Three-Compartment Model Analysis with Minimal Sampling Points in the Caco-2 Permeability Assay

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INTRODUCTION

Evaluation of the apparent permeability coefficient ($P_{\text{app}}$) in a transcellular transport study is widely used in drug development to predict drug absorption in humans.\(^1\) The lag often shown before reaching the linear range in the receiver compartment limits the range of the linear phase,\(^2\) and several proposals have been made to modify the equation to overcome the issue in calculating $P_{\text{app}}$.\(^3\) However, these approaches are not often applied because the calculations are complex.\(^4\)

There are several reports of using three-compartment model analysis in a transcellular transport assay to analyze the concentration profiles.\(^5\) Furthermore, $P_{\text{app}}$ can be appropriately calculated from a three-compartment model.\(^6\) However, multiple sampling is required to evaluate the concentration profiles of donor, intracellular, and receiver compartments. In particular, obtaining sequential data on concentration in the intracellular compartment requires a lot of wells because one well per sample is needed. Therefore, the data set should be minimized to a single well when using the three-compartment model for drug discovery.

The aim of this study was to establish a more convenient method of three-compartment model analysis with minimal sampling points in the Caco-2 permeability assay. Ten structurally diverse compounds with passive diffusion were used in a Caco-2 permeability assay, and the concentration–time profiles were calculated by both the conventional method and a method that uses one data point from each compartment, called the simplified method.

MATERIALS AND METHODS

Materials 4-Acetamidophenol [2,6-$^3$H] (acetaminophen), doxepin hydrochloride [N-methyl-$^3$H], fluoxetine hydrochloride [N-methyl-$^3$H], and ibuprofen RS [ring-$^3$H] were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). Fluconazole [1H], atenolol [ring-$^3$H], and metoprolol [1H] were purchased from Moravek Inc. (Brea, CA, U.S.A.). Corticosterone [1,2,6,7-$^3$H (N)], imipramine hydrochloride [benzene ring-$^3$H (N)], and propranolol L-[4-$^3$H] were purchased from PerkinElmer, Inc. (Waltham, MA, U.S.A.). These compounds, which were structurally diverse, were selected to provide a wide range of log $P$, $F_\text{a}$ in humans, and Caco-2 permeability.\(^3\) Fasted state simulated intestinal fluid (FaSSIF) was purchased from Celeste Corp (Easton, MD, U.S.A.). Other reagents were purchased from the same companies as previously reported.\(^3\)

Caco-2 Permeability Assay The Caco-2 permeability assay was performed under the same conditions as previously reported.\(^3\) Briefly, preincubation was carried out at 37°C for 30 min by adding Hank’s balanced salt solution (HBSS)/N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer to the donor side and HBSS/HEPES buffer containing 4% bovine serum albumin (pH 7.4) to the receiver side. The permeability study was initiated by adding FaSSIF/HEPES buffer (pH 7.4) containing 10 μM of compound to the donor side. After incubation for 0, 30, 60, 90, and 120 min, samples were taken from the donor, cell, and receiver sides ($n = 3$).

Model Fitting Analysis of the Caco-2 Cell Transport Assay The theory of three-compartment model analysis and the equations were the same as previously reported\(^3\) (Fig. 1). Briefly, the mass-balance equations can be described as follows:

\[ \begin{align*} \frac{d}{dt} N_t & = -P_{\text{app}} F_\text{a} C_{\text{d}} - P_{\text{app}} (1 - F_\text{a}) C_{\text{i}} + \frac{1}{V_{\text{i}}} \frac{d}{dt} Y_{\text{i}} \\ \frac{d}{dt} Y_{\text{i}} & = -k_{\text{i}} Y_{\text{i}} + \frac{1}{V_{\text{i}}} \frac{d}{dt} N_{\text{i}} \\ \frac{d}{dt} C_{\text{r}} & = -P_{\text{app}} F_\text{a} C_{\text{d}} + P_{\text{app}} (1 - F_\text{a}) C_{\text{i}} + k_{\text{i}} Y_{\text{i}} \end{align*} \]
Fig. 1. Schematic Diagram Illustrating Transcellular Transport of Drugs across the Cell Monolayer

\( C_1, C_2, \) and \( C_3 \) are the total drug concentrations in the donor, intracellular, and receiver, respectively; \( V_1, V_2, \) and \( V_3 \) are the volume of the donor, intracellular, and receiver compartments, respectively; \( PS_1 \) is the influx permeability clearance of total drug across the apical membrane; \( PS_2 \) is the efflux permeability clearance of total drug across the basolateral membrane; and \( PS_3 \) is the influx permeability clearance of total drug across the basolateral membrane.

\[
V_1 \frac{dC_1}{dt} = -PS_1 \cdot C_1 + PS_2 \cdot C_2
\]

(1)

\[
V_2 \frac{dC_2}{dt} = PS_1 \cdot C_1 + PS_4 \cdot C_3 - (PS_2 + PS_3) \cdot C_2
\]

(2)

\[
V_3 \frac{dC_3}{dt} = -PS_4 \cdot C_3 + PS_3 \cdot C_2
\]

(3)

where \( C_1, C_2, \) and \( C_3 \) are the total compound concentration in the donor, intracellular, and receiver compartments, respectively; \( V_1, V_2, \) and \( V_3 \) are the volume of the donor, intracellular, and receiver compartments, respectively; \( PS_1 \) is the influx permeability clearance of total drug across the apical membrane, \( PS_2 \) is the efflux permeability clearance of total drug across the apical membrane, \( PS_3 \) is the efflux permeability clearance of total drug across the basolateral membrane, and \( PS_4 \) is the influx permeability clearance of total drug across the basolateral membrane. The unbound molecules were regarded as having identical permeability clearance (basolateral membrane. The unbound molecules were regarded as having identical permeability clearance (basolateral membrane. The unbound molecules were regarded

\[ B_1 = -\frac{C_1(0)}{\beta(\alpha - \beta)} \left\{ \alpha^2 \left( \frac{PS_1 \times fu_3}{fu_1} + \frac{PS_2 \times fu_2}{fu_1} + \frac{PS_1 \times fu_1}{fu_3} \right) \right\} + \frac{PS_1 \times fu_3}{fu_1} \frac{PS_2 \times fu_2}{fu_1} \frac{PS_1 \times fu_1}{fu_3} \frac{PS_1 \times fu_3}{fu_1} \]

(8)

\[ A_2 = \frac{C_1(0)}{\alpha(\beta - \alpha)} \frac{PS_1}{fu_1} \left( -\alpha + \frac{PS_1 \times fu_1}{V_3} \right) \]

(9)

\[ B_3 = \frac{C_1(0)}{\beta(\alpha - \beta)} \frac{PS_1 \times PS_2 \times fu_2}{fu_1} \frac{PS_1 \times PS_3 \times fu_2}{fu_1} \]

(10)

\[ A_3 = \frac{C_1(0)}{\alpha(\beta - \alpha)} \frac{PS_1 \times PS_2 \times fu_2}{fu_1} \frac{PS_2 \times fu_2}{V_2 \times V_3} \]

(11)

\[ \alpha = \frac{1}{2} \left\{ \frac{PS_1}{fu_1} \frac{PS_2 \times fu_2}{fu_1} + \frac{PS_1 \times fu_2}{fu_1} + \frac{PS_1 \times fu_1}{fu_3} \right\} + z \]

(13)

\[ \beta = \frac{1}{2} \left\{ \frac{PS_1}{fu_1} \frac{PS_2 \times fu_2}{fu_1} + \frac{PS_1 \times fu_2}{fu_1} + \frac{PS_1 \times fu_1}{fu_3} \right\} + z \]

(14)

\[ \frac{1}{2} \left\{ \frac{PS_1}{fu_1} \frac{PS_2 \times fu_2}{fu_1} + \frac{PS_1 \times fu_2}{fu_1} + \frac{PS_1 \times fu_1}{fu_3} \right\} \]

(15)

\[ t = \frac{1}{\alpha} \]

(16)

The permeability coefficients in the direction of apical to basal (\( P_{\text{app AtoB}} \), cm/s) were described by the equation below:

\[ P_{\text{app AtoB}} = PS_1 \left( \frac{PS_2 + PS_3}{PS_4} \right) \frac{S}{S} \]

(17)

Where \( S \) is 0.33 cm², which is the surface area of each well assuming a 24-well device. \( V_1 \) was 250 µL, and \( V_3 \) was 600 µL. \( V_2 \) was calculated to be 0.9 µL based on the height of Caco-2 cells (27.2 µm) measured by a confocal microscope and the surface area of each well. The concentration profiles were fitted to Eqs. (4)–(6), and the values of \( fu_2 \) and \( PS_1/fu_1 \) were optimized assuming \( PS_1/fu_1 = PS_2/fu_2 = PS_3/fu_3 = PS_4/fu_4 \) and...
1. Fitting by the conventional method used all collected data. The simplified method used only data from the donor compartment at 0 min and from the intracellular and receiver compartments at 120 min. Because the compound concentrations in the receiver compartment were much lower than those in the donor compartment, fitting was performed using the solver function in Excel 2010 (Microsoft, Redmond, WA, U.S.A.) with a weight of $1/y^2$ to minimize the difference between the observed and predicted concentrations.

### Statistical Analysis

The correlation between observed concentrations and predicted concentrations was evaluated by the Spearman-test using Prism 7.00 for Windows (GraphPad

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**Table 1. Parameters Calculated by the Conventional and Simplified Methods for Ten Compounds in the Caco-2 Permeability Assay**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$pK_a$</th>
<th>$c \log P$</th>
<th>$fu_2$</th>
<th>$PS/fu_1$</th>
<th>$P_{app}$</th>
<th>Lag time</th>
<th>$fu_2$</th>
<th>$PS/fu_1$</th>
<th>$P_{app}$</th>
<th>Lag time</th>
<th>Difference in value compared to conventional method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>9.5</td>
<td>0.611</td>
<td>0.302</td>
<td>15.6</td>
<td>23.6</td>
<td>1.6</td>
<td>0.282</td>
<td>15.8</td>
<td>24.0</td>
<td>1.7</td>
<td>−7%</td>
</tr>
<tr>
<td>Atenolol</td>
<td>9.6</td>
<td>0.561</td>
<td>1.22</td>
<td>0.585</td>
<td>0.886</td>
<td>11</td>
<td>1.14</td>
<td>0.552</td>
<td>0.837</td>
<td>12</td>
<td>−7% −6%</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>n.d.</td>
<td>2.15</td>
<td>0.119</td>
<td>45.7</td>
<td>69.2</td>
<td>1.4</td>
<td>0.113</td>
<td>64.9</td>
<td>98.3</td>
<td>1.0</td>
<td>−5% 42%</td>
</tr>
<tr>
<td>Doxepin</td>
<td>8.0</td>
<td>3.57</td>
<td>0.0132</td>
<td>39.8</td>
<td>60.3</td>
<td>0.93</td>
<td>0.0131</td>
<td>49.3</td>
<td>74.7</td>
<td>11</td>
<td>−1% 24%</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1.2</td>
<td>1.22</td>
<td>1.15</td>
<td>22.3</td>
<td>33.8</td>
<td>0.29</td>
<td>1.23</td>
<td>24.4</td>
<td>36.9</td>
<td>0.25</td>
<td>7% 9%</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>9.5</td>
<td>4.19</td>
<td>0.0147</td>
<td>14.0</td>
<td>21.3</td>
<td>34</td>
<td>0.0144</td>
<td>14.0</td>
<td>21.2</td>
<td>34</td>
<td>−2% 0%</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>5.2</td>
<td>3.83</td>
<td>1.11</td>
<td>96.1</td>
<td>146</td>
<td>0.070</td>
<td>1.47</td>
<td>933</td>
<td>1413</td>
<td>0.0055</td>
<td>32% 871%</td>
</tr>
<tr>
<td>Imipramine</td>
<td>9.5</td>
<td>3.98</td>
<td>0.0107</td>
<td>33.4</td>
<td>50.6</td>
<td>19</td>
<td>0.0102</td>
<td>40.5</td>
<td>61.3</td>
<td>16</td>
<td>−4% 21%</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>9.7</td>
<td>1.79</td>
<td>0.108</td>
<td>29.2</td>
<td>44.2</td>
<td>2.4</td>
<td>0.108</td>
<td>33.0</td>
<td>50.1</td>
<td>2.1</td>
<td>0% 13%</td>
</tr>
<tr>
<td>Propanolol</td>
<td>9.5</td>
<td>2.80</td>
<td>0.0180</td>
<td>34.2</td>
<td>51.8</td>
<td>11</td>
<td>0.0171</td>
<td>40.3</td>
<td>61.1</td>
<td>10</td>
<td>−5% 18%</td>
</tr>
</tbody>
</table>

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(a) Three-compartment model analysis using 0, 30, 60, 90, 120 min data of donor, intracellular, and receiver compartments for fitting. (b) Three-compartment model analysis using 0 min of donor and 120 min of intracellular and receiver compartments for fitting. (c) $pK_a$ values were referenced from the package inserts of each marketed drug. (d) The anomaly of a fraction greater than 1 is explained in the Results & Discussion section. (e) $P_{app} = PS_2/PS_1(fu_2/PS_1)$ (f) Lag time $= 1/\alpha$ (g) Percent errors vs. conventional method = ((value of simplified method) − (value of conventional method))/ (value of conventional method) × 100. (h) Corticosterone is not ionized.
RESULTS AND DISCUSSION

The Caco-2 permeability assay in this study was performed on ten compounds selected for their diversity in terms of log $P$, $F_a$ in humans, chemical structure, and Caco-2 permeability. Although the three-compartment model is useful for describing more complicated phenomena, such as transporter-mediated membrane permeation, all ten compounds permeate through passive diffusion. Compounds with only passive diffusion were purposefully selected for this preliminary study to develop the modified method. The recovery of the ten compounds ranged from 104–116% of the initial amount in the donor compartment (data not shown). Of the ten compounds, the most appropriate for calculating $P_{app}$ by three-compartment model analysis is fluoxetine, because its concentration in the receiver compartment shows a lag time before reaching the linear range, due to such influences as intracellular distribution and adsorption to the cell membrane. In the Caco-2 permeability assay of fluoxetine, the fitted curves of the donor, intracellular, and receiver compartments were well described by both the conventional method and the simplified method (Figs. 2a–c). The values for lag time, $f_{u_2}$ and $P_{app}$ parameters calculated by the simplified method were 34 min, 0.0144, and $21.2 \times 10^{-6}$ cm/s, respectively (Table 1). These values were comparable to the ones calculated by the conventional method. The $f_{u_1}$ was assumed to be 1 in this study, but the actual value of $f_{u_1}$ might have been different because all compounds were added to the donor side with a buffer containing FaSSIF. Since lipophilic compounds may form micelles with FaSSIF and thus affect $f_{u_1}$, the actual value of $f_{u_1}$ would need to be measured for three-compartment model analysis. The fitted curves of nine other compounds transported by passive diffusion were also well described by the conventional method.
There was significant correlation between the observed and predicted concentrations for the ten compounds, including fluoxetine (Spearman $r_s = 0.998$, $p < 0.0001$, data not shown). The values for lag time, $t_{lag}$ and $P_{app}$ parameters ranged from 0.070 (ibuprofen) to 34 (fluoxetine) min, from 0.0107 (imipramine) to 1.22 (atenolol), and from 0.886 (atenolol) to 146 (ibuprofen) $\times 10^{-6}$ cm/s, respectively (Table 1), which demonstrates the diversity of the compounds. The calculated $t_{lag}$ for atenolol, fluconazole, and ibuprofen exceeded 1. The intracellular concentration was calculated using 0.9 $\mu$L as the cell volume, but it might actually have been a little larger, because the culture conditions may have changed the cell volume.\(^{11)}\)

The curves fitted by the simplified method using one point in each compartment described all the compounds except ibuprofen. There was significant correlation between the observed and the predicted concentrations for these nine compounds (Spearman $r_s = 0.997$, $p < 0.0001$, Fig. 3). The percent error of the parameter values for $t_{lag}$ and $P_{app}$ calculated by the simplified method were between $-7$ and $7\%$ and $-6$ and $42\%$, respectively, of those obtained by the conventional method. These results show that the parameters calculated by the simplified method were comparable with those by the conventional method. Although the simplified method uses only one well to calculate $P_{app}$, the experiment would be carried out in duplicate or triplicate to account for variations. On the other hand, the fitted curve of ibuprofen calculated using the simplified method differed significantly from observed concentrations (Figs. 3, 4). Because ibuprofen has high membrane permeability,\(^{12)}\) sink conditions were not maintained, and the donor and intracellular concentrations of ibuprofen decreased quickly (Figs. 4a–c). In the drug discovery stage, there are compounds that are ‘beyond the rule of five,’ having not only passive diffusion, but also transporter mediated permeation or the influence of an unstirred layer.\(^{13)}\) In this study, only compounds with passive diffusion within the ‘rule of five’ were evaluated, and the simplified method needs to be confirmed using transporter substrates and compounds that are highly permeable and lipophilic. Additionally, accuracy of the simplified method might be lower than the conventional method, as the 2 unknown parameters were optimized using only 3 points.

In conclusion, a simplified method for appropriately calculating $P_{app}$ using the three-compartment model in the Caco-2 permeability assay was developed. The simplified method can be effectively performed with a small data set obtained from one-well experiments, which allows efficient compound screening in the early stage of drug discovery.

**Conflict of Interest** M. Nagayasu, and K. Ozeki are employees of Chugai Pharmaceutical Co., Ltd. There are no other conflicts of interest.

**REFERENCES**


