

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”[§]

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ABSTRACT

We investigated whether human serum albumin (HSA) in suspended human hepatocytes would affect the uptake clearance of anionic drugs with high binding to HSA and improve the extrapolation of in vivo hepatic clearance from in vitro uptake clearance by the hepatocytes via the “albumin-mediated” hepatic uptake mechanism. The uptake clearances for total forms (PS_{inf}) and for unbound forms ($PS_{\text{u,inf}}$) of 11 anionic drugs [all of which were organic anion-transporting polypeptide (OATP) substrates] were determined with suspended human hepatocytes in varying concentrations of HSA. The fraction of unbound drugs (f_u) was determined using an equilibrium dialysis at the various HSA concentrations. The PS_{inf} values decreased with increasing concentrations of HSA, whereas the unbound uptake clearances ($PS_{\text{u,inf}(+) = PS_{\text{inf}}/f_u$) in the presence

of HSA increased substantially, thus demonstrating the “albumin-mediated” hepatic uptake mechanism. The relationships between PS_{inf} and HSA concentration were well described by the previously proposed facilitated-dissociation model, in which the drug–albumin complex interacts with the cell surface, enhancing the dissociation of the complex and providing unbound drug for hepatic uptake. Furthermore, the $PS_{\text{u,inf}(+)}$ values in in vivo conditions (at 5% HSA) were predicted from those obtained in isolated hepatocytes on the basis of the facilitated-dissociation model, revealing compatibility with the overall hepatic intrinsic clearance in vivo. We conclude that the “facilitated-dissociation” model is useful for describing the “albumin-mediated” hepatic uptake phenomenon of OATP drugs and to predict hepatic uptake clearance in vivo.

Introduction

It is very important that the pharmacokinetic features of new chemical entities in humans be adequately predicted during early stages of drug discovery and development. The prediction of human hepatic clearance is crucial because the liver is the major organ responsible for elimination of a variety of endogenous and exogenous

chemicals via metabolism and/or biliary excretion. Currently, human-derived reagents, such as human hepatic microsomes, cytosols, and suspended hepatocytes, are available for in vitro studies, greatly improving the quantitative in vitro-to-in vivo extrapolation (IVIVE) for hepatic clearance values (Watanabe et al., 2011; Izumi et al., 2017). Compared with anion drugs, anionic drugs with high plasma binding tend to have poor predictive accuracy for human hepatic clearances, because they require mechanistic elucidation and quantitative improvement (Watanabe et al., 2011; Izumi et al., 2017).

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ABBREVIATIONS: $CL_{\text{h,u,inf,all}}^{\text{vivo}}$, the in vivo unbound overall hepatic intrinsic clearance; f_p , plasma-unbound fraction; f_u , HSA-unbound fraction; HSA, human serum albumin; IVIVE, in vitro-to-in vivo extrapolation; OATP, organic anion transporting polypeptide; $PS_{\text{b,inf}}$, the hepatic uptake clearance for the unbound drug dissociated from the drug–albumin complex at the surface; PS_{inf} , the hepatic uptake clearance for total drug; $PS_{\text{u,inf}}$, the hepatic uptake clearance for unbound drug; $PS_{\text{u,inf}(+)}$, the hepatic unbound uptake clearance in the presence of the albumin; PRV, pravastatin; R, hepatic uptake clearance; RSV, rosuvastatin; SF, scaling factor.

pH-partition mechanisms. This adjustment procedure improved the predictive accuracy of hepatic clearances for drugs that bind avidly to albumin.

We recently demonstrated that organic anion-transporting polypeptide (Oatp/OATP) substrates were taken up by primary cultured rat hepatocytes, and human suspended hepatocytes in the presence of albumin, to a much greater extent than could be expected on the basis of their unbound concentration and the “free drug” hypothesis (Miyachi et al., 2018). The enhancement of the hepatic uptake (often referred to as “albumin-mediated” hepatic uptake) has been described in the “facilitated-dissociation” model, in which the drug–albumin complex interacts with the cell surface, enhancing the dissociation of the complex and providing unbound drug for hepatic uptake (Tsao et al., 1988a). Considering the “albumin-mediated” hepatic uptake, IVIVE for hepatic uptake clearance improves prediction accuracy. Using an isolated perfused rat liver system, Bounakta et al. (2018) and Poulin et al. (2017) also demonstrated that the IVIVE method considering the “albumin-mediated” hepatic uptake mechanism improved the predictive accuracy of *in vivo* hepatic clearance compared with their empirical IVIVE that considered pH and albumin concentration differences between the plasma and liver. Together, these results provide solid evidence supporting a kinetic model for describing the “albumin-mediated” hepatic uptake phenomenon for a drug avidly bound to albumin, which is important for robust IVIVE.

In the present study, our first objective was to investigate the effect of human serum albumin (HSA) on the uptake clearances of 11 drugs known as OATP substrates using suspended human hepatocytes. Our second objective was to improve the prediction of quantitative IVIVE for hepatic uptake clearance values by considering “albumin-mediated” hepatic uptake mechanisms on the basis of the “facilitated-dissociation” model.

Materials and Methods

Materials. Pitavastatin calcium salt, atorvastatin calcium trihydrate, rosuvastatin (RSV) calcium salt, fluvastatin sodium salt, and pravastatin (PRV) sodium salt were purchased from Wako Pure Chemicals (Kyoto, Japan). Cerivastatin sodium salt and repaglinide were purchased from LKT Laboratories (St. Paul, MN). Glibenclamide (GLB), nateglinide, and valsartan were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Bosentan was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Human plasma and HSA (fatty acid free) were purchased from MilliporeSigma (St. Louis, MO). All other reagents used were of analytical or high-performance liquid chromatography grade.

Unbound Fraction in Human Plasma and HSA. Plasma protein binding of 11 compounds was determined in 100% human plasma and six different concentrations (0%, 0.125%, 0.25%, 0.5%, 1%, and 5% or 0, 18.8, 37.6, 75.2, 150, and 752 μM) of HSA by equilibrium dialysis with a Rapid Equilibrium Dialysis (RED) Device (Thermo Fisher Scientific, Rockford, IL). Various concentrations of HSA were prepared with transporter uptake buffer (pH 7.4). The final concentration of compounds in human plasma or HSA samples was 3 μM . Aliquots (100 μl) of each sample and phosphate-buffered saline or transporter uptake buffer (300 μl) were added to the equilibrium dialysis device. The plate was put into a 37°C incubator and shaken gently for 20 hours. Aliquots of samples (25- μl) and 50- μl aliquots of buffers were then taken and mixed with acetonitrile containing an internal standard. The mixture was vigorously mixed and centrifuged at 4°C. An aliquot (5 μl) of each supernatant was injected into a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system (Supplemental Table S1).

Uptake Studies in Suspended Human Hepatocytes. The uptake rate of all compounds was determined using pooled cryopreserved human hepatocytes (Lot TFF, 20 mixed-gender donors) (BioIVT, Baltimore, MD) by a centrifugal filtration technique described previously (Hirano et al., 2004). Immediately prior to the uptake experiment, the cryopreserved hepatocytes (1 ml suspension) were thawed at 37°C, then suspended in 50 ml of prewarmed

cryopreserved hepatocyte recovery media thawing medium (APSciences, Inc., Columbia, MD) and centrifuged (100 *g*) for 2 minutes at 4°C, followed by removal of the supernatants. The hepatocytes were gently resuspended in ice-cold Krebs-Henseleit buffer to give 2.0×10^6 viable cells/ml on ice. The uptake studies were initiated by adding an equal volume of prewarmed buffer containing two sets of drugs: 1) pitavastatin, atorvastatin, RSV, fluvastatin, cerivastatin, and PRV; 2) glibenclamide, valsartan, repaglinide, bosentan, and nateglinide, with six different concentrations of HSA (0%, 0.125%, 0.25%, 0.5%, 1%, and 5%), added to the cell suspension after a 5-minute preincubation at 37°C, resulting in a final substrate concentration of 3 μM in 1×10^6 cells/ml. The reported Michaelis constant for the transporter (K_m) value of 11 drugs ranged from 0.18 to 76.7 μM (Supplemental Table S2). The final free concentration of substrate in the presence of HSA would still be lower than 3 μM .

After incubation for 0.25 and 1.25 minutes, the reactions were terminated by separating the cells from the substrate solution. The hepatic uptake study was conducted using an oil-spin method described previously in detail (Miyachi et al., 2018). The concentrations of medium and cells were analyzed by LC–MS/MS (Supplemental Table S1). Our results for the uptake clearances in the absence of HSA were comparable with reported values (Supplemental Table S2), indicating that the competition among drugs in the cassette mixture was minimal in our hepatic uptake experimental conditions.

Kinetic Analysis. The unbound fraction (f_u) for each drug in steady-state conditions using equilibrium dialysis was calculated using the following equation:

$$\frac{1}{f_u} = 1 + \frac{n[Alb]}{K_d} \quad (1)$$

where n , $[Alb]$ and K_d represent the number of binding sites on the albumin, the HSA concentration (micromolar), and the dissociation constant (micromolar) between drug and HSA, respectively. The n values for some OATP1B1 substrates such as statins, antidiabetic drugs, and anti-HIV drugs have been reported to be 0.87–1.1 (Seedher and Kanojia, 2008; Gulati et al., 2009; Shi et al., 2017). For the simplicity of eq. 1, the n value was assumed to be unity. The molecular concentration of HSA was calculated using 66,500 as the molecular weight.

The initial uptake rates were obtained from the slopes of the time courses within 0.25–1.25 minutes using linear regression analysis. The uptake clearances for total (PS_{inf}) and unbound forms ($PS_{\text{u,inf}}$) of each drug were calculated by dividing the initial uptake velocity by the total and unbound drug concentrations in the incubation buffer at the various concentrations of HSA.

In our previous study, the “albumin-mediated” hepatic transport phenomenon was analyzed using the “facilitated-dissociation” model, in which the interaction between the cell surface and the drug–albumin complex enhances the dissociation of drug from the complex (Tsao et al., 1988a). A facilitation mechanism has been postulated to the effect that the interaction of the drug–albumin complex with the surface of hepatocytes induces a conformational change in albumin, resulting in enhanced dissociation of drug from the complex, providing unbound drug near the surface. Therefore, the uptake of highly albumin-bound drugs by hepatocytes includes the pathway of unbound drugs and the additional pathway of drugs dissociated from drug–albumin complexes near the surface. We assumed that unbound albumin and drug-bound albumin compete for the same binding site(s) on the surface of hepatocytes with the same affinity, which is the dissociation constant ($K_{d,m}$) between albumin and the surface of hepatocytes. The fraction of albumin bound to the surface of the hepatocytes (λ) is expressed as:

$$\lambda = \frac{B_{\text{max}}}{K_{d,m} + [Alb]} \quad (2)$$

where B_{max} represents the capacity of albumin-binding sites on the surface of hepatocytes. The equations for the uptake rate (v) of drug derived by Tsao et al. (1988a) are as follows:

$$v = PS_{\text{inf}} \cdot c_0 = PS_{\text{u,inf}} \cdot f_u \cdot c_0 + PS_{\text{b,inf}} \cdot \lambda \cdot (1 - f_u) \cdot c_0 \quad (3)$$

$$PS_{\text{inf}} = PS_{\text{u,inf}} \cdot f_u + PS_{\text{b,inf}} \cdot \lambda \cdot (1 - f_u) \quad (4)$$

Substituting eqs. 1 and 2 into eq. 4 yields

$$PS_{\text{inf}} = PS_{\text{u,inf}} \cdot \frac{K_d}{K_d + [Alb]} + PS_{\text{b,inf}} \cdot \frac{B_{\text{max}}}{K_{d,m} + [Alb]} \cdot \frac{[Alb]}{K_d + [Alb]} \quad (5)$$

where c_0 represents the total concentration of drug; PS_{inf} , $PS_{\text{u,inf}}$, and $PS_{\text{b,inf}}$ represent the uptake clearance for the total drug, unbound drug, and unbound drug dissociated from the drug–albumin complex at the surface, respectively. On the basis of the “facilitated-dissociation” model, the uptake clearance of unbound drug ($PS_{\text{inf}}^{\text{(free)}}$) and the uptake clearance of unbound drug associated from the drug–albumin complex ($PS_{\text{inf}}^{\text{(bound)}}$) at the cell surface were estimated from the first and second terms of the right-hand side of eq. 5, respectively, as follows.

$$PS_{\text{inf}}^{\text{(free)}} = \frac{PS_{\text{u,inf}} \cdot K_d}{K_d + [Alb]} \quad (6)$$

$$PS_{\text{inf}}^{\text{(bound)}} = \frac{PS_{\text{b,inf}} \cdot B_{\text{max}}}{K_{\text{d,m}} + [Alb]} \cdot \frac{[Alb]}{K_d + [Alb]} \quad (7)$$

In eq. 7, $PS_{\text{b,inf}} \cdot B_{\text{max}}$ represents the parameter of the clearance capacity for the uptake of “facilitated-dissociation” drugs, and is designated as $V_{\text{B,max}}$. The unbound uptake clearance in the presence of HSA, ($PS_{\text{u,inf}}^{\text{(+)}}$), was calculated using the following equation modified from eqs. 1 and 5.

$$PS_{\text{u,inf}}^{\text{(+)}} = \frac{PS_{\text{inf}}}{f_u} = PS_{\text{u,inf}} + \frac{V_{\text{B,max}} \cdot [Alb]}{K_{\text{d,m}} + [Alb]} \cdot \frac{1}{K_d} \quad (8)$$

This equation implies that $PS_{\text{u,inf}}^{\text{(+)}}$ shows saturation as $[Alb]$ increases. The K_d values of each compound were estimated using eq. 1, and $PS_{\text{u,inf}}$, $K_{\text{d,m}}$, and $V_{\text{B,max}}$ were obtained with eq. 5 using nonlinear least-squares fitting software (Napp, version 2.31, The University of Tokyo Hospital, Japan) (Hisaka and Sugiyama, 1998).

Extrapolation to In Vivo Hepatic Clearances from the In Vitro Uptake Clearance by Suspended Hepatocytes. The in vitro unbound hepatic uptake clearance (microliters per 10^6 cells per milliliter) was scaled up to in vivo (milliliters per minute per kilogram body weight) using the following physiologic scaling factors: 1.2×10^8 cells/g liver and 25.7 g liver/kg body weight (Davies and Morris, 1993; Miyauchi et al., 1993).

In vivo hepatic overall intrinsic clearance, representing the elimination of unbound drug ($CL_{\text{h,u,int,all}}^{\text{vivo}}$) from circulating blood, was calculated from the reported in vivo hepatic clearance (Supplemental Table S3) using the dispersion model (eqs. 9–12) (Roberts and Rowland, 1986) and the well-stirred model (eqs. 9 and 13) (Pang and Rowland, 1977).

$$CL_{\text{h,B}} = Q_h \times (1 - F_h) \quad (9)$$

For the dispersion model:

$$F_h = \frac{4a}{(1+a)^2 \cdot \exp\left\{\frac{(a-1)}{2D_N}\right\} - (1-a)^2 \cdot \exp\left\{-\frac{(a+1)}{2D_N}\right\}} \quad (10)$$

$$a = (1 + 4R_N \cdot D_N)^{1/2} \quad (11)$$

$$R_N = f_B \cdot CL_{\text{h,u,int,all}}^{\text{vivo}} \quad (12)$$

For the well-stirred model:

$$F_h = Q_h / \left(Q_h + f_B \cdot CL_{\text{h,u,int,all}}^{\text{vivo}} \right) \quad (13)$$

Where $CL_{\text{h,B}}$, Q_h , D_N , and f_B are the hepatic clearance, hepatic blood flow rate (20.7 ml/min per kilogram body weight) (Davies and Morris, 1993), the dispersion number (0.17) (Roberts and Rowland, 1986), and the unbound fraction in the blood, respectively. For almost all of the drugs used, it has been suggested that hepatic uptake is a rate-determining step for hepatic elimination (Watanabe et al., 2010; Izumi et al., 2017). According to the extended clearance concept, in vivo hepatic intrinsic clearance for 11 OATP substrates could be well accounted for by the in vitro hepatic uptake clearance, regardless of the involvement of hepatic metabolism.

Results

Unbound Fractions in Human Plasma and HSA. We determined the unbound fraction (f_p) of 11 drugs in human plasma. These values were used for the calculation of blood unbound fraction as f_p/R_B

(where R_B is the blood partitioning; Table 2). The f_u values were determined in the presence of HSA at six concentrations (0%, 0.125%, 0.25%, 0.5%, 1%, and 5%). The f_p values for almost all drugs except RSV and PRV were less than 0.05 (0.000787–0.0308), whereas those for RSV and PRV were 0.134 and 0.563, respectively. The f_p values were very close to f_u values in the presence of 5% HSA, a normal albumin concentration (Table 2). The K_d values of drugs were obtained by nonlinear least-squares fitting using eq. 1 (Fig. 1). For nine of the drugs, these were 0.490–27.1 μM , and for RSV and PRV they were 94.5 and 667 μM , respectively (Table 1). Most of the drugs used here are highly bound to albumin in plasma (bound form >95%), whereas two drugs, RSV and PRV, showed low protein binding, with f_p values of 0.134 for RSV and 0.563 for PRV (Fig. 1; Table 1). These results were consistent with the f_p values reported previously (Colussi et al., 1997; Hatorp, 2002; Blanchard et al., 2005; Watanabe et al., 2010, 2011), indicating that the major binding protein in the plasma is indeed albumin. The recovery of the measurement for 11 drugs in the uptake experiments was more than 92%, irrespective of HSA in the buffer. The nonspecific adsorption to the wall of test tubes was assumed to be minimal.

Effect of Albumin on Uptake Clearances by Isolated Human Hepatocytes. The PS_{inf} (hepatic uptake clearance for total drug) values of drugs at various concentrations of HSA (0.125%, 0.25%, 0.5%, and 1%) were determined from the initial slopes of the uptake volume (Supplemental Fig. S1). However, the PS_{inf} value in the presence of 5% HSA could not be determined robustly because of the avid binding of these drugs to albumin, resulting in no significant time-dependent uptake volume by hepatocytes over 0.25–1.25 minutes. The $PS_{\text{u,inf}}^{\text{(+)}}$ (the hepatic unbound uptake clearance in the presence of the albumin) value was estimated by dividing the PS_{inf} value by the f_u value at the various concentrations of HSA, whereas the $PS_{\text{u,inf}}$ value was obtained in the absence of HSA. The PS_{inf} values of almost all drugs except PRV were decreased in association with increases in the concentration of HSA, whereas the $PS_{\text{u,inf}}^{\text{(+)}}$ values were increased substantially (Figs. 2 and 3; Supplemental Fig. S2), thus demonstrating that these 10 drugs with high binding to the albumin show “albumin-mediated” hepatic uptake. Nevertheless, PRV with its low protein binding to albumin ($f_p = 0.563$) did not show any change in the PS_{inf} and $PS_{\text{u,inf}}^{\text{(+)}}$ values, irrespective of the concentration of HSA (Fig. 3; Supplemental Fig. S2). Furthermore, the relationships between the PS_{inf} values and HSA concentrations for the 10 drugs except for PRV were simultaneously fitted to eq. 5 on the basis of the “facilitated-dissociation” model (Tsao et al., 1988a), and the estimated $K_{\text{d,m}}$ value was $45.2 \pm 13.0 \mu\text{M}$ (\pm calculated S.D.). The $PS_{\text{u,inf}}$ values obtained in the absence of HSA were very close to those of $PS_{\text{u,inf}}$ estimated in the fitting analysis by the “facilitated-dissociation” model. These estimated parameters are summarized in Table 1. The PS_{inf} values in the presence of a normal physiologic albumin concentration (5%) were predicted according to eq. 5 by the “facilitated-dissociation” model. The predicted PS_{inf} values were much greater than those obtained on the basis of the “free drug” hypothesis (Fig. 3). The contributions of “albumin-mediated” uptake clearance ($PS_{\text{inf}}^{\text{(bound)}}$) to total uptake clearance for these 10 drugs were estimated to be 55–98%, according to eqs. 6 and 7 (Table 1).

Extrapolation to In Vivo Hepatic Clearances from In Vitro Uptake Data. The $PS_{\text{u,inf}}$ values of 10 drugs were extrapolated to the $PS_{\text{u,inf}}^{\text{vivo}}$ values (ml/min/kg body weight). As shown in Table 2, the $f_B \cdot PS_{\text{u,inf}}^{\text{vivo}}$ values whose basis was the “free drug” hypothesis were underestimated substantially compared with the $f_B \cdot CL_{\text{h,u,int,all}}^{\text{vivo}}$ (the in vivo unbound overall hepatic intrinsic clearance) values obtained from hepatic clearance by the dispersion model (Fig. 4A) and the well-stirred model (Fig. 5A). The $PS_{\text{u,inf}}^{\text{(+)}}$ values for 10 drugs at

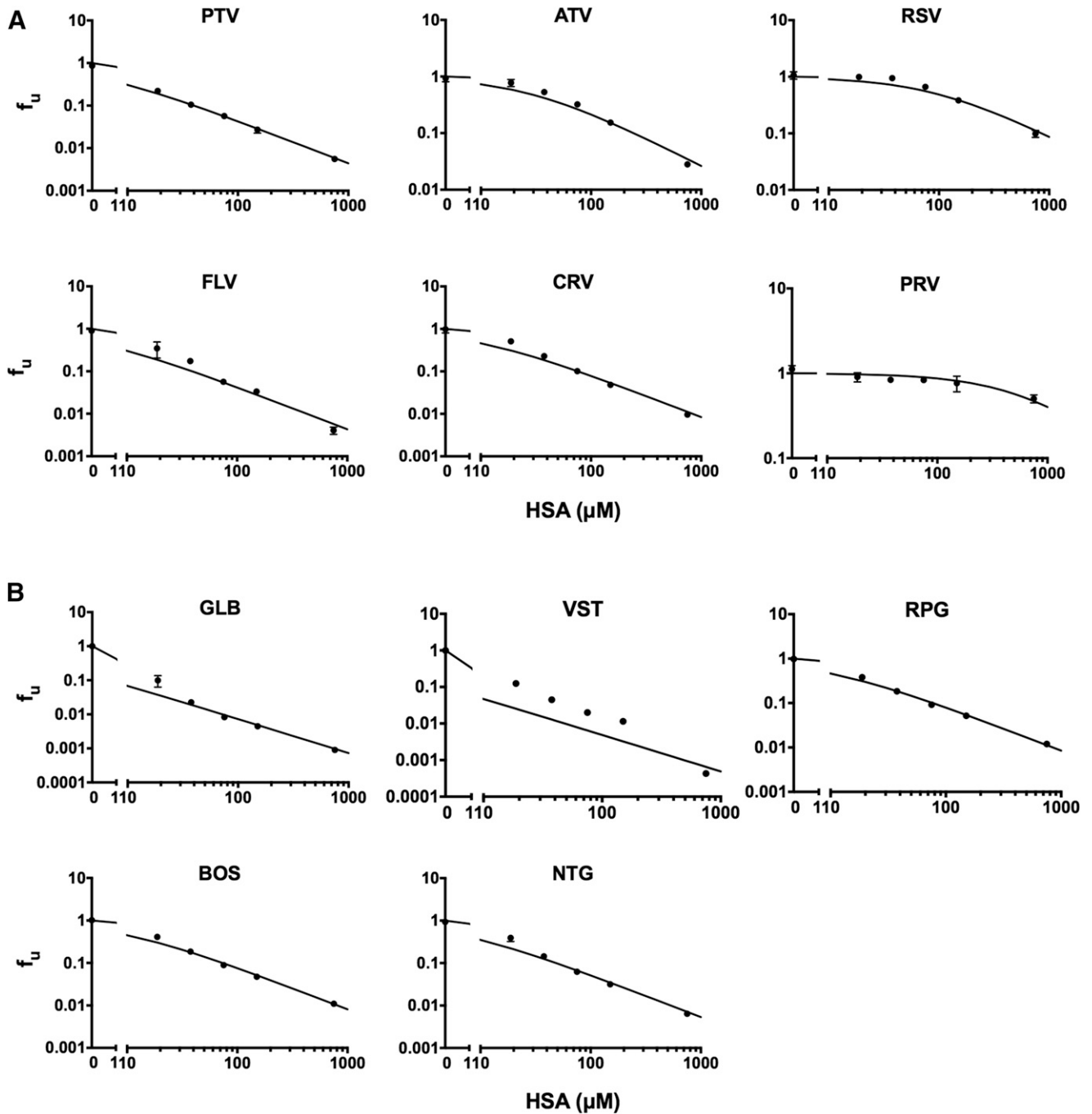


Fig. 1. The unbound fraction (f_u) of 11 drugs in HSA at 0%, 0.125%, 0.25%, 0.5%, 1%, and 5%. The filled circles represent the observed HSA f_u values (mean \pm S.D., $n = 4$) in the absence and presence of HSA, which concentrations were 18.8, 37.6, 75.2, 150, and 752 μM . The solid lines represent the fitted line estimated from eq. 1. (A) and (B) show sets A and B, respectively. Key: PTV, pitavastatin; ATV, atorvastatin; FLV, fluvastatin; CRV, cerivastatin; GLB, glibenclamide; VST, valsartan; RPG, repaglinide; BOS, bosentan; NTG, nateglinide.

5% HSA estimated on the basis of the “facilitated-dissociation” model (eq. 8) were also extrapolated to the in vivo value per body weight ($PS_{u,inf}^{vitro}$, ml/min/kg) (Table 2). The $f_B \cdot PS_{u,inf}^{vitro}$ values at 5% HSA showed similarities to the $f_B \cdot CL_{h,u,int,all}^{vivo}$ values estimated from the hepatic clearance regardless of the dispersion or well-stirred model (Figs. 4B and 5B; Table 2). The “albumin-mediated” uptake factors or enhancement of the hepatic uptake clearance (R) in the physiologic albumin concentration range were estimated as the ratios of $PS_{u,inf}$ (+) values at 5% HSA to $PS_{u,inf}$ values in the absence of HSA; the estimated R values were 2.44–63.8 (Table 2).

As shown in Figs. 4B and 5B, the “albumin-mediated” uptake factor improved the prediction of IVIVE, but the relationship between $f_B \cdot PS_{u,inf}^{vitro}$ and $f_B \cdot CL_{h,u,int,all}^{vivo}$ still showed some underestimation in the IVIVE from the in vitro hepatic uptake clearance. The ratios of $f_B \cdot CL_{h,u,int,all}^{vivo}$ whose basis was the dispersion and well-stirred models to $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA were 0.63–5.57 and 0.63–7.11, respectively, and the average value was 2.44 and 3.19, respectively (Table 2). Furthermore, the $f_B \cdot PS_{u,inf}^{vitro}$ values at 5% HSA were corrected by the mean value (2.44 and 3.19) designated as scaling factor (SF), giving a better relationship between $f_B \cdot PS_{u,inf}^{vitro}$

TABLE 1

Unbound fractions in plasma and 5% HSA, and the estimated contribution of “albumin-mediated” uptake of 11 drugs based on Tsao’s model

Data are presented as the mean or mean \pm S.D.

Drugs	f_p	$f_{u,5\% \text{ HSA}}$	K_d^a	$PS_{u,inf}$		$K_{d,m}^d$	$V_{B,max}^c$	PS_{inf}		Contribution (%) of “albumin-mediated” uptake ^f
				Observed ^b	Calculated ^e			Free ^e	Bound ^d	
Pitavastatin	0.00541	0.00558	4.44 \pm 0.21	36.2 \pm 11.4	36.3 \pm 3.1	45.2 \pm 13.0	225 \pm 79	0.213	0.281	56.9
Atorvastatin	0.0308	0.0281	27.1 \pm 3.2	24.2 \pm 3.7	23.5 \pm 2.5		1090 \pm 258	0.842	1.32	61.0
Rosuvastatin ^g	0.134	0.0988	94.5 \pm 12.1	4.01 \pm 1.61	3.76 \pm 0.58		480 \pm 145	0.448	0.535	54.4
Fluvastatin	0.00428	0.00405	4.33 \pm 0.75	62.1 \pm 22.9	62.4 \pm 4.1		1090 \pm 193	0.356	1.36	79.3
Cerivastatin	0.00734	0.00961	8.48 \pm 0.93	77.5 \pm 23.3	74.1 \pm 4.56		1160 \pm 239	0.864	1.45	62.6
Pravastatin	0.563	0.502	667 \pm 121	3.55 \pm 2.17	NA		NA	NA	NA	NA
Glibenclamide	0.000787	0.000897	0.730 \pm 0.104	32.2 \pm 4.7	32.3 \pm 3.0		98.9 \pm 35.7	0.0312	0.124	79.9
Valsartan	0.00165	0.000431	0.490 \pm 0.210	1.87 \pm 0.65	1.87 \pm 0.72		40.1 \pm 18.3	0.00122	0.0502	97.6
Repaglinide	0.00676	0.0120	8.55 \pm 0.44	39.2 \pm 10.7	40.0 \pm 3.3		1640 \pm 271	0.441	2.04	82.2
Bosentan	0.00814	0.0110	8.16 \pm 0.55	23.2 \pm 5.7	23.4 \pm 2.5		796 \pm 157	0.249	0.988	79.8
Nateglinide	0.00497	0.00641	5.39 \pm 0.58	12.5 \pm 3.0	12.7 \pm 1.8		155 \pm 57	0.0886	0.193	68.5

f_p , plasma-unbound fraction; f_u , HSA-unbound fraction; HSA, human serum albumin; K_d , the dissociation constant between drug and HSA; $K_{d,m}$, the dissociation constant for the binding of HSA to the cell surface; NA, not applicable; $PS_{u,inf}$, the hepatic uptake clearance for unbound drug; $V_{B,max}$, the clearance capacity for the uptake of facilitated-dissociation drugs.

^aThese values were obtained from in vitro HSA binding experiments for each drug by eq. 1, and they were fixed when fitting with Tsao’s model.

^bThese values were observed in the suspended hepatocyte uptake study at 0% HSA.

^cThese values were estimated using Tsao’s model (eq. 5) and the observed PS_{inf} at the various concentration of HSA.

^dUptake clearance by bound form at 5% HSA, calculated using eq. 7.

^eUptake clearance by the “free drug” theory at 5% HSA, calculated using eq. 6.

^fCalculated by $PS_{inf(bound)} / (PS_{inf(free)} + PS_{inf(bound)}) \times 100(\%)$.

^g $PS_{u,inf}$ and $V_{B,max}$ for rosuvastatin were estimated using Tsao’s model with a fixed value (45.2 μM) of $K_{d,m}$.

SF and $f_B \cdot CL_{h,u,int,all}^{vivo}$ within a 3-fold range of difference regardless of estimated models of $f_B \cdot CL_{h,u,int,all}^{vivo}$ (Fig. 4C; Table 2).

Discussion

Human-derived reagents, such as hepatic microsomes, cytosols and suspended hepatocytes, are now available for in vitro studies (Chiba et al., 2009; Shitara et al., 2013), resulting in success in the prediction of hepatic clearances for new chemical entities at early stages of drug discovery and development. Here, we investigated whether the in vitro hepatic uptake clearance by suspended human hepatocytes could be extrapolated quantitatively to estimate the in vivo hepatic clearances for 11 drugs known as OATP substrates.

Previously, in vitro hepatic uptake clearance with cryopreserved human hepatocytes showed good prediction of in vivo hepatic clearance for some OATP substrates (Watanabe et al., 2010, 2011; Kimoto et al., 2012). Nevertheless, the predictions of human hepatic clearances in some anionic drugs with high binding to albumin were poor, showing underestimation of IVIVE for hepatic uptake clearances. Consistent with this notion, Figs. 4A and 5A show the underestimation of IVIVE for the hepatic uptake clearances regardless of estimated models of $f_B \cdot CL_{h,u,int,all}^{vivo}$. We hypothesized that “albumin-mediated” hepatic uptake would result in poor predictions of IVIVE because uptake studies

in human hepatocytes have usually been conducted in the absence of serum albumin. Recently, using primary cultured rat hepatocytes and suspended human hepatocytes, we demonstrated that IVIVE for hepatic clearance for Oatp/OATP substrates with high protein binding was robustly improved by an “albumin-mediated” hepatic uptake mechanism considered in the analysis (Miyachi et al., 2018), thereby suggesting that the underestimation in IVIVE could be attributed to an “albumin-mediated” hepatic uptake mechanism. In the present study, we further investigated the effect of HSA on the hepatic uptake clearance of 11 anionic OATP substrates by using suspended human hepatocytes in the absence and presence of various concentrations of HSA, and the improvement in the prediction of quantitative IVIVE for hepatic uptake clearance values by considering “albumin-mediated” hepatic uptake mechanisms that used the “facilitated-dissociation” model as a basis.

The PS_{inf} values of 11 drugs by human hepatocytes were determined in the presence of HSA, and the $PS_{u,inf}(+)$ values were obtained by dividing the PS_{inf} by f_u for the various concentrations of HSA. Except for PRV, all drugs showed decreases in the PS_{inf} values along with increases in HSA concentration (Figs. 2 and 3), whereas there was a substantial increase in $PS_{u,inf}(+)$ value (Fig. 2B; Supplemental Fig. S2), which invalidated the “free drug” hypothesis, suggesting that “albumin-

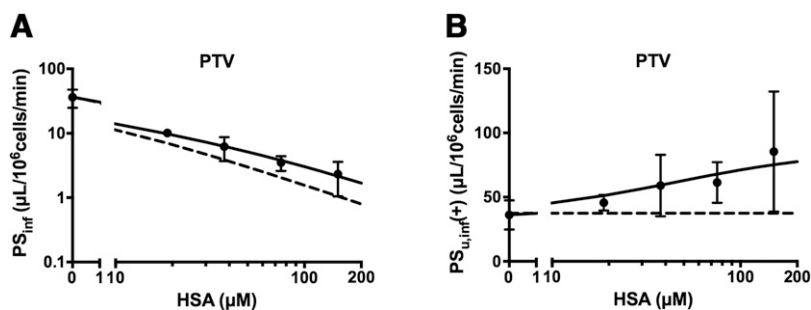


Fig. 2. The uptake clearances for total form (PS_{inf}) (A) and the unbound uptake clearance in the presence of HSA [$PS_{u,inf}(+)$] (B) of pitavastatin (PTV) in suspended human hepatocytes. The filled circles, solid lines, and broken lines represent the observed or calculated values (mean \pm S.D., $n = 8$), the fitted line by Tsao’s model, and the theoretical line with the “free drug” theory as a basis, respectively.

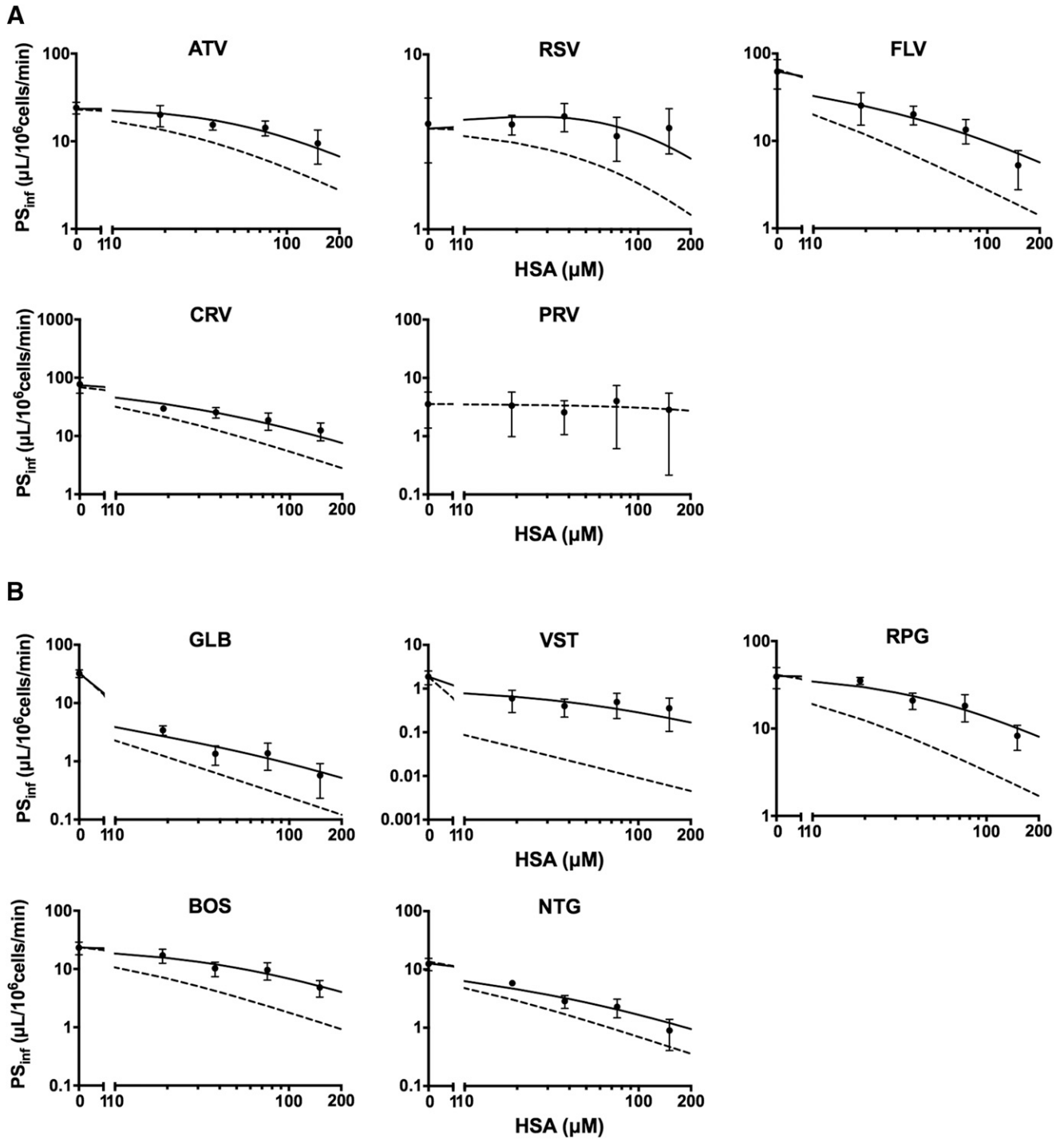


Fig. 3. The observed uptake clearances for total form (PS_{inf}) of clinical OATP substrates in suspended human hepatocytes. The filled circles, solid lines, and broken lines represent the observed PS_{inf} (mean \pm S.D., $n = 8$), the fitted line by Tsao's model (eq. 5), and the theoretical line with the “free drug” theory as a basis, respectively. (A) and (B) represent sets A and B, respectively. Key as in Fig. 1. Notes: 1) When fitting for RSV by Tsao's model, only $PS_{u,inf}$ and $V_{B,max}$ were estimated with a fixed $K_{d,m}$ value (Table 1). 2) The fitting by Tsao's model was not performed for PRV.

mediated” hepatic uptake for various OATP substrates with high protein binding was also observed in human hepatocytes. Nevertheless, PRV with low protein binding to albumin ($f_p = 0.563$) did not show any change in the PS_{inf} and $PS_{u,inf}$ (+) values, irrespective of the concentrations of HSA (Fig. 3; Supplemental Fig. S2).

In the present study, the $PS_{u,inf}$ (+) was composed of the uptake clearances for unbound drug and unbound drug dissociated from the drug–albumin complex near the cell surface (eq. 8). If “albumin-mediated”

uptake is involved in hepatic uptake, the $PS_{u,inf}$ (+) value would be greater than the $PS_{u,inf}$ value by the uptake clearance of unbound drug dissociated from the drug–albumin complex. It should be noted that $PS_{u,inf}$ (+) is a hybrid parameter accounting for the “albumin-mediated” uptake clearance attributable to the hepatic uptake of unbound drug dissociated from the drug–albumin complex.

Previously, the hepatic uptake of a number of endogenous and exogenous ligands and OATP substrates has been shown to exhibit the

TABLE 2
Kinetic parameters for drug uptake in suspended hepatocytes

Drugs	$PS_{\text{unbound}}^{\text{in vitro}}$ (+) ^c				$f_b \cdot PS_{\text{unbound}}^{\text{in vitro}}$				Dispersion model				Well-stirred model			
	0% HSA ^a	5% HSA ^b	0% HSA	5% HSA	0% HSA	5% HSA	R value ^f	$f_b \cdot CL_{\text{albumin,all}}^{\text{in vitro}}$ ^g	Ratio ^h (in vivo/in vitro)	$f_b \cdot PS_{\text{unbound}}^{\text{in vitro}} \cdot SF$ at 5% HAS	$f_b \cdot CL_{\text{albumin,all}}^{\text{in vitro}}$ ^g	Ratio ^h (in vivo/in vitro)	$f_b \cdot PS_{\text{unbound}}^{\text{in vitro}} \cdot SF$ at 5% HAS	$f_b \cdot CL_{\text{albumin,all}}^{\text{in vitro}}$ ^g	Ratio ^h (in vivo/in vitro)	$f_b \cdot PS_{\text{unbound}}^{\text{in vitro}} \cdot SF$ at 5% HAS
	$\mu\text{l}/\text{min}/10^6$ cells	$\mu\text{l}/\text{min}/10^6$ cells	$\mu\text{l}/\text{min}/10^6$ cells	$\mu\text{l}/\text{min}/10^6$ cells				ml/min/kg		ml/min/kg		ml/min/kg		ml/min/kg		ml/min per kilogram
Pitavastatin	36.2	0.493	36.2	88.3	1.04	2.54	2.44	14.1	5.57	14.1	5.57	6.20	18.0	7.11	7.11	18.0
Atorvastatin	24.2	2.16	24.2	77.0	3.48	11.1	3.18	25.3	2.28	25.3	2.28	27.1	39.1	3.53	3.53	39.1
Rosuvastatin ^g	4.01	0.983	4.01	9.94	2.41	5.98	2.48	20.6	3.44	20.6	3.44	14.6	29.2	4.89	4.89	29.2
Fluvastatin	62.1	1.72	62.1	424	1.32	9.03	6.83	27.3	3.02	27.3	3.02	22.1	43.6	4.83	4.83	43.6
Cerivastatin	77.5	2.31	77.5	240	0.0127	9.37	3.10	5.96	0.636	5.96	0.636	22.9	6.59	0.703	0.703	6.59
Pravastatin	3.55	NA	3.55	NA	11.0	NA	NA	22.9	NA	22.9	NA	NA	33.9	NA	NA	33.9
Gibencloamide	32.2	0.155	32.2	173	0.00171	0.170	5.38	2.47	2.71	2.47	2.71	2.23	2.58	2.83	2.83	2.58
Valsartan	1.87	0.0514	1.87	119	0.00301	0.0174	63.8	0.698	0.630	0.698	0.630	2.71	0.701	0.633	0.633	0.701
Repaglinide	39.2	2.48	39.2	207	0.0109	1.32	5.27	19.0	2.72	19.0	2.72	17.0	26.3	3.78	3.78	26.3
Bosentan	23.2	1.24	23.2	113	0.00981	0.703	4.86	3.14	0.919	3.14	0.919	8.34	3.31	0.969	0.969	3.31
Nateglinide	12.5	0.282	12.5	43.9	0.00994	0.382	3.52	3.37	2.50	3.37	2.50	3.29	3.58	2.66	2.66	3.58

$CL_{\text{albumin,all}}$, the in vivo unbound hepatic intrinsic clearance; f_b , blood-unbound fraction; NA, not applicable; PS_{unbound} , the hepatic uptake clearance for total drug; $PS_{\text{unbound}}^{\text{in vitro}}$, the hepatic uptake clearance for unbound drug; SF, Scaling factor.

^aObtained mean value from the suspended hepatocyte uptake study without HSA ($PS_{\text{unbound}}^{\text{in vitro}} = PS_{\text{unbound}}^{\text{observed}}$).

^bPredicted by Tsao's model in the presence of 5% HSA using the observed data at 0–1% HSA (eq. 5; Table 1).

^cCalculated by dividing $PS_{\text{unbound}}^{\text{in vitro}}$ by f_{un} , where f_{un} is 1 in the absence of HSA.

^dCalculated by dividing f_b by K_B , where K_B is the blood partitioning.

^eScaled-up from in vitro $PS_{\text{unbound}}^{\text{in vitro}}$ (+) using the following physiologic scaling factors (1.2×10^8 cells/g of liver, 25.7 g of liver/kg of body weight).

^fR values, the "albumin-mediated" uptake factor, was calculated as the ratio of $f_b \cdot PS_{\text{unbound}}^{\text{in vitro}}$ at 0% HSA to that at 5% HSA.

^gThese data were calculated using the dispersion model or the well-stirred model using the reported hepatic clearance. The details are shown in Supplemental Table S3.

^hCalculated by $f_b \cdot CL_{\text{albumin,all}}^{\text{in vitro}} / f_b \cdot PS_{\text{unbound}}^{\text{in vitro}}$; the mean value is 2.44 for the dispersion model and 3.19 for the well-stirred model, which was used as the scaling factor (SF).

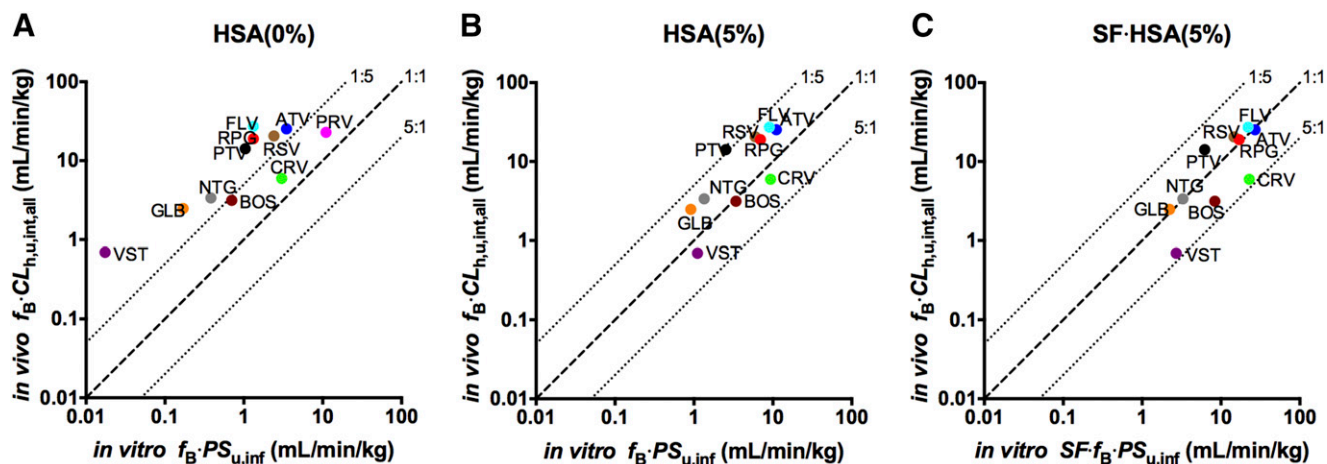


Fig. 4. Comparison between the uptake clearance ($f_B \cdot PS_{u,inf}^{vitro}$) at 0% HSA (A), the predicted $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA (B), and the adjusted $f_B \cdot PS_{u,inf}^{vitro}$ by SF (C) in suspended human hepatocytes and the observed hepatic overall intrinsic clearance ($f_B \cdot CL_{h,u,int,all}^{vivo}$) by the dispersion model. The $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA was predicted by Tsao’s model, the adjusted $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA was calculated by the predicted $f_B \cdot PS_{u,inf}^{vitro}$ and SF is 2.44, the average value of the ratios of $f_B \cdot CL_{h,u,int,all}^{vivo}$ to $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA. The dashed and dotted lines represent the line of unity and the lines of the 1:5 and 5:1 correlations, respectively. Key as in Fig. 1.

kinetics of “albumin-mediated” hepatic transport (Weisiger et al., 1981, 1984; Forker et al., 1982; Forker and Luxon, 1983; Tsao et al., 1986, 1988b; Pond et al., 1992; Fujino et al., 2018; Miyauchi et al., 2018). We have analyzed the “albumin-mediated” hepatic uptake transport, which uses the “facilitated-dissociation” model as a basis, in which the interaction of the drug–albumin complex with the surface of hepatocytes enhances the dissociation of the drug from the complex and provides unbound drug that is more available for hepatic uptake (Tsao et al., 1988a). The relationships between the PS_{inf} values and HSA concentrations in all 10 drugs except PRV were simultaneously fitted to eq. 5. $K_{d,m}$, a drug-independent parameter showing the dissociation constant between the albumin (unbound albumin or drug-bound albumin) and the surface of hepatocytes, was estimated to be 45 μM regardless of the drug tested, which is consistent with results reported in other studies (Weisiger et al., 1981; Forker and Luxon, 1983). $V_{B,max}$, a drug-dependent parameter showing the clearance capacity for the uptake of “facilitated-dissociation” drug, was estimated at various values (Table 1). The PS_{inf} values at the normal blood concentration of HSA (5%) are a key parameter of the quantitative IVIVE of hepatic uptake

clearances; unfortunately, these values could not be determined robustly because the drugs bind avidly to the albumin, resulting in no time-dependent uptake volume by hepatocytes during 0.25–1.25 minutes in the presence of 5% HSA. To predict IVIVE quantitatively for hepatic uptake clearances by isolated hepatocytes, we therefore estimated PS_{inf} in the presence of 5% HSA according to eq. 5 on the basis of the “facilitated-dissociation” model (Table 2). The PS_{inf} values with the “facilitated-dissociation” model as a basis were higher than values whose basis was the “free drug” hypothesis (Fig. 3; Table 2). The contributions of “albumin-mediated” uptake to the total uptake clearance of 10 drugs were 54%–98%, whereas rosuvastatin with its relatively low protein binding property (0.134 of f_B) exhibited the minimum value for “albumin-mediated” hepatic uptake contribution (Table 1). In light of these findings, albumin facilitates the hepatic uptake of the bound form of OATP substrate drugs with high protein binding (approximately >90%), a major pathway of hepatic uptake in the normal range of albumin concentration in the blood, thereby violating the “free drug” hypothesis. Therefore, we believe that the “albumin-mediated” hepatic uptake mechanism may improve the prediction accuracy for IVIVE.

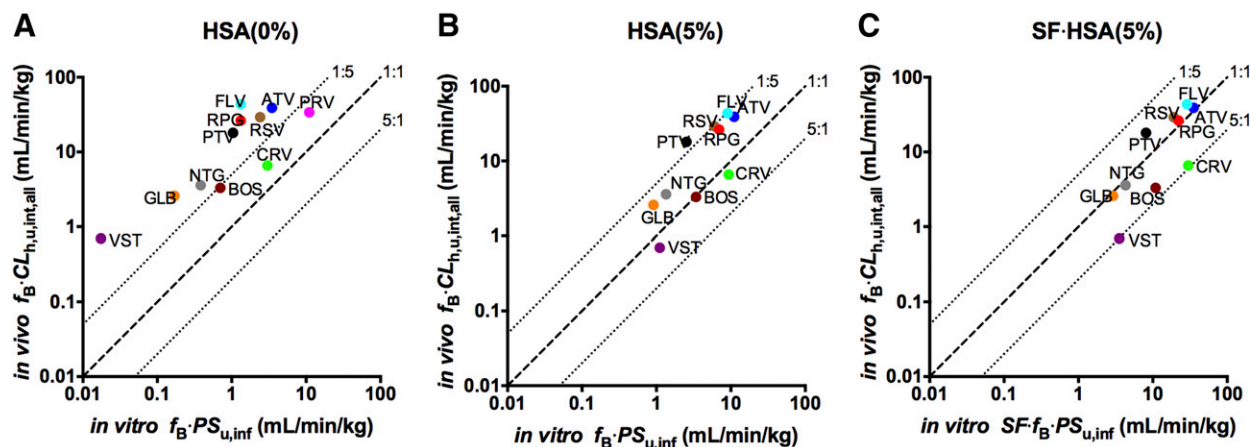


Fig. 5. Comparison between the uptake clearance ($f_B \cdot PS_{u,inf}^{vitro}$) at 0% HSA (A), the predicted $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA (B), and the adjusted $f_B \cdot PS_{u,inf}^{vitro}$ by SF (C) in suspended human hepatocytes and the observed hepatic overall intrinsic clearance ($f_B \cdot CL_{h,u,int,all}^{vivo}$) by the well-stirred model. The $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA was predicted by Tsao’s model, the adjusted $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA was calculated by the predicted $f_B \cdot PS_{u,inf}^{vitro}$ and SF is 3.19, the average value of the ratios of $f_B \cdot CL_{h,u,int,all}^{vivo}$ to $f_B \cdot PS_{u,inf}^{vitro}$ at 5% HSA. The dashed and dotted lines represent the line of unity and the lines of the 1:5 and 5:1 correlations, respectively. Key as in Fig. 1.

Regardless of estimated models of $f_B \cdot CL_{h,u,int,all}^{vivo}$, the $f_B \cdot PS_{u,inf}^{vivo}$ values estimated from in vitro $PS_{u,inf}$ (+) values at 5% HSA correlated better with the $f_B \cdot CL_{h,u,int,all}^{vivo}$ values estimated from the hepatic clearances (Fig. 4B; Fig. 5B; Table 2), suggesting that the underestimation of IVIVE for the hepatic uptake clearances could be adjusted sufficiently by “albumin-mediated” uptake factors, the ratio of $f_B \cdot PS_{u,inf}^{vivo}$ at 0% HSA to that at 5% HSA (R), or by enhancement of the hepatic uptake clearance in the normal range of albumin concentrations; the R values were estimated to be 2.44–63.8 (Table 2). Nevertheless, the relationship between $f_B \cdot PS_{u,inf}^{vivo}$ and $f_B \cdot CL_{h,u,int,all}^{vivo}$ continued to show slight underestimation of IVIVE from the in vitro hepatic uptake clearances, although the “albumin-mediated” uptake factor improved the prediction. The ratios of $f_B \cdot CL_{h,u,int,all}^{vivo}$ with the dispersion and well-stirred models as bases to $f_B \cdot PS_{u,inf}^{vivo}$ at 5% HSA were 0.63–5.57 and 0.63–7.11, respectively, and the mean values were 2.44 and 3.19, respectively (Table 2). The ratios of the in vivo-to-in vitro for hepatic clearance exhibited substrate dependency. Although drugs are only taken up by OATP1B1, the affinities for OATP1B1 of each drug ranged widely from low to high (the reciprocal of the K_m values, summarized in Supplemental Table S2). Furthermore, the relationship between the values of “albumin-mediated” uptake factors (R) and the affinities of drug for OATP1B1 exhibited a tendency by which the higher the affinity for the transporter, the more albumin effectively enhanced uptake (Miyachi et al., 2018). Furthermore, the $f_B \cdot PS_{u,inf}^{vivo}$ values at 5% HSA were corrected by the mean value designated as SF , resulting in a better relationship between $f_B \cdot PS_{u,inf}^{vivo} \cdot SF$ and $f_B \cdot CL_{h,u,int,all}^{vivo}$ within a 3-fold range of differences (Fig. 4C; Fig. 5C; Table 2) for both the dispersion and well-stirred models.

Alternatively, the underestimation of IVIVE for hepatic uptake clearance also could be partially explained by multifactorial mechanisms, including physiologic factors such as pH (Poulin and Haddad, 2015) and albumin concentration in the interstitial fluids (Poulin et al., 2016), experimental conditions (Badolo et al., 2011), and kinetic models describing the liver disposition (Iwatsubo et al., 1996). As shown in Figs. 4B and 5B, some discrepancy remained between $f_B \cdot CL_{h,u,int,all}^{vivo}$ and $f_B \cdot PS_{u,inf}^{vivo}$, and the SF values could be made up with various factors described above. The comprehensive review by Bowman and Benet (2018) has investigated the plausible mechanisms involved in the SF value of IVIVE for hepatic uptake clearance. Determining how these factors are involved in the SF values requires further investigation.

Recently, we reported an improvement in the accuracy of prediction of intrinsic hepatic clearance for three antidiabetic drugs that are substrates of both OATP1Bs and CYP2Cs by considering the “albumin-mediated” uptake in the in vitro measurements of OATP1B-mediated hepatic uptake and CYP2C-mediated metabolism (Fujino et al., 2018). Therefore, it is important to note that a kinetic model for describing the “albumin-mediated” hepatic uptake phenomenon for a drug avidly bound to albumin is useful for its robust IVIVE.

In conclusion, albumin facilitates the hepatic uptake of anionic drugs known as OATP substrates with high protein binding to hepatocytes, and the predicted $PS_{u,inf}$ (+) values at 5% HSA estimated by the “facilitated-dissociation” model provide a better extrapolation for in vivo hepatic intrinsic clearances. Furthermore, the ratios of $f_B \cdot CL_{h,u,int,all}^{vivo}$ to $f_B \cdot PS_{u,inf}^{vivo}$ at 5% HSA or the “albumin-mediated” factors are applicable and useful for quantifying IVIVE from the hepatic uptake clearances by isolated human hepatocytes.

Authorship Contributions

Participated in research design: Kim, Lee, Miyachi, Sugiyama.

Conducted experiments: Kim, Lee.

Contributed new reagents or analytic tools: Kim, Lee, Miyachi, Sugiyama.

Performed data analysis: Kim, Lee, Miyachi, Sugiyama.

Wrote or contributed to the writing of the manuscript: Kim, Lee, Miyachi, Sugiyama.

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