs to test whether protein addition may have had a larger effect on poorly soluble drugs. For hepatocyte incubations without serum when predicting $CL_{H,b}$, 39.5% accurate predictions within twofold were obtained for BDDCS class 1 and 3 drugs, whereas 21.4% were obtained for BDDCS class 2 and 3 drugs (Table 5). The addition of serum had minimal effects for BDDCS class 1 and 3 drugs (39.5% to 43.2%), whereas for BDDCS

class 2 and 4 drugs, predictability increased from 21.4% to 37.9%. For hepatocyte $CL_{H,p}$ values, the addition of serum had little effect on correct predictability of class 1 and 3 drugs (without serum 20.9%; with serum 20.5%), but as for the blood clearance values, predictability as seen in Table 5 was lower for the class 2 and 4 drugs (12.5%) and increased markedly with the addition of serum (40.0%). For microsome data (Table 6), all 24 drugs are BDDCS class 1 and 2. There are only 7 class 1 evaluations to be considered, and therefore it is difficult to draw conclusions with confidence, but for the 17 class 2 drug evaluations, $CL_{H,B}$ correct percentages increased from 17.6% to 29.4% with the addition of serum, whereas $CL_{H,P}$ correct percentages increased from 23.5% to 41.2% (Table 6).

TABLE 1

The effect of protein addition on hepatocyte clearance predictability within twofold of observed $CL_{H,b}$ (or $CL_{H,p}$) for all drugs and separated for low (≤ 0.3), intermediate (>0.3–<0.7), and high (≥ 0.7) extraction ratio drugs

	Predicted/Observed $CL_{H,b}$				Predicted/Observed $CL_{H,p}$			
	Without Serum		With Serum		Without Serum		With Serum	
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
All drugs	67.6% (n = 48 of 71)	32.4% (n = 23 of 71)	58.9% (n = 43 of 73)	41.1% (n = 30 of 73)	82.1% (n = 55 of 67)	17.9% (n = 12 of 67)	72.5% (n = 50 of 69)	27.5% (n = 19 of 69)
Low ER	68.7% (n = 22 of 32)	31.3% (n = 10 of 32)	53.1% (n = 17 of 32)	46.9% (n = 15 of 32)	77.3% (n = 17 of 22)	22.7% (n = 5 of 22)	50.0% (n = 11 of 22)	50.0% (n = 11 of 22)
Moderate ER	65.5% (n = 19 of 29)	34.5% (n = 10 of 29)	67.7% (n = 21 of 31)	32.3% (n = 10 of 31)	73.3% (n = 11 of 15)	26.7% (n = 4 of 15)	68.7% (n = 11 of 16)	31.3% (n = 5 of 16)
High ER	70.0% (n = 7 of 10)	30.0% (n = 3 of 10)	50.0% (n = 5 of 10)	50.0% (n = 5 of 10)	90.0% (n = 27 of 30)	10.0% (n = 3 of 30)	90.3% (n = 28 of 31)	9.68% (n = 3 of 31)

TABLE 2

The effect of protein addition on microsome clearance predictability within twofold of observed $CL_{H,b}$ (or $CL_{H,p}$) for all drugs and separated for low (≤ 0.3), intermediate (>0.3–<0.7), and high (≥ 0.7) extraction ratio drugs

| | Predicted/Observed $CL_{H,b}$ | | | | Predicted/Observed $CL_{H,p}$ | | | |
| | Without Serum | | With Serum | | Without Serum | | With Serum | |
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
All drugs	79.2% (n = 19 of 24)	20.8% (n = 5 of 24)	62.5% (n = 15 of 24)	37.5% (n = 9 of 24)	79.2% (n = 19 of 24)	20.8% (n = 5 of 24)	66.7% (n = 16 of 24)	33.3% (n = 8 of 24)
Low ER	75.0% (n = 6 of 8)	25.0% (n = 2 of 8)	75.0% (n = 6 of 8)	25.0% (n = 2 of 8)	75.0% (n = 6 of 8)	25.0% (n = 2 of 8)	62.5% (n = 5 of 8)	37.5% (n = 3 of 8)
Moderate ER	71.4% (n = 5 of 7)	28.6% (n = 2 of 7)	57.1% (n = 4 of 7)	42.9% (n = 3 of 7)	40.0% (n = 2 of 5)	60.0% (n = 3 of 5)	0.00% (n = 0 of 5)	100% (n = 5 of 5)
High ER	88.9% (n = 8 of 9)	11.1% (n = 1 of 9)	55.6% (n = 5 of 9)	44.4% (n = 4 of 9)	100% (n = 11 of 11)	0.00% (n = 0 of 11)	100% (n = 11 of 11)	0.00% (n = 0 of 11)

The association between IVIVE predictability and charge class was also conducted with and without serum addition (Tables 7 and 8). For the hepatocyte results of $CL_{H,b}$ without serum, predictability within twofold for acid, basic, and neutral drugs was 11.1%, 45.2%, and 35.0%, respectively, and in the presence of serum were 31.6%, 46.9%, and 40.0%, respectively (Table 7). Zwitterions are also listed in the tables, but there were so few compounds that they are not addressed here. Results for prediction of $CL_{H,p}$ were similar, with predictability within twofold for acid, base, and neutral drugs without protein addition of 5.9%, 22.6%, and 23.5% and in the presence of protein, these values were 33.3%, 28.1%, and 17.6%. These results indicate that the addition

of serum into hepatocyte incubations seems to improve IVIVE for acidic drugs, but never more than 34%. Basic and neutral drugs give higher predictability without serum addition, and there was little improvement of IVIVE with protein addition. For the subset of acidic OATP rate limited substrates, as reported by Kim et al. (2019), addition of protein increased estimated clearance predictions, but only three of the 10 drugs tested with HSA addition then yielded values within twofold predictability. In the microsomal results (Table 8) of $CL_{H,b}$ and $CL_{H,P}$, predictability was higher for acidic drugs than for neutral drugs, exhibiting no effect on neutral drug predictability. Only one weakly basic drug, midazolam, was investigated in microsomes, and its
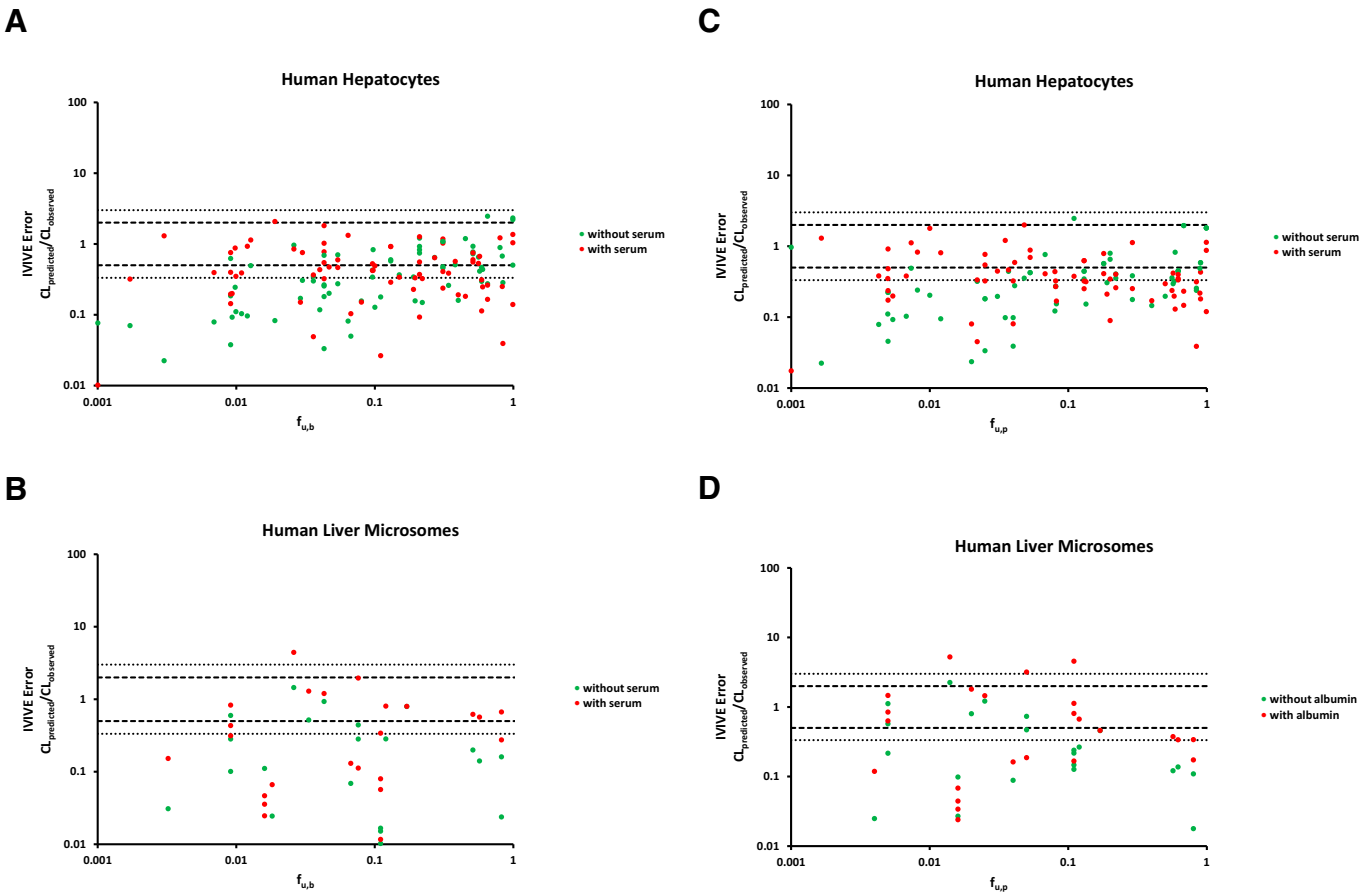
Fig. 2. Comparison of IVIVE error (predicted in vivo clearance/observed in vivo clearance) from the metabolic stability incubations with and without serum/albumin addition versus $f_{u,b}$ for (A) hepatocytes and (B) microsomes and $f_{u,p}$ for (C) hepatocytes and (D) microsomes. Dashed lines indicate within twofold, and dotted lines indicate within threefold predictability. Green dots are incubations without serum/albumin addition; red dots are incubations to which serum/albumin has been added.

TABLE 3

The effect of protein addition on the hepatocyte predictability within twofold of observed $CL_{H,b}$ (or $CL_{H,p}$) for all drugs and separated by protein-binding category

	Predicted/Observed $CL_{H,b}$				Predicted/Observed $CL_{H,p}$			
	Without Serum		With Serum		Without Serum		With Serum	
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
All drugs	67.6% (n = 48 of 71)	32.4% (n = 23 of 71)	58.9% (n = 43 of 73)	41.1% (n = 30 of 73)	82.1% (n = 55 of 67)	17.9% (n = 12 of 67)	72.5% (n = 50 of 69)	27.5% (n = 19 of 69)
$f_u < 0.1$	85.3% (n = 29 of 34)	14.7% (n = 5 of 34)	60.0% (n = 21 of 35)	40.0% (n = 14 of 35)	91.2% (n = 31 of 34)	8.82% (n = 3 of 34)	62.9% (n = 22 of 35)	37.1% (n = 13 of 35)
$f_u > 0.1$	51.4% (n = 19 of 37)	48.6% (n = 18 of 37)	57.9% (n = 22 of 38)	42.1% (n = 16 of 38)	72.7% (n = 24 of 33)	27.3% (n = 9 of 33)	82.4% (n = 28 of 34)	17.6% (n = 6 of 34)

predictability became within twofold with protein addition. These results indicate that the addition of albumin in microsomal incubations improves IVIVE for acidic drugs, although IVIVE success is still quite low, never exceeding 50% in the presence of protein.

Finally, we investigated the effect that protein addition had on the magnitude of increase of in vivo intrinsic clearance ($CL_{int, in vivo}$) values in hepatocyte and microsomal incubations (Fig. 3 and Table 9) to evaluate the potential for protein-facilitated uptake that could occur in hepatocytes (due to the presence of membranes and transporters) but not in the microsomal incubations. In hepatocytes, serum addition exhibited decreased $CL_{int, in vivo}$ in 42.5% of all drugs examined. The percentage of compounds that showed this decrease was greater in the BDDCS class 1 (51.2%) than the class 2 (33.3%) compounds, reflecting the inaccuracy of poorly soluble compounds without serum addition and the greater increase in predictability seen with poorly soluble compound when protein was added. With the addition of serum into hepatocyte incubations, 31.7% of BDDCS class 1 drugs increased $CL_{int, in vivo}$ more than twofold, whereas 55.6% of BDDCS class 2 drugs increased $CL_{int, in vivo}$ more than twofold. As seen in Fig. 3B, protein addition had a much greater increase in predictability for microsome measurements than for hepatocyte measurements. With the addition of albumin into microsomal incubations, 42.9% of BDDCS class 1 drugs displayed increases in $CL_{int, in vivo}$ of more than twofold, whereas for BDDCS class 2 drugs this value was 76.5%. These results highlight that the addition of serum/albumin into the hepatocyte/microsomal incubations tended to markedly increase (greater than twofold) unbound intrinsic clearance of a larger percentage of the poorly soluble BDDCS class 2, in comparison with the highly soluble BDDCS class 1 drugs.

Discussion

Accurate prediction of in vivo hepatic clearance is an integral part of successful and efficient drug development. Although IVIVE methodologies are commonly used to predict in vivo hepatic clearance, it has been demonstrated for decades by many investigators that there are significant limitations in predictability of clearance. A tendency for significant underprediction for primarily metabolized drugs has been reported (Bowman and Benet, 2016; Wood et al., 2017; Benet and Sodhi, 2020) and for drugs where clearance is rate limited by hepatic uptake (Zou et al., 2013; Kim et al., 2019). Moreover, it has been reported that the predictability of in vivo hepatic clearance is poor for the drugs that are highly bound to protein in blood (Francis et al., 2021). As we (Bowman and Benet, 2018) and Poulin et al. (2016) have reviewed, when conducting in vitro studies, many groups have reported that adding plasma or serum to hepatocyte incubations and adding albumin to microsome incubations can cause decreases in free Michaelis constant (K_m) values and improved IVIVE results (Ludden et al., 1997; Carlile et al., 1999; Baba et al., 2002; Shibata et al., 2002; Tang et al., 2002; Blanchard et al., 2004, 2005, 2006; Rowland et al., 2007, 2008a,b; Chao et al., 2009; Wattanachai et al., 2011, 2012, 2015; Gill et al., 2012; Mao et al., 2012; Walsky et al., 2012; Palacharla et al., 2017; Fujino et al., 2018; Bowman et al., 2019; Bteich et al., 2019; Kim et al., 2019; Nishimuta et al., 2019) (values presented in Supplemental Tables 1 and 2). Here, we review these 24 studies together to test the validity of this conclusion. For each of these investigations, we emphasize the importance of measuring incubational binding for all CL_{int} determinations, to accurately predict clearance based on the in vitro conditions in each laboratory as well as to account for nonspecific binding or high protein binding characteristics inherent for certain compounds. We also acknowledge the potential challenges of accurately measuring in vitro intrinsic clearance values for highly bound drugs in the presence of protein (particularly those that exhibit low in vitro turnover) and for poorly soluble or highly lipophilic drugs in the absence of protein due to the low amounts of available drug in the incubation.

First, as seen for all drugs in Table 1, the addition of serum only yields minimal increases in twofold predictability for hepatocyte blood clearance measurements (32.4% twofold predictability without serum addition and 41.1% with serum addition) and hepatocyte plasma

TABLE 4

The effect of protein addition on the microsome predictability within twofold of observed $CL_{H,b}$ (or $CL_{H,p}$) for all drugs and separated by protein-binding category

	Predicted/Observed $CL_{H,b}$				Predicted/Observed $CL_{H,p}$			
	Without Serum		With Serum		Without Serum		With Serum	
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
All drugs	79.2% (n = 19 of 24)	20.8% (n = 5 of 24)	62.5% (n = 15 of 24)	37.5% (n = 9 of 24)	79.2% (n = 19 of 24)	20.8% (n = 5 of 24)	66.7% (n = 16 of 24)	33.3% (n = 8 of 24)
$f_u < 0.1$	71.4% (n = 10 of 14)	28.6% (n = 4 of 14)	71.4% (n = 10 of 14)	28.6% (n = 4 of 14)	64.3% (n = 9 of 14)	35.7% (n = 5 of 14)	71.4% (n = 10 of 14)	28.6% (n = 4 of 14)
$f_u > 0.1$	90.0% (n = 9 of 10)	10.0% (n = 1 of 10)	50.0% (n = 5 of 10)	50.0% (n = 5 of 10)	100% (n = 10 of 10)	0.00% (n = 0 of 10)	70.0% (n = 7 of 10)	30.0% (n = 3 of 10)

TABLE 5

The effect of protein addition on hepatocyte predictability within twofold observed for $CL_{H,b}$ (or $CL_{H,p}$) by high solubility BDDCS class 1 and 3 drugs and low solubility BDDCS class 2 and 4 drugs

	Predicted/Observed $CL_{H,b}$				Predicted/Observed $CL_{H,p}$			
	Without Serum		With Serum		Without Serum		With Serum	
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
BDDCS class 1 and 3	60.5% (n = 26 of 43)	39.5% (n = 17 of 43)	56.8% (n = 25 of 44)	43.2% (n = 19 of 44)	70.1% (n = 34 of 43)	20.9% (n = 9 of 43)	79.5% (n = 35 of 44)	20.5% (n = 9 of 44)
BDDCS class 2 and 4	88.6% (n = 22 of 28)	21.4% (n = 6 of 28)	52.1% (n = 18 of 29)	37.9% (n = 11 of 29)	87.5% (n = 21 of 24)	12.5% (n = 3 of 24)	60.0% (n = 15 of 25)	40.0% (n = 10 of 25)

clearance measurements (17.9% without serum and 27.5% with serum). Slightly higher percentage increases are observed for microsomal measurements, but still not good predictability as seen in Table 2 (20.8% without albumin versus 37.5% with albumin addition for blood measurements, and 20.8% without versus 33.3% with albumin addition for plasma measurements). From the 73 individual blood clearance predictions with human hepatocytes for 52 different drugs and the 24 individual blood clearance predictions with human microsomes for 15 different drugs, seven drugs were evaluated in both hepatocytes (14 predictions; 28.6% predicted within twofold without serum and this increased to 42.9% with serum addition) and microsomes (12 predictions; 25.0% predicted within twofold without albumin addition and this increased to 33.3% with albumin addition) as seen in Supplemental Tables 1 and 2. The percentage of predictions within twofold for this subset were very similar to that reported in Tables 1 and 2 for the entire data set. Comparison of low, moderate, and high extraction ratio for all drugs in Tables 1 and 2 yielded no obvious consistent differences based on ER for the four different measurements (hepatocyte and microsome, blood and plasma values). We believe that it is relevant that no dependence on ER was found, as low ER compounds would provide clearance measurements that are the same for all the hepatic models of organ elimination.

In Fig. 2 and Tables 3 and 4, we tested the hypothesis that predictability of in vivo hepatic clearance is poor for drugs that are highly bound to protein in blood as proposed by Soars et al. (2007) by using the cutoff of $f_{u,p} < 0.1$. For hepatocyte incubations, IVIVE success of $CL_{H,b}$ within twofold for highly bound drugs increases from 14.7% without serum to 40.0% with serum, whereas for poorly bound drugs, IVIVE success decreases from 48.6% without serum to 42.1% with serum (Table 3). Similar results were observed for plasma measurements. In contrast, for microsomal incubations, IVIVE success for blood measurements within twofold for highly bound drugs does not change with albumin (28.6%), whereas poorly bound drugs IVIVE success increases from 10% without albumin to 50% with albumin (Table 4). Again, similar results are seen with plasma measurements. However, the contrasting trends for microsomes versus hepatocytes were

surprising as it was expected that serum addition for highly bound compounds would improve IVIVE predictions in both matrices.

Most recently, Francis et al. (2021) also reported increased predictability in the presence of protein for highly protein bound drugs. There are significant differences between the Francis et al. (2021) analysis and the material presented here that readers should consider. Francis et al. (2021) evaluated both human and rat hepatocyte data, but no microsome measurements for 22 drugs, of which nine were from the published study of Kim et al. (2019) for acidic Extended Clearance Classification System (ECCS) (Varma et al., 2015) 1B substrates where clearance is rate limited by hepatic OATP uptake. The present analysis of 52 drugs for 73 experimental human hepatocyte investigations, includes all 22 drugs analyzed by Francis et al. (2021) from 36 experimental human hepatocyte measurements. Thus, it is possible that better predictability occurs for highly bound ECCS class 1B drugs (OATP substrates), but our results suggest this is not carried over to all highly bound drugs.

IVIVE success with and without protein addition was investigated with respect to BDDCS class (Wu and Benet, 2005). Both BDDCS class 1 and 2 drugs are primarily metabolized due to their high permeability characteristics; however, BDDCS class 1 drugs have high solubility, whereas BDDCS class 2 drugs have poor solubility. It has been proposed that the high solubility characteristics of BDDCS class 1 drugs allow for rapid membrane passage of drug at high soluble concentrations, deeming any potential involvement of xenobiotic transporters clinically insignificant. However, due to the low solubility characteristics of BDDCS class 2 drugs, the potential for transporter involvement cannot be ruled out as passive diffusion may not occur at high enough concentrations to overwhelm any potential transporter-mediated involvement in drug disposition. Thus, the primarily metabolized BDDCS class 2 drugs may or may not also be transporter substrates. BDDCS class 3 and 4 drugs are expected to be substrates for transporter mediated processes but differ in terms of solubility.

In the present study, the addition of serum into the hepatocyte incubations did not improve in vivo $CL_{H,b}$ prediction for highly soluble BDDCS class 1 and 3 (39.5% without serum; 43.2% with serum) (Table 5). Poorly soluble BDDCS class 2 and 4 drugs exhibited lower

TABLE 6

The effect of protein addition on microsome predictability within twofold observed for $CL_{H,b}$ (or $CL_{H,p}$) by high solubility BDDCS class 1 drugs and low solubility BDDCS class 2 drugs

	Predicted/Observed $CL_{H,b}$				Predicted/Observed $CL_{H,p}$			
	Without Serum		With Serum		Without Serum		With Serum	
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
BDDCS class 1	71.4% (n = 5 of 7)	28.6% (n = 2 of 7)	42.9% (n = 3 of 7)	57.1% (n = 4 of 7)	85.7% (n = 6 of 7)	14.3% (n = 1 of 7)	85.7% (n = 6 of 7)	14.3% (n = 1 of 7)
BDDCS class 2	82.4% (n = 14 of 17)	17.6% (n = 3 of 17)	70.6% (n = 12 of 17)	29.4% (n = 5 of 17)	66.5% (n = 13 of 17)	23.5% (n = 4 of 17)	58.8% (n = 10 of 17)	41.2% (n = 7 of 17)

TABLE 7

The effect of protein addition on hepatocyte predictability within twofold observed for $CL_{H,b}$ (or $CL_{H,p}$) by drugs categorized as acids, bases, neutrals, or zwitterions

| | Predicted/Observed $CL_{H,b}$ | | | | Predicted/Observed $CL_{H,p}$ | | | |
| | Without Serum | | With Serum | | Without Serum | | With Serum | |
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
Acids	88.9% (n = 16 of 18)	11.1% (n = 2 of 18)	68.4% (n = 13 of 19)	31.6% (n = 6 of 19)	94.1% (n = 16 of 17)	5.9% (n = 1 of 17)	66.7% (n = 12 of 18)	33.3% (n = 6 of 18)
Bases	54.8% (n = 17 of 31)	45.2% (n = 14 of 31)	53.1% (n = 17 of 32)	46.9% (n = 15 of 32)	77.4% (n = 24 of 31)	22.6% (n = 7of 31)	71.9% (n = 23 of 32)	28.1% (n = 9 of 32)
Neutrals	75.0% (n = 13 of 20)	35.0% (n = 7 of 20)	60.0% (n = 12 of 20)	40.0% (n = 8 of 20)	76.5% (n = 13 of 17)	23.5% (n = 4 of 17)	82.4% (n = 14 of 17)	17.6% (n = 3 of 17)
Zwitterions	100% (n = 2 of 2)	0.00% (n = 0 of 2)	50.0% (n = 1 of 2)	50.0% (n = 1 of 2)	100% (n = 2 of 2)	0.00% (n = 0 of 2)	50.0% (n = 1 of 2)	50.0% (n = 1 of 2)

predictability without serum (21.4%), which increased to a comparable 37.9% with serum addition (Table 5). We had speculated that the presence of protein in in vitro incubations may be enhancing assay conditions for low-solubility compounds, by preventing nonspecific binding and potentially boosting drug concentrations available for metabolism. We also analyzed the effect of albumin addition in the microsomal incubations on IVIVE predictability, where the potential for protein-facilitated uptake is not a possibility due to the lack of cell membranes and transporters in such incubations. No data for BDDCS class 3 and 4 drugs are available. In Table 6 for the microsome incubations, improvement of IVIVE success is noted with serum addition, with a marked increase from 28.6% to 57.1% for BDDCS class 1 blood measurements but no change from 14.3% for plasma measurements. However, only seven BDDCS class 1 drug measurements were available in microsomes (versus 17 for BDDCS class 2) so the class 1 change in blood measurements may be suspect. Class 2 compounds displayed increases in IVIVE predictability with albumin addition (from 17.6% to 29.4% for blood values and 23.5% to 41.2% for plasma values), although overall predictability within twofold is still quite low with protein addition.

In Fig. 3, we plot the individual unbound intrinsic clearance values from hepatocyte incubations (Fig. 3A) and microsome incubations (Fig. 3B) with respect to BDDCS class, visually highlighting the tendency of the addition of albumin into the incubations to increase unbound intrinsic clearance of both BDDCS class 1 and class 2 drugs in both hepatocyte and microsomal incubations. Although the mechanism of the protein effect in hepatocytes has frequently been cited as protein-facilitated uptake, in microsomes it has been suggested that the protein effect is due to albumin trapping the unsaturated long-chain fatty acids that can inhibit the activity of cytochrome P450s and UDP-

glucuronosyltransferases (Rowland et al., 2007, 2008a,b; Wattanachai et al., 2011, 2012, 2015; Palacharla et al., 2017). The unsaturated long-fatty acids are present in the hepatocytes, but most of them are trapped to fatty acid-binding proteins, and thus it is thought that the concentration of the unsaturated long-fatty acids would not be high enough to inhibit cytochrome P450 and UDP-glucuronosyltransferase isoforms activities (Fujino et al., 2018). It would be expected that trapping of the inhibitory fatty acids via protein addition in microsomal incubations would increase in vitro unbound CL_{int} values of BDDCS class 1 and 2 drugs equally, as both classes are primarily metabolized. The microsomal data in Table 6 confirms that the protein effect is apparent for both BDDCS class 1 and 2; however, a greater percentage of class 2 drugs displayed increases in unbound intrinsic clearance greater than twofold (70.6% versus 42.9%), suggesting that additional factors (such as improvement of solubility) may also be contributing.

Our laboratory has recently pointed out (Benet and Sodhi, 2020) that although values of $f_{u,b}$ are required to predict in vivo blood clearance using the IVIVE method, there is potential for error in calculation of $f_{u,b}$ from experimental determination of $f_{u,p}$ and measurements of R_{BP} ratios. Experimental $f_{u,b}$ values are usually calculated by eq. 5.

$$f_{u,b} = \frac{f_{u,p}}{R_{BP}} \tag{5}$$

To derive eq. 5, one must assume that free drug concentrations are equal in the blood cells and the plasma. However, transporters have been identified in the blood cell membrane, and this assumption may not hold for transporter substrates expressed in erythrocytes. Therefore, it is possible that this inaccuracy of $f_{u,b}$ may impact poor IVIVE prediction (Benet and Sodhi, 2020). And thus, we also conducted the analysis

TABLE 8

The effect of protein addition on microsome predictability within twofold observed for $CL_{H,b}$ (or $CL_{H,p}$) by drugs categorized as acids, bases, neutrals, or zwitterions

| | Predicted/Observed $CL_{H,b}$ | | | | Predicted/Observed $CL_{H,p}$ | | | |
| | Without Serum | | With Serum | | Without Serum | | With Serum | |
	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold	Over Twofold	Within Twofold
Acids	75.0% (n = 9 of 12)	25.0% (n = 3 of 12)	50.0% (n = 6 of 12)	50.0% (n = 6 of 12)	66.7% (n = 8 of 12)	33.3% (n = 4 of 12)	50.0% (n = 6 of 12)	50.0% (n = 6 of 12)
Bases	0.00% (n = 0 of 1)	100% (n = 1 of 1)	0.00% (n = 0 of 1)	100% (n = 1 of 1)	0.00% (n = 0 of 1)	100% (n = 1 of 1)	0.00% (n = 0 of 1)	100% (n = 1 of 1)
Neutrals	88.9% (n = 8 of 9)	11.1% (n = 1 of 9)	88.9% (n = 8 of 9)	11.1% (n = 1 of 9)	100% (n = 9 of 9)	0.00% (n = 0 of 9)	88.9% (n = 8 of 9)	11.1% (n = 1 of 9)
Zwitterions	100% (n = 2 of 2)	0.00% (n = 0 of 2)	100% (n = 2 of 2)	0.00% (n = 0 of 2)	100% (n = 2 of 2)	0.00% (n = 0 of 2)	100% (n = 2 of 2)	0.00% (n = 0 of 2)

A

Human Hepatocytes

B

Human Liver Microsome
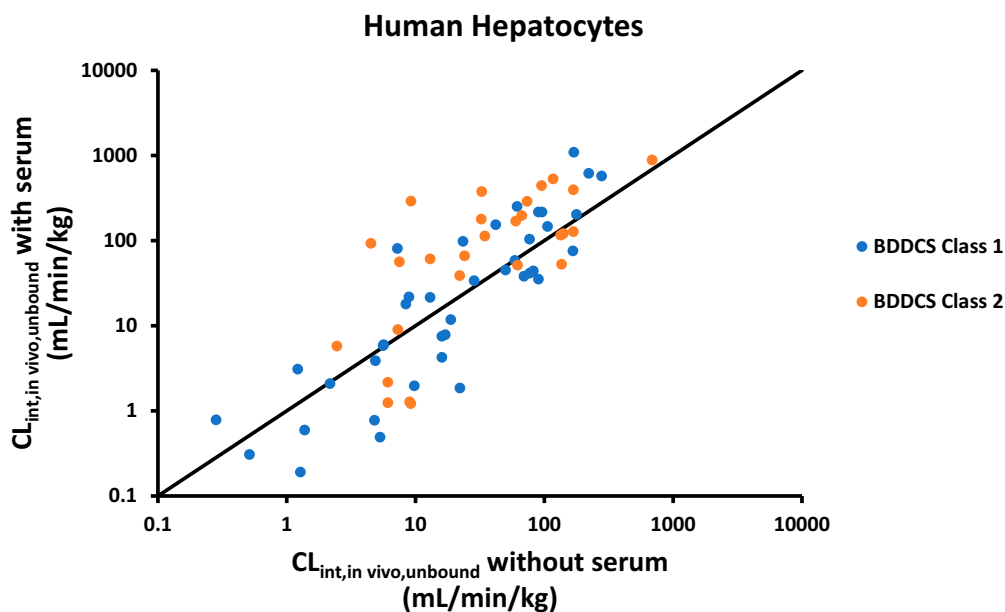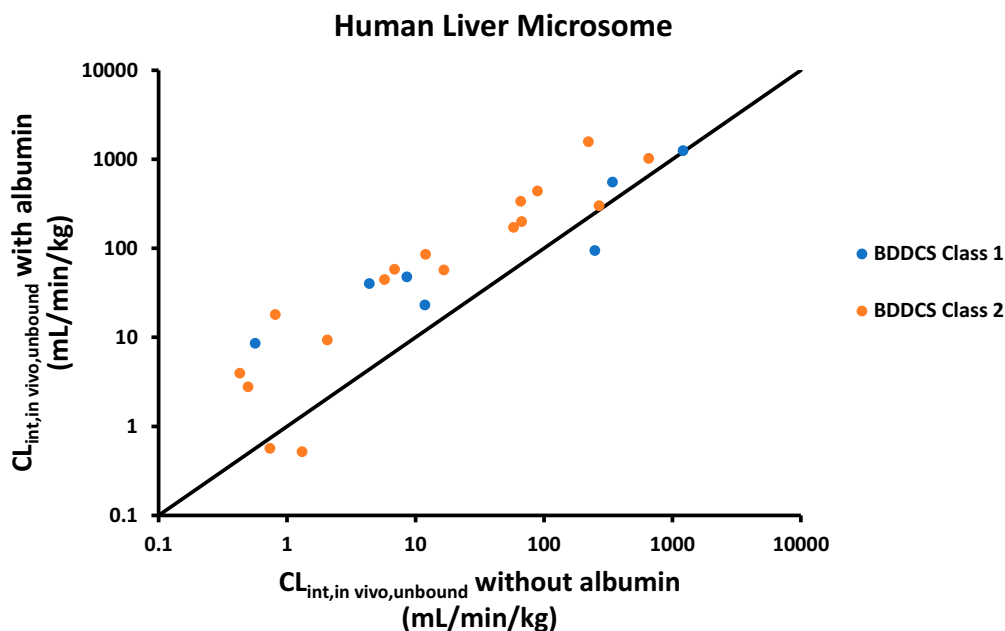
Fig. 3. Comparison of unbound intrinsic clearance from (A) hepatocyte (B) microsomal incubations with and without serum/albumin addition for BDDCS class 1 drugs (blue dots) and class 2 drugs (orange dots). Solid line is line of identity.

of $CL_{H,p}$ IVIVE to avoid using $f_{u,b}$. However, the $CL_{H,p}$ analyses did not result in improved IVIVE predictions as compared with the $CL_{H,b}$ analysis. We generally have greater faith in the blood level measurements. When attempting to use the plasma values, we had to assume that plasma flow was (1-hematocrit) multiplied by hepatic blood flow with a resulting plasma flow of 11.4 ml/min/kg as given in eq. 3. It is generally accepted that the $CL_{H,p}$ value cannot exceed $Q_{H,p}$. However, we found that 18 of the 49 drugs for which plasma measurements were analyzed, $CL_{H,p}$ values were greater than the $Q_{H,p}$ value of 11.4 mL/ min/kg (Supplemental Tables 1 and 2). In contrast, three of the 49 drugs reported $Q_{H,b}$ values greater than 20.7 mL/min/kg. The difference in frequency of the plasma clearances exceeding plasma hepatic flow is sixfold greater than when it occurred for the blood clearances exceeding blood flow.

We also investigated the effect of charge class on the predictability within twofold of observed in vivo hepatic clearance as depicted in Table 7 for hepatocyte measurements and Table 8 for microsome measurements. In the presence of protein, there appears to be a modest

TABLE 9

Analysis of reported data from hepatocyte and microsomal incubations for comparison of the change of predicted in vivo CL_{int} ($CL_{int, in\ vitro, with\ protein}$/$CL_{int, in\ vitro, without\ protein}$) in terms of BDDCS class.

		$CL_{int,in\ vivo,with\ protein}$/$CL_{int,in\ vivo,without\ protein}$			
		All	Decrease	Increase < Twofold	Increase > Twofold
Hepatocytes	All drugs	73	42.5% (31 of 73)	15.1% (11 of 73)	42.5% (31 of 73)
	BDDCS class 1	41	51.2% (21 of 41)	17.1% (7 of 41)	31.7% (13 of 41)
	BDDCS class 2	27	33.3% (9 of 27)	11.1% (3 of 27)	55.6% (15 of 27)
	BDDCS class 3	3	33.3% (1 of 3)	33.3% (1 of 3)	33.3% (1 of 3)
	BDDCS class 4	2	0.00% (0 of 2)	0.00% (0 of 2)	100% (2 of 2)
Microsomes	All drugs	24	12.5% (3 of 24)	20.8% (5 of 24)	66.7% (16 of 24)
	BDDCS class 1	7	14.3% (1 of 7)	42.9% (3 of 7)	42.9% (3 of 7)
	BDDCS class 2	17	11.8% (2 of 17)	11.8% (2 of 17)	76.5% (13 of 17)

increase in predictability for neutrals in hepatocytes but not for microsomes and a doubling of the percentage accuracy for acids in both hepatocytes and microsomes, but accuracy is never greater than 50%. For bases, serum addition caused little improvement in accuracy in hepatocytes. Only one base was studied in microsomes. It is interesting to note that the IVIVE success in the presence of protein between hepatocytes and microsomes was similar (ranging from 31.6%–50%) for acids, a class of drugs for which the rate-limiting hepatic uptake is well-documented throughout the literature (Kim et al., 2019). One may have anticipated that if rate-limiting steps based on the Extended Clearance Model (as we reviewed, Benet et al., 2018) or ECCS were relevant, that there would be differences between the IVIVE success of hepatocytes (where the possibility of rate-limited uptake exists) versus microsomes (where there is no such possibility), yet IVIVE success was approximately 31.6%–50% for both matrices in the presence of protein.

Returning to ECCS class 1B drugs, it is instructive to note that Bi et al. (2021) investigated eight ECCS class 1B drugs in human hepatocytes (with the addition of plasma compared with buffer) that were also reported by Kim et al. (2019) for studies in human hepatocytes (with the addition of 5% HSA compared with buffer). Both Bi et al. (2021) and Kim et al. (2019) report that adequate predictions can only be achieved by using a scaling factor, i.e., 2.44 (Kim et al., 2019) and 3.8–5.3 (Bi et al., 2021). This approximately twofold difference in scaling factor reflects that significant differences in predictability are found in different, highly respected laboratories for the same drugs and emphasizes the need for future studies comparing the effects of different amounts of added protein as well as different experimental systems. It is important to note that the unbound CL_{int} of Kim et al. (2019) for uptake of OTAP substrates was determined using the oil-spin method, whereas CL_{int} values of the other data sets cited in our analysis were mainly determined by the conventional hepatocyte stability assay (measuring the compound disappearance in hepatocyte incubation). The two methods could lead to significant differences in underestimation of hepatic clearance for hepatic uptake transporter substrates, although neither method gives IVIVE predictions within twofold with protein addition. Even more recently, the Sugiyama laboratory, guided by its understanding of the facilitated-dissociation model obtained from the 10 highly protein-bound anionic drugs analyzed by Kim et al. (2019), proposed a simplified method to predict the hepatic uptake clearance in vivo (in the presence of the physiologic 5% albumin concentration) for these 10 drugs (Miyauchi et al., 2021). In this simplified method, Miyauchi et al. (2021) introduced the concept of the Extent of Facilitated Dissociation (EFD) where the increase of intrinsic permeability clearance of unbound drug via influx into hepatocytes at percentage of albumin concentration ($PS_{u,inf,\ 5\%}$) for these 10 anionic substrates may be predicted from the measured intrinsic uptake clearance in the absence of albumin ($PS_{u,inf,0\%}$) using eq. 6.

$$PS_{u,inf,5\%} = PS_{u,inf,0\%} \cdot \left(1 + \frac{EFD}{K_d}\right) \qquad (6)$$

They reported that an EFD value of 11 μM and a dissociation equilibrium constant for the binding of a ligand to albumin value of 46 μM could be used for all the 10 substrates rather than measuring the 5% albumin PS value. Miyauchi et al. (2021) report that the prediction of hepatic clearance obtained by the simplified method was comparable to those obtained by the previously proposed method utilizing the measurement of the protein binding and hepatic uptake at varying concentrations of albumin. Whether this facilitated dissociation model for a particular subset of drugs (highly protein-bound OATP substrates) (Kim et al., 2019; Miyauchi et al., 2021) and other studies proposing protein-facilitated uptake via the presence of an albumin-specific receptor on the hepatocyte surface, rate-limiting dissociation of ligand from a protein-ligand complex, rate-limiting diffusion of ligand through an unstirred water layer, interactions with the hepatocyte cell surface (as reviewed by Poulin et al., 2016 and Bowman and Benet, 2018), as well as our proposed transporter-induced protein binding shift (Bowman et al., 2019), we emphasize that all of these studies do not include the appropriate controls, i.e., drugs giving good IVIVE predictions without the addition of protein. For example, another very recent article by Bteich et al. (2021) shows excellent improvement in hepatic clearance predictivity for fluoxetine and perampanel using a newly proposed extrapolation approach, but like other proposed methodologies does not validate the approach by testing a drug showing good predictability without protein addition. We are not suggesting that this last study and others do not show increased predictability with the addition of protein, just that the conclusions regarding the proposed hypotheses being tested by these investigators (regardless of the methodology used) can only be validated by also including drugs that exhibit good predictability without protein addition. In all the analyses reviewed thus far, there appears to be minimal support for the hypothesis that protein addition provides a clinically meaningful increase in IVIVE predictability when all published drugs investigated in hepatocyte and microsomal incubations are considered.

When a presentation such as this proposes that a well-accepted methodology has little use, the scientific community, of course, questions the data upon which this conclusion is made. Here, we only review the published data. However, in our very recently accepted manuscript (Benet and Sodhi, 2021), we report that the original concept of the "albumin mediated hepatic uptake" is based on invalid assumptions and that, in fact, there is no theoretical basis upon which IVIVE should be expected to work, as it doesn't. However, the scattered literature data upon which our conclusions here are based probably requires that prospective, well-controlled experimental studies in a single laboratory will be needed to rigorously determine whether adding protein in any form to in vitro

determinations of clearance improves the IVIVE and whether any improvement can be tied to chemical structure, physical properties (e.g., solubility, charge), and clearance mechanism, before the scientific community abandons the concept. We would welcome such a study.

Conclusions

The present manuscript demonstrates for the available experimental data that addition of protein to in vitro incubations of hepatocytes and microsomes does not improve IVIVE predictability in a useful manner. Analyzing published data for 60 different drugs and 97 experimental comparisons (with 17 drugs being investigated from two to seven times), we confirmed the marked underprediction of clearance previously reported. The addition of protein into hepatocyte incubations has been reported to improve the predictability for high clearance (extraction ratio) drugs and highly protein-bound drugs. However, we could not validate any relevant improved predictability within twofold by the addition of serum to hepatocyte incubations or albumin to microsomal incubations. This was the case when investigating all measurements, or when subdividing analyses by extraction ratio, degree of protein binding, BDDCS class, examining ECCS class 1B drugs only, or drug charge.

For the 73 human hepatocyte studies, adding protein decreased predictability in 42.5% of the evaluations. Manipulating characteristics of small data sets of similar compounds and adding scaling factors can appear to yield good predictability, but the carryover of these methods to alternate drug classes and different laboratories is not evident. Improvement in predictability of poorly soluble compounds is greater than that for soluble compounds but not to a meaningful extent. Overall, we cannot confirm that protein addition improves IVIVE predictability to any meaningful degree when considering all drugs and different subsets.

Authorship Contributions

Participated in research design: Kameyama, Sodhi, Benet.

Performed data analysis: Kameyama.

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

References

Baba T, Touchi A, Ito K, Yamaguchi Y, Yamazoe Y, Ohno Y, and Sugiyama Y (2002) Effects of serum albumin and liver cytosol on CYP2C9- and CYP3A4-mediated drug metabolism. *Drug Metab Pharmacokinet* **17**:522–531.

Bachmann K, Byers J, and Ghosh R (2003) Prediction of in vivo hepatic clearance from in vitro data using cryopreserved human hepatocytes. *Xenobiotica* **33**:475–483.

Benet LZ, Bowman CM, Liu S, and Sodhi JK (2018) The extended clearance concept following oral and intravenous dosing: theory and critical analyses. *Pharm Res* **35**:242.

Benet LZ, Broccatelli F, and Oprea TI (2011) BDDCS applied to over 900 drugs. *AAPS J* **13**:519–547.

Benet LZ and Sodhi JK (2020) Investigating the theoretical basis for in vitro-in vivo extrapolation (IVIVE) in predicting drug metabolic clearance and proposing future experimental pathways. *AAPS J* **22**:120.

Benet LZ and Sodhi JK (2021) Can in vitro-in vivo extrapolation (IVIVE) be successful? Recognizing the incorrect clearance assumptions. *Clin Pharmacol Ther* (published ahead of print) 10.1002/cpt.2482.

Bi Y-A, Ryu S, Tess DA, Rodrigues AD, and Varma MVS (2021) Effect of human plasma on hepatic uptake of organic anion-transporting polypeptide 1B substrates: studies using transfected cell and primary human hepatocytes. *Drug Metab Dispos* **49**:72–83.

Blanchard N, Alexandre E, Abadie C, Lavé T, Heyd B, Mantion G, Jaeck D, Richert L, and Coassolo P (2005) Comparison of clearance predictions using primary cultures and suspensions of human hepatocytes. *Xenobiotica* **35**:1–15.

Blanchard N, Hewitt NJ, Silber P, Jones H, Coassolo P, and Lavé T (2006) Prediction of hepatic clearance using cryopreserved human hepatocytes: a comparison of serum and serum-free incubations. *J Pharm Pharmacol* **58**:633–641.

Blanchard N, Richert L, Notter B, Delobel F, David P, Coassolo P, and Lavé T (2004) Impact of serum on clearance predictions obtained from suspensions and primary cultures of rat hepatocytes. *Eur J Pharm Sci* **23**:189–199.

Bowman CM and Benet LZ (2016) Hepatic clearance predictions from in vitro-in vivo extrapolation and the biopharmaceutics drug disposition classification system. *Drug Metab Dispos* **44**:1731–1735.

Bowman CM and Benet LZ (2018) An examination of protein binding and protein-facilitated uptake relating to in vitro-in vivo extrapolation. *Eur J Pharm Sci* **123**:502–514.

Bowman CM, Chen E, Chen L, Chen YC, Liang X, Wright M, Chen Y, and Mao J (2020) Changes in organic anion transporting polypeptide uptake in HEK293 overexpressing cells in the presence and absence of human plasma. *Drug Metab Dispos* **48**:18–24.

Bowman CM, Okochi H, and Benet LZ (2019) The presence of a transporter-induced protein binding shift: a new explanation for protein-facilitated uptake and improvement for in vitro-in vivo extrapolation. *Drug Metab Dispos* **47**:358–363.

Bteich M, Poulin P, and Haddad S (2019) The potential protein-mediated hepatic uptake: discussion on the molecular interactions between albumin and the hepatocyte cell surface and their implications for the in vitro-to-in vivo extrapolations of hepatic clearance of drugs. *Expert Opin Drug Metab Toxicol* **15**:633–658.

Bteich M, Poulin P, and Haddad S (2021) Comparative assessment of extrapolation methods based on the conventional free drug hypothesis and plasma protein-mediated hepatic uptake theory for the hepatic clearance predictions of two drugs extensively bound to both albumin and alpha-1-acid glycoprotein. *J Pharm Sci* **110**:1385–1391.

Carlile DJ, Hakooz N, Bayliss MK, and Houston JB (1999) Microsomal prediction of in vivo clearance of CYP2C9 substrates in humans. *Br J Clin Pharmacol* **47**:625–635.

Chao P, Barminko J, Novik E, Han Y, Maguire T, and Cheng K-C (2009) Prediction of human hepatic clearance using an in vitro plated hepatocyte clearance model. *Drug Metab Lett* **3**:296–307.

Francis LJ, Houston JB, and Hallifax D (2021) Impact of plasma protein binding in drug clearance prediction: a database analysis of published studies and implications for in vitro-in vivo extrapolation. *Drug Metab Dispos* **49**:188–201.

Fujino R, Hashizume K, Aoyama S, Maeda K, Ito K, Toshimoto K, Lee W, Ninomiya SI, and Sugiyama Y (2018) Strategies to improve the prediction accuracy of hepatic intrinsic clearance of three antidiabetic drugs: application of the extended clearance concept and consideration of the effect of albumin on CYP2C metabolism and OATP1B-mediated hepatic uptake. *Eur J Pharm Sci* **125**:181–192.

Gill KL, Houston JB, and Galetin A (2012) Characterization of in vitro glucuronidation clearance of a range of drugs in human kidney microsomes: comparison with liver and intestinal glucuronidation and impact of albumin. *Drug Metab Dispos* **40**:825–835.

Hallifax D, Foster JA, and Houston JB (2010) Prediction of human metabolic clearance from in vitro systems: retrospective analysis and prospective view. *Pharm Res* **27**:2150–2161.

Hosey CM, Chan R, and Benet LZ (2016) BDDCS predictions, self-correcting aspects of BDDCS assignments, BDDCS assignment corrections, and classification for more than 175 additional drugs. *AAPS J* **18**:251–260.

Kim SJ, Lee KR, Miyauchi S, and Sugiyama Y (2019) Extrapolation of in vivo hepatic clearance from in vitro uptake clearance by suspended human hepatocytes for anionic drugs with high binding to human albumin: improvement of in vitro-to-in vivo extrapolation by considering the "albumin-mediated" hepatic uptake mechanism on the basis of the "facilitated-dissociation model". *Drug Metab Dispos* **47**:94–103.

Lombardo F, Berellini G, and Obach RS (2018) Trend analysis of a database of intravenous pharmacokinetic parameters in humans for 1352 drug compounds. *Drug Metab Dispos* **46**:1466–1477.

Ludden LK, Ludden TM, Collins JM, Pentikis HS, and Strong JM (1997) Effect of albumin on the estimation, in vitro, of phenytoin Vmax and Km values: implications for clinical correlation. *J Pharmacol Exp Ther* **282**:391–396.

Mao J, Mohutsky MA, Harrelson JP, Wrighton SA, and Hall SD (2012) Predictions of cytochrome P450-mediated drug-drug interactions using cryopreserved human hepatocytes: comparison of plasma and protein-free media incubation conditions. *Drug Metab Dispos* **40**:706–716.

Miyauchi S, Masuda M, Kim S-J, Tanaka Y, Lee K-R, Iwakado S, Nemoto M, Sasaki S, Shimono K, Tanaka Y, et al. (2018) The phenomenon of albumin-mediated hepatic uptake of organic anion transport polypeptide substrates: prediction of the in vivo uptake clearance from the in vitro uptake by isolated hepatocytes using a facilitated-dissociation model. *Drug Metab Dispos* **46**:259–267.

Miyauchi S, Kim S, Lee W, and Sugiyama Y (2021) Consideration of albumin-mediated hepatic uptake for highly protein-bound anionic drugs: bridging the gap of hepatic uptake clearance between in vitro and in vivo. *Pharmacol Ther* **229**:107938. 10.1016/j.pharmthera.2021.107938.

Nishimuta H, Watanabe T, and Bando K (2019) Quantitative prediction of human hepatic clearance for P450 and non-P450 substrates from in vivo monkey pharmacokinetics study and in vitro metabolic stability tests using hepatocytes. *AAPS J* **21**:20.

Palacharla RC, Uthukam V, Manoharan A, Ponnamaneni RK, Padala NP, Boggavarapu RK, Bhyrapuneni G, Ajjala DR, and Nirogi R (2017) Inhibition of cytochrome P450 enzymes by saturated and unsaturated fatty acids in human liver microsomes, characterization of enzyme kinetics in the presence of bovine serum albumin (0.1 and 1.0% w/v) and in vitro-in vivo extrapolation of hepatic clearance. *Eur J Pharm Sci* **101**:80–89.

Poulin P, Burczynski FJ, and Haddad S (2016) The role of extracellular binding proteins in the cellular uptake of drugs: impact on quantitative in vitro-to-in vivo extrapolations of toxicity and efficacy in physiologically based pharmacokinetic-pharmacodynamic research. *J Pharm Sci* **105**:497–508.

Rowland A, Elliot DJ, Knights KM, Mackenzie PI, and Miners JO (2008a) The "albumin effect" and in vitro-in vivo extrapolation: sequestration of long-chain unsaturated fatty acids enhances phenytoin hydroxylation by human liver microsomal and recombinant cytochrome P450 2C9. *Drug Metab Dispos* **36**:870–877.

Rowland A, Gaganis P, Elliot DJ, Mackenzie PI, Knights KM, and Miners JO (2007) Binding of inhibitory fatty acids is responsible for the enhancement of UDP-glucuronosyltransferase 2B7 activity by albumin: implications for in vitro-in vivo extrapolation. *J Pharmacol Exp Ther* **321**:137–147.

Rowland A, Knights KM, Mackenzie PI, and Miners JO (2008b) The "albumin effect" and drug glucuronidation: bovine serum albumin and fatty acid-free human serum albumin enhance the glucuronidation of UDP-glucuronosyltransferase (UGT) 1A9 substrates but not UGT1A1 and UGT1A6 activities. *Drug Metab Dispos* **36**:1056–1062.

Shibata Y, Takahashi H, Chiba M, and Ishii Y (2002) Prediction of hepatic clearance and availability by cryopreserved human hepatocytes: an application of serum incubation method. *Drug Metab Dispos* **30**:892–896.

Soars MG, McGinnity DF, Grime K, and Riley RJ (2007) The pivotal role of hepatocytes in drug discovery. *Chem Biol Interact* **168**:2–15.

Sodhi JK and Benet LZ (2021) Successful and unsuccessful prediction of human hepatic clearance for lead optimization. *J Med Chem* **64**:3546–3559.

Tang C, Lin Y, Rodrigues AD, and Lin JH (2002) Effect of albumin on phenytoin and tolbutamide metabolism in human liver microsomes: an impact more than protein binding. *Drug Metab Dispos* **30**:648–654.

Varma MV, Steyn SJ, Allerton C, and El-Kattan AF (2015) Predicting clearance mechanism in drug discovery: Extended Clearance Classification System (ECCS). *Pharm Res* **32**:3785–3802.

Walsky RL, Bauman JN, Bourcier K, Giddens G, Lapham K, Negahban A, Ryder TF, Obach RS, Hyland R, and Goosen TC (2012) Optimized assays for human UDP-glucuronosyltransferase (UGT) activities: altered alamethicin concentration and utility to screen for UGT inhibitors. *Drug Metab Dispos* **40**:1051–1065.

Wattanachai N, Polasek TM, Heath TM, Uchaipichat V, Tassaneeyakul W, Tassaneeyakul W, and Miners JO (2011) In vitro-in vivo extrapolation of CYP2C8-catalyzed paclitaxel 6α-hydroxylation: effects of albumin on in vitro kinetic parameters and assessment of interindividual variability in predicted clearance. *Eur J Clin Pharmacol* **67**:815–824.

Wattanachai N, Tassaneeyakul W, Rowland A, Elliot DJ, Bowalgaha K, Knights KM, and Miners JO (2012) Effect of albumin on human liver microsomal and recombinant CYP1A2 activities: impact on in vitro-in vivo extrapolation of drug clearance. *Drug Metab Dispos* **40**:982–989.

Wattanachai N, Tassaneeyakul W, and Tassaneeyakul W (2015) The effects of bovine serum albumin on kinetic characterization of human liver microsomal CYP2C19 and CYP2E1 activities. *Thai J Pharmacol* **37**:27–40.

Wood FL, Houston JB, and Hallifax D (2017) Clearance prediction methodology needs fundamental improvement: trends common to rat and human hepatocytes/microsomes and implications for experimental methodology. *Drug Metab Dispos* **45**:1178–1188.

Wu CY and Benet LZ (2005) Predicting drug disposition via application of BCS: transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm Res* **22**:11–23.

Zou P, Liu X, Wong S, Feng MR, and Liederer BM (2013) Comparison of in vitro-in vivo extrapolation of biliary clearance using an empirical scaling factor versus transport-based scaling factors in sandwich-cultured rat hepatocytes. *J Pharm Sci* **102**:2837–2850.

Address correspondence to: Dr. Leslie Z. Benet, University of California San Francisco, 513 Parnassus Avenue, Room HSE-1164, Campus Box 0912, San Francisco, CA 94143. E-mail: leslie.benet@ucsf.edu

SUPPLEMENTAL INFORMATION

Does Addition of Protein to Hepatocyte or Microsomal In Vitro Incubations Provide A Useful Improvement in In Vitro-In Vivo Extrapolation (IVIVE)

Predictability?

Tsubasa Kameyama, Jasleen K. Sodhi and Leslie Z. Benet

Drug Metabolism and Disposition, Manuscript # DMD-AR-2021-000677

Table S-1 Pharmacokinetic parameters of drugs from human hepatocytes and in vivo measures

Drug	BDDCS	Charge	$f_{u,b}$ [a]	$f_{u,p}$ [a]	$f_{u,hep}$ Without Serum	$f_{u,hep}$ With Serum [c]	Observed $CL_{H,b}$ [a] ml/min/kg	Observed $CL_{H,p}$ [b] ml/min/kg	Predicted $CL_{int,in\,vivo,unbound}$ [d] (mL/min/kg) Without Serum	Predicted $CL_{int,in\,vivo,unbound}$ With Serum	Predicted $CL_{H,b}$ (mL/min/kg) Without Serum	Predicted $CL_{H,b}$ With Serum	Predicted $CL_{H,p}$ (mL/min/kg) Without Serum	Predicted $CL_{H,p}$ With Serum	Reference
Carbamazepine	2	neutral	0.31	0.26	0.84 [a]	-	1.6	-	2.46 [c]	5.77	0.734	1.65	0.605	1.32	
Phenytoin	2	Acid	0.11	0.11	0.84 [a]	-	5.3	0.37	9.00 [c]	1.28	0.945	0.14	0.911	0.139	
Procainamide	3	Base	0.84 [e]	0.84	0.92 [f]	-	10 [e]	10	3.93 [c]	0.477	2.85	0.393	2.56	0.387	(1)
Quinidine	1	Base	0.21	0.2	0.64 [a]	-	4.1	4	22.1 [c]	1.85	3.79	0.38	3.18	0.357	
Theophylline	1	neutral	0.45	0.59	0.96 [a]	-	0.47	0.86	1.28 [c]	0.191	0.559	0.0854	0.707	0.111	
Antipyrine	1	neutral	0.99	0.99	1 [c]	1	0.55	0.64	1.38 [c]	0.595	1.28	0.573	1.22	0.56	(2)

Bosentan	2	Acid	0.064	0.035	1[c]	0.026	3.5	2.1	4.51[c]	93.1	0.285	4.63	0.156	2.53
Mibefradil	2	Acid	0.012[c]	0.012[c]	1[c]	0.012	4[e]	4	32.7[c]	377	0.385	3.71	0.379	3.24
Midazolam	1	Base	0.043	0.025	0.817[c]	0.021	9.2	5.3	61.5[c]	252	0.317	2.17	0.348	2.19
Naloxone	1	neutral	0.51	0.62	1[c]	0.596	18	23	69.5[c]	38.1	2.35	7.12	1.36	4.06
Oxazepam	2	neutral	0.043	0.048	0.8[c]	0.05	1.2	1.1	7.49[c]	56.5	13.1	10	9.01	7.69
Alprazolam	1	Base	0.31	0.29	0.843[c]	0.581	0.78	0.74	1.22[c]	3.09	0.371	0.917	0.343	0.832
Antipyrine	1	neutral	0.99	0.99	0.907[c]	0.655	0.55	0.64	0.283[c]	0.784	0.277	0.748	0.274	0.727
Caffeine	1	neutral	0.65	0.68	1[c]	0.834	1.2	1.4	0.514[c]	0.308	0.329	0.198	0.339	0.206
Carbamazepine	2	neutral	0.31	0.26	0.84[c]	0.411	1.6	-	6.1[c]	1.25	1.73	0.38	1.39	0.316
Chlorpromazine	1	Base	0.043	0.037	0.395[c]	0.082	11	16	278[c]	573	7.58	11.3	5.41	7.41
Clozapine	2	Base	0.054	0.053	0.481[c]	0.1	4.1	2.5	61.9[c]	51.4	2.88	2.45	2.55	2.2
Desipramine	1	Base	0.21	0.18	0.819[c]	0.357	11	11	178[c]	203	13.3	13.9	8.41	8.69
Diazepam	1	neutral	0.036	0.022	0.643[c]	0.33	0.57	0.38	4.79[c]	0.778	0.171	0.028	0.104	0.0171
Diclofenac	2	Acid	0.0091	0.005	0.05[c]	0.011	7.7	3.5	688[c]	887	4.81	5.81	2.64	3.19
Diltiazem	1	Base	0.22	0.22	0.894[c]	0.684	12	13	8.9[c]	21.8	1.79	3.89	1.67	3.37
Imipramine	1	Base	0.13	0.13	0.821[c]	0.32	13	13	96[c]	217	7.79	11.9	5.96	8.12
Indomethacin	2	Acid	0.019	0.01	0.307[c]	0.015	2.1	1.3	9.2[c]	291	0.173	4.36	0.0913	2.32
Labetalol	1	Base	0.38	0.5	0.928[c]	0.463	14	23	28.5[c]	33.8	7.11	7.93	6.33	6.81
Lorazepam	1	neutral	0.08	0.082	0.474[c]	0.246	1.1	1	2.17[c]	2.09	0.172	0.166	0.175	0.169
Naloxone	1	neutral	0.51	0.62	0.834[c]	0.852	18	23	82.2[c]	44	13.9	10.8	9.32	8.04
Naproxen	2	Acid	0.001	0.001	0.028[c]	0.631	0.12	0.07	9.17[c]	1.22	0.00917	0.00122	0.00916	0.00122
Nitrendipine	2	zwitterion	0.029	0.02	0.347[c]	0.055	20	25	141[c]	121	3.41	3.01	2.26	2
Pindolol	1	Base	0.56	0.56	1[c]	0.992	3.7	7.7	4.88[c]	3.88	2.41	1.97	2.2	1.83

(3)

Sildenafil	1	Base	0.04	0.04	0.681[c]	0.139	7.6	9	23.4[c]	97.9	0.895	3.29	0.864	2.92	
Timolol	1	Base	0.59	0.9	1[c]	0.858	9.7	8.5	5.65[c]	5.99	2.87	3.02	3.52	3.66	
Verapamil	1	Base	0.096	0.081	0.838[c]	0.442	16	18	76.9[c]	104	5.44	6.74	4.03	4.84	
Bufuralol	1	Base	0.19[e]	0.19	0.66[f]	-	8.9[e]	8.9	18.8[c]	11.8	3.04	2.03	2.72	1.88	
Diclofenac	2	Acid	0.0091	0.005	0.94[a]	-	7.7	3.5	32.4[c]	179	0.291	1.51	0.16	0.829	(4)
Midazolam	1	Base	0.043	0.025	0.92[a]	-	9.2	5.3	7.23[c]	80.7	0.306	2.97	0.178	1.71	
Alprenolol	1	Base	0.27	0.22	0.85[a]	-	14	15	59[a]	58.4	9	8.95	6.07	6.04	
Carbamazepine	2	neutral	0.31	0.26	0.84[a]	-	1.6	-	6.1[a]	2.17	1.73	0.652	1.39	0.538	
Clozapine	2	Base	0.054	0.053	0.7[a]	-	4.1	2.5	22[a]	38.8	1.12	1.9	1.06	1.74	
Desipramine	1	Base	0.21	0.18	0.16[a]	-	11	11	77[a]	41.4	9.08	6.12	6.26	4.5	
Diclofenac	2	Acid	0.0091	0.005	0.94[a]	-	7.7	3.5	168[a]	128	1.42	1.11	0.782	0.608	
Flumazenil	1	neutral	0.6	0.58	0.94[a]	-	15	16	16[a]	7.53	6.56	3.71	5.12	3.16	
Gemfibrozil	2	Acid	0.026	0.014	0.93[a]	-	3.1	-	134[a]	116	2.98	2.62	1.61	1.42	
Haloperidol	2	Base	0.1	0.08	0.56[a]	-	9.6	7.8	13[a]	61	1.22	4.71	0.953	3.42	(5)
Imipramine	1	Base	0.13	0.13	0.18[a]	-	13	13	90[a]	217	7.48	11.9	5.77	8.12	
Ketanserin	2	Base	0.097	0.068	0.87[a]	-	9.7	6.7	136[a]	52.9	8.06	4.11	5.11	2.73	
Midazolam	1	Base	0.043	0.025	0.92[a]	-	9.2	5.3	42[a]	154	1.66	5.02	0.961	2.88	
Nifedipine	2	zwitterion	0.03	0.041	0.79[a]	-	5.4	7.3	60[a]	169	1.66	4.07	2.02	4.31	
Omeprazole	1	neutral	0.067	0.04	0.87[a]	-	11	8.4	8.4[a]	18	0.548	1.14	0.326	0.676	
Ranitidine	3	Base	0.8	0.84	0.95[a]	-	2.7	9.6	3.4[a]	4.89	2.4	3.29	2.28	3.02	
Zaleplon	2	neutral	0.4	0.4	0.92[a]	-	16	16	7.3[a]	8.99	2.56	3.06	2.32	2.73	
Antipyrine	1	neutral	0.99	0.99	0.95[a]	-	0.55	0.64	1.3[a]	0.0778	1.21	0.0768	1.16	0.0765	(6)
Caffeine	1	neutral	0.65	0.68	0.96[a]	-	1.2	1.4	5.3[a]	0.491	2.95	0.314	2.74	0.324	

Diazepam	1	neutral	0.036	0.022	0.54 [a]	-	0.57	0.38	5.6 [a]	5.84	0.2	0.208	0.122	0.127	
Diclofenac	2	Acid	0.0091	0.005	0.94 [a]	-	7.7	3.5	168 [a]	395	1.42	3.07	0.782	1.69	
Imipramine	1	Base	0.13	0.13	0.18 [a]	-	13	13	90 [a]	35.2	7.48	3.74	5.77	3.26	
Lidocaine	1	Base	0.34	0.29	0.9 [a]	-	14	16	13 [a]	21.5	3.64	5.41	2.83	4.03	
Metoprolol	1	Base	0.83	0.89	0.9 [a]	-	12	13	16 [a]	4.24	8.09	3.01	6.33	2.84	
Naloxone	1	neutral	0.51	0.62	0.93 [a]	-	18	23	167 [a]	75.8	16.7	13.5	10.3	9.18	
Phenacetin	2	neutral	0.57	0.57	0.91 [a]	-	20	21	24 [a]	66.3	8.24	13.4	6.22	8.76	
Propranolol	1	Base	0.15	0.13	0.63 [a]	-	15	12	50 [a]	45	5.51	5.09	4.14	3.87	
Quinidine	1	Base	0.21	0.2	0.64 [a]	-	4.1	4	17 [a]	7.83	3.04	1.52	2.62	1.38	
Timolol	1	Base	0.59	0.9	0.9 [a]	-	9.7	8.5	9.8 [a]	1.97	4.52	1.1	4.97	1.53	
Verapamil	1	Base	0.096	0.081	0.52 [a]	-	16	18	106 [a]	147	6.82	8.41	4.9	5.83	
Atorvastatin	2	Acid	0.0467 [c]	0.0308 [c]	0.93 [f]	-	13.5 [c]	8.91 [c]	66.8 [c]	197	2.71	6.38	1.74	3.97	
Bosentan	2	Acid	0.00981 [c]	0.0081 [c]	0.81 [a]	-	2.85 [c]	2.37 [c]	73.6 [c]	289	0.697	2.5	0.569	1.95	
Cerivastatin	1	Acid	0.0127 [c]	0.0073 [c]	0.90 [f]	-	5 [c]	2.9 [c]	221 [c]	617	2.47	5.69	1.42	3.24	
Fluvastatin	1	Acid	0.0069 [c]	0.0043 [c]	0.94 [f]	-	14 [c]	8.68 [c]	170 [c]	1090	1.11	5.52	0.683	3.31	
Glibenclamide	2	Acid	0.00171 [c]	0.0008 [c]	0.87 [f]	-	2.3 [c]	1.06 [c]	95.0 [c]	444	0.161	0.732	0.0743	0.339	(7)
Nateglinide	2	Acid	0.00994 [c]	0.005 [c]	0.93 [f]	-	3.05 [c]	1.53 [c]	34.5 [c]	113	0.337	1.07	0.169	0.535	
Pitavastatin	4	Acid	0.00932 [c]	0.0054 [c]	0.93 [f]	-	9.64 [c]	5.59 [c]	100 [c]	227	0.891	1.92	0.516	1.11	
Repaglinide	2	Acid	0.0109 [c]	0.0068 [c]	0.86 [f]	-	11.6 [c]	7.19 [c]	117 [c]	531	1.2	4.52	0.74	2.73	
Rosuvastatin	3	Acid	0.195 [c]	0.134 [c]	0.96 [f]	-	12.1 [c]	8.35 [c]	10.7 [c]	25.6	1.9	4.02	1.28	2.63	
Valsartan	4	Acid	0.00301 [c]	0.0017 [c]	0.95 [f]	-	0.678 [c]	0.373 [c]	5.05 [c]	306	0.0152	0.883	0.00833	0.484	

[a] Wood et al., 2017; [b] Lombardo et al., 2018; [c] The values were cited from the studies in the reference column;

[d] Values were calculated using consistent scaling factors. The physiological scaling factor utilized for hepatocellularity was 120 x 10^6 hepatocytes/g liver and the liver weight was 21.4 g/kg body weight;

$$f_{u,hep} = \frac{1}{1+125 \cdot V_R \cdot 10^{0.072 \cdot logP/D^2 + 0.067 \cdot logP/D - 1.126}} \qquad (S1)$$

Log P/D is either the log P value for basic and neutral drugs or the log D value for acidic drug; and V_R is the volume ratio of hepatocytes to medium (0.005 for 1×10^6 cells/ml).

Table S-2 Pharmacokinetic parameters of drugs from human microsomes and in vivo measures

Drug	BDDCS	Charge	$f_{u,b}$	$f_{u,p}$	$f_{u,inc}$ [d]		Observed		Predicted						Reference
							$CL_{H,b}$	$CL_{H,p}$ [b]	$CL_{int,in\ vivo,umbound}$ [d] (mL/min/kg)		$CL_{H,b}$ (mL/min/kg)		$CL_{H,p}$ (mL/min/kg)		
					Without	With	(ml/min/kg)	(ml/min/kg)	Without	With	Without	With	Without	With	

					Albumin	Albumin			Albumin	Albumin	Albumin	Albumin	Albumin	Albumin	
Diclofenac	2	Acid	0.0091 [a]	0.005 [a]	0.84-0.90	0.002-0.029	7.7 [a]	3.5	268	300	0.377	0.941	0.206	0.515	(10)
Tolbutamide	2	Acid	0.076 [a]	0.05 [a]	0.93-0.99	0.011-0.095	0.35 [a]	0.21	1.31	0.519	1.21	0.178	1.16	0.177	
Phenytoin	2	Acid	0.11 [a]	0.11 [a]	0.36	0.25	5.3 [a]	0.37	0.428	3.94	2.95	0.278	2.74	0.288	(11)
Diclofenac	2	Acid	0.0091 [a]	0.005 [a]	0.98	0.02	7.7 [a]	3.5	651	1020	9.08	6.12	6.26	4.5	
Gemfibrozil	2	Acid	0.026 [a]	0.014 [a]	0.90	0.01	3.1 [a]	1.7	222	1580	7.48	11.9	5.77	8.12	
Mycophenolic acid	2	Acid	0.0333 [c]	0.02 [b]	0.83	0.18	3.88 [c]	2.33	66.9	199	1.73	0.652	1.39	0.538	(12)
Naloxone	1	neutral	0.51 [a]	0.62 [a]	1.00	0.96	18 [a]	23	8.56	47.6	1.66	5.02	0.961	2.88	
Propofol	2	neutral	0.0182 [c]	0.016 [b]	0.69	0.25	40.9 [c]	36	58	172	1.66	4.07	2.02	4.31	
Telmisartan	2	Acid	0.00323 [c]	0.004 [b]	0.65	0.07	6.77 [c]	8.4	65.8	338	1.42	1.11	0.782	0.608	
Phenytoin	2	Acid	0.11 [a]	0.11 [a]	1	0.126	5.3 [a]	0.37	0.809	18	0.355	0.818	0.389	0.877	(13)
Coumarin	1	neutral	0.17 [c]	0.17 [c]	0.92	0.89	23.6 [c]	23.6	1210	1250	0.151	0.702	0.154	0.699	
Diclofenac	2	Acid	0.0091 [a]	0.005 [a]	0.93	0.026	7.7 [a]	3.5	89	439	1.66	10.1	0.961	5.74	(14)
Midazolam	1	Base	0.043 [a]	0.025 [a]	1	0.025	9.2 [a]	5.3	342	553	16.7	13	10.3	8.99	
Phenacetin	2	neutral	0.57 [a]	0.57 [a]	1	0.97	20 [a]	21	5.73	44.4	5.61	3.71	4.6	3.24	
Zidovudine	1	zwitterion	0.82 [a]	0.8 [a]	1	1	19 [a]	25	0.565	8.53	1.12	1.9	1.06	1.74	(15)

Phenytoin	2	Acid	0.11[a]	0.11[a]	1	0.51	5.3[a]	0.37	0.736	0.565	0.0688	0.445	0.0389	0.251	(16)
Propofol	2	neutral	0.016[c]	0.016[b]	0.7	0.2	36[c]	36	6.85	58.2	2.98	2.62	1.61	1.42	(17)
Phenytoin	2	acid	0.11[a]	0.11[a]	1	0.2	5.3[a]	0.37	0.496	2.78	0.548	1.14	0.326	0.676	(18)
Tolbutamide	2	Acid	0.076[a]	0.05[a]	1	0.012-0.098	0.35[a]	0.21	2.05	9.33	9	8.95	6.07	6.04	
Estradiol	1	neutral	0.016[c]	0.016[b]	0.14	0.039	30[c]	30	248	94.2	2.4	3.29	2.28	3.02	
Propofol	2	neutral	0.016[c]	0.016[b]	1	0.17	36[c]	36	12	85.6	6.56	3.71	5.12	3.16	(19)
Zidovudine	1	zwitterion	0.82[a]	0.8[a]	0.69	0.69	19[a]	25	4.37	40.2	1.22	4.71	0.953	3.42	
Paclitaxel	2	neutral	0.12[c]	0.12[b]	0.66	0.44	6.4[c]	6.4	16.6	57.2	0.863	0.204	1.07	0.263	(20)
Omeprazole	1	neutral	0.067[a]	0.04[a]	1	0.26	11[a]	8.4	11.8	23.1	5.51	7.8	4.14	5.56	(21)

[a] Wood et al., 2017; [b] Lombardo et al., 2018;

[c] Values were derived from the quotient of $f_{u,p}$ or $CL_{H,p}$ and blood-to-plasma partitioning ratio (R_{BP}). For the drugs that did not have reported R_{BP} values, it was assumed to be equal to 1 for a basic or neutral compound and 0.55 (1-hematocrit) for an acidic compound; [d] Values were cited from the studies in the reference column.

REFERENCES

1. Bachmann K, Byers J, Ghosh R. Prediction of in vivo hepatic clearance from in vitro data using cryopreserved human hepatocytes. Xenobiotica. 2003;33(5):475–83.

2. Blanchard N, Hewitt NJ, Silber P, Jones H, Coassolo P, Lave T. Prediction of hepatic clearance using cryopreserved human hepatocytes: a comparison of serum and serum-free incubations. J Pharm Pharmacol. 2006;58:633–41.

3. Chao P, Barminko J, Novik E, Han Y, Maguire T, Cheng K-C. Prediction of human hepatic clearance using an in vitro plated hepatocyte clearance model. Drug Metab Lett. 2009;3:296–307.

4. Mao J, Mohutsky MA, Harrelson JP, Wrighton SA, Hall SD. Predictions of cytochrome P450-mediated drug-drug interactions using cryopreserved human hepatocytes: Comparison of plasma and protein-free media incubation conditions. Drug Metab Dispos. 2012;40(4):706–16.

5. Nishimuta H, Watanabe T, Bando K. Quantitative prediction of human hepatic clearance for P450 and non-P450 substrates from in vivo monkey pharmacokinetics study and in vitro metabolic stability tests using hepatocytes. AAPS J. 2019;21:20.

6. Shibata Y, Takahashi H, Chiba M, Ishii Y. Prediction of hepatic clearance and availability by cryopreserved human hepatocytes: An application of serum incubation method. Drug Metab Dispos. 2002;30:892–6.

7. Kim S, Lee K, Miyauchi S, Sugiyama Y. Extrapolation of in vivo hepatic clearance from in vitro uptake clearance by suspended human hepatocytes for anionic drugs with high binding to human albumin: Improvement of in vitro-to-in vivo extrapolation by considering the "albumin-mediated" hepatic uptake mechanism on the basis of the "facilitated-dissociation model". Drug Metab Dispos. 2019;47:94-103.

8. Wood FL, Houston JB, Hallifax D. Clearance prediction methodology needs fundamental improvement: Trends common to rat and human hepatocytes/microsomes and implications for experimental methodology. Drug Metab Dispos. 2017;45:1178–1188.

9. Lombardo F, Berellini G, Obach RS. Trend analysis of a database of intravenous pharmacokinetic parameters in humans for 1352 drug compounds. Drug Metab Dispos. 2018;46:1466–77.

10. Baba T, Touchi A, Ito K, Yamaguchi Y, Yamazoe Y, Ohno Y, et al. Effects of serum albumin and liver cytosol on CYP2C9- and CYP3A4-mediated drug metabolism. Drug Metab Pharmacokinet. 2002;17:522–31.

11. Carlile DJ, Hakooz N, Bayliss MK, Houston JB. Microsomal prediction of in vivo clearance of CYP2C9 substrates in humans. Br J Clin Pharmacol. 1999;47:625–35.

12. Gill KL, Houston JB, Galetin A. Characterization of in vitro glucuronidation clearance of a range of drugs in human kidney microsomes: Comparison with liver and intestinal glucuronidation and impact of albumin. Drug Metab Dispos. 2012;40:825–35.

13. Ludden LK, Ludden TM, Collins JM, Pentikis HS, Strong JM. Effect of albumin on the estimation, in vitro, of phenytoin Vmax and Km values: Implications for clinical correlation1. J Pharmacol Exp Ther. 1997;282:391–6.

14. Palacharla RC, Uthukam V, Manoharan A, Ponnamaneni RK, Padala NP, Boggavarapu RK, et al. Inhibition of cytochrome P450 enzymes by saturated and unsaturated fatty acids in human liver microsomes, characterization of enzyme kinetics in the presence of bovine serum albumin (0.1 and 1.0% w/v) and in vitro – in vivo extrapolation of hepatic clearance. Eur J Pharm Sci. 2017;101:80–9.

15. Rowland A, Gaganis P, Elliot DJ, Mackenzie PI, Knights KM, Miners JO. Binding of inhibitory fatty acids is responsible for the enhancement of UDP-glucuronosyltransferase 2B7 activity by albumin: Implications for in vitro-in vivo extrapolation. J Pharmacol Exp Ther. 2007;321:137–47.

16. Rowland A, Elliot DJ, Knights KM, Mackenzie PI, Miners JO. The "albumin effect" and in vitro-in vivo extrapolation: Sequestration of long-chain unsaturated fatty acids enhances phenytoin hydroxylation by human liver microsomal and recombinant cytochrome P450 2C9. Drug Metab Dispos. 2008;36:870–7.

17. Rowland A, Knights KM, Mackenzie PI, Miners JO. The "albumin effect" and drug glucuronidation: Bovine serum albumin and fatty acid-free human serum albumin enhance the glucuronidation of UDP-glucuronosyltransferase (UGT) 1A9 substrates but not UGT1A1 and UGT1A6 activities. Drug Metab Dispos. 2008;36:1056–62.

18. Tang C, Lin YUH, Rodrigues AD, Lin JH. Effect of albumin on phenytoin and tolbutamide metabolism in human liver microsomes: An impact more than protein binding. Drug Metab Dispos. 2002;30:648–54.

19. Walsky RL, Bauman JN, Bourcier K, Giddens G, Lapham K, Negahban A, et al. Optimized assays for human UDP-glucuronosyltransferase (UGT) activities: Altered alamethicin concentration and utility to screen for UGT Inhibitors. Drug Metab Dispos. 2012;40:1051–65.

20. Wattanachai N, Polasek TM, Heath TM, Uchaipichat V, Tassaneeyakul W, Tassaneeyakul W, et al. In vitro-in vivo extrapolation of CYP2C8-catalyzed paclitaxel 6α-hydroxylation: Effects of albumin on in vitro kinetic parameters and assessment of interindividual variability in predicted clearance. Eur J Clin Pharmacol. 2011;67:815–24.

21. Wattanachai N, Tassaneeyakul W, Tassaneeyakul W. The effects of bovine serum albumin on kinetic characterization of human liver microsomal CYP2C19 and CYP2E1 activities. Thai J Pharmacol. 2015;37:27-40.