Evaluation of Absorbability of Poorly Water-Soluble Drugs: Validity of the Use of Additives

Etsushi Watanabe,∗,† Rika Sudō,‡ Masayuki Takahashi,‡ and Masahiro Hayashi§
Department of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Science University of Tokyo,1 12 Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162-8626, Japan and Pharmaceutical Formulation Research Laboratory, Daiichi Pharmaceutical Co., LTD.§ 16–13, Kita-Kasai 1-chome, Edogawa-ku, Tokyo 134–8630, Japan. Received October 5, 1999; accepted March 23, 2000

Apparent membrane permeation coefficients (Papp) of poorly water-soluble drugs such as indomethacin (IDM) and triamterene (TAT) were obtained by the chamber method using an isolated rat intestinal tissue after solubilization of the drugs by additives. For the additives, sodium deoxycholate (DOC), polyethylene glycol 600 (PEG 600), dimethylsulfoxide (DMSO), ethanol (EtOH), propylene glycol (PG), and rat bile were examined. Their concentrations were determined in ranges considered to be appropriate from the results of in vitro experiments and physiological findings. From the correspondence between this membrane permeability and in vivo bioavailability, we evaluated the validity of our in vitro experiment. On the basis of these evaluations, it was shown that 5% DMSO and 10% PEG 600, which did not affect the membrane integrity, were most appropriate additives for chamber experiments. Papp of IDM was greater than that of TAT, indicating that the order corresponded with that of in vivo bioavailability after oral administration of their PEG 600 solutions. Accordingly, it was concluded that Papp obtained by our in vitro system can be used to assess the in vivo bioavailability.

Key words membrane permeability; in vivo bioavailability; in vivo absorbability; isolated rat intestine; poorly water-soluble drug; Ussing-type chamber

The bioavailability of drugs is determined by their membrane permeability and intraluminal concentration.1,2) Useful drugs for oral administration should have high intraluminal solubility and high membrane permeability. Even a drug with low intraluminal solubility may be developed for clinical use by pharmaceutical modifications if its membrane permeability is sufficiently high. However, as the molecular weights of many drug candidates have recently been increasing by the progress of high throughput screening (HTS) and combinatorial chemistry, drug candidates tend to increase their lipophilicity and reduce water solubility.3) Accordingly, it is important to obtain accurate information on the absorbability of poorly water-soluble drugs.

First, for evaluation of the intestinal membrane permeability of poorly water-soluble drugs, it is important to completely dissolve the drug and prevent precipitation during the experiment. Therefore, low solubility would pose a great problem in evaluating the membrane permeability. One of the methods to overcome this problem is the use of an additive that facilitates dissolution. Second, for evaluation of membrane permeability, the chamber experiment using isolated intestinal tissues and the membrane permeation test system using Caco-2 cell monolayer is widely used. Caco-2 layer has a tighter junctional structure and higher transepithelial electrical resistance than an isolated intestinal tissue. For this reason, Caco-2 model is too sensitive to the changes in the tight junction induced by additives. Actually a number of studies have been conducted on the structural changes of the intestinal tissue such as changes in the paracellular route induced by absorption enhancers.4) With highly soluble drugs, relationships between the membrane permeability in Caco-2 system and the bioavailability in humans have been investigated.5,6–8) A relationship between membrane permeability and in vivo bioavailability has also been investigated in an isolated intestinal tissue similar to Caco-2.5,6) However, in the present study, Caco-2 model is not always suitable to the in vitro system with additives. Junctional structure of the isolated intestinal tissue used in the present study is more leaky, and the mucus layer on the membrane surface has its resistance to the effects of additives. These characteristics favor studies by the in vitro chamber system using the isolated tissue in the presence of additives.

Precipitation in the lumen limits the study of a relationship between membrane permeability and in vivo absorbability for poorly water-soluble drugs. Thus, membrane permeability should be evaluated after solubilization of poorly water-soluble drugs by using additives. In the present study, additives that do not affect the membrane integrity of the isolated rat intestinal tissue mounted in the diffusion chamber were selected. Sodium deoxycholate (DOC), polyethylene glycol 600 (PEG 600), dimethylsulfoxide (DMSO), ethanol (EtOH), propylene glycol (PG), and bile were examined as additives. Effects of the additives on the membrane integrity were evaluated by measurement of released enzymes such as lactate dehydrogenase (LDH) and alkaline phosphatase (ALP), membrane conductance, which is a reciprocal of transepithelial resistance. And effects of the additives on the membrane permeability through the paracellular route were evaluated by the apparent permeability coefficients (Papp) of fluorescein isothiocyanate-labeled dextran 4000 (FD4) and m-cresol purple (MCP). Indomethacin (IDM)9,10) and triamterene (TAT)11–13) were used as representative poorly water-soluble drugs. Finally, the validity of an in vitro experiment to the bioavailability of such drugs was evaluated on the basis of correspondence between Papp and in vivo absorbability after oral administration of a solubilized drug.

MATERIALS AND METHODS

Chemicals IDM, TAT, FD4 and antipyrine (APy) were purchased from Sigma Chemical Co. (MO, U.S.A.). MCP was purchased from Acros Chimica N.V. (Geel, Belgium).
### Table 1. HPLC Conditions for the Determination of m-Cresol Purple, Antipyrine, Indomethacin and Triamterene

<table>
<thead>
<tr>
<th>Drug</th>
<th>Internal standard</th>
<th>Stationary phase</th>
<th>Eluent</th>
<th>Wavelength of detection (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-Cresol purple</td>
<td>—</td>
<td>Symmetry</td>
<td>50 mm Phosphate buffer (pH 2.5) – acetonitrile (75:25)</td>
<td>420</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>—</td>
<td>Symmetry</td>
<td>100 mm Ammonium acetate – acetonitrile (75:25)</td>
<td>280</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Hexyl p-hydroxybenzoate</td>
<td>Symmetry</td>
<td>1% Acetic acid solution – acetonitrile (40:60)</td>
<td>240</td>
</tr>
<tr>
<td>Triamterene</td>
<td>Methyl p-hydroxybenzoate</td>
<td>L-column ODS</td>
<td>20 mm DIammonium hydrogen phosphate – methanol (55:45)</td>
<td>260</td>
</tr>
</tbody>
</table>

LDH CII (#271-54101) and Alp enzyme kit (#274-04401) were purchased from Wako Pure Chem. (Osaka, Japan). Other chemicals used were of analytical grade.

**Animals** Male Wistar rats older than 7 weeks of age (body weight about 200–250 g) were used. The rats were fasted for about 24 h with free access to water.

**In Vitro Permeation Study by the Ussing-Type Chamber Method** Transport studies were carried out using modified Ringer's solution (pH 7.4) in an atmosphere of a 95% O₂/5% CO₂ gas mixture. Rats were anesthetized with urethane and the abdominal wall was cut open along the midline. A 2 to 3 cm jejunal segment was excised and placed in warm Ringer's solution. The epithelium was then exposed *via* a longitudinal incision along the mesenterium, and the underlying muscle layer was removed. Finally the epithelium was mounted onto an Ussing-type chamber having a tissue surface area of 0.64 cm². Ringer's solution (5 ml) was added to both the donor (mucosal) and the receiver (serosal) chambers. After 10 min preincubation, drug solution (5 ml) was added to the donor chamber and the permeation study was started. Samples (100 µl) were withdrawn from both sides at regular intervals up to 100 min. Electrical parameters were measured by the methods of Yamashita et al. and Sawada et al.

**Enzyme Release from the Intestinal Tissue** To assess the membrane damage induced by the additives on the mucosal side, the activities of released enzymes were determined. In the *in vitro* permeation study, the mucosal side buffer was assayed for LDH and Alp activity at the zero time and 100 min after the start of incubation with additives.

**Solubilities and Distribution Coefficients of Poorly Water-Soluble Drugs** Apparent solubilities of poorly water-soluble drugs were calculated as follows. Two mg of a poorly water-soluble drug was mixed with 1 ml of either distilled water, Japanese Pharmacopoeia (JP) 1st-fluid (JP1, pH 1.2), JP 2nd-fluid (JP2, pH 6.8), polyethylene glycol 600 (PEG 600), miglyol 812 or rat bile. Bile was withdrawn from the rat bile duct under urethane anesthesia. The collected bile was freeze-dried and stored at −80 °C until experiment. The mixed solution was sonicated for 5 min, then stirred with a vortex mixer for 30 s and left standing for 5 min. This treatment was repeated 5 times. The mixture was filtered (pore size, 0.5 µm) and the drug concentration in the filtrate was measured by HPLC. Drug distribution coefficients [D] were calculated from their distribution in octanol to phosphate buffer saline (PBS) (pH 6.8) or Ringer's solution (pH 7.4).

**In Vivo Absorption Study** IDM or TAT was solubilized in PEG 600 solution and the solution was further dissolved in distilled water of a 2-fold volume to make a final concentration of 5 mg/ml. In addition, the drug solution was injected intravenously *via* the jugular vein at a dose of 5 mg/kg body weight. These drugs were also administered orally with an oral sonde at a dose of 10 mg/kg. For oral administration, a PEG 600 solution of drug was used immediately after preparation. Five hundred microliters blood samples were taken from the jugular vein 0, 0.5, 1, 1.5, 2, 4 and 6 h after i.v. administration and 0.5, 1, 2, 3, 4, 8 and 24 h after oral administration. The blood was centrifuged and the plasma was stored at 3 °C until assay. The assay for drug concentration was performed within 1 week.

**Assays** The Alp activity was determined with the Alp enzyme kit by the Bessey–Lowry method. The LDH activity was determined with the LDH CII kit, *i.e.*, by spectrophotometric determination (λ=560 nm) of diformazan formation. FD4 concentration was determined using a fluorescent microplate reader (Ex. 480 nm, Em. 520 nm). MCP and APY concentrations were determined using HPLC. The experimental characteristics of the various HPLC methods are outlined in Table 1.

The plasma drug concentrations after oral or intravenous administration were assayed as follows: 100 µl of rat plasma, 100 µl of internal standard solution (Table 1) and 1.1 ml of methanol were mixed well, and centrifuged for 10 min at 14000 rpm. One milliliter of the supernatant was collected and placed in another tube, and evaporated in a centrifugal evaporator. The dried residue was dissolved in 100 µl of mobile phase, and the concentration of drug in the solution was determined by HPLC (Table 1).

**Data Analysis** The apparent permeability coefficients (D<sub>app</sub>) per unit membrane surface area were calculated by dividing the steady-state appearance rate of drugs in the serosal side by the product of the initial concentration of the sample on the mucosal side and the surface area of the membrane. The area under the plasma concentration curve (AUC) was calculated by the trapezoidal rule. The absolute bioavailability (BA) was determined by dividing the AUC value normalized with the oral dose (AUC<sub>p.o.</sub> ) by that normalized with the intravenous dose (AUC<sub>i.v.</sub>).

**Statistical Analysis** Results are expressed as means± S.D. For multiple group analysis of variance, one-factor ANOVA was used. The location of variance was determined using Student's t-test in comparison with the control. A p value less than 0.05 was regarded as statistically significant.

**RESULTS**

**Effects of Additives on Enzyme Release from the Intestinal Tissue** Figure 1 shows the results of enzyme release measurement on the mucosal side 100 min after addition of the additives. The release of Alp was significantly increased in comparison with the control (no additive) for the DOC and bile groups. Also the release of LDH was significantly increased in 10% DMSO, 10% PG and 100 mm DOC. For further permeation and absorption experiments, 5%
DMSO and 10% PEG 600 were mainly employed.

**Effects of Additives on Membrane Conductance**

An increase in membrane conductance (ΔG) indicates an opening of tight junction. Increases in ΔG 100 min after addition of the additives were observed in the DOC group (Fig. 2). No changes in ΔG were induced by the addition of additives other than DOC (Fig. 2).

**Effects of Additives on P_{app} of FD4, MCP and APY**

No significant changes in P_{app} of FD4, a high molecular and paracellular permeate compound, were observed except for the increases in the presence of 20% PEG 600 and 100 mM DOC compared with the control group (no additive) (Fig. 3). MCP is a highly water-soluble pH indicator with a molecular weight of 382.3. Since it is mildly acidic with pK_a = 2.5, it is considered to be mostly ionized at neutral pH and its main permeation route is considered to be the paracellular route. No significant difference in P_{app} of MCP from the control was observed with any additives (Fig. 3). Also with APY, a representative transcellular permeate drug, no significant change in P_{app} from the control was found by the addition of DMSO and PEG 600 (Fig. 4).

**Solubilities and Distribution Coefficients of Poorly Water-Soluble Drugs**

The apparent solubility of IDM was less than 100 μg/ml in distilled water, JP1, miglyol 812 and rat bile and about 218 μg/ml in JP2 (Table 2). The apparent solubility of TAT was similar to that of IDM in distilled water and miglyol 812, and 7 times smaller than that of IDM in JP2. However, the solubility of TAT in rat bile was 15 times greater than that of IDM (Table 2). The apparent solubility of both IDM and TAT in PEG 600 was more than 2000 μg/ml (Table 2). The value of logarithmic distribution coefficient (log D) was somewhat smaller for IDM than for TAT (Table 2).

**Effects of Additives on P_{app} of IDM and TAT**

With IDM at 1 mM as an initial concentration, no significant difference in P_{app} was observed between the 10% PEG 600 group and the control (no additive) group (Fig. 5). At the initial IDM concentration of 5 mM, P_{app} in the 10% PEG 600 group was similar to that of 1 mM IDM, but P_{app} in the control group was significantly reduced (Fig. 5). P_{app} of TAT at 0.2 mM as the initial concentration showed no significant difference between the 10% PEG 600 group and the control group, but P_{app} of 1 mM TAT was smaller than that of 0.2 mM TAT in the control while the difference was not significant in the 10% PEG 600 group (Fig. 5).

**Effects of Additives on in Vivo BA of IDM and TAT**

AUC values after intravenous and oral administration of IDM and TAT were listed in Table 3. The BA values of IDM and TAT after oral administration of their PEG 600 solutions were about 95% and 23%, respectively (Table 3).

**DISCUSSION**

The concentrations of the additives were selected in the ranges considered to be appropriate on the basis of the results of *in vivo* experiments and physiologic findings. From the secretion rate of gastric juice (180 ml/h), daily volume of bile secretion (700—1000 ml), and daily volume of intestinal juice secretion (3000 ml) in humans, the bile concentration in the intestine 1 h after oral administration of the drug was assumed to be diluted about 10 times. It is known that DOC is one of the components of bile and contained in bile at about 100 mM, and therefore its concentration in the intestinal lumen was assumed to be 10 mM from the above data.
In our in vivo oral administration studies in rats, PEG 600 was used at the dose of 400 μl/rat. If the gastric volume of a rat is assumed to be about 2 ml from the ratio of the human gastric volume of 500 ml to the body weight, the concentration of PEG 600 is considered to have been diluted about 5 times. Therefore, PEG 600 was used at concentrations less than 20% of the original solution. The concentrations of the other additives were selected at similar levels to those of PEG 600 or less.

The release of Alp and LDH is considered to represent toxicity to the brush border membrane and the epithelial membrane, respectively. The release of Alp was increased by 10% bile or 10 mM DOC which was considered to be near the intestinal luminal concentration. DOC is known to disintegrate the mucus layer by its surfactant activity, and the release of Alp markedly increased with the increase in the concentration of DOC (Fig. 1). Considering such increases in the release of Alp and LDH, 10% DMSO, 10% PG, DOC and bile should not be used for solubilization of poorly water-soluble drugs in the present system. Consequently, as additives with excellent solubilizing ability (Table 2) and no cell membrane-damaging property, 5% DMSO, 10% PEG 600 and 20% PEG 600 were examined with 10 and 100 mM DOC as positive controls for the study of effects on the paracellular route of permeation.

From the increase in membrane conductance, a widening of the paracellular route was suggested in the presence of 100 mM DOC (Fig. 2). Also the increase in FD4 permeation suggested an enlargement of the paracellular route by 100 mM DOC (Fig. 3). As for the reason why the MCP transport was not significantly affected by any additives (Fig. 3), the effect on the passage of such a small compound with a MW of about 400 was considered to be too small in the intrinsically leaky intestinal tissue. In the 100 mM DOC group, short circuit currents were overloaded during the experiment in some cases (data not shown), suggesting that DOC damages the transepithelial route as well as the paracellular route. On the basis of these results, 10% PEG 600 and 10% DMSO, which did not affect the paracellular route, were selected to examine their effects on the transepithelial route. These additives had no effect on the transepithelial route since the $P_{app}$ of APY was not changed (Fig. 4).

As a representative additive, the effects of 10% PEG 600 on the $P_{app}$ of IDM and TAT was examined in detail. $P_{app}$ is decreased if these drugs precipitate during the permeation experiment. With IDM at 5 mM as the initial mucosal concentration, $P_{app}$ in the control was decreased in comparison with the other three groups (Fig. 5). Also with 1 mM TAT, $P_{app}$ in the control was significantly reduced (Fig. 5). The mucosal TAT concentration in the control 100 min after initiation of the permeation experiment at the initial concentration of 1 mM became lower than 1 mM, but not in the system of 10% PEG 600 (data not shown). These results suggested that the above decrease in $P_{app}$ of 1 mM TAT in the control was due to its precipitation during the experiment. On the other hand, $P_{app}$ of IDM at 1 mM as the initial mucosal concentration showed no change. IDM has pH-dependent dissolution characteristics despite its poor solubility, and its apparent solubility in neutral media is relatively high, e.g. about 0.6 mM in JP2 (pH 6.8) (Table 2). IDM is a mildly acidic compound and is dissolved by ionization. Therefore, its solubility is considered to have been increased in the Ringer's solution used in the chamber experiment (pH 7.4) compared with JP2. This speculation is supported by the finding that there was no difference in the transport of 1 mM IDM between the control (no additive) and 10% PEG 600 groups (Fig. 5). TAT is a non-pH-dependent and poorly water-soluble drug, and its apparent solubility near pH 7 is more than 0.1 mM (Table 2).
Table 2. Apparent Solubilities and Distribution Coefficients

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indomethacin</th>
<th>Triamterene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image" alt="Indomethacin Structure" /></td>
<td><img src="image" alt="Triamterene Structure" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solubility µg/ml (mm)</th>
<th>Dist. water</th>
<th>JP1</th>
<th>JP2</th>
<th>PEG 600</th>
<th>Miglyol 812</th>
<th>Rat bile</th>
<th>Octanol/PBS (pH 6.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>357.79</td>
<td>21.8 (0.06)</td>
<td>0.1 (0.00)</td>
<td>218.2 (0.61)</td>
<td>&gt;2000 (&gt;5.59)</td>
<td>2.0 (0.01)</td>
<td>48.0 (0.13)</td>
</tr>
<tr>
<td>log D₆₈₁</td>
<td>253.27</td>
<td>27.7 (0.11)</td>
<td>29.5 (0.12)</td>
<td>31.2 (0.12)</td>
<td>&gt;2000 (&gt;7.90)</td>
<td>3.0 (0.01)</td>
<td>721.6 (2.85)</td>
</tr>
</tbody>
</table>

Fig. 5. Effects of Additives on Pₘₚ of Indomethacin (IDM) and Triamterene (TAT)
IDM: ■, 1 mm; □, 5 mm; TAT: ■, 0.2 mm; □, 1 mm. Data represent the means ± S.D. of 3 experiments. * p<0.05.

Table 3. Absolute Bioavailability of Indomethacin (IDM) and Triamterene (TAT)

<table>
<thead>
<tr>
<th>Administration Dose (mg/kg)</th>
<th>IDM</th>
<th>TAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.v.</td>
<td>p.o.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>AUC (µg/ml-h)</td>
<td>384.50</td>
<td>726.59</td>
</tr>
<tr>
<td>BA (%)</td>
<td>94.5</td>
<td>23.0</td>
</tr>
</tbody>
</table>

The solutions for i.v. administration were prepared with 50% PEG 600. For oral administration, the drugs were dissolved in undiluted PEG 600.

With TAT at 0.2 mm, which is the minimal measurable concentration, Pₘₚ of TAT was identical between the control and the 10% PEG 600 groups (Fig. 5). From these results, it is suggested that 0.2 mm TAT remains in solution differently from 1 mm TAT. Accordingly, Pₘₚ could be obtained with 1 mm IDM and 0.2 mm TAT without the additives, in the same manner as with water-soluble drugs. However, for 5 mm IDM and 1 mm TAT, additives such as 10% PEG 600 were shown to be necessary.

The Pₘₚ values of IDM and TAT, which were obtained without precipitation during the experiment, were about 9x10⁻⁶ cm/s and 4x10⁻⁶ cm/s, respectively (Fig. 5). The value of IDM is almost equal to that of highly membrane-permeable propranolol obtained in our experiment (9.2x10⁻⁶ cm/s; BA=90%). On the other hand, the value of TAT was lower than that of atenolol¹ (6x10⁻⁶ cm/s; BA=50%). Accordingly, it could be stated that IDM is highly permeable but TAT is less permeable, comparing with these poorly water-soluble drugs under the perfectly solubilizing condition.

The BA values of IDM and TAT were about 95% and 23%, respectively (Table 3). The hepatic extraction ratio of TAT examined by comparing the AUC values after intraportal and intravenous administration was less than 0.4 (data not shown), indicating that the absorption ratio of TAT cannot exceed 0.4 if the hepatic first pass effect was considered. These in vivo results corresponded well with the data of estimation of in vitro permeability, indicating that the use of additives is helpful for evaluation of absorbability of poorly water-soluble drugs. It was also shown that Pₘₚ of poorly water-soluble drugs determined in the above in vitro experiment can be discussed in the same manner as that of water-soluble drugs.

The poorly water-soluble drugs used in the present study raise the problem of precipitation at high doses and marked decrease in absorbability. Therefore, for precise estimation of their absorbability, the exact rates of their precipitation and permeability should be determined by chamber experiments at concentrations that correspond to their in vivo intraluminal concentrations expected from their dosages. However, as
long as these drugs are in solution, the degree of absorbability can be estimated by comparing the $P_{app}$ value with that of highly soluble compound such as propranolol and atenolol\(^1\) at concentrations where precipitation does not occur during the experiment.

In the present study, several additives were shown not to affect the membrane permeability of drugs when their concentrations were about 10% or less. Also, the membrane permeability of poorly water-soluble drugs can be obtained in the same manner as that of water-soluble drugs by avoiding their precipitation during the experiment with the help of additives, and the membrane permeability was shown to closely reflect their in vivo absorbability. More detailed evaluation using a large variety of poorly water-soluble drugs is needed.

**Acknowledgements** The authors would like to thank Mr. Noriuki Kinoshita, Mr. Masaru Nakamura, Miss Maho Kojima and Miss Chiaki Kobayashi for their technical assistance.

**REFERENCES**


