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

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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:

$$\frac{dX}{dt} = (f_{0,ion} \cdot PS_{\text{act,inf}} + f_{0,ion} \cdot PS_{\text{dif,inf,ion}} + f_{0,ion} \cdot PS_{\text{diff,inf,ion}}) \cdot C_{\text{medium}} - (f_{i,ion} \cdot PS_{\text{act,eff}} + f_{i,ion} \cdot PS_{\text{diff,eff,ion}} + f_{i,ion} \cdot PS_{\text{diff,eff,ion}} + CL_{\text{int}}) \cdot C_{\text{cell,unbound}}$$  
(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:

$$K_{p,uu,\text{true}} = \frac{C_{\text{cell,unbound}}}{C_{\text{medium}}} = \frac{f_{0,ion} \cdot PS_{\text{act,inf}} + f_{0,ion} \cdot PS_{\text{dif,inf,ion}} + f_{0,ion} \cdot PS_{\text{diff,inf,ion}}}{f_{i,ion} \cdot PS_{\text{act,eff}} + f_{i,ion} \cdot PS_{\text{diff,eff,ion}} + f_{i,ion} \cdot PS_{\text{diff,eff,ion}} + CL_{\text{int}}}$$  
(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 nonionized drugs, Equation S4 is given:

$$ PS_{\text{diff,inf,ion}} = PS_{\text{diff,eff,ion}} $$

(Eq. S4)

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

$$ PS_{\text{diff,eff,ion}} = \exp\left(\frac{z \cdot F \cdot \Delta \Psi}{R \cdot T}\right) \cdot PS_{\text{diff,inf,ion}} = \Phi \cdot PS_{\text{diff,inf,ion}} $$

(Eq. S5)

where R, T, z, F, and $\Delta \Psi$ are the gas constant, absolute temperature, valency of the ion, Faraday constant, and plasma membrane potential, respectively. We define the $\Phi$ 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:

$$ K_{p,uu,\text{true}} = \frac{f_{0,\text{ion}} \cdot PS_{\text{act,inf}} + f_{0,\text{ion}} \cdot PS_{\text{diff,inf,ion}} + f_{0,\text{uion}} \cdot PS_{\text{diff,inf,ion}}}{f_{i,\text{ion}} \cdot PS_{\text{diff,eff,ion}} + f_{i,\text{uion}} \cdot PS_{\text{diff,eff,ion}}} $$

(Eq. S6)

Because the previously published method (11) assumed that $PS_{\text{diff,inf}}$ is equal to $PS_{\text{diff,eff}}$, $K_{p,uu,V0}$ is expressed as follows:

$$ K_{p,uu,V0} = \frac{f_{0,\text{ion}} \cdot PS_{\text{act,inf}} + f_{0,\text{ion}} \cdot PS_{\text{diff,inf,ion}} + f_{0,\text{uion}} \cdot PS_{\text{diff,inf,ion}}}{f_{0,\text{ion}} \cdot PS_{\text{diff,inf,ion}} + f_{0,\text{uion}} \cdot PS_{\text{diff,inf,ion}}} $$

(Eq. S7)

Substituting Equations S4 and S5 into Equation S7 gives:
\[ K_{p,uu,v0} = \frac{f_{o,ion} \cdot PS_{act,inf} + (\lambda \cdot f_{o,ion} + f_{o,uion}) \cdot PS_{diff,inf, unin}}{\left(\lambda \cdot f_{o,ion} + f_{o,uion}\right) \cdot PS_{diff,inf, unin}} \]  

(Eq. S8)

By contrast, the present method provides \( K_{p,uu,ss} \) according to Equation 2. At a steady state, C/M ratio_{37ºC} is expressed as follows:

\[ C/M \text{ ratio}_{37ºC} = \frac{f_{o,ion} \cdot PS_{act,inf} + (\lambda \cdot f_{o,ion} + f_{o,uion}) \cdot PS_{diff,inf, unin}}{\left(\Phi \cdot \lambda \cdot f_{i,ion} + f_{i,uion}\right) \cdot PS_{diff,inf, unin}} \cdot \frac{1}{f_{T,cell,37ºC}} \]  

(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:

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

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

\[ K_{p,uu,ss} = \frac{\left(f_{o,ion} \cdot PS_{act,inf} + (\lambda \cdot f_{o,ion} + f_{o,uion}) \cdot PS_{diff,inf, unin}\right) \cdot \left(\lambda \cdot f_{i,ion} + f_{i,uion}\right)}{\left[\left(\Phi \cdot \lambda \cdot f_{i,ion} + f_{i,uion}\right) \cdot PS_{diff,inf, unin}\right] \cdot \left(\lambda \cdot f_{o,ion} + f_{o,uion}\right)} \]  

(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[D3]methylinulin</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>
</tbody>
</table>

Mean ± S.D. 3.68 ± 1.37

**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 (PS_{diff,infinity,Caco-2} and λ) 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), rosuvastatin (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 rosuvastatin; 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 rosuvastatin) were used for fitting.