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

Seiji Miyauchi, Masayuki Masuda, Soo-Jin Kim, Yuudai Tanaka, Kyeong Ryoon Lee, Shouko Iwakado, Momoko Nemoto, Shotaro Sasaki, Kazumi Shimono, Yoshio Tanaka, and Yuichi Sugiyama

Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan (S.M., M.M., Y.T., S.I., M.S., K.S., Y.T.)

Sugiyama Laboratory, RIKEN Innovation Center, RIKEN Cluster for Industry Partnerships, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan (S.K., K.L., Y.S.)
Running Title Page

Running title: IVIVE for “Albumin-mediated” hepatic uptake

Corresponding Author: Yuichi Sugiyama, PhD
Sugiyama Laboratory, RIKEN Innovation Center, RIKEN Cluster for Industry Partnerships, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan
Phone: +81-45-503-9211
FAX: +81-45-503-9190
E-mail: ychi.sugiyama@riken.jp

Number of text pages: 24
Number of tables: 2
Number of supplementary table: 1
Number of figures: 8
Number of supplementary figure: 1
Number of references: 38
Number of words in the Abstract: 211
Number of words in the Introduction: 753
Number of words in the Discussion: 1488

Abbreviations: ANS, 1-anilino 8-naphthalene sulfonate; BSA, bovine serum albumin; Hepes, N-2-hydroxy-ethylpiperazine-N'2-ethanesulfonic acid; HSA, human serum albumin; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; IVIVE, in vitro-to-in vivo extrapolation; Mes, 2-(N-morpholino) ethanesulfonate; PBS, phosphate buffered saline; PSinf, uptake clearances for total ligand concentration; PSu,inf, uptake clearances for unbound ligand concentration; PTV, pitavastatin; SD, Sprague Dawley; Tris, Tris(hydroxymethyl) aminomethane.
Abstract

The effects of bovine serum albumin (BSA) and human serum albumin (HSA) on the unbound hepatic uptake clearance \( (P_{S_u,\text{inf}}) \) of the organic anion transporting polypeptide (Oatp/OATP) substrates 1-anilino-8-naphthalene sulfonate (ANS) and pitavastatin (PTV) were determined using primary cultured rat hepatocytes and isolated human hepatocytes, respectively. The \( P_{S_u,\text{inf}} \) value of hepatocytes was estimated by dividing the initial uptake rate of these anions by their unbound concentrations. The \( P_{S_u,\text{inf}} \) values for ANS and PTV were enhanced in the presence of albumin, thereby demonstrating the phenomenon of “albumin-mediated” hepatic uptake. We previously constructed a “facilitated-dissociation” model, in which the interaction of the ligand–albumin complex with the cell surface enhanced the dissociation of that complex to provide unbound ligand for uptake to the hepatocytes [\textit{J Pharmacokinet Biopharm} 16:165–181 (1988)]. That model was able to describe accurately the relationship between the enhancement of the \( P_{S_u,\text{inf}} \) values and the albumin concentration. By considering the enhancement of hepatic uptake clearance by albumin, using this “facilitated-dissociation” model we could predict accurately the \( P_{S_u,\text{inf}} \) \textit{in vivo} from that obtained in isolated hepatocytes. In the light of these findings, we suggest that the “facilitated-dissociation” model is applicable to describing the phenomenon of “albumin-mediated” hepatic uptake via organic anion transporters and to evaluating hepatic uptake clearance \textit{in vivo}. 
Introduction

The liver efficiently eliminates a variety of endogenous and exogenous organic anions from the blood circulation, which then undergo metabolism and/or biliary excretion in the liver. The hepatic uptake process is the most important step in their overall disposition by the liver. According to the conventional “free-drug” hypothesis, it has been assumed that the level of unbound ligand governs the hepatic uptake rate (Wilkinson and Shand, 1975; Shand et al., 1976). However, it is a distinguishing feature of the liver that the hepatic uptake of organic anions is very efficient despite these organic anions being avidly bound to serum albumin, a phenomenon referred to as “albumin-mediated” hepatic uptake (Weisiger et al., 1981; Forker and Luxon, 1983; Bass and Pond, 1988; Tsao et al., 1988b; Meijer and van der Sluijs, 1989; Ichikawa et al., 1992). Consequently, it has been argued that the hepatic uptake of organic anions is more efficient than can be accounted for by their unbound concentration and the “free-drug” hypothesis.

Ample evidence of the efficient elimination of organic anions has led to the proposal of several models to explain the efficient hepatic uptake. These include (1) the dissociation of ligand–albumin complexes catalyzed by their interaction with the cell surface (Forker and Luxon, 1981; Weisiger et al., 1981; Forker et al., 1982; Forker and Luxon, 1983; Tsao et al., 1988a), (2) the rate-limiting dissociation of ligand–albumin complexes in the extracellular fluid (Weisiger et al., 1984; Weisiger, 1985; van der Sluijs et al., 1987), and (3) the rate-limiting diffusion of ligand through the unstirred water layer adjacent
to the cell surface (Bass and Pond, 1988; Weisiger et al., 1991; Ichikawa et al., 1992; Pond et al., 1992).

The albumin-mediated hepatic uptake effect has been demonstrated previously using various experimental systems including isolated hepatocytes (Weisiger et al., 1981; Tsao et al., 1988b; Burczynski et al., 2001), primary cultured hepatocytes (Burczynski et al., 1989), isolated perfused liver (Forker and Luxon, 1981; Forker and Luxon, 1983; Tsao et al., 1988a) and isolated basolateral membrane vesicles (Blitzer and Lyons, 1985), also suggesting that the albumin-mediated effect might be driven by a general mechanism, irrespective of species and experimental systems.

Recently, the development of the quantitative in vitro to in vivo extrapolation (IVIVE) method has allowed prediction of the uptake clearance in vivo from the uptake rate obtained in human cryopreserved hepatocytes, and the parameters estimated using this technique have been extensively used for the prediction of human pharmacokinetics. IVIVE of the hepatic uptake clearance with cryopreserved human hepatocytes provided good predictions of the uptake of some organic anions (Watanabe et al., 2010; Watanabe et al., 2011; Kimoto et al., 2012). However, for other organic anions that tend to bind avidly to serum albumin, the accuracy of the predictions was poor (Watanabe et al., 2010; Watanabe et al., 2011). It is possible that the “albumin-mediated” hepatic uptake mechanism is responsible for these poor predictions by IVIVE, because the uptake by human hepatocytes has usually been determined in the absence of serum albumin. Recently, Poulin et al. have developed an adjustment method for estimating quantitatively the unbound fraction on the surface of hepatocytes by using the
plasma-to-whole liver concentration ratio of extracellular serum albumin, which improved the predictive accuracy of IVIVE for a variety of drugs (Poulin and Haddad, 2015; Poulin et al., 2016). Therefore, adequate prediction of hepatic uptake clearance by IVIVE requires adjustment for the “albumin-mediated” uptake factor, the enhancement of hepatic uptake seen in physiological albumin concentrations.

In the present study, we investigated whether bovine serum albumin (BSA) and human serum albumin (HSA), respectively, enhanced the unbound uptake clearance of 1-anilino-8-naphthalene sulfonate (ANS) by primary cultured rat hepatocytes and of pitavastatin (PTV) by human isolated hepatocytes. Both organic anions have been demonstrated to be taken up efficiently by the liver via organic anion transporting polypeptides (Oatp/OATP), despite their avid binding to serum albumin in the blood circulation (Sugiyama et al., 1983; Chung et al., 1990a; Chung et al., 1990b; Watanabe et al., 2010; Watanabe et al., 2011; Kimoto et al., 2012). We here demonstrate the role of the “albumin-mediated” hepatic uptake phenomenon in uptake of these anions by rat and human hepatocytes. Furthermore, we have determined whether the relationship between the enhancement of the unbound hepatic clearance and the albumin concentration could be predicted by a “facilitated-dissociation” model constructed previously (Tsao et al., 1988a), in which the interaction of the ligand–albumin complex with the cell surface enhances the dissociation of the complex, resulting in more unbound ligand being available for uptake by hepatocytes.
Materials and Methods

Materials

Chemicals including ANS, BSA (fraction V), HSA, Krebs–Henseleit buffer, and PTV were purchased from Sigma-Aldrich (St. Louis, MO). Cryopreserved human hepatocytes (lot 109) were purchased from BD Biosciences (Woburn, MA). Six-week-old Sprague Dawley (SD) rats were purchased from Sankyo Laboratory Corp. Inc. (Tokyo, Japan). All other chemicals used were of the highest purity available from Wako Chemical Inc. (Tokyo, Japan).

Measurements of ANS uptake by primary cultured rat hepatocytes

Hepatocytes were isolated from the livers of 6-week-old male SD rats that were given free access to food and water. The isolation procedure was basically the same as previously reported (Miyauchi et al., 1993; Masuda et al., 2014). Cell viability, determined by trypan blue exclusion, ranged from 95% to 98%. Cells were suspended at a density of $3.3 \times 10^5$ cells/ml in William’s E medium supplemented with dexamethasone (5 nM), insulin (10 nM), 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. A 3-ml aliquot of cell suspension was added to each well of a 6-well plate. The culture medium was exchanged with fresh medium 4 h after the initial seeding. Cells were cultured for an additional 16 h at 37 °C in 5% CO$_2$/95% air and then used for ANS uptake experiments. The primary cultured hepatocytes were used for the uptake experiments only.
after being cultured for less than 22 h. The experimental protocol for the use of the animals was approved by Toho University Animal Care and Use Committee (approval number: 16-53-258).

Uptake of ANS in primary cultured hepatocytes was measured at 37 °C in uptake buffer (pH 7.5) as described previously (Masuda et al., 2014). The composition of the uptake buffer was 140 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM glucose, and 10 mM Hepes/Tris adjusted to pH 7.5. Cells in each well were washed with the prewarmed uptake buffer and were preincubated for 5 min at 37 °C. After aspiration of the uptake buffer, uptake was initiated by the addition of a 1-ml aliquot of uptake buffer containing ANS and BSA at various concentrations as indicated in the figure legends. At the designated time, the buffer was aspirated, and cells were immediately washed twice with ice-cold uptake buffer. The cells were then dissolved in a 1-ml aliquot of 1% (w/v) Triton-X. The dissolved cells were diluted 10-fold with ethanol, and the samples were centrifuged at 18000 g for 10 min by a tabletop centrifuge. This aliquot of the supernatant was used to measure ANS concentration. The fluorescence intensity resulting from ANS uptake to the cells was measured at 470 nm (excitation 370 nm) using an Enspire multiplate fluorescence reader (PerkinElmer Inc., Waltham, MA) (Sugiyama et al., 1983; Chung et al., 1990a).

**Measurement of PTV uptake by suspended human hepatocytes**

The rate of uptake of PTV by suspended human hepatocytes was determined using cryopreserved human hepatocytes and a centrifugal filtration technique as described previously (Watanabe et al.,...
2010; Watanabe et al., 2011). For the uptake experiments, cryopreserved hepatocytes were thawed in a water bath at 37 °C then placed on ice, after which they were resuspended in Krebs–Henseleit buffer. Cell viability was determined by trypan blue exclusion, and the suspension was diluted to \(2 \times 10^6\) cells/ml. Then, 150-μl aliquots of cells were placed in test plates and prewarmed in a 37 °C incubator for 3 min. Uptake incubations were initiated by the addition of 150 μl of prewarmed 10 μM PTV, with and without HSA, which resulted in a final substrate concentration of 5 μM and a cell density of \(1 \times 10^6\) cells/ml in a 300-μl incubation volume. After a designated time (0.25 and 1.25 min), the reactions were terminated by separating the cells from the medium using a centrifugal filtration technique. For this purpose, the 80-μl aliquot of incubation mixture was placed in a 0.4-ml centrifuge tube (Sarstedt, Numbrecht, Germany) containing 50-μl of 5 M ammonium acetate under a 100-μl layer of an oil mixture (density 1.05, mixture of silicone oil and mineral oil; Sigma-Aldrich). Samples were then centrifuged for 10 s in a microfuge (Beckman Coulter). During the centrifugation, the hepatocytes rapidly passed through the oil layer into the aqueous solution (5 M ammonium acetate). The tubes were frozen in liquid nitrogen immediately after centrifugation and stored at –20 °C until analysis. The bottom of the tube was sliced off, and the contents were transferred to a new tube. Protein in the aqueous solution was precipitated with 3 volumes of acetonitrile/methanol (50/50, v/v) and removed by centrifugation at 15,000 g at 4 °C for 10 min. The supernatants from the top (medium) and the bottom (cells) of the tube were analyzed by liquid
chromatography coupled with tandem mass spectrometry (LC–MS/MS).

PTV was analyzed on a Shimadzu LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Samples (5 μl) were injected onto a Kintex C18 column (2.1 \times 100 \text{ mm}, 2.6 μm; Phenomenex, Torrance, CA) and eluted by a mobile phase with initial conditions of 5% solvent B, followed by a linear gradient of 5% solvent B to 80% solvent B over 3.0 min (solvent A, 0.1% formic acid in water; solvent B, 100% acetonitrile) at a flow rate of 0.3 ml/min. The column and autosampler tray temperature were maintained at 40 °C and 4 °C, respectively. For PTV, the mass spectrometer was operated in positive turbo-ion spray model at the transitions (m/z) 422 to 290.

**Measurement of ANS binding to BSA by the equilibrium dialysis method**

A dialysis cell comprising two chambers was divided by a 15 kDa cutoff dialysis membrane (Spectrum Medical Industries. Inc., Los Angeles, CA). Five milliliters of uptake buffer with or without added BSA was put into each chamber. ANS was added to the side containing BSA. After equilibrium was achieved at 37 °C (more than 22 h), the aliquot of the sample in each cell was diluted more than 10-fold with EtOH, and ANS concentrations were measured as described above. The recovery of ANS was greater than 98%.

**Measurement of PTV binding to HSA by the equilibrium dialysis method**

The binding of PTV to HSA was determined using a single-use RED (rapid equilibrium dialysis)
device (Thermo Scientific, Rockford, IL). In this study, PTV was added at a final concentration of 5 μM to PBS (phosphate buffered saline) buffer pH 7.4 containing HSA at 0, 0.125, 0.25, 0.5 and 1%, and then incubated at 37 °C for 24 h on an orbital shaker. According to the manufacturer’s instructions, 300 μl of the mixture was placed into the sample chamber, and the dialysis buffer (i.e., PBS, pH 7.4) was placed into the buffer chamber. After equilibrium was achieved (i.e., after 20 h), the concentrations of PTV in both chambers were measured using LC-MS/MS analysis, as described above. The recovery of PTV was approximately 92–98%.

**Kinetic analyses**

The initial uptake rates were calculated from the slopes of the time courses from 1 to 3 min for ANS and from 0.25 to 1.25 min for PTV, using linear regression analysis. The uptake clearances ($PS_{u,inf}$ and $PS_{inf}$) for unbound and total ligand concentrations were estimated by dividing the initial uptake rate by the unbound and total ligand concentrations, respectively. Saturation kinetics of the initial uptake rates were analyzed by fitting the concentration dependency of the initial uptake rate of ANS to a Michaelis–Menten-type equation with a saturable component. The kinetic parameters ($V_{max}$, the maximum uptake rate; $K_m$, Michaelis constant) were determined by an iterative nonlinear least-squares method using Sigmaplot (v.13, Systat Software Inc., San Jose, CA).

**Kinetic modeling**
We used two models in this study: a conventional “free-drug” model and the “facilitated-dissociation” model (Tsao et al., 1988a). The models used here are based on the assumption that the association and dissociation of ligand and albumin were rapid enough to achieve equilibrium.

The two models are summarized below.

**A conventional model of hepatic uptake involving a transporter**

In the conventional “free-drug” model (Fig. 1), it is assumed that rapid equilibrium of the binding of a ligand to albumin can be achieved in the extracellular bulk fluid. The hepatic uptake of the ligand is assumed to comprise transporter-mediated uptake and passive diffusion across the membrane, which correspond to the saturable and nonsaturable components, respectively, in the Michaelis–Menten equation. The uptake rate is described by the following equation:

\[
v = \frac{V_{\text{max}} \cdot c_i}{K_m + c_i} + P_s \cdot c_i \quad (1)
\]

where \(c_i\) is the unbound ligand in the extracellular fluid; \(V_{\text{max}}\) and \(K_m\) represent the maximum uptake rate and Michaelis constant, respectively; and \(P_s\) is the passive diffusion coefficient. Under linear conditions of transporter kinetics, Eq. 1 can be reduced to

\[
v = \left(\frac{V_{\text{max}}}{K_m} + P_s\right) \cdot c_i \quad (2)
\]

In the initial uptake by hepatocytes, the following mass–balance equation holds:

\[
c_0 V_{\text{ext}} = (c_b + c_i) V_{\text{ext}} = (\alpha + 1)c_i V_{\text{ext}} \quad (3)
\]
where \( c_s \) and \( c_b \) represent the concentrations for the total ligand and the ligand bound to albumin, respectively; the ratio of \( c_b \) to \( c_s \) is designated as \( \alpha \) and \( V_{ext} \) is the extracellular fluid volume.

Substituting Eq. 3 into Eq. 2 gives:

\[
v = \left( \frac{V_{max}}{K_m} + P_d \right) \cdot \frac{1}{1 + \alpha} \cdot c_0.
\]

(4)

The uptake clearances for the total ligand concentration (\( PS_{inf} \)) and the unbound ligand concentration (\( PS_{u,inf} \)) can be described as follows.

\[
PS_{inf} = \left( \frac{V_{max}}{K_m} + P_d \right) \cdot \frac{1}{1 + \alpha}
\]

(5)

\[
PS_{u,inf} = \left( \frac{V_{max}}{K_m} + P_d \right)
\]

(6)

The ratio (\( R \)) of \( PS_{u,inf} \) in the presence of albumin (\( PS_{u,inf} (+) \)) to that in the absence of albumin (\( PS_{u,inf} (-) \)) can be derived from Eq. 6:

\[
R = \frac{PS_{u,inf} (+)}{PS_{u,inf} (-)} = 1
\]

(7)

where the \( R \) value in the conventional model usually represents 1, irrespective of the presence of albumin.

A “facilitated-dissociation” model of the dissociation of unbound ligand from the ligand–albumin complex

In our previous study, the “albumin-mediated” hepatic transport phenomenon was characterized using isolated hepatocytes and isolated perfused liver, and was analyzed using a “facilitated-dissociation” model, in which the interaction of the ligand–albumin complex with the
surface of hepatocytes enhances dissociation of the ligand from the complex (Tsao et al., 1988a; Tsao et al., 1988b). As shown in Fig. 2, the mechanism of this facilitation has been postulated to be that an interaction between the ligand–albumin complex and the surface of hepatocytes induces a conformational change in albumin, which results in enhancement of the dissociation of ligand from the ligand–albumin complex near the cell surface and consequently effective uptake of the ligand (Forker and Luxon, 1981; Forker and Luxon, 1983; Tsao et al., 1988a). If we presume that the unbound albumin and the ligand-bound albumin compete for the same binding sites on the surface of hepatocytes, the fraction of albumin bound to the surface of the hepatocytes (\( \lambda \)) is given by the following equation:

\[
\lambda = \frac{B_{\text{max}}}{K_{d,m} + [Alb]}
\]

(8)

where \( B_{\text{max}} \) and \( K_{d,m} \) represent the capacity of albumin binding sites on the surface of hepatocytes and the dissociation constant of the bound albumin from the surface, respectively, and \([Alb]\) represents the concentration of albumin. The governing equations for the uptake rate of ligand (\( \nu \)) derived by Tsao et al. (Tsao et al., 1988a) are as follows:

\[
\nu = PS_{\text{inf}} \cdot c_0 = P_m \cdot f_u \cdot c_0 + P_{\text{B,influx}} \cdot \lambda \cdot (1 - f_u) \cdot c_0
\]

(9)

\[
PS_{\text{inf}} (+) = P_n + P_{\text{B,influx}} \cdot \lambda \cdot \left( \frac{1}{f_u} - 1 \right)
\]

(10)

where \( c_0 \) represents the total concentration of ligand; \( PS_{\text{inf}}, PS_{\text{inf}} (+), P_m \) and \( P_{\text{B,influx}} \) represent the uptake clearances for the total and unbound ligands in the presence of albumin, the
permeability clearances for the unbound ligand, and the unbound ligand dissociated from the ligand-albumin complex at the surface, respectively; \( f_u \) represents the fraction of the unbound ligand in the extracellular fluid. \( P_m \) and \( P_{\text{H, influx}} \) values include the transporter-mediated transport and passive diffusion. The \( f_u \) value is expressed as the following equation:

\[
f_u = \frac{1}{1 + \frac{n[Alb]}{K_d}}
\]

(11)

where \( n \) and \( K_d \) represent the number of binding sites on the albumin and the dissociation constant of the ligand, respectively.

The ratio \( R \) of \( PS_{\text{u, inf}}^{(+)} \) in the presence of albumin \( (PS_{\text{u, inf}}^{(+)}) \) to that in the absence of albumin \( (PS_{\text{u, inf}}^{(-)}) \) can be derived from Eqs 8, 10 and 11, as follows:

\[
R = \frac{PS_{\text{u, inf}}^{(+)}}{PS_{\text{u, inf}}^{(-)}} = 1 + \frac{P_{\text{H, influx}}}{P_m} \cdot \frac{1}{f_u} \cdot \frac{1}{K_d} + \frac{P_{\text{H, influx}} \cdot B_{\text{max}}[Alb]}{K_d + [Alb]}
\]

(12)

where \( \frac{P_{\text{H, influx}}}{P_m} \cdot B_{\text{max}} \) represents the parameters with regard to the relative capacity for the interaction of the albumin with the surface of hepatocytes and is designated as \( r \cdot B_{\text{max}} \).

Alternatively, we also analyzed the obtained data using the model derived by Forker et al. (Forker and Luxon, 1983), which was categorized as a “facilitated-dissociation” model, but was more similar to a model based on the “albumin receptor” theory. According to this model, the ratio of \( PS_{\text{u, inf}}^{(+)} \) to \( PS_{\text{u, inf}}^{(-)} \) can be expressed, as follows:
Extrapolation of the in vivo hepatic uptake clearance from the uptake clearance measured using isolated hepatocytes.

The unbound hepatic uptake clearances for ANS and PTV in vivo (per body weight) were scaled up from the in vitro unbound hepatic uptake clearance using the following scaling factors: $1.2 \times 10^8$ cells/g liver, 40.0 g liver/kg body weight for rat, and 25.7 g liver/kg body weight for human (Davies and Morris, 1993; Miyauchi et al., 1993).

In vivo hepatic overall intrinsic clearance, which represents the elimination of unbound ligands ($CL_{in vivo}$) from circulating blood, was also estimated from the in vivo hepatic clearance using a dispersion model as follows:

\[
CL_{in vivo} = \frac{Q_h \times (1 - F_h)}{1 + \frac{4a}{(1 + a)^2 \cdot \exp\left\{\frac{(a - 1)}{2D_N}\right\} \cdot (1 - a)^2 \cdot \exp\left\{\frac{(a + 1)}{2D_N}\right\}}
\]

\[
a = (1 + 4R_N \cdot D_N)^{1/2}
\]

\[
R_h = f_n \cdot CL_{in vivo}
\]

where $CL_{in vivo}$, $Q_h$, $D_N$ and $f_n$ are the hepatic clearance, hepatic blood flow rate, the dispersion number and unbound fraction in the blood, respectively. The $Q_h$ values for rat and human are 55.2
ml/min/kg and 20.7 ml/min/kg, respectively (Davies and Morris, 1993). The $D_N$ value was set at 0.17.

The hepatic uptake clearance *in vivo* was estimated based on Eqs. 14–17, using the dispersion model described previously (Roberts and Rowland, 1986; Iwatsubo et al., 1996).
Results

Initial uptake rate of ANS by primary cultured rat hepatocytes in the absence and presence of BSA

The uptake rate of ANS by primary cultured rat hepatocytes in the absence of BSA was measured, and the results are summarized in Fig. 3. The ANS uptake increased linearly over 3 min, and the initial uptake rate was determined from the uptake over 1–3 min. The relationship between the initial uptake rate and concentration of ANS showed saturation, and the data fit the Michaelis–Menten equation describing a single saturable process; this was supported by the linearity of the Eadie–Hofstee plot shown in the inset. The kinetic parameters $K_m$ (Michaelis–Menten constant) and $V_{max}$ (maximal uptake rate) were $53.7 \pm 18.2 \mu$M and $3.7 \pm 0.8$ nmol/min/mg protein, respectively ($\pm$ calculated SD). These estimated $K_m$ and $V_{max}$ values were similar to those reported for isolated rat hepatocytes (Chung et al., 1990a). The ANS uptake by primary cultured hepatocytes determined in the presence of various concentrations of BSA is summarized in Fig. 4. ANS binds avidly to albumin, and the presence of BSA caused a remarkable decrease in its uptake. The hepatic uptake clearance ($P_{S_{inf}}$) and the unbound uptake clearance ($P_{S_{u,inf}}$) were estimated by dividing the uptake rate by the initial concentration and unbound concentrations, respectively, and are depicted in Fig. 5 and summarized in Table I (a). First, to confirm whether the ANS uptake by Oatp was linear within the range of 20 $\mu$M, we determined the ANS uptake rates at various concentrations of ANS. As shown in Fig. 4, the hepatic uptake clearances for ANS in the range of 2–20 $\mu$M remained unchanged (did not differ significantly), being consistent
with the estimated $K_m$ value. We then conducted the uptake experiments under conditions of varying ANS and BSA concentrations while maintaining the ratio of ANS to BSA, or keeping the unbound concentration of ANS constant at approximately 2.4 µM. These experimental conditions followed those developed by Weisiger et al. (Weisiger et al., 1981), who were the first to propose the “albumin receptor” theory.

The unbound concentrations were determined using the equilibrium dialysis method. The $P_S\text{inf}$ value of ANS decreased markedly as the concentration of BSA increased, in association with the decrease in the unbound fraction. In contrast, the $P_S\text{u,inf}$ value of ANS was enhanced markedly as the concentration of BSA increased, although the unbound concentration was kept at approximately 2.4 µM (Fig. 5 (b)). In other words, the $P_S\text{u,inf}$ value in the presence of albumin was much higher than could be accounted for by the unbound concentration. The extent of the enhancement in the $P_S\text{u,inf}$ values was saturated at the highest concentration of BSA. These kinetic features are distinctive of the “albumin-mediated” hepatic transport phenomenon (Weisiger et al., 1981; Forker and Luxon, 1983).

Alternatively, ANS uptake by primary cultured hepatocytes was determined in the presence of varying BSA concentrations while the ANS concentration was kept constant at 20 µM under linear kinetic conditions. As shown in Fig. 6, the $P_S\text{inf}$ values decreased as BSA concentration increased, whereas the $P_S\text{u,inf}$ values increased consistently with those in the uptake experiments under conditions where the unbound concentration of ANS was kept constant. We determined the albumin-mediated
hepatic uptake under two experimental conditions: where ANS and BSA concentrations were varied while unbound ANS concentration was kept constant, or where the ANS concentration was kept at 20 µM, although the BSA concentrations were increased. Both experimental conditions demonstrated similar relationships between the albumin concentration and $PS_{u,\inf}$ enhancement, as shown below (Fig. 8 (a)).

Initial uptake rate of PTV by isolated human hepatocytes in the absence and presence of HSA

The rate of uptake of PTV by isolated human hepatocytes was measured in the absence and presence of HSA, and the results are summarized in Fig. 7 and Table I (b). The PTV uptake increased linearly over 1.5 min, and the initial uptake rate was determined from the uptake over 0.25–1.25 min. Similar to ANS uptake by primary cultured rat hepatocytes, the $PS_{inf}$ value was estimated by dividing the uptake rate by the initial concentration. The $PS_{inf}$ value of PTV decreased substantially as the concentration of HSA increased. In contrast, the $PS_{u,inf}$ value clearly increased as the HSA concentration increased, and the $PS_{u,inf}$ values were saturated at 1% (w/v) HSA. Therefore, the “albumin-mediated” hepatic transport phenomenon was also observed in the uptake of PTV by human hepatocytes, known to be mediated by OATP1B1 (Watanabe et al., 2010).

Analysis by the previous “facilitated-dissociation” model of the albumin dependency of the ratio of the unbound uptake clearance in the presence of albumin to that in its absence ($R$)
The “facilitated-dissociation” model is well known to describe adequately the hepatic uptake rate of a highly bound ligand in the presence of albumin (Forker and Luxon, 1981; Forker and Luxon, 1983; Tsao et al., 1988a). We therefore performed curve fitting of the albumin concentration-dependency of the $R$ values (Fig. 8), based on Eq. 12. The “facilitated-dissociation” model provided adequately fitting curves for the albumin dependency (Fig. 8). The relative capacity for the interaction of albumin with the surface of hepatocytes ($r \cdot B_{\text{max}}$) and the dissociation constant of ANS from the bound albumin at the surface ($K_{\text{d,m}}$) were estimated to be $3.51 \pm 1.07 \mu M$ and $24.4 \pm 17.5 \mu M$ (± calculated SD), respectively (Table II). The values of $r \cdot B_{\text{max}}$ and $K_{\text{d,m}}$ for PTV were estimated to be $48.8 \pm 11.6 \mu M$ and $199 \pm 61 \mu M$ (± calculated SD), respectively (Table II). As an alternative, we analyzed the albumin concentration-dependency of the $R$ values using the model developed by Forker et al (Forker and Luxon, 1981; Forker and Luxon, 1983). As shown in supplementary Fig. 1, this model also provided adequately fitting curves for the albumin dependency. The values of $B_{\text{max}}$ and $K_{\text{d,m}}$ for ANS were estimated to be $3.71 \pm 1.22 \mu M$ and $31.8 \pm 22.0 \mu M$ (± calculated SD), respectively. The values of $B_{\text{max}}$ and $K_{\text{d,m}}$ values for PTV were estimated to be $58.7 \pm 21.9 \mu M$ and $275 \pm 131 \mu M$ (± calculated SD), respectively (supplementary Table I).

**Extrapolation of the in vivo hepatic uptake clearance from the in vitro hepatic uptake clearance obtained using isolated hepatocytes**
The *in vivo* hepatic uptake clearances of unbound ligand for ANS and PTV (\(PS_{u,inf}^{vivo}\)) were extrapolated from the *in vitro* hepatic uptake clearance obtained using isolated hepatocytes, and the results are summarized in Table II. The values of \(PS_{u,inf}^{vivo}\) for ANS and PTV were estimated to be 263 ± 22 and 83.0 ± 20.3 ml/min/kg, respectively. The overall *in vivo* hepatic intrinsic clearances (\(CL_{h,u,int,all}^{vivo}\)) for the unbound ligands were also determined from Eqs 14–17 to be 867 and 1520 ml/min/kg, respectively. The ratios of \(CL_{h,u,int,all}^{vivo}\) to \(PS_{u,inf}^{vivo}\) for ANS and PTV were estimated to be 3.30 and 18.3, respectively, indicating a poor predictive accuracy by IVIVE. The \(R\) values in the range of physiological concentrations of albumin were 2.70 and 10.6, respectively, which are close to the ratios of \(CL_{h,u,int,all}^{vivo}\) to \(PS_{u,inf}^{vivo}\).
Discussion

In this study, we used primary cultured rat hepatocytes and isolated human hepatocytes to determine whether BSA and HSA enhanced the $PS_{u,inf}$ of ANS and PTV, respectively. These organic anions have been shown to be taken up by the liver via a transporter-mediated process (Sugiyama et al., 1983; Chung et al., 1990a; Chung et al., 1990b; Watanabe et al., 2010; Watanabe et al., 2011; Kimoto et al., 2012). As shown in Figs 5 and 6, the presence of albumin prominently enhanced $PS_{u,inf}$ values of hepatocytes. This enhancement of the $PS_{u,inf}$ values was saturated at high concentrations of albumins, indicating that the “albumin-mediated” hepatic uptake phenomenon was being observed in rat and human hepatocytes (Figs 5 (b) and 6 (b)).

As discussed earlier, several mechanisms have been proposed to account for the enhancement of the hepatic uptake clearance of unbound ligand. In the present study, the “albumin-mediated” hepatic uptake transport of ANS and PTV was analyzed using a “facilitated-dissociation” model, in which contact of the ligand–albumin complex with the surface of hepatocytes enhances the dissociation of the ligand from the complex and provides the hepatocytes with more unbound ligand for uptake (Fig. 2). We therefore performed curve fitting using the governing equation, Eq. 12. The calculated line fitted well with the data for the enhancement ratios of both ANS and PTV uptake (Fig. 7). The values of $r \cdot B_{max}$ and $K_{z,u}$ for ANS were estimated to be 4 μM and 20 μM, respectively. The values of $r \cdot B_{max}$ and $K_{z,u}$ for PTV were estimated to be 50 μM and 200 μM, respectively. These values of $K_{z,u}$ were
compatible with those reported in other studies (Weisiger et al., 1981; Forker and Luxon, 1983; Elmadhoun et al., 2001). Various models previously proposed can explain the kinetic features; the albumin-dependent enhancement of $PS_{u,inf}$ and its saturation in the presence of higher concentrations of albumin and provide the enhancement ratio of $PS_{u,inf}$ in the presence of physiological concentrations of albumin, although it is difficult to distinguish the most adequate of the various models. Among these model, the “facilitated-dissociation” model developed by Forker et al. (Forker and Luxon, 1983) was most similar to a model based on the albumin receptor theory. The model also provided a good prediction of the relationship between the enhancement of $PS_{u,inf}$ and the albumin concentrations (supplementary Fig. 1), with $K_{d,m}$ values of 30 $\mu$M and 300 $\mu$M, respectively (supplementary Table I), compatible with our model. Either mechanism can explain the kinetic features of the albumin-mediated hepatic uptake and the enhancement ratio of $PS_{u,inf}$ value under physiological levels of albumin.

Blitzer and Lyons (Blitzer and Lyons, 1985) directly demonstrated, using rat basolateral liver plasma membrane vesicles, that albumin enhanced Na$^+$-dependent bile acid uptake. They proposed as a possible mechanism that the binding of albumin–bile acid complex to the liver plasma membrane might facilitate the interaction of bile acid with its transporter and enhance the bile acid available for uptake, implying that the bile-acid transporter might function as the albumin receptor and facilitate the dissociation of the albumin–bile acid complex. If the transporter were involved in the interaction of albumin with the surface of hepatocytes, this could explain why the $R$ values differed so markedly between ANS and PTV, although their unbound
fractions in the blood were similar. According to a kinetic study by Blitzer and Lyons (Blitzer and Lyons, 1985), albumin reduced the bile-acid transporter $K_m$ value; in other words, the binding of albumin to the liver plasma membrane enhanced the affinity of the bile acid for its transporter. PTV shows a relatively high affinity for OATP (Kimoto et al., 2012), whereas ANS shows a relatively low affinity for Oatp (Chung et al., 1990b), leading us to hypothesize that the higher the affinity for the transporter, the more effective is the albumin-mediated enhancement. Further development of another model including the interaction between the albumin–organic anion complex and the transporter is required to demonstrate its mechanism.

There are several published reports concerning the interaction of albumin with the surface of hepatocytes. Weisiger et al. demonstrated directly using isolated rat hepatocytes that $^{125}$I-labeled albumin bound to the surface of hepatocytes with a dissociation constant of 25 μM (Weisiger et al., 1981). Horie et al. also demonstrated using spectrophotometric methods (electron spin resonance and absorption spectrum) that the interaction of albumin and isolated rat hepatocytes induced a conformational change in albumin (Horie et al., 1988). From another perspective, Burczynski et al., using several proteins including modified albumins, demonstrated that an ionic interaction between extracellular protein and hepatocytes occurs at the cell surface, which enhances palmitate uptake by hepatocytes (Burczynski et al., 2001; Elmadhoun et al., 2001). However, the physiological and biochemical relevance of this interaction remains unknown, and its mechanism requires further investigation.
Recently, based on the “free-drug” hypothesis, quantitative IVIVE of hepatic uptake clearance using cryopreserved human hepatocytes resulted in good predictions of the uptake of some organic anions (Watanabe et al., 2010). However, the uptake of other organic anions that bind avidly to serum albumin was poorly predicted (Watanabe et al., 2010; Watanabe et al., 2011). Hypothesizing that the “albumin-mediated” hepatic uptake mechanism might be a factor in these poor predictions, we estimated the $R$ values in physiological albumin concentrations (5% albumin) on the basis of the “facilitated-dissociation” model. The $R$ values for ANS and PTV were estimated to be 2.7 and 11, respectively, implying that the albumin-mediated hepatic uptake mechanism should be considered \textit{in vivo}. Furthermore, the unbound hepatic uptake clearances ($PS_{\text{unb}}$) for ANS and PTV \textit{in vivo} (per body weight) were extrapolated from the \textit{in vitro} unbound hepatic uptake clearances (Davies and Morris, 1993) to be 260 ml/min/kg and 83 ml/min/kg, respectively (Table 2). The hepatic uptake clearance ($CL_{\text{h.u.int.all}}^{\text{vivo}}$) was estimated from \textit{in vivo} kinetic parameters using a dispersion model, as described previously (Roberts and Rowland, 1986; Iwatsubo et al., 1996). The $CL_{\text{h.u.int.all}}^{\text{vivo}}$ values were determined to be 870 ml/min/kg and 1500 ml/min/kg, respectively. The ratios ($R_{\text{vivo/vitro}}$) of $CL_{\text{h.u.int.all}}^{\text{vivo}}$ to $PS_{\text{unb}}^{\text{vivo}}$ for ANS and PTV were estimated to be 3 and 18, respectively, indicating that the huge discrepancy seen in IVIVE could be attributed mainly to “albumin-mediated” hepatic uptake and could be largely corrected by using the $R$ value estimated with the “facilitated-dissociation” model. In the present study, however, IVIVE taking into account the albumin-mediated hepatic uptake provided good
predictions for only the two organic anions ANS and PTV; therefore, further investigations of other organic anions that are highly bound to albumin are required to elucidate how albumin-mediated hepatic uptake might be involved in IVIVE.

Alternative reasons also could be considered to raise poor predictions of IVIVE; (i) pH difference between intracellular and extracellular spaces (Poulin and Haddad, 2015), ii) the concentration difference in between the plasma and liver (Poulin et al., 2016), iii) the activity heterogeneity of the human cryopreserved hepatocytes (Badolo et al., 2011), and (iv) the kinetic model describing the behavior of anion in the liver (Iwatsubo et al., 1996). Poulin et al. (Poulin et al., 2016) developed a useful procedure for quantifying the unbound ligand concentration under in vivo conditions by taking into account pH difference between intracellular and extracellular spaces and the concentration difference in between the plasma and liver and pH-partition mechanisms. This empirical method provides an improvement of quantitative IVIVE for a range of drugs that bind avidly to albumin (Poulin et al., 2012; Poulin and Haddad, 2015; Poulin et al., 2016). Very recently, Bounakta et al. (Bounakta et al., 2017) and Poulin et al. (Poulin et al., 2017) demonstrated using an isolated perfused rat liver system that the IVIVE method based on the albumin-mediated uptake mechanism provideed the most accurate predictions of in vivo hepatic clearance as compared with other empirical IVIVE considering pH and albumin concentration differences between the plasma and liver. Thus, it is important to note that a kinetic model for describing the “albumin-mediated” hepatic uptake phenomenon for a ligand avidly bound to albumin is
indispensable for its robust IVIVE.

In conclusion, we have demonstrated that the unbound hepatic uptake clearance of the organic anions ANS and PTV was substantially enhanced by the presence of albumin and that this enhancement was saturated at a high concentration of albumin. Therefore, “albumin-mediated” hepatic transport was demonstrated to play a role in uptake of these anions by rat and human hepatocytes. The relationship between the enhancement of the unbound hepatic clearance and the albumin concentration could be accurately predicted by the “facilitated-dissociation” model constructed previously, in which the interaction of the ligand–albumin complex with the cell surface enhances the dissociation of the complex to make unbound ligand available for uptake to the hepatocytes. By considering this enhancement of hepatic uptake clearance by albumin, we could also predict the uptake clearance in vivo from that observed in isolated hepatocytes. In light of these findings, we propose that the “facilitated-dissociation” model can be applied to describing the phenomenon of “albumin-mediated” hepatic uptake via organic anion transporters and to evaluating hepatic uptake clearance in vivo.
Author Contributions

Participated in research design: Miyauchi, Masuda, Kim, Shimono, and Sugiyama

Conducted experiments: Miyauchi, Masuda, Kim, Yuudai Tanaka, Iwakado, Nemoto, Sasaki and Lee

Performed data analysis: Miyauchi, Masuda, Kim, Lee, Shimono, Sasaki and Sugiyama

Wrote or contributed to the writing of the manuscript: Miyauchi, Kim, Yoshio Tanaka, and Sugiyama
REFERENCES


Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. Pharm


Electrophysiological characterization of human Na(+)/taurocholate cotransporting polypeptide (hNTCP) heterologously expressed in Xenopus laevis oocytes. Arch Biochem Biophys 562:115-121.


Footnotes

This work was supported by Grant-in-Aid for Scientific Research (C) and (S) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology [26460044] and [24229002], respectively.

Figure legends

Figure 1 A conventional model of hepatic uptake via a transporter
In the conventional model, it is assumed that rapid equilibrium in the binding of a ligand to albumin is maintained in the extracellular bulk fluid. The unbound and bound ligands are designated as $c_b$ and $c_f$, respectively. The ratio of $c_b$ to $c_f$ is designated as $\alpha$. The uptake rate of a ligand is presumed to be governed not only by transport-mediated uptake but also by passive diffusion uptake (Eq. 1).

Figure 2 The “facilitated-dissociation” model
The uptake of highly albumin-bound ligands to hepatocytes comprises a pathway for the unbound ligands and an additional pathway for the albumin-bound ligands. The interaction of the ligand–albumin complex with the surface of hepatocytes makes more unbound ligand available for uptake than would be expected according to the conventional model. It was assumed that the unbound and ligand-bound albumin compete for the same binding site(s) on the surface of hepatocytes with the same affinity (dissociation constant, $K_{d,m}$).

Figure 3 Concentration-dependency of ANS uptake by primary cultured rat hepatocytes
Panel (a): Time course of ANS uptake by rat hepatocytes. The uptake rate of ANS was measured for 3 min at 37 °C and pH 7.5. Each point represents the mean ± SE (n = 4). Panel (b): Concentration-dependency of the rate of uptake of ANS. The rate of uptake of ANS was measured for 3 min at 37 °C and pH 7.5 in the presence of increasing ANS concentrations (2–200 μM). The inset figure shows the Eadie–Hofstee plot of ANS uptake. Lines fitted to the nonlinear or linear forms of the Michaelis–Menten equation are shown. $K_m$ (Michaelis constant) and $V_{max}$ (maximal uptake rate) were estimated to be 53.7 ± 18.2 μM and 3.7 ± 0.8 nmol/min/mg protein, respectively (± calculated SD).

Figure 4 Effect of BSA on ANS uptake by primary cultured rat hepatocytes
The uptake of ANS was measured for 3 min at 37 °C and pH 7.5. Each point represents the mean ± SE (n = 4).

Figure 5 The effect of BSA on unbound uptake clearance ($PS_{u,inf}$) of ANS by rat hepatocytes
Panel (a): Uptake clearance ($PS_{inf}$) of ANS. The uptake clearance was estimated by dividing the uptake rate by the ANS concentration in the medium (Fig. 4). Panel (b): Unbound uptake clearance ($PS_{u,inf}$) of ANS. The values of $PS_{u,inf}$ were estimated by normalizing the value of $PS_{inf}$ (panel (a)) to the unbound fraction of ANS. The unbound concentration of ANS in the presence of BSA was kept at 2.4 μM. Enhancement of the unbound uptake clearance of ANS was saturated at higher concentrations of BSA. Comparison of the uptake clearances in the presence and absence of albumin were performed with Student’s t-test. (*), significantly different from control (P < 0.05); (**), significantly different from control (P < 0.01).
Figure 6 The effect of BSA on unbound uptake clearance (PS_{u,inf}) of ANS by rat hepatocytes
Panel (a): Uptake clearance (PS_{inf}) of ANS. The uptake clearance was estimated by dividing the uptake rate by the 20 μM ANS concentration in the medium. Panel (b): Unbound uptake clearance (PS_{u,inf}) of ANS. The values of PS_{u,inf} were estimated by normalizing the value of PS_{inf} (panel (a)) to the unbound fraction of ANS. Enhancement of the unbound uptake clearance of ANS was saturated at higher concentrations of BSA. Comparison of the uptake clearances in the presence and absence of albumin were performed with Student’s t-test. (*), significantly different from control (P < 0.05); (**), significantly different from control (P < 0.01).

Figure 7 The effect of HSA on unbound uptake clearance (PS_{u,inf}) of PTV by human hepatocytes
Panel (a): Uptake clearance (PS_{inf}) of PTV. The uptake rate of 5 μM PTV was measured for 75 sec at 37°C and pH 7.5. The uptake clearance was estimated by dividing the uptake rate by the PTV concentration in the medium. Each bar represents the mean ± SE (n = 4). Panel (b): Unbound uptake clearance (PS_{u,inf}) of PTV. The values of PS_{u,inf} were estimated by normalizing the value of PS_{inf} (panel (a)) to the unbound fraction of PTV. The enhancement of the unbound uptake clearance of PTV was saturated at higher concentrations of HSA. Comparison of the uptake clearances in the presence and absence of albumin were performed with Student’s t-test. (*), significantly different from control (P < 0.05); (**), significantly different from control (P < 0.01).

Figure 8 Curve-fitting of the relationship between the albumin concentration and the R values for ANS uptake (a) and PTV uptake (b) analyzed by the “facilitated-dissociation” model.
The R values for ANS and PTV were estimated from the data shown in Figs 5 and 6, and 7, respectively.
The values of \frac{n}{K_d} for ANS and PTV were estimated by the equilibrium dialysis method to be 0.501 and 0.248 μM^{-1}, respectively. The relationships were fitted to Eq. 12 using a nonlinear least-squares method. The fitting curve for ANS and PTV adequately predicted the observed data. The lines in the figures show the fitted lines determined by analysis using the “facilitated-dissociation” model.
### Table I (a). Relationship between BSA concentration (unbound fraction) and $PS_{u,inf}$ of ANS

<table>
<thead>
<tr>
<th>BSA (%)</th>
<th>0</th>
<th>0.016</th>
<th>0.094</th>
<th>0.25</th>
<th>0.51</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbound fraction</td>
<td>1</td>
<td>0.486</td>
<td>0.126</td>
<td>0.0487</td>
<td>0.0231</td>
</tr>
</tbody>
</table>

$PS_{u,inf}$

(μl/min/mg protein)

- 60.3 ± 5.0
- 69.1 ± 12.9
- 84.0 ± 4.6
- 113 ± 15
- 130 ± 10

$PS_{u,inf}$

(μl/min/mg protein)

- 62.7 ± 1.4
- 83.9 ± 0.4
- 120 ± 14
- 171 ± 19
- 159 ± 33

*a Data shown in Fig. 5. ANS hepatic uptakes were determined as ANS and BSA concentrations were varied, while keeping the unbound concentration of ANS constant at approximately 2.4 μM.

*b Data shown in Fig. 6. ANS uptakes were determined with varying BSA concentrations while ANS concentration was kept constant at 20 μM.

### Table I (b). Relationship between albumin concentration (unbound fraction) and $PS_{u,inf}$ of PTV

<table>
<thead>
<tr>
<th>HSA (%)</th>
<th>0</th>
<th>0.125</th>
<th>0.25</th>
<th>0.50</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unbound fraction</td>
<td>1</td>
<td>0.237</td>
<td>0.0929</td>
<td>0.0413</td>
<td>0.0182</td>
</tr>
</tbody>
</table>

$PS_{u,inf}$

(μl/min/10^6 cells)

- 26.9 ± 6.6
- 52.6 ± 13.3
- 85.8 ± 20.4
- 109 ± 41
- 170 ± 60

Data shown in Fig. 7. PTV uptakes were determined with varying HSA concentrations while PTV concentration was kept constant at 5 μM.
Table II. Extrapolation of the in vivo hepatic uptake clearance from the uptake clearance obtained using isolated hepatocytes and its comparison with the in vivo hepatic overall intrinsic clearance

<table>
<thead>
<tr>
<th>Ligands</th>
<th>$PS_{\text{sad}}^{a)}$ (µl/min/10^6 cells)</th>
<th>$PS_{\text{vivo}}^{b)}$ (ml/min/kg)</th>
<th>$f_B^{c)}$</th>
<th>Estimated parameters by the “facilitated-dissociation” model $^{d)}$</th>
<th>$CL_{\text{h,B}}^{e)}$ (ml/min/kg)</th>
<th>$CL_{\text{h,int,all}}^{f)}$ (ml/min/kg)</th>
<th>$R_{\text{vivo/vitro}}^{g)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS</td>
<td>54.8 ± 4.5$^h)$</td>
<td>263 ± 22</td>
<td>0.00796</td>
<td>$3.51 ± 1.07^{b)}$</td>
<td>24.4 ± 17.5$^{b)}$</td>
<td>2.70</td>
<td>6.43</td>
</tr>
<tr>
<td>PTV</td>
<td>26.9 ± 6.6</td>
<td>83.0 ± 20.3</td>
<td>0.00933</td>
<td>48.8 ± 11.6$^{b)}$</td>
<td>199 ± 61$^{b)}$</td>
<td>10.6</td>
<td>9.64</td>
</tr>
</tbody>
</table>

$PS_{\text{sad}}$, the hepatic uptake clearance in the absence of the albumin by isolated hepatocytes; $PS_{\text{vivo}}$, the in vivo hepatic uptake clearance extrapolated from the in vitro hepatic uptake by isolated hepatocytes; $f_B$, unbound fraction in the blood; $CL_{\text{h,B}}$, the hepatic clearance for the blood; $CL_{\text{h,int,all}}$, the in vivo hepatic overall intrinsic clearance; $R_{\text{vivo/vitro}}$, the ratio of $CL_{\text{h,int,all}}$ to $PS_{\text{vivo}}$.

$^a$ Data shown in Figs 5, 6 and 7.

$^b$ Calculated using the following physiological scaling factors: $1.2 \times 10^8$ cells/g of liver, 40 g liver/kg body weight for rat, 25.7 g liver/kg body weight for human (Davies and Morris, 1993)

$^c$ Calculated by dividing the unbound fraction in the plasma by the blood-to-plasma concentration ratio (Chung et al., 1990a; Watanabe et al., 2010).

$^d$ The parameters estimated by the fitting curves in Fig. 8; $r \cdot B_{\text{max}}$, the relative capacity for the interaction of albumin with the surface of hepatocytes; $K_{d,m}$, the dissociation constant of the bound albumin from the surface; $R_{5\%}$, $R$ value in the presence of 5% albumin.

$^e$ Values from the literature (Chung et al., 1990a; Watanabe et al., 2010).

$^f$ Calculated using a dispersion model as described previously (Roberts and Rowland, 1986; Iwatsubo et al., 1996).

$^g$ Calculated using a dispersion model as described previously (Roberts and Rowland, 1986; Iwatsubo et al., 1996).

$^h$ Values from the literature (Chung et al., 1990a; Watanabe et al., 2010).

$^i$ Calculated using a dispersion model as described previously (Roberts and Rowland, 1986; Iwatsubo et al., 1996).

$^j$ Converted the unit of $PS_{\text{sad}}$ to µl/min/10^6 cells using a scaling factor of $1.1 \times 10^6$ cells/mg protein.

$^{h)}$ ± calculated SD
Figure 1

rapid equilibrium

\[ \alpha = \frac{c_b}{c_f} \]

Extracellular bulk fluid

transporter

passive diffusion
The vicinity of the surface on hepatocytes

\[ P_{B,\text{influx}} \]

\[ P_m \]
Figure 4

[Graph showing the uptake of ANS (nmol/mg protein) over time (min) for different concentrations of ANS and BSA.]

- 50 μM ANS
- 20 μM ANS
- 5 μM ANS
- 5 μM ANS + 0.016% BSA
- 20 μM ANS + 0.094% BSA
- 100 μM ANS + 0.51% BSA
- 2 μM ANS
Figure 8

A

Ratio of unbound uptake clearance in the presence to that in the absence of albumin (R)

[Albumin] (μM)

B

[Albumin] (μM)

Fig 5 data
Fig 6 data

Downloaded from dmd.aspetjournals.org on January 6, 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

Seiji Miyauchi, Masayuki Masuda, Soo-Jin Kim, Yuudai Tanaka, Kyeong Ryoon Lee, Shouko Iwakado, Momoko Nemoto, Shotaro Sasaki, Kazumi Shimono, Yoshio Tanaka, and Yuichi Sugiyama


Sugiyama Laboratory, RIKEN Innovation Center, RIKEN Cluster for Industry Partnerships, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan (S.K., K.L., Y.S.)

Corresponding Author: Yuichi Sugiyama, PhD
Sugiyama Laboratory, RIKEN Innovation Center, RIKEN Cluster for Industry Partnerships, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan
Phone: +81-45-503-9211
FAX: +81-45-503-9190
E-mail: ychi.sugiyama@riken.jp

Journal: Drug metabolism and disposition

The material includes Supplementary Figure 1 and supplementary Table I.
Supplementary Figure 1 Curve-fitting of the relationship between the albumin concentration and the R values for ANS uptake (a) and PTV uptake (b) analyzed by the model developed by Forker et al.

The R values for ANS and PTV were estimated from the data shown in Figs 5 and 6, and 7, respectively. The values of \( \frac{n}{K_d} \) for ANS and PTV were estimated by the equilibrium dialysis method to be 0.501 and 0.248 \( \mu \text{M}^{-1} \), respectively. The relationships were fitted to Eq. 13 using a nonlinear least-squares method. The fitting curve for ANS and PTV adequately predicted the observed data. The lines in the figures show the fitted lines determined by analysis using the model developed by Forker et al (Forker and Luxon, 1983).
Supplementary Figure 1

A

B

Ratio of unbound uptake clearance in the presence to that in the absence of albumin (R)

[Albumin] (μM)

Fig 5 data

Fig 6 data
**Supplementary Table I.** Estimated parameters by the model developed by Forker *et al* (Forker and Luxon, 1983).

Estimated parameters by the model developed by Forker *et al.* a)

<table>
<thead>
<tr>
<th>Ligands</th>
<th>$B_{\text{max}}$</th>
<th>$K_{d,m}$</th>
<th>$R_{5%}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS</td>
<td>$3.71 \pm 1.22^{c)}$</td>
<td>$31.8 \pm 22.0^{c)}$</td>
<td>2.79</td>
</tr>
<tr>
<td>PTV</td>
<td>$58.7 \pm 21.9^{c)}$</td>
<td>$275 \pm 131^{c)}$</td>
<td>11.8</td>
</tr>
</tbody>
</table>

a) Data shown in Figs 5, 6 and 7.

b) The parameters estimated by the fitting curves in supplementary Fig. 1; $B_{\text{max}}$, the capacity for the interaction of albumin with the surface of hepatocytes; $K_{d,m}$, the dissociation constant of the bound albumin from the surface; $R_{5\%}$, $R$ value in the presence of 5% albumin;

c) $\pm$ calculated SD