Comparison of Methods for Estimating Unbound Intracellular-to-Medium Concentration Ratios in Rat and Human Hepatocytes Using Statins

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ABSTRACT

It is essential to estimate concentrations of unbound drugs inside the hepatocytes to predict hepatic clearance, efficacy, and toxicity of the drugs. The present study was undertaken to compare predictability of the unbound hepatocyte-to-medium concentration ratios ($K_{p,uu}$) by two methods based on the steady-state cell-to-medium total concentration ratios at 37°C and on ice ($K_{p,uu,ss}$ and $K_{p,uu,V0}$), respectively, because of organic anion-transporting polypeptide (OATP)–mediated uptake (Nezasa et al., 2003). Poorly metabolized statins were used as test drugs because of their concentration uptake via organic anion-transporting polypeptides. $K_{p,uu,ss}$ values of these statins provided less interexperimental variation than the $K_{p,uu,V0}$ values, because only data at longer time are required for $K_{p,uu,ss}$ compared with true $K_{p,uu}$ ($K_{p,uu,true}$), considering the inside-negative membrane potential and ionization of the drugs in hepatocytes and medium. Membrane potentials were approximately −30 mV in human hepatocytes at 37°C and almost abolished on ice. Theoretical equations considering the membrane potentials indicate that $K_{p,uu,ss}$ values for the statins are 0.85- to 1.2-fold $K_{p,uu,true}$, whereas $K_{p,uu,V0}$ values are 2.2- to 3.1-fold $K_{p,uu,true}$, depending on the ratio of the passive permeability of the ionized to nonionized forms. In conclusion, $K_{p,uu,ss}$ values of anions are similar to $K_{p,uu,true}$ when the inside-negative membrane potential is considered. This suggests that $K_{p,uu,ss}$ is preferable for estimating the concentration of unbound drugs inside the hepatocytes.

Introduction

According to the free drug hypothesis, only unbound drug is believed to interact with metabolic enzymes and pharmacological/toxicological target proteins. Knowing the intracellular unbound drug concentration is essential to estimate accurately the risk of drug–drug interactions involving drug-metabolizing enzymes and canalicular efflux transporters, and efficacy and toxicity of drugs when their targets are intracellular proteins. Extracellular unbound drug concentration is frequently assumed to be equivalent to the intracellular unbound concentration, especially when drugs are neutral or nonionized and freely permeable across the cytoplasmic membrane (Smith et al., 2010). However, this assumption cannot be applied to drugs whose tissue uptake is dominated by active transporters (Smith et al., 2005; Shugarts and Benet, 2009; Giacomini et al., 2010; Niemi et al., 2011; Shitara et al., 2013), in which their intracellular unbound concentration of drugs could be higher than the extracellular unbound concentration. For instance, unbound concentration of pravastatin and rosuvastatin in the rat liver are 11- to 16-fold (Yamazaki et al., 1993; Nezasa et al., 2003) and 15-fold higher than unbound plasma concentrations, respectively, because of organic anion-transporting polypeptide (OATP)–mediated uptake (Nezasa et al., 2003).

Methods to estimate the unbound hepatocyte-to-medium concentration ratio ($K_{p,u,u}$) are needed to predict the magnitude of drug–drug interactions involving drug-metabolizing enzymes, efflux transporters, and other intrahepatic target proteins in the liver. Brown et al. (2010) investigated the impact of transporters on the inhibition constant ($K_i$) values of cytochrome P450 inhibitors by comparing their inhibitory effects using rat liver microsomes and freshly isolated rat hepatocytes. Using the hepatocytes, $K_i$ values of clarithromycin and enoxacin with known hepatic transporter involvement were markedly smaller than those using the microsomes, which was consistent with their high cell-to-medium total concentration ratios (C/M ratios). The International Transporter Consortium published a review summarizing strategies to estimate intracellular drug concentrations (Chu et al., 2013). Among

ABBREVIATIONS: 1Ψ, membrane potential; AFE, average fold error; $C_{L_uptake}$, uptake clearance by hepatocytes; C/M ratio, cell-to-medium total concentration ratio; $f_{T,cell}$, unbound fraction in hepatocytes at steady state; $f_{T,cell,V0}$, unbound fraction in hepatocytes based on initial uptake rate; $f_{T,homogenate}$, unbound fraction in liver homogenates; HBSS, Hank’s balanced salt solution; $K_f$, inhibition constant; $K_{f,M}$, Michaelis-Menten constant; $K_{p,uu}$, unbound hepatocyte-to-medium concentration ratio; $K_{p,uu,ss}$, unbound hepatocyte-to-medium concentration ratio based on steady-state uptake; $K_{p,uu,V0}$, theoretically true unbound hepatocyte-to-medium concentration ratio; $K_{p,V0}$, unbound hepatocyte-to-medium concentration ratio based on initial uptake rate; MRP, multidrug resistance-associated protein; OATP, organic anion-transporting polypeptide; $PS_{dif}$, influx intrinsic clearance by passive diffusion through sinusoidal membrane; $TPP^+$, tetraphenylphosphonium; $V_{max}$, maximum transport rate.

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T.Y., K.T., and T.N. contributed to this research equally.

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them, a strategy to estimate $K_{p,uu}$ based on the initial uptake rate ($K_{p,us,V0}$) calculated using active transport clearance ($V_{max}/K_m$), and passive diffusion clearance at various concentrations (Yabe et al., 2011) was introduced. In addition, we have proposed an alternative strategy to estimate $K_{p,uu}$ under steady-state conditions ($K_{p,us,ss}$), which can be calculated by dividing the C/M ratio at 37°C by that at a low temperature (on ice) or in the presence of ATP depletors, when active transport is stopped (Yamazaki et al., 1992; Shitara et al., 2013). Yamazaki et al. (1993) demonstrated that the uptake of pravastatin by rat hepatocytes was more greatly reduced at a low temperature than by ATP depletion.

In the present study, steady-state uptake of typical OATP substrates, pitavastatin, rosuvastatin, and pravastatin in rat and human hepatocytes was investigated at 37°C and on ice to evaluate their $K_{p,uu,ss}$. In general, $K_{p,uu}$ can be described by the C/M ratio of drugs in hepatocytes as follows:

$$\frac{C}{M} \text{ ratio} = \frac{C_{\text{cell}}}{C_{\text{medium}}} = \frac{C_{\text{cell,unbound}}/f_T}{C_{\text{medium,unbound}}/f_T} = K_{p,uu} \cdot \frac{f_B}{f_T}$$ (1)

where $f_B$ is the unbound fraction of the drugs in the blood (in vivo) or in the incubation medium (in vitro), and $f_T$ is the unbound fraction of the drugs in the hepatocytes. The unbound fraction in hepatocytes at the steady state ($f_{T,cell,ss}$) obtained using our method should be validated because intracellular binding to cytosolic proteins/cellular organelles might be altered at low temperatures. Therefore, $f_{T,cell,ss}$ were compared with the unbound fraction in liver homogenates ($f_{T,\text{homogenate}}$) measured by equilibrium dialysis using human liver samples. The $K_{p,uu,ss}$ obtained were then compared with $K_{p,uu,ss}$ obtained in both rat and human hepatocytes. The difference between $K_{p,uu,ss}$ or $K_{p,uu,Vo}$ and true $K_{p,uu}$ ($K_{p,uu,\text{true}}$) is discussed in the context of theoretical equations, considering the membrane potential ($\Delta \Psi$) in hepatocytes and the fraction of ionized drugs at the designated pH. Finally, a method to predict $K_{p,uu,\text{true}}$ from $K_{p,uu,ss}$ and $K_{p,uu,Vo}$ obtained experimentally is proposed.

**Materials and Methods**

**Chemicals.** [3H]Pitavastatin, [3H]rosuvastatin calcium, and [3H]pravastatin calcium were obtained from American Radiolabeled Chemicals (St. Louis, MO). [3H]Diazepam was obtained from PerkinElmer Life Sciences (Boston, MA). Unlabeled diazepam, pitavastatin calcium, rosuvastatin calcium, and pravastatin sodium were obtained from Wako Pure Chemicals (Osaka, Japan). All other reagents and solvents were purchased from Invitrogen (Carlsbad, CA), Sigma-Aldrich (St. Louis, MO), and Wako Pure Chemicals.

**Animals.** Male Sprague–Dawley rats were purchased from Charles River Japan (Shiga, Japan) and acclimatized for 7 days before the experiments. The rats were housed under conditions of controlled temperature and humidity with a 12-hour light/dark cycle with free access to standard laboratory rodent food (CE-2; CLEA Japan, Tokyo, Japan) and water. All animal experiments were approved by the Experimental Animal Care and Use Committee of the Mitsubishi Tanabe Pharma (Saitama, Japan) and conducted in accordance with the Declaration of Helsinki and the guidelines of the ethics committee.

**Isolation of Rat Hepatocytes.** Hepatocytes were isolated from male Sprague–Dawley rats (7–9 weeks old) using a procedure described previously (Baur et al., 1975). Isolated hepatocytes were suspended in albumin-free Krebs–Henseleit buffer with 12.5 mM HEPES (pH 7.4), and cell viabilities were determined using a trypan blue exclusion test. Hepatocytes obtained from three independent preparations with >80% viability were used for the uptake studies described below.

**Preparation of Human Hepatocytes.** Human biologic samples were obtained ethically, and their research use was in accordance with the terms of informed consent. Cryopreserved human hepatocytes from a donor (Lot Hu8075) were purchased from Life Technologies (Carlsbad, CA). Pooled cryopreserved human hepatocytes from 20 mixed-sex donors (Lot TFF) were purchased from BioreclamationIVT (Baltimore, MD). Pooled cryopreserved human hepatocytes from 50 mixed-sex donors (Lot HUE50C) were purchased from Thermo Fisher Scientific (Waltham, MA). These hepatocytes were suspended in albumin-free Krebs–Henseleit buffer with 12.5 mM HEPES (pH 7.4), and viabilities were determined using a trypan blue exclusion test. Hepatocytes obtained from three independent preparations with >80% viability were used for the uptake studies described below.

**Determination of the Intracellular Volume of Hepatocytes.** The intracellular volume of rat hepatocytes (3.68 ± 1.37 μL/106 cells) was estimated using published methods (Supplemental Table 1A) (Baur et al., 1975; Kletzien et al., 1975; Eaton and Klaassen, 1978; Kristensen and Folk, 1984; Yamazaki et al., 1992; Miyachichi et al., 1993; Reinoso et al., 2001; Halifax and Houston, 2006). In brief, to determine the intracellular volume of human hepatocytes, cryopreserved human hepatocytes (Lot Hu8075) were suspended in Krebs–Henseleit buffer (pH 7.4) at 6.0 ± 10 μL/viable cells/mL and preincubated at 37°C for 5 minutes. A reaction was initiated by adding an equal volume of buffer containing [3H]water and [14C]dextran at final concentrations of 2.5 μCi/mL and 0.5 μCi/mL, respectively. After incubation at 37°C for 10 minutes, during which the distribution of [3H]water and [14C]dextran reached a steady state, aliquots were removed and added to a narrow tube containing silicon–mineral oil (density: 1.015; Sigma-Aldrich) over aqueous 2 M sodium hydroxide, followed by centrifugation through the silicon–mineral oil layer to separate the cells from the medium. After the basic bottom layer was neutralized with 2 M hydrochloric acid, radioactivities in both cells and medium were determined using a Tri-Carb liquid scintillation counter (PerkinElmer, Shelton, CT). Thereby, the intracellular volume of human hepatocytes was estimated to be 2.28 ± 0.33 μL/106 cells (Supplemental Table 1B).

**Determination of $K_{p,uu,ss}$ and $f_{T,cell,ss}$ in Rat and Human Hepatocytes Based on Steady-State Uptake.** To determine the incubation time for steady-state uptake into hepatocytes, transport studies were performed by an oil–spin method (Iga et al., 1979) using suspended hepatocytes. Hepatocytes were suspended in Krebs–Henseleit buffer (pH 7.4) at 2.0 ± 10 μL/viable cells/mL and preincubated at 37°C for 5 minutes. A reaction was initiated by adding an equal volume of buffer containing each drug (pitavastatin, rosuvastatin, pravastatin, or diazepam previously used as a neutral drug with high membrane permeability for evaluating the uptake into isolated rat hepatocytes) (Ichikawa et al., 1992) at 1 μM. After incubation at 37°C for 0.5, 2, 5, 15, and 30 minutes (rat hepatocytes), 0.5, 1.5, 30, and 60 minutes (human hepatocytes, Lot Hu8075), 0.5, 1.5, 30, and 60 minutes (human hepatocytes, Lot TFF), aliquots were removed and added to a narrow tube containing silicon–mineral oil over aqueous 2 M sodium hydroxide and centrifuged through the silicon–mineral oil layer to separate the cells from the medium. To provide low temperature values, the uptake studies were performed on ice. After the basic bottom layer was neutralized with 2 M hydrochloric acid, radioactivity in both cells and medium was measured using the liquid scintillation counter.

The unbound hepatocyte-to-medium concentration ratio ($K_{p,uu}$) based on the steady-state uptake ratio at 37°C and on ice ($K_{p,uu,ss}$) and the unbound fraction in hepatocytes based on the steady-state uptake ($f_{T,cell,ss}$) were defined as described in eqs. 2 and 3, respectively, based on the C/M ratio at 37°C and on ice. A part of the method to obtain $f_{T,cell,ss}$ was reported previously (Yoshikado et al., 2016).

$$K_{p,uu,ss} = \frac{C_{\text{medium,37°C}}}{C_{\text{cell,37°C}}}$$

$$f_{T,cell,ss} = \frac{C_{\text{cell,unbound,37°C}}}{C_{\text{medium,37°C}}}$$

$$f_{T,cell,ss} = \frac{C_{\text{cell,unbound,37°C}}}{C_{\text{medium,37°C}}} = \frac{1}{\frac{C}{M} \text{ ratio}_{37°C}}$$ (3)

The following assumptions were made in calculating $K_{p,uu,ss}$ and $f_{T,cell,ss}$ using eqs. 2 and 3: the active uptake in hepatocytes is abolished on ice (i.e., $C_{\text{cell,unbound,37°C}}$ is equal to $C_{\text{cell,37°C}}$), and $f_{T,cell,ss}$ is independent of temperature (i.e., $f_{T,cell,ss}$ is equal to $f_{T,cell,37°C}$).

**Determination of $K_{p,uu,Vo}$, $f_{T,cell,Vo}$ and Other Kinetic Parameters in Rat and Human Hepatocytes Based on Initial Uptake Rate.** To evaluate the initial uptake rate in pooled human hepatocytes, hepatocytes were preincubated for 5 minutes and then incubated for 0.5–1.5 or 0.5–2 minutes, as shown in the transport studies described above.
The uptake clearance by hepatocytes (CL\textsubscript{uptake}) was determined by the slope of the plot of C/M ratio versus time, and the initial uptake rate (v) was calculated by multiplying CL\textsubscript{uptake} with the initial substrate concentration. According to a method reported previously (Yabe et al., 2011), v can be calculated using eq. 4:

\[ v = \frac{V\text{\textsubscript{max}} \cdot S}{K\text{\textsubscript{m}} + S} + PS\text{\textsubscript{dif}} \cdot S, \]  

where \( V\text{\textsubscript{max}} \) is the maximum uptake rate, \( K\text{\textsubscript{m}} \) is the Michaelis constant, \( PS\text{\textsubscript{dif}} \) is the passive diffusion clearance, and \( S \) is the substrate concentration in the medium. These kinetic parameters were optimized by fitting the equation to observed data using Phoenix WinNonlin version 6.3 (Pharsight Certara, St. Louis, MO). Because CL\textsubscript{uptake} consists of active uptake clearance (PS\textsubscript{act}) and passive diffusion clearance (PS\textsubscript{dif}), assuming that PS\textsubscript{dif} for the cellular uptake is equal to that for the efflux, \( K\text{p,uu} \) and \( fT\text{cell} \) based on initial uptake rate (\( K\text{p,uu} \text{V0} - fT\text{cell}\text{V0}) \) can be calculated using eqs. 5 and 6 (Yabe et al., 2011):

\[ K\text{p,uu} \text{V0} = \frac{PS\text{act} + PS\text{dif}}{PS\text{act} \cdot PS\text{dif}} \]  

\[ fT\text{cell}\text{V0} = \frac{K\text{p,uu} \text{V0}}{C/M \text{ratio c,}37 \text{C}/C176 C} \]  

where the C/M ratio\textsubscript{37°C}/C176 in rat hepatocytes was obtained at 30 minutes, and that in human hepatocytes was obtained at 60 minutes.

**Determination of the Unbound Fraction in Human Liver Homogenates (fT\textsubscript{homogenate}) Using Equilibrium Dialysis.** Human liver samples were obtained from the Human and Animal Bridging Research Organization (Tokyo, Japan) with approval of the Ethics Committees of University of Tokyo and Human and Animal Bridging Research Organization. Three lots of liver samples were pooled and homogenized in 66.7 mM isotonic phosphate buffer at 1:3 (w/v) producing 25% homogenates. By diluting these homogenates, 12.5% and 6.25% homogenates were also prepared. Diazepam, pitavastatin, pravastatin, and rosuvastatin (final concentrations: 0.2 μM) were added to the compartment containing homogenates in a Rapid Equilibrium Dialysis plate (Thermo Fisher Scientific) and incubated for 12 hours at 37°C, or on ice.

**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>( fT\text{act} )</th>
<th>( fT\text{act} )</th>
<th>( fT\text{act} )</th>
<th>( fT\text{act} )</th>
<th>( pK\text{a} )</th>
<th>( PS\text{act,inf,\text{Caco-2}} \times 10^{-6} )</th>
<th>( \lambda' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitavastatin</td>
<td>0.857</td>
<td>0.143</td>
<td>0.905</td>
<td>0.095</td>
<td>4.46</td>
<td>3487 ± 1403</td>
<td>0.0282 ± 0.0204</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.986</td>
<td>0.014</td>
<td>0.991</td>
<td>0.009</td>
<td>4.6</td>
<td>714 ± 68</td>
<td>0.0112 ± 0.0035</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.986</td>
<td>0.014</td>
<td>0.991</td>
<td>0.009</td>
<td>4.6</td>
<td>111 ± 9</td>
<td>0.188 ± 0.038</td>
</tr>
</tbody>
</table>

*The \( fT\text{act} \) and \( fT\text{act} \) were calculated based on Henderson-Hasselbalch equation assuming that intracellular pH and medium pH are 7.2 and 7.4, respectively. The \( fT\text{act} \) and \( fT\text{act} \) were subtracted \( fT\text{act} \) and \( fT\text{act} \) from one, respectively.

*Fixed at values obtained from the manufacturer’s Interview Forms.

*The PS\text{act,inf,\text{Caco-2}} and \( \lambda' \) were determined by fitting eq. 9 to the pH-dependent permeation data of statins observed in Caco-2 cells (Supplemental Fig. 1).

**Fig. 1.** Uptake of diazepam (1 μM; A), pitavastatin (1 μM; B), rosuvastatin (1 μM; C), and pravastatin (1 μM; D) by rat hepatocytes measured after incubation at 37°C for 0.5–30 minutes. The data are presented as mean ± S.D. (n = 3).
The presence of each radiolabeled statin (10, 5.5, 6.0, 6.5, and 7.4 for the apical side, and 7.4 for the basal side). Subsequently, determined using a liquid scintillation counter.

examine the integrity of the monolayer. The radioactivity on the basal side was (both sides). Lucifer yellow (300 μL) was used as a paracellular marker to determination of the nonionized drug, as follows:

\[
PS_{\text{dif,inf}} = \frac{1 - f_{\text{ion}}}{f_{\text{ion}}} \cdot PS_{\text{dif,inf,ion}} + f_{\text{ion}} \cdot PS_{\text{dif,inf,uion}}
\]

(7)

where the subscripts ion and uion represent the ionized and unionized (nonionized) form of a drug, respectively, and \( f_{\text{ion}} \) and \( f_{\text{uion}} \) are fractions of ionized and nonionized drug outside the cells, respectively. Subsequently, \( \lambda \) was defined as the ratio of passive diffusion influx clearance of the ionized drug to that of the nonionized drug, as follows:

\[
\lambda = \frac{PS_{\text{dif,inf,uion}}}{PS_{\text{dif,inf,ion}}}
\]

(8)

It is assumed that the \( \lambda \) value is not changed by temperature: the effect of low temperature on \( PS_{\text{dif,inf,ion}} \) is assumed to be the same as on \( PS_{\text{dif,inf,uion}} \), although these passive diffusion clearances should be affected by the change in membrane fluidity at low temperature (Kanduser et al., 2008). A part of the method to obtain \( \lambda \) was reported previously (Yoshikado et al., 2016). Using \( \lambda \), eq. 7 can be converted as follows:

\[
PS_{\text{dif,inf}} = \left(1 - f_{\text{ion}}\right) \cdot PS_{\text{dif,inf,ion}} + f_{\text{ion}} \cdot PS_{\text{dif,inf,uion}}
\]

(9)

\[
\Delta V = \left(1 - f_{\text{ion}}\right) \cdot \frac{C_{\text{phys}}}{C_{\text{abol}}} + f_{\text{ion}} \cdot \frac{C_{\text{phys}}}{C_{\text{abol}}}
\]

(10)

where \( R, T \), and \( F \) are the gas constant, absolute temperature, and Faraday constant, respectively. \( C_{\text{phys}} \) and \( C_{\text{abol}} \) are the \( C/M \) under physiologic conditions and when the \( \Delta V \) is abolished, respectively. The selective abolition of \( \Delta V \) can be achieved by rendering the cytoplasmic membrane permeable with 20 μM amphotericin B (Saito et al., 1992). To measure the \( C/M \) ratio of \( TPP^+ \), transport studies were performed using an oil–spin method (Iga et al., 1979) with cryopreserved human hepatocytes (Lot HUE50C). Hepatocytes were suspended in Krebs–Henseleit buffer (pH 7.4) at 2.0 × 10⁻³ v/v, and preincubated at 37° C for 5 minutes. The reaction was initiated by adding an equal volume of buffer containing \( TPP^+ \) (final: 3 μM) and amphotericin B (final: 20 μM). After incubation at 37° C for 0.5, 15, 30, and 60 minutes, aliquots were removed and added to a narrow tube containing silicone–mineral oil over a 2.5 M ammonium acetate, and then centrifuged to separate the cells from the medium. To examine the effect of low temperature on \( \Delta V \), uptake studies were also performed on ice. The concentrations of \( TPP^+ \) in the cells and the medium were determined using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system equipped with a Nexera UHPLC and a LCMS-8050 triple-quadrupole liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan). A Kinetex C18 column (2.6 μm, 3 mm × 100 mm; Phenomenex, Torrance, CA) was used, and UHPLC was conducted under gradient mobile-phase conditions with a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile as solvents (5.0 mL/min; 0.5–3.5 minutes 95:5 v/v, 3.5–5.5 minutes 15:85 v/v, 5:95 v/v, 5:95 v/v, 6:5 minutes 95:5 v/v, 6:5 minutes 95:5 v/v, and 6:1–6.5 minutes 95:5 v/v). The UHPLC eluent was introduced into the MS in positive electrospray ionization (ESI) mode. \( TPP^+ \) was quantified in multiple reaction monitoring mode (339.10 > 183.10, Q1 prebias: −300 V, CE: −54.0, Q3 prebias: −170 V), in which chlorpropamide was used as an internal standard (277.20 > 111.05; Q1 prebias: −18.0 V, CE: −32.0, Q3 prebias: −20.0 V).

**Evaluation of the Correlation Between \( K_p_{\text{phys}} \) Values Examined by the Two Different Methods.** To quantitatively evaluate the correlation between \( K_p_{\text{phys}} \) and \( K_p_{\text{mol,Vo}} \), commonly employed accuracy test criteria, the average fold errors (AFE), were calculated, as shown in eq. 11.

---

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>C/M Ratio</th>
<th>( K_{p_{\text{phys}}} )</th>
<th>( f_{\text{cell}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>On ice</td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>58.9 ± 7.3</td>
<td>69.3 ± 9.7</td>
<td>0.851 ± 0.024</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>324 ± 174</td>
<td>29.6 ± 11.5</td>
<td>0.810 ± 0.44</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>7.4 ± 5.5</td>
<td>11.5 ± 8.0</td>
<td>0.383 ± 0.167</td>
</tr>
</tbody>
</table>

*Values are shown as the mean ± S.D. (n = 3).*

\( PS_{\text{dif,inf}} = \left(1 - f_{\text{ion}}\right) \cdot \frac{C_{\text{phys}}}{C_{\text{abol}}} + f_{\text{ion}} \cdot \frac{C_{\text{phys}}}{C_{\text{abol}}}
\)

(10)

The \( f_{\text{ion}} \) and \( f_{\text{cell}} \) for each statin were estimated using the pH of the medium (7.4) and pKa of the drug obtained from the manufacturer’s Information Forms, based on the Henderson–Hasselbalch equation (Table 1). Then, \( \lambda \) and \( PS_{\text{dif,inf,uion}} \) in Caco-2 cells were optimized by fitting eq. 9 to the pH-dependent membrane permeation of statins (Supplemental Fig. 1). Phoenix WinNonlin software version 6.3 (Pharsight Certara) was used to optimize the parameters. The obtained \( \lambda \) values for pitavastatin, rosuvastatin, and pravastatin are shown in Table 1.

### Table 3

Kinetic parameters for the initial uptake rates of pitavastatin, rosuvastatin, and pravastatin in rat hepatocytes

<table>
<thead>
<tr>
<th>Drug</th>
<th>( V_{\max} )</th>
<th>( K_s )</th>
<th>( V_{\max}/K_s )</th>
<th>( PS_{u/N} )</th>
<th>( K_{p_{\text{phys}}} )</th>
<th>( f_{\text{cell}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/10⁶ Cells</td>
<td>μM</td>
<td>μL/min/10⁶ Cells</td>
<td>μL/min/10⁶ Cells</td>
<td>μM</td>
<td></td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>893 ± 124</td>
<td>5.43 ± 1.29</td>
<td>164 ± 4</td>
<td>6.0 ± 1.18</td>
<td>55.8 ± 29.9</td>
<td>0.172 ± 0.131</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>250 ± 50</td>
<td>2.86 ± 1.12</td>
<td>87.4 ± 62.3</td>
<td>1.76 ± 1.15</td>
<td>50.7 ± 48.0</td>
<td>0.649 ± 0.688</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>178 ± 11</td>
<td>20.3 ± 1.4</td>
<td>8.77 ± 0.81</td>
<td>1.16 ± 0.08</td>
<td>8.56 ± 0.87</td>
<td>0.685 ± 0.428</td>
</tr>
</tbody>
</table>

*The initial uptake rate in rat hepatocytes was calculated from the uptake of [¹³C]pitavastatin, [³H]rosuvastatin, and [¹⁴C]pravastatin for 0.5–1.5 minutes. Kinetic parameters are obtained by fitting to the data at seven concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 μM for all statins; Supplemental Fig. 2) using eq. 4.*

*The \( K_{p_{\text{phys}}} \) and \( f_{\text{cell}} \) were calculated using eqs. 5 and 6, respectively. C/M ratios at 37°C (Table 2) were used for the calculation of the \( f_{\text{cell}} \).
\[
AFE = 10^{\frac{\sum \log K_p}{n}}
\]  

(11)

**Results**

**Determination of \( K_{p,uu,ss} \) and \( f_{cell,ss} \) in Rat Hepatocytes.** The time-dependent uptake of diazepam (a lipophilic neutral compound) and pitavastatin, rosuvastatin, and pravastatin (substrates of hepatic OATPs) in rat hepatocytes was monitored. The uptake of diazepam in rat hepatocytes reached a steady state instantaneously because of its high permeability, whereas the uptake of pitavastatin, rosuvastatin, and pravastatin gradually increased over time and reached the steady state within 30 minutes (Fig. 1). On ice, C/M ratios of pitavastatin, rosuvastatin, and pravastatin were significantly smaller than the ratios at 37°C, whereas that for diazepam was not different from that at 37°C (Table 2). The \( K_{p,uu,ss} \) for diazepam was 0.85, and the values for

---

**Fig. 2.** Uptake of diazepam (1 μM; A and E), pitavastatin (1 μM; B and F), rosuvastatin (1 μM; C and G), and pravastatin (1 μM; D and H) by human hepatocytes measured after incubation at 37°C for 0.5–60 minutes. (A–D) Cryopreserved human hepatocytes from a single donor (Lot Hu8075) were used. (E–H) Pooled cryopreserved human hepatocytes from 20 mixed-sex donors (Lot TFF) were used. The data are presented as mean ± S.D. (n = 3).
pitavastatin, rosuvastatin, and pravastatin were approximately 11, 13, and 6.7, respectively (Table 2), confirming that these statins were concentrated in the hepatocytes by an active transport system. The calculated $f_{T,cell,V0}$ for pitavastatin, rosuvastatin, and pravastatin were approximately 0.038, 0.15, and 0.60, respectively.

**Determination of $K_{p,uu,ss}$ and $f_{T,cell,ss}$ in Human Hepatocytes.** We investigated the time-dependent uptake of dzipam, pitavastatin, rosuvastatin, and pravastatin by human hepatocytes prepared from single donor (Lot Hu8075) (Fig. 2, A—D) and pooled human hepatocytes from 20 mixed-sex donors (Lot TFF) (Fig. 2, E—H). The uptake of all these statins increased over time and reached a steady state within 60 minutes, whereas that of dzipam reached a peak instantaneously. On ice, the C/M ratios for pitavastatin, rosuvastatin, and pravastatin were reduced, whereas that for dzipam was not dependent on temperature (Table 4).

> **Table 4**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lot</th>
<th>C/M Ratio$^d$</th>
<th>$K_{p,uu}^{ss}$</th>
<th>$f_{T,cell}^{ss}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>Hu8075</td>
<td>216 ± 20</td>
<td>1.19 ± 0.51</td>
<td>0.00553 ± 0.00115</td>
</tr>
<tr>
<td>TFF$^c$</td>
<td>124 ± 53</td>
<td>303 ± 22</td>
<td>0.409 ± 0.247</td>
<td>0.00330 ± 0.00024</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>Hu8075</td>
<td>471 ± 88</td>
<td>13.4 ± 2.7</td>
<td>0.0284 ± 0.00021</td>
</tr>
<tr>
<td>TFF$^c$</td>
<td>150 ± 2.6</td>
<td>21.8 ± 3.2</td>
<td>6.92 ± 1.02</td>
<td>0.0460 ± 0.0068</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Hu8075</td>
<td>52.2 ± 8.6</td>
<td>11.6 ± 2.2</td>
<td>0.222 ± 0.020</td>
</tr>
<tr>
<td>TFF$^c$</td>
<td>27.2 ± 2.5</td>
<td>4.28 ± 0.53</td>
<td>6.36 ± 1.06</td>
<td>0.234 ± 0.029</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>Hu8075</td>
<td>3.73 ± 0.73</td>
<td>1.84 ± 0.12</td>
<td>2.03 ± 0.54</td>
</tr>
<tr>
<td>TFF$^c$</td>
<td>2.65 ± 1.29</td>
<td>2.07 ± 0.64</td>
<td>1.28 ± 0.89</td>
<td>0.484 ± 0.151</td>
</tr>
</tbody>
</table>

$^a$Isolated cryopreserved human hepatocytes (Lot Hu8075) were incubated with diazepam (0.2 μM), pitavastatin (0.1 μM), rosuvastatin (0.5 μM), and pravastatin (1 μM).

$^b$Pooled cryopreserved human hepatocytes from 0.5, 1, 3, 10, 30, and 100 μM for pitavastatin; 0.5, 1, 3, 10, 30, and 100 μM for rosuvastatin; and 1 μM for pravastatin.

$^d$C/M ratios at 37°C and on ice were calculated using the uptake data at 30 minutes and 60 minutes, respectively (Fig. 2).

$^e$The $K_{p,uu,ss}$ and $f_{T,cell,ss}$ were calculated from C/M ratios using eqs. 2 and 3, respectively.

**Determination of $K_{p,uu,V0}$ and $f_{T,cell,V0}$ in Rat Hepatocytes.** Based on a method reported previously (Yabe et al., 2011), the kinetic parameters ($V_{\text{max}}$, $K_m$, and $P_{\text{Sat}}$) for pitavastatin, rosuvastatin, and pravastatin were determined by fitting eq. 4 to their initial uptake rate by cells from Lot TFF (Supplemental Fig. 2). The obtained $K_{p,uu,V0}$ values for pitavastatin, rosuvastatin, and pravastatin were approximately 56, 51, and 8.6, respectively (Table 3). The calculated $f_{T,cell,V0}$ for pitavastatin, rosuvastatin, and pravastatin were approximately 0.17, 0.65, and 0.69, respectively.

**Determination of $K_{p,uu,V0}$ and $f_{T,cell,V0}$ in Human Hepatocytes.** Based on a method reported previously (Yabe et al., 2011), the kinetic parameters ($V_{\text{max}}$, $K_m$, and $P_{\text{Sat}}$) for pitavastatin and rosuvastatin were determined by fitting eq. 4 to their initial uptake rate by cells from Lot Hu8075 and Lot TFF, respectively (Table 4). The obtained $f_{T,cell,V0}$ values were approximately 0.028 and 0.046 for pitavastatin, 0.22 and 0.23 for rosuvastatin, and 0.55 and 0.48 for pravastatin in cells from Lot Hu8075 and Lot TFF, respectively.

**Table 5**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lot</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>$V_{\text{max}}/K_m$</th>
<th>$P_{\text{Sat}}$</th>
<th>$K_{p,uu,V0}$</th>
<th>$f_{T,cell,V0}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitavastatin</td>
<td>Hu8075</td>
<td>403 ± 84</td>
<td>4.77 ± 1.80</td>
<td>84.5 ± 36.4</td>
<td>0.388 ± 0.912</td>
<td>219 ± 521</td>
<td>0.465 ± 1.110</td>
</tr>
<tr>
<td></td>
<td>TFF$^c$</td>
<td>148 ± 66.1</td>
<td>1.78 ± 1.04</td>
<td>83.1 ± 61.1</td>
<td>4.36 ± 0.70</td>
<td>20.1 ± 14.4</td>
<td>0.134 ± 0.096</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Hu8075</td>
<td>198 ± 50</td>
<td>21.5 ± 7.8</td>
<td>92.1 ± 4.07</td>
<td>0.0462 ± 0.1547</td>
<td>200 ± 673</td>
<td>3.83 ± 12.91</td>
</tr>
<tr>
<td></td>
<td>TFF$^c$</td>
<td>23.0 ± 8.6</td>
<td>4.49 ± 1.72</td>
<td>5.12 ± 2.75</td>
<td>2.07 ± 0.279</td>
<td>3.47 ± 1.41</td>
<td>0.128 ± 0.053</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>Hu8075</td>
<td>93.8 ± 56.0</td>
<td>127 ± 81</td>
<td>0.739 ± 0.645</td>
<td>0.0137 ± 0.0922</td>
<td>54.8 ± 363.7</td>
<td>14.7 ± 97.5</td>
</tr>
<tr>
<td></td>
<td>TFF$^c$</td>
<td>Not determined$^e$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Initial uptake rate in isolated cryopreserved human hepatocytes (Lot Hu8075) was calculated from the uptake of [3H]pitavastatin, [3H]rosuvastatin, and [3H]pravastatin for 0.5—2.0 minutes. Kinetic parameters are obtained by fitting to the data at several concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 μM for pitavastatin; 0.1, 0.3, 1, 3, 10, 30, and 300 μM for rosuvastatin; 1, 3, 100, and 300 μM for pravastatin; Supplemental Fig. 3, A—C) using eq. 4.

$^c$Initial uptake rate in pooled cryopreserved human hepatocytes for 0.5, 1, 3, 10, 30, and 300 μM for pitavastatin; 0.5, 1, 3, 10, 30, and 300 μM for rosuvastatin; and 1, 3, 10, and 300 μM for pravastatin; Supplemental Fig. 3, D and E) using eq. 4.

$^d$The $K_{p,uu,V0}$ and $f_{T,cell,V0}$ were calculated using eqs. 5 and 6, respectively. C/M ratios at 37°C (Table 4) were used for the calculation of the $f_{T,cell,V0}$.
values of $K_{p,uu,V0}$ and $f_{T,cell,V0}$ exhibited greater differences between lots.

**Comparison of $K_{p,uu}$ and $f_{T,cell}$ Obtained by Different Methods.**

In rat hepatocytes, $K_{p,uu,V0}$ values for pitavastatin, rosuvastatin, and pravastatin were, respectively, 5.1, 5.1, and 1.2 times those for $K_{p,uu,ss}$ (Fig. 3A). By contrast, in human hepatocytes (Lot Hu8075), $K_{p,uu,V0}$ values for pitavastatin, rosuvastatin, and pravastatin were, respectively, 16, 17, and 43 times those for $K_{p,uu,ss}$ (Fig. 3B). The differences between $K_{p,uu,V0}$ and $K_{p,uu,ss}$ in human hepatocytes from Lot TFF were smaller than those in Lot Hu8075: $K_{p,uu,V0}$ values for pitavastatin and rosuvastatin were, respectively, 2.9 and 0.55 times those for $K_{p,uu,ss}$ in cells from Lot TFF (Fig. 3B).

The $f_{T,cell,V0}$ values tended to be higher than $f_{T,cell,ss}$ in both rat (Fig. 3C) and human hepatocytes (Fig. 3D), except for rosuvastatin in human hepatocytes (Lot TFF). Both $K_{p,uu,V0}$ and $f_{T,cell,V0}$ values thus exhibited quite larger interlot and interexperimental variabilities compared with $K_{p,uu,ss}$ and $f_{T,cell,ss}$ values (comparison between Tables 2 and 3 for rats and Tables 4 and 5 for humans).

**Comparison of $f_{T,cell}$ with the Measured Unbound Fraction in Human Liver Homogenates.** For diazepam, pitavastatin, rosuvastatin, and pravastatin, the measured unbound fractions in human liver homogenates ($f_{T,homogenate}$) were measured using equilibrium dialysis (Table 6). The $f_{T,homogenate}$ values for these drugs obtained at 37°C ($f_{T,homogenate,37°C}$) were close to those obtained on ice ($f_{T,homogenate,ice}$). Moreover, the $f_{T,homogenate,ice}$ values for pitavastatin and rosuvastatin were comparable to those for $f_{T,cell,ss}$ (Table 4); the difference between $f_{T,homogenate,ice}$ and $f_{T,cell,ss}$ for pravastatin was within threefold, whereas there was a larger discrepancy between $f_{T,homogenate,ice}$ and $f_{T,cell,ss}$ for diazepam.

**Measurement of the $\Delta\Psi$ Using TPP**. To evaluate whether $\Delta\Psi$ might affect the ratio of $PS_{diff}/PS_{diff,eff}$ and, accordingly, $K_{p,uu}$, the time-dependent uptake of TPP was examined in human hepatocytes under physiologic conditions and with amphotericin B, which is reported to abolish $\Delta\Psi$ specifically by 10-minute incubation with isolated rat hepatocytes (Saito et al., 1992). The C/M ratio of TPP gradually increased over time and reached the steady state between 30 and 60 minutes (Fig. 4). On ice, the C/M ratio of TPP was significantly smaller than that at 37°C. In addition, in the presence of amphotericin B, the C/M ratio of TPP was decreased significantly at 37°C compared with the condition without amphotericin B, whereas the C/M ratio on ice was not significantly changed with amphotericin B (Fig. 4). The C/M ratio (37°C) in the presence of amphotericin B at 60 minutes was lower than that at 30 minutes, suggesting some additional effects of amphotericin B (i.e., cytotoxicity) other than the loss of the $\Delta\Psi$. Therefore, based on the obtained data at 30 minutes and

<table>
<thead>
<tr>
<th>Drug</th>
<th>$f_{T,homogenate}$</th>
<th>$f_{T,cell,ss}$</th>
<th>$f_{T,cell,V0}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>On Ice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.0259 ± 0.0018</td>
<td>0.0242 ± 0.0016</td>
<td>1.07 ± 0.10</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.0301 ± 0.0017</td>
<td>0.0344 ± 0.0018</td>
<td>0.875 ± 0.067</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.237 ± 0.037</td>
<td>0.206 ± 0.025</td>
<td>1.15 ± 0.23</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.183 ± 0.022</td>
<td>0.167 ± 0.020</td>
<td>1.10 ± 0.19</td>
</tr>
</tbody>
</table>
eq. 10, $\Delta \Psi$ values were calculated to be approximately −30 mV at 37°C and 5 mV on ice (Table 7).

Using $\Psi = \exp(\alpha F \Delta \Psi/R/T)$ calculated from $\Delta \Psi$ (Supplemental Equation 5) and physicochemical parameters calculated for statins (Table 1), theoretical values of $K_{p,uu,true}$, $K_{p,uu,V0}$, and $K_{p,uu,ss}$ were calculated by Supplemental Equations 6, 8, and 11, respectively (Table 8). $R_{ss/true}$ was calculated by eq. 14 and $R_{V0/true}$ was calculated by eq. 15:

$$K_{p,uu,V0,corrected} = \frac{K_{p,uu,V0}}{R_{V0/true}}$$

$$K_{p,uu,ss,corrected} = \frac{K_{p,uu,ss}}{R_{ss/true}}$$

where $f_{i,ion}$ and $f_{o,ion}$ are fractions of the ionized and nonionized forms of the drug in hepatocytes, respectively, with the use of intracellular pH (7.2) and pKa. $R_{V0/true}$ was calculated to be 2.2−3.1 for statins, whereas $R_{ss/true}$ was 0.85−1.2 (Table 8). Figure 5 also shows that simulated $R_{ss/true}$ was closer to 1 than $R_{V0/true}$ when $\alpha$ values for pitavastatin, rosuvastatin, and pravastatin were approximately 0.028, 0.011, and 0.19, respectively (Table 1). Furthermore, experimentally obtained $K_{p,uu,V0}$ and $K_{p,uu,ss}$ for statins were corrected using the calculated $R_{V0/true}$ and $R_{ss/true}$ values (Table 8) and eqs. 14 and 15:

**Discussion**

We previously proposed a method to estimate $K_{p,uu,ss}$ for anions in hepatocytes based on their steady-state C/M ratio at 37°C and the C/M ratio after suppressing active transport (Yamazaki et al., 1992; Shitara et al., 2013). Another method reported by Yabe et al. (2011) is to estimate $K_{p,uu,V0}$ based on initial uptake rates at various concentrations. $K_{p,uu,ss}$ values for pitavastatin, rosuvastatin, and pravastatin in rat and human hepatocytes, which were obtained with less interexperiment variation compared with $K_{p,uu,V0}$ values in this study, tended to be smaller than the corresponding $K_{p,uu,V0}$ values (Fig. 3, A and B). Considering the measured inside-negative $\Delta \Psi$ (Table 7) and subsequent asymmetric diffusional clearance for influx and efflux, $K_{p,uu,V0,corrected}$ in rat and human hepatocytes approached $K_{p,uu,ss,corrected}$ (Fig. 6).

There exists a practical advantage of the method to obtain the $K_{p,uu,ss}$ value over the method to obtain the $K_{p,uu,V0}$ value. Interlot and interexperimental variabilities are much less for $K_{p,uu,ss}$ than for $K_{p,uu,V0}$ (Tables 2−5), because uptake data at longer time (e.g., 30−60 minutes, almost at steady state) are enough to obtain $K_{p,uu,ss}$, whereas initial uptake rates (e.g., 0.5−1.5 or 0.5−2.0 minutes) are needed for $K_{p,uu,V0}$, and such a method requires quite accurate techniques for rapid samplings.

In the extended clearance concept, $K_{p,uu,true}$ is described as in eq. 16 (Shitara et al., 2013):

$$K_{p,uu,true} = \frac{PS_{act,inf} + PS_{diff,inf} + C_{L,inf,met} + C_{L,ext,bile}}{PS_{act,eff} + PS_{diff,eff} + C_{L,inf,met} + C_{L,ext,bile}}$$

where $PS_{act,inf}$, $PS_{act,eff}$, $C_{L,inf,met}$, and $C_{L,ext,bile}$ represent the active influx clearance, the active efflux clearance, the intrinsic clearance for metabolism, and the intrinsic clearance for biliary excretion in an unchanged form, respectively. Based on eq. 16, a number of explanations can be considered for why $K_{p,uu,V0}$ values for pitavastatin, rosuvastatin, and pravastatin tended to be larger than $K_{p,uu,ss}$ in both rat and human hepatocytes (Fig. 3, A and B).
(i) As PSact,inf is estimated by initial uptake rate for the calculation for Kp,uu,V0, it may include not only active transport, but also facilitated diffusion, which is also transporter mediated, but not by active (concentrated) transport. Thus, in this case, Kp,uu,V0 can be larger than Kp,uu,ss. However, in the case of statins, hepatic OATPs are thought to be major uptake transporters, and the impact of facilitated diffusion on their overall uptake may be negligible.

(ii) The existence of non-negligible metabolism during the measurement of steady-state uptake (30–60 minutes) of a drug may lead to smaller Kp,uu,ss than Kp,uu,V0 values, because metabolism might be negligible during short incubation times (~2 minutes) for the measurement of initial uptake rates. Considering that pitavastatin, rosuvastatin, and pravastatin are generally classified as statins metabolized poorly in humans (Shitara and Sugiyama, 2006), it is unlikely that metabolism accounts for the overestimation of Kp,uu,V0. However, a recent report suggested that pitavastatin undergoes lactonization by uridine 5'-diphospho-glucuronosyltransferases more extensively than other statins (Schirris et al., 2015), which may explain, at least in part, the overestimation of Kp,uu,V0 compared with Kp,uu,ss (Fig. 3, A and B). In addition, pentenoic acid derivative was reported to be the major metabolite of rosuvastatin in rats (Nezasa et al., 2002; He et al., 2014). In our experiments using rat hepatocytes, the remaining amount of rosuvastatin after the 30-minute incubation was 87% of the initial amount, whereas little loss of rosuvastatin was observed after the 60-minute incubation in human hepatocytes. Therefore, the contribution of metabolism to the elimination of rosuvastatin in rats might be larger than that in humans, and the metabolism of rosuvastatin in rat hepatocytes might only slightly influence the estimation of its Kp,uu,ss.

(iii) PSact,eff and CLint,bile cannot be fully estimated by the short-term uptake of drugs. Thus, if a drug is a substrate of basolateral/apical efflux transporters, Kp,uu,V0 may be overestimated. The expression of apical efflux transporters [P-glycoprotein, multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein] and basolateral efflux transporters (MRP3 and MRP4) was partly retained in cryopreserved human hepatocytes compared with fresh liver biopsies (Lundquist et al., 2014), which might contribute, at least in part, to the drug efflux from hepatocytes and lead to a discrepancy between Kp,uu,V0 and Kp,uu,ss.

(iv) In most work, PSdif,inf and PSdif,eff are conventionally assumed to be equal for kinetic consideration of the cellular transport (Yabe et al., 2011), although PSdif,eff might be larger than PSdif,inf in the case of anions because of the inside-negative ΔΨ in normal cells. We calculated Kp,uu,ss by taking

![Fig. 5. Theoretical simulation of Kp,uu,V0/Kp,uu,true (= Rtrue/true, blue) and Kp,uu,V0/Kp,uu,true (= Rtrue/true, red) using eqs. 12 and 13. (A) The solid line represents the simulation result for pitavastatin. (B) The broken line represents simulation results for rosuvastatin and pravastatin. Arrows represent the λ for statins estimated from our experiments with Caco-2 cells.](image)

![Fig. 6. Comparison of Kp,uu,ss,corrected and Kp,uu,V0,corrected corrected by eqs. 14 and 15 using Rtrue and Rtrue/(Rtrue + Rfalse) (Table 8). (A) Closed symbols: original Kp,uu,V0 in rat hepatocytes were obtained from a previous report (Yabe et al., 2011). Open symbols: original Kp,uu,V0 values were obtained in the present study. (B) Gray symbols: original Kp,uu,V0 values were obtained in human hepatocytes from a single donor (Lot Hu8075). Open symbols: original Kp,uu,V0 values were obtained in pooled human hepatocytes from 20 mixed-sex donors (Lot. TFF). 1, pitavastatin; 2, rosuvastatin; 3, pravastatin. Solid and dashed lines denote unity and threefold boundaries, respectively. The data are presented as mean ± S.D. for the x- and y-axis (n = 3).](image)
asymmetric $\text{PS}_{\text{diff}}$ into consideration with the assumption that $
abla$ is almost abolished on ice, as we confirmed experimentally (Table 8). Conversely, to calculate $K_{\text{p,uu,V0}}$, $\text{PS}_{\text{diff,eff}}$ is set to be the same as $\text{PS}_{\text{diff,inf}}$ as shown in Supplementary Equation 8. Therefore, theoretically, $K_{\text{p,uu,VO}}$ should be larger than $K_{\text{p,uu,SS}}$ in the case of anions, which might mostly explain the observed discrepancy between $K_{\text{p,uu,VO}}$ and $K_{\text{p,uu,VO}}$.

In this study, two assumptions were made. First, $f_{\text{T,cell,SS}}$ is not affected by temperature; second, $\Delta\psi$ is abolished on ice. To investigate the first assumption, we sought to evaluate whether $f_{\text{T,cell,SS}}$ was largely dependent on temperature, as mentioned in previous reports (Sugano et al., 1989), and Weinman et al., (1989), and Shitara et al., (2013). We found that values for $f_{\text{T,share,ice}}$ on ice were comparable with those for $f_{\text{T,share,37°C}}$ (Table 6), which supports the first assumption, at least for the statins tested. In addition, $f_{\text{T,cell,SS}}$ for rosuvastatin and pitavastatin measured by equilibrium dialysis were similar to $f_{\text{T,share,37°C}}$ whereas $f_{\text{T,cell,SS}}$ for pravastatin was larger than $f_{\text{T,share,37°C}}$ (Tables 4 and 6), although the mechanism behind this has not yet been clarified. By contrast, $f_{\text{T,cell,V0}}$ for rosuvastatin and pravastatin in human hepatocytes exceeded 1 with large S.D. (Table 5), suggesting that it might be difficult to obtain reliable values for $f_{\text{T,cell,V0}}$ based on the rate of initial uptake. Indeed, Yabe et al. (2011) estimated $f_{\text{T,cell,V0}}$ for some drugs, including pravastatin, not by eq. 6, but by using in silico prediction with logD to obtain reasonable values for $f_{\text{T,cell,V0}}$.

The second assumption was investigated using TPP+ uptake studies with and without amphotericin B in human hepatocytes (Fig. 4), according to a method reported previously (Saito et al., 1992). Calculated $\Delta\psi$ at 37°C was similar to reported values (−35 to −39 mV) obtained in isolated rodent hepatocytes (Bradford et al., 1985; Edmondson et al., 1985; Fitz and Schwarzschild, 1987; Wondergem and Castillo, 1988; Weinman et al., 1989), and $\Delta\psi$ was almost abolished on ice under steady-state conditions at 3 minutes (Table 7). Collectively, our assumptions are valid for anions.

For the estimation of $K_{\text{P,uu,SS}}$, ATP depletors such as rotenone were also used to stop the active uptake (Yamazaki et al., 1992); however, it is difficult to optimize experimental conditions for the exposure to ATP depletors to abolish the active uptake without affecting cell viability, and the effect of ATP depletion is often required to be insufficient to maintain viability (Yamazaki et al., 1993). Thus, this approach may lead to underestimation of $K_{\text{P,uu,SS}}$. The use of transporter inhibitors is another strategy to stop the active transport. However, before this, we should understand the major transport mechanism of the drugs to be tested, and confirm that the contribution of other transporters to the overall active uptake is negligible. In the case of statins, because Na+-taurocholate cotransporting polypeptide is known to partly play a role in their hepatic uptake (Bi et al., 2013), the inhibition of Na+-taurocholate cotransporting polypeptide in addition to OATPs is required; otherwise, insufficient inhibition of the hepatic uptake causes an underestimation of $K_{\text{P,uu,SS}}$.

In conclusion, $K_{\text{P,uu,SS}}$ of statins were estimated in rat and human hepatocytes using two different methods based on steady-state uptake ($K_{\text{P,uu,SS}}$) and initial uptake rate ($K_{\text{P,uu,V0}}$). Considering the inside-negative $\nabla$ and subsequent asymmetric diffusional clearance for influx and efflux, in theory, $K_{\text{P,uu,SS}}$ should be similar to true $K_{\text{P,uu,SS}}$, whereas $K_{\text{P,uu,V0}}$ should be higher compared with $K_{\text{P,uu,SS}}$. This can explain, at least in part, the discrepancies observed between $K_{\text{P,uu,SS}}$ and $K_{\text{P,uu,V0}}$ for statins in the present study. Using the estimated $K_{\text{P,uu,SS}}$ Value and the ratio of diffusional uptake to active uptake determined in vitro, we can mathematically describe the relationship among permeation clearances across the sinusoidal membrane of hepatocytes, which can be applied for the construction of a physiologically-based pharmacokinetic model.

Although our current approach should be valid from the viewpoint of kinetic theory, we will need to practically validate our method for the in vitro–in vivo extrapolation of $K_{\text{P,uu}}$. One of the approaches is to compare the observed liver-to-blood total concentration ratio of positron emission tomography probes (e.g., $^{11}$C)dehydropavastatin (Ijuin et al., 2012) in humans with that predicted from experimentally estimated hepatic $K_{\text{P,uu}}$ and their unbound fractions in blood and hepatocytes. Another approach is to confirm whether the discrepancy of in vitro values with regard to the medium concentration of inhibitors obtained with hepatocytes and recombinant enzymes can be well explained by in vitro $K_{\text{P,uu}}$ when target proteins are located inside the hepatocytes (e.g., inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase by statins, inhibition of metabolic enzymes and efflux transporters by inhibitor drugs). Such validations should strengthen our theoretical considerations for in vitro $K_{\text{P,uu}}$ estimation.

Authorship Contributions
Participated in research design: Yoshikado, Toshimoto, Nakada, Maeda, Sugiyama.
Conducted experiments: Yoshikado, Nakada, Ikejiri.
Performed data analysis: Yoshikado, Toshimoto, Nakada.
Wrote or contributed to the writing of the manuscript: Yoshikado, Toshimoto, Nakada, Kisuhara, Maeda, Sugiyama.

References


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Title: Comparison of methods for estimating unbound intracellular-to-medium concentration ratios in rat and human hepatocytes using statins

Authors: Takashi Yoshikado, Kota Toshimoto, Tomohisa Nakada, Kazuaki Ikejiri, Hiroyuki Kusuhara, Kazuya Maeda, and Yuichi Sugiyama

Journal: Drug Metabolism and Disposition

Supplemental Text: Theoretical derivation of the equations for $K_{p,uu}$ considering the plasma membrane potential of cells and the fraction of drugs ionized

$C_{\text{cell,unbound}}$ and $C_{\text{medium}}$ are defined as unbound concentrations in cells and in medium, respectively. $PS_{\text{act}}, PS_{\text{dif}}$, and $CL_{\text{int}}$ are active transport clearance, passive diffusion clearance, and hepatic intrinsic clearance (metabolism + biliary excretion clearance in an unchanged form), respectively. One differential equation for the mass balance in the cell is as follows:

$$\frac{dX}{dt} = \left( f_{o,\text{ion}} \cdot PS_{\text{act,inf}} + f_{o,\text{ion}} \cdot PS_{\text{dif,inf,ion}} + f_{o,\text{ion}} \cdot PS_{\text{dif,inf,ion}} \right) \cdot C_{\text{medium}} - \left( f_{i,\text{ion}} \cdot PS_{\text{act,eff}} + f_{i,\text{ion}} \cdot PS_{\text{dif,eff,ion}} + f_{i,\text{ion}} \cdot PS_{\text{dif,eff,ion}} + CL_{\text{int}} \right) \cdot C_{\text{cell,unbound}}$$

(Eq. S1)

where $X$ is the amount of a drug in the cell. In a steady state, theoretically true $K_{p,uu}$ ($K_{p,uu,\text{true}}$) can be described as follows by converting Equation S1:

$$K_{p,uu,\text{true}} = \frac{C_{\text{cell,unbound}}}{C_{\text{medium}}} = \frac{f_{o,\text{ion}} \cdot PS_{\text{act,inf}} + f_{o,\text{ion}} \cdot PS_{\text{dif,inf,ion}} + f_{o,\text{ion}} \cdot PS_{\text{dif,inf,ion}}}{f_{i,\text{ion}} \cdot PS_{\text{act,eff}} + f_{i,\text{ion}} \cdot PS_{\text{dif,eff,ion}} + f_{i,\text{ion}} \cdot PS_{\text{dif,eff,ion}} + CL_{\text{int}}}$$

(Eq. S2)

Assuming that the intrinsic clearances for metabolism and biliary excretion clearance in an unchanged form and active efflux are negligible, substituting $CL_{\text{int}}$ and $PS_{\text{act,eff}}$ with zero gives:
For passive diffusion of non-ionized drugs, Equation S4 is given:

\[ PS_{\text{diff, inf, ion}} = PS_{\text{diff, eff, ion}} \]  \hspace{1cm} (Eq. S4)

Based on the Nernst equation, passive diffusion of ionized drugs at a steady state is expressed as follows:

\[ PS_{\text{diff, eff, ion}} = \exp\left(\frac{z \cdot F \cdot \Delta \Psi}{R \cdot T}\right) \cdot PS_{\text{diff, inf, ion}} = \Phi \cdot PS_{\text{diff, inf, ion}} \]  \hspace{1cm} (Eq. S5)

where R, T, z, F, and ΔΨ are the gas constant, absolute temperature, valency of the ion, Faraday constant, and plasma membrane potential, respectively. We define the Φ value as “exp(z\cdot F \cdot \Delta \Psi / R \cdot T)” to show the following calculations simply. Substituting Equations 8, S4, and S5 into Equation S3 gives:

\[ K_{p, uu, true} = \frac{f_{\text{o, ion}} \cdot PS_{\text{act, inf}} + f_{\text{o, ion}} \cdot PS_{\text{diff, inf, ion}} + f_{\text{o, uion}} \cdot PS_{\text{diff, inf, uion}}}{f_{\text{i, ion}} \cdot PS_{\text{diff, eff, ion}} + f_{\text{i, uion}} \cdot PS_{\text{diff, eff, uion}}} \]  \hspace{1cm} (Eq. S6)

Because the previously published method (11) assumed that PS_{\text{diff, inf}} is equal to PS_{\text{diff, eff}}, \( K_{p, uu, V_0} \) is expressed as follows:

\[ K_{p, uu, V_0} = \frac{f_{\text{o, ion}} \cdot PS_{\text{act, inf}} + f_{\text{o, ion}} \cdot PS_{\text{diff, inf, ion}} + f_{\text{o, uion}} \cdot PS_{\text{diff, inf, uion}}}{f_{\text{o, ion}} \cdot PS_{\text{diff, inf, ion}} + f_{\text{o, uion}} \cdot PS_{\text{diff, inf, uion}}} \]  \hspace{1cm} (Eq. S7)

Substituting Equations S4 and S5 into Equation S7 gives:
By contrast, the present method provides \( K_{p,uu,ss} \) according to Equation 2. At a steady state, C/M ratio\(_{37\degree C} \) is expressed as follows:

\[
C/M \text{ ratio}_{37\degree C} = \frac{f_{o,ion} \cdot PS_{act,inf} + (\lambda \cdot f_{o,ion} + f_{0,uion}) \cdot PS_{diff,inf,union}}{(\phi \cdot f_{i,ion} + f_{i,uion}) \cdot PS_{diff,inf,union}} \cdot \frac{1}{f_{T,cell,37\degree C}}
\]

(Eq. S9)

Because the active transport and \( \Delta \Psi \) are diminished on ice (\( PS_{act,inf} = 0; \ \phi = 1 \)), and it is assumed that the \( \lambda \) value is not changed by temperature, C/M ratio\(_{on \ ice} \) is expressed as follows:

\[
C/M \text{ ratio}_{on \ ice} = \frac{\lambda \cdot f_{0,ion} + f_{0,uion}}{\lambda \cdot f_{i,ion} + f_{i,uion}} \cdot \frac{1}{f_{T,cell,ice} \cdot f_{T,cell,37\degree C}}
\]

(Eq. S10)

Although the passive diffusion clearance should be affected by the change in membrane fluidity at low temperature (Kanduser et al., 2008), the term for passive diffusion clearance does not appear in Equation S10.

Because \( f_{T,\text{homogenate,}37\degree C} \) is almost equal to \( f_{T,\text{homogenate,on ice}} \) (Table 6), substituting Equations S9 and S10 into Equation 2 gives:

\[
K_{p,uu,ss} = \frac{f_{o,ion} \cdot PS_{act,inf} + (\lambda \cdot f_{o,ion} + f_{0,uion}) \cdot PS_{diff,inf,union}}{(\phi \cdot f_{i,ion} + f_{i,uion}) \cdot PS_{diff,inf,union}} \cdot \frac{1}{f_{T,cell,37\degree C}}
\]

(Eq. S11)
Supplemental Table 1. Intracellular volumes of rat and human hepatocytes.

### A) Rat

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
<th>Intracellular volume µL/10⁶ cells or µL/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]water and [¹⁴C]dextran</td>
<td>Baur et al., 1975</td>
<td>3.0</td>
</tr>
<tr>
<td>3-O-methyl-D-glucose</td>
<td>Kletzien et al., 1975</td>
<td>2.8</td>
</tr>
<tr>
<td>[¹⁴C]dextran</td>
<td>Eaton and Klassen, 1978</td>
<td>2.5</td>
</tr>
<tr>
<td>hydroxy[¹⁴C]methyl-inulin</td>
<td>Kristensen and Folke, 1984</td>
<td>2.9</td>
</tr>
<tr>
<td>[³H]water and [¹⁴C]dextran</td>
<td>Yamazaki et al., 1992</td>
<td>4.3</td>
</tr>
<tr>
<td>[³H]water and [¹⁴C]inulin</td>
<td>Miyauchi et al., 1993</td>
<td>5.2</td>
</tr>
<tr>
<td>[³H]water and [¹⁴C]sucrose</td>
<td>Reinoso et al., 2001</td>
<td>3.9</td>
</tr>
<tr>
<td>[³H]water and [¹⁴C]sucrose</td>
<td>Hallifax and Houston, 2006</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>3.68 ± 1.37</td>
</tr>
</tbody>
</table>

### B) Human (examined in this study)

<table>
<thead>
<tr>
<th>[³H]Water space µL/10⁶ cells</th>
<th>Adherent fluid volume µL/10⁶ cells</th>
<th>Intracellular volume µL/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.14 ± 0.31</td>
<td>1.86 ± 0.10</td>
<td>2.28 ± 0.33</td>
</tr>
</tbody>
</table>

Values are shown as the mean ± S.D. (n = 3).
**Supplemental Figure 1.** The pH-dependent membrane permeation clearance of pitavastatin (A), rosuvastatin (B), and pravastatin (C) across Caco-2 cell monolayers was measured in an apical-to-basal direction with an apical pH of 5.5, 6.0, 6.5, and 7.4, and a basal pH of 7.4 in the presence of rifamycin SV (100 µM), cyclosporin A (10 µM), and Ko143 (10 µM). Parameters in Table 1 ($P_{\text{diff,inf,uion,Caco-2}}$ and $\lambda$) were determined by fitting Equation 9 to the observed data. The data are presented as mean + SD (n = 3). The lines represent simulated values using fitted parameters in Equation 9.
Supplemental Figure 2. Concentration-dependent uptake of pitavastatin (A), rosuvastatin (B), and pravastatin (C) by rat hepatocytes. Kinetic parameters in Table 3 were determined by fitting Equation 4 to the data observed at 7 concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 µM). The data are presented as mean + SD for the X- and Y-axis (n = 3). The lines represent simulated values using fitted parameters.
Supplemental Figure 3. Concentration-dependent uptake of pitavastatin (A, D), rosvavastatin (B, E), and pravastatin (C) by human hepatocytes. Kinetic parameters in Table 5 were determined by fitting Equation 4 to the observed data. The data are presented as mean ± SD for the X- and Y-axis (n = 3). The lines represent simulated values using fitted parameters. (A–C) Cryopreserved human hepatocytes from a single donor (Lot. Hu8075) were used. The data at various concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 µM for pitavastatin; 0.1, 0.3, 1, 3, 10, 30, and 300 µM for rosvavastatin; and 1, 3, 100, and 300 µM for pravastatin) were used for fitting. (D, E) Pooled cryopreserved human hepatocytes from 20 mixed-sex donors (Lot. TFF) were used. The data at various concentrations (0.5, 1, 5, 10, 30, 100, and 300 µM for pitavastatin and rosvavastatin) were used for fitting.