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Scale-up of *in vitro* permeation assay data to human intestinal permeability using pore theory

Makoto Kataoka[a,*], Katsuaki Iwai[a], Yoshie Masaoka[a], Toshiyasu Sakane[b], Shinji Sakuma[a], Shinji Yamashita[a,**]

[a] Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan
[b] Department of Biopharmaceutics, Kyoto Pharmaceutical University, 5 Misasagi-Nakauchicho, Yamashina-ku, Kyoto 607-8414, Japan

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ABSTRACT

The aim of this study is to establish a theoretical method for the prediction of human intestinal permeability from *in vitro* permeation assay. Pore radius and porosity/length and ion selectivity of the paracellular pathway were calculated using the Renkin function using permeabilities of mannitol and urea and potential difference study to evaluate paracellular permeability in Caco-2 cell monolayer; they were calculated to be 5.91 Å, 7.51 cm^{-1} and 2.75, respectively. These values in the human epithelium were calculated from the reported intestinal permeability. The area factor, which can correct the difference in the transcellular permeability between Caco-2 cell monolayer and human epithelium, was obtained using the ratio of permeability of high lipophilicity compounds (human/Caco-2) and was calculated to be 13.3. Paracellular and transcellular permeabilities of 9 compounds in human epithelium were estimated on the basis of the characteristics of the paracellular pathway using physicochemical properties of compounds and the area factor, respectively. Human intestinal permeabilities were predicted by the sum of estimated transcellular and paracellular permeabilities. A linear correlation whose slope and intercept were nearly 1 and 0, respectively, was observed between predicted and reported human intestinal permeabilities. We successfully predicted human intestinal permeability from *in vitro* data.

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1. Introduction

Many experimental techniques, not only *in vivo*/*in situ* experiments with rodents, dogs and monkeys, but also *in vitro* experiments with several kinds of cultured cell lines, have been used to estimate the intestinal absorption of drug candidates. In particular, monolayers of Caco-2 cells (Caco-2 cell monolayers), originated from human colorectal adenocarcinoma, are now widely accepted and used as a potent *in vitro* model to predict oral absorption in humans (Hidalgo et al., 1989; Artursson and Karlsson, 1991; Yamashita et al., 1997). For the last two decades, many protocols to culture Caco-2 cell monolayers and to measure their permeability have been reported (Chong et al., 1997; Lentz et al., 2000; Yamashita et al., 2002). Although these reports clearly indicate a significant correlation between drug permeabilities and fractions of absorbed dose in humans, the absolute permeability of each drug is quite different among reports. For instance, the mannitol permeability to Caco-2 cell monolayers ranges from 0.38×10^{-6} to 3.23×10^{-6} cm/s (Yazdanian et al., 1998; Rubas et al., 1993; Chong et al., 1996). Not only experimental conditions, such as volume and agitation of solutions, but also the structure of monolayers, such as those of microvillus and tight junction, may cause considerable differences because Caco-2 cells differentiate and then form the microvillus structure in the apical membrane and tight junction between cells during an appropriate culture period after seeding onto semipermeable membrane.

There are transcellular and paracellular routes as drug permeation pathways across epithelial models and the epithelial membrane in human intestine. Permeation via the transcellular route could depend on the lipophilicity of compounds, while permeation via the paracellular route could depend on the molecular size and charge. By analyzing the permeation of compounds through each permeation pathway, permeabilities of compounds across various membranes could be characterized, regardless of the membrane types that are used for the assay.

In this study, we investigated the contribution of transcellular and paracellular routes to the membrane permeation of compounds using pore theory. In addition, we attempted to predict the human intestinal permeability from *in vitro* membrane permeation assay.

* Corresponding author. Tel.: +81 72 866 3126; fax: +81 72 866 3126.
** Corresponding author. Tel.: +81 72 866 3125; fax: +81 72 866 3126.
E-mail addresses: makoto@pharm.setsunan.ac.jp (M. Kataoka), shinji@pharm.setsunan.ac.jp (S. Yamashita).

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2. Theory

The apparent permeability (P_{app}) of passive transport across the intestinal membrane or model membranes is the sum of permeabilities of transcellular and paracellular transport (P_{trans} and P_{para}, respectively):

$$P_{app} = P_{trans} + P_{para} \quad (1)$$

For estimation of the passive permeability through the paracellular pathway, the Renkin function was used. Adson et al. (1994) described a quantitative approach to estimate passive transport through the paracellular pathway. This approach utilized the size, the diffusion coefficient and the charge (neutral, cationic or anionic) of the drug molecule (Eqs. (2)–(4)):

$$\text{neutral} \quad P_{para}(\text{neutral}) = \frac{\varepsilon D}{\delta} F\left(\frac{r}{R}\right) \quad (2)$$

$$\text{cation} \quad P_{para}(\text{cation}) = \frac{\varepsilon D}{\delta} F\left(\frac{r}{R}\right)\left(\frac{\kappa}{1-e^{-\kappa}}\right) \quad (3)$$

$$\text{anion} \quad P_{para}(\text{anion}) = \frac{\varepsilon D}{\delta} F\left(\frac{r}{R}\right)\left(\frac{\kappa}{e^{\kappa}-1}\right) \quad (4)$$

where $\varepsilon, D, r, R, F(r/R)$ and κ are the porosity, the diffusion coefficient of the molecule in the aqueous solution, the radius of the molecule, the apparent radius of the pore, the molecular sieving factor and the dimensionless electrochemical energy function across the pore with length δ, respectively.

The diffusion coefficient (D) in the aqueous solution is estimated using the equation of Stokes–Einstein (Eq. (5)):

$$D = \frac{k_B T}{6\pi\eta r} \quad (5)$$

where k_B, T, η and π are the Boltzmann constant, the absolute temperature, the viscosity of water and the circular constant, respectively.

The molecular radius (r) of drugs was estimated from their molecular volumes using Eq. (6):

$$r = \sqrt[3]{\frac{3V}{4\pi}} \quad (6)$$

where V is McGowan's molecular volume. V of each drug was estimated using simulation software (QMPRplus™, SimulationPlus, Inc., CA).

The molecular sieving function for cylindrical and spherical molecules is calculated on the basis of the Renkin function (Eq. (7)):

$$F\left(\frac{r}{R}\right) = \left(1-\frac{r}{R}\right)^2\left(1-2.104\left(\frac{r}{R}\right)+2.09\left(\frac{r}{R}\right)^3-0.95\left(\frac{r}{R}\right)^5\right) \quad (7)$$

Eq. (7) with each molecular radius of mannitol and urea were substituted to Eq. (8). The pore radius of a monolayer can be obtained by solving this formula:

$$\frac{P_{man}}{P_{urea}} = \frac{D_{man}\cdot F(r_{man}/R)}{D_{urea}\cdot F(r_{urea}/R)} \quad (8)$$

where P_{man} and P_{urea} are the permeabilities of mannitol and urea, respectively. D_{man} and D_{urea} are the diffusion coefficients of mannitol and urea, respectively. r_{man} and r_{urea} are the molecular radii of mannitol and urea, respectively.

The transcellular permeability to human intestine was predicted using an area factor, which is a correction coefficient for the difference in the absorptive surface area between Caco-2 cell monolayers and human intestine. The permeability in the paracellular pathway in human intestine was estimated by above equations. Then, the permeability to the human intestine was calculated as follows:

$$\text{Human intestinal permeability} = S_f \cdot P_{\text{trans Caco-2}} + P_{\text{para human}} \quad (9)$$

where S_f is the area factor, $P_{\text{trans Caco-2}}$ is the transcellular permeability to Caco-2 cell monolayers and $P_{\text{para human}}$ is the estimated paracellular permeability to the human intestine.

3. Materials and methods

3.1. Materials

Caco-2 cell line was purchased from American Type Culture Collection (Rockville, MD) at passages 17. Dulbecco's modified Eagle medium (D-MEM) was obtained from Sigma–Aldrich (St. Louis, MO). Non-essential amino acids (NEAA), fetal bovine serum (FBS), L-glutamate, trypsin (0.25%)–EDTA (1 mM) and antibiotic–antimycotic mixture (10,000 U/mL penicillin G, 10 mg/mL streptomycin sulfate and 25 μg/mL amphotericin B in 0.85% saline) were purchased from Gibco Laboratories (Lenexa, KS). Entero-STIM™ Differentiation Medium and MITO+™ Serum Extender were obtained from Becton Dickinson Bioscience (Bedford, MA). [^{14}C]D-Mannitol and [^{14}C]urea were purchased from PerkinElmer (Waltham, MA). Atenolol, carbamazepine, cimetidine, furosemide, ketoprofen, metoprolol, piroxicam, propranolol and ranitidine were obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents used were of the highest purity.

3.2. Preparation of Caco-2 cell monolayers

Caco-2 cells were cultured at 37 °C in humidified air with 5% CO_2 atmosphere using D-MEM supplemented with 10% FBS, 1% L-glutamate, 1% NEAA and 0.5% antibiotic–antimycotic solution (basic culture medium, BCM) in culture flasks (Becton Dickinson Bioscience, Bedford, MA). Cells were routinely subcultured at 90% confluence with trypsin–EDTA and seeded at a density of 90×10^4 cells per flask (75 cm^2).

In the traditional cell culture, Caco-2 cells were seeded onto polycarbonate filters (1.0 μm pore, 0.9 cm^2 growth area) in a cell culture insert (Becton Dickinson Bioscience, Bedford, MA) at a density of 6.4×10^4 cells/insert. The culture medium (0.8 mL in the insert and 2.0 mL in the well) was replaced every 48 h for the initial 6 days and every 24 h thereafter for 18–21 days.

In the short-term cell culture, 3-day and 5-day Caco-2 cell monolayers were prepared according to short-term methods established by Chong et al. (1997) and Yamashita et al. (2002), respectively. Cells were seeded on fibrillar collagen-precoated inserts (1.0 μm pore, 0.9 cm^2 growth area, Becton Dickinson Bioscience, Bedford, MA) at a density of 40×10^4 cells/insert. BCM was used as seeding medium and medium was replaced with Differentiation Medium (Entero-STIM™ Differentiation Medium with 0.1% MITO+™ Serum Extender) every 24 h after seeding for the 3-day Caco-2 cell monolayer and every 24 h from 2 days after seeding for the 5-day Caco-2 cell monolayer. At appropriate days after seeding, all Caco-2 cell monolayers were utilized for the transport study.

3.3. Drug transport study

Apical to basal permeabilities of compounds were measured in the presence of the pH gradient condition (apical pH: 6.5 and basal pH: 7.4). As the medium for the drug transport study (transport medium), Hank's balanced salts solution (HBSS) supplemented with 25 mM glucose was used in all studies after adjusting the pH to 6.5 or 7.4 with MES or HEPES. After both sides of the monolayers were incubated for 20 min with compound-free transport

medium at 37 °C, the medium containing each compound was introduced to the apical side. Thereafter, sample aliquots were taken from the basal side every 20 min for 1 h. The volume of the basal solution was maintained by adding an equal volume of fresh transport medium. Initial concentrations of drugs applied to the apical side were 10 μM for [^{14}C]D-mannitol and [^{14}C]urea and 100 μM for other compounds. All experiments were performed at 37 °C. The integrity of the monolayer was checked by measuring the transepithelial electrical resistance (TEER) with the Millicell-ERS system (Millipore Corporation, Bedford, MA) before and after the transport study. Significant difference in TEER value before and after the transport study was not observed (data not shown).

The apical-to-basal permeability (apparent permeability, P_{app} (cm/s)) of each compound was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0} \quad (10)$$

where dQ/dt is the appearance rate of compounds in the basal side (μmol/s), C_0 is the initial drug concentration in the apical side (μM) and A is the surface area of the monolayer (cm^2).

3.4. Diffusion potential across the membrane

The membrane was mounted between Ussing chambers, together with a support filter. The membrane in the chamber was equilibrated with the standard Ringer solution for 25 min. After both sides of the membrane were washed with fresh standard Ringer solution, the NaCl dilution potential was measured, initially by introducing 75 mM NaCl solution to the apical side of the membrane. After reaching a steady state, the change in the potential difference was monitored. The membrane (both sides) was washed and re-equilibrated with the standard Ringer solution. Then, 150 mM KCl solution was introduced to the apical side to measure the Na^+/K^+ bi-ionic potential. In all measurements of the diffusion potential, the basal solution was the standard Ringer solution.

3.5. Calculation of ion relative selectivity

Permeability ratios among Na^+, K^+ and Cl^- were calculated from two diffusion potentials, as described by Moreno and Diamond (1975), on the basis of the Goldman–Hodgkin–Katz equation:

$$\Delta V = \frac{RT}{F}\ln\frac{P_{K\gamma K}[K]_m + P_{Na\gamma Na}[Na]_m + P_{Cl\gamma Cl}[Cl]_m}{P_{K\gamma K}[K]_s + P_{Na\gamma Na}[Na]_s + P_{Cl\gamma Cl}[Cl]_s} \quad (11)$$

where P_i is the relative permeability coefficient for ion i, γ_i is the ion activity coefficient and $[i]_m$ and $[i]_s$ are ion concentrations in apical and basal solutions, respectively. ΔV represents the diffusion potential (NaCl dilution potential or Na^+/K^+ bi-ionic potential), which was corrected for asymmetric junction potentials developed at the tips of salt bridges on the basis of equations described by Barry and Diamond (1970). R, T and F are gas constant, absolute temperature and Faraday constant, respectively. Since the γ value of each ion is impossible to determine, the Guggenheim assumption (Barry and Diamond, 1970) that $\gamma = \gamma^+ = \gamma^-$ was employed. The ion permeability was determined as the permeability ratio of Cl^- to Na^+ (P_{Cl}/P_{Na}).

3.6. Analytical methods

The concentration of radiolabeled compounds in the sample was determined using a liquid scintillation counter (LSC 3500, Aloka, Tokyo, Japan). Unlabeled compounds in the sample were analyzed with a reversed-phase HPLC system (LC-10Avp Shimadzu Co., Kyoto, Japan) equipped with a variable wavelength ultraviolet detector (SPD-10Av, Shimadzu Co., Kyoto, Japan). Each compound was trapped in a C18 column (J'sphere ODS-H80 75 mm × 4.6 mm I.D., YMC, Kyoto, Japan) and was eluted with a mobile phase. HPLC conditions used to determine the concentration of each compound are listed in Table 1.

Table 1
HPLC conditions for determination of compounds.

Compounds	Wavelength (nm)	Mobile phase composition (A:B)
Atenolol	226	97:3
Carbamazepine	285	75:25
Cimetidine	230	70:30
Furosemide	280	72:28
Ketoprofen	260	65:35
Metoprolol	225	85:15
Piroxicam	326	68:32
Propranolol	228	79:21
Ranitidine	320	97:3

Column temperature and flow rate were 40 °C and 1.0 mL/min, respectively. Mobile phase A was water with 50 mM phosphate (pH 2.5). Mobile phase B was acetonitrile.

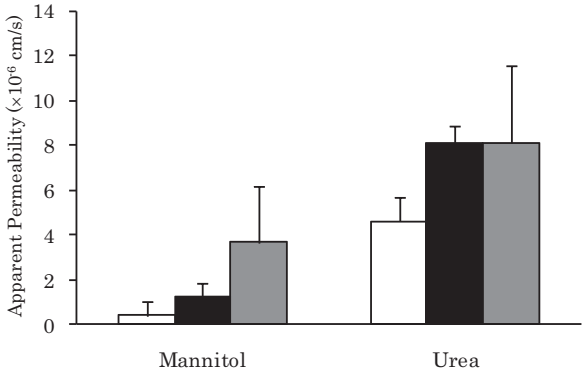

Fig. 1. Permeability of mannitol and urea to various membranes. The data are expressed as the mean ± s.d. of at least three independent experiments. Open, closed and shaded bars express the values obtained with 21-day, 5-day and 3-day Caco-2 cell monolayers, respectively.

4. Results

4.1. Characterization of paracellular pathway in various membranes

4.1.1. Pore radius, porosity and length of paracellular pathway

Fig. 1 shows permeabilities of mannitol and urea to Caco-2 cell monolayers obtained by various culture methods. The permeabilities of urea are much higher than those of mannitol regardless of the membrane used. The permeation of each compound across Caco-2 cell monolayers was increased with the decrease in the culture period. The pore radius of the paracellular pathway in each membrane was estimated from the permeability ratio of mannitol and urea on the basis of the Renkin function with physicochemical properties of both compounds (Table 2). Calculated pore radii in 21-day, 5-day and 3-day Caco-2 cell monolayers are shown in Table 3.

Table 2
Physicochemical properties of mannitol and urea.

Compound	M.W.	Molecular volume[a] (cm^3/mol)	r (Å)	D (×10^{-6} cm^2/s)
Mannitol	180	182	4.16	7.88
Urea	60.6	56.0	2.81	11.7

[a] Predicted with QMPRPlus™ (SimulationPlus, Inc.).

Table 3
Characteristics of various membranes.

Membrane	Pore radius (Å)	ε/δ (cm^{-1})	P_{Cl}/P_{Na}	U_{Cl}/U_{Na} [d]	κ
21-Day Caco-2[a]	5.91	7.15	0.102	1.61	2.75
5-Day Caco-2[b]	7.58	5.47	0.374	1.61	1.45
3-Day Caco-2[c]	18.5	1.41	–	–	–

[a] Traditional culture method.
[b] Modified original short-term culture method constructed by Yamashita et al. (2002).
[c] Original short-term culture method (Chong et al., 1997).
[d] The free mobility ratio in an aqueous solution of Cl^- relative to that of Na^+ (Artursson et al., 1993).

ε/δ in each membrane was calculated as a hybrid parameter of porosity and length of paracellular pathway on the basis of the Renkin function using the permeability of mannitol and pore radius (Table 3).

4.1.2. Ion selectivity in paracellular pathway

In the experiment on the diffusion potential, 3-day Caco-2 cell monolayer was not suitable for mounting between the chambers owing to the short period of culture. Thus, the measurement of the diffusion potential was performed on the two other membranes. The permeability ratios of Cl^- and Na^+ ions in the two membranes were calculated from the diffusion potential and are summarized in Table 3, together with the free mobility ratio of these ions in aqueous solution. Although the mobility of Cl^- ion in aqueous solution is higher than that of Na^+ ion, the permeation of Cl^- ion through the pore in all membranes is slower than that of Na^+ ion, indicating that the paracellular pathway clearly shows cation selectivity; the ion selectivity (κ) was elucidated from the mobility ratios of Cl^- and Na^+ ions in membranes and aqueous solution (Table 3).

4.2. Contribution of transcellular pathway to in vitro membrane permeation

Fig. 2A shows apparent permeability of atenolol and propranolol to various Caco-2 cell monolayers. Using various parameters for the paracellular pathway, pore radius, ε/δ and κ, the paracellular permeabilities of atenolol and propranolol were calculated on the basis of the Renkin function (Fig. 2B). The calculated paracellular permeability of atenolol was similar to that of propranolol in all monolayers because both compounds are positively charged in a physiologic condition and their molecular volumes are similar. The contribution of the transcellular pathway to the apparent permeability was evaluated from observed and estimated paracellular permeabilities of atenolol and propranolol to each monolayer. As shown in Fig. 2C, the contribution of the transcellular permeability was significantly different between atenolol and propranolol. The contribution of transcellular permeability to the permeation of atenolol was only 0.0–0.1%, while that of propranolol was 98.3–99.2%.

4.3. Analysis of human intestinal permeability of various compounds

The pore size of the paracellular pathway in the human intestine was reported to be 10 Å (Fine et al., 1995). The value of the permeability of mannitol (0.28×10^{-4} cm/s) to the human intestine was provided by Prof. Amidon (University of Michigan). Thus, the relationship between porosity and length of the pore (ε/δ) was estimated to be 3.79 cm^{-1} from Eq. (2). Assuming that atenolol permeate through only paracellular pathway in the human intestine, the ion selectivity in the human intestine can be estimated

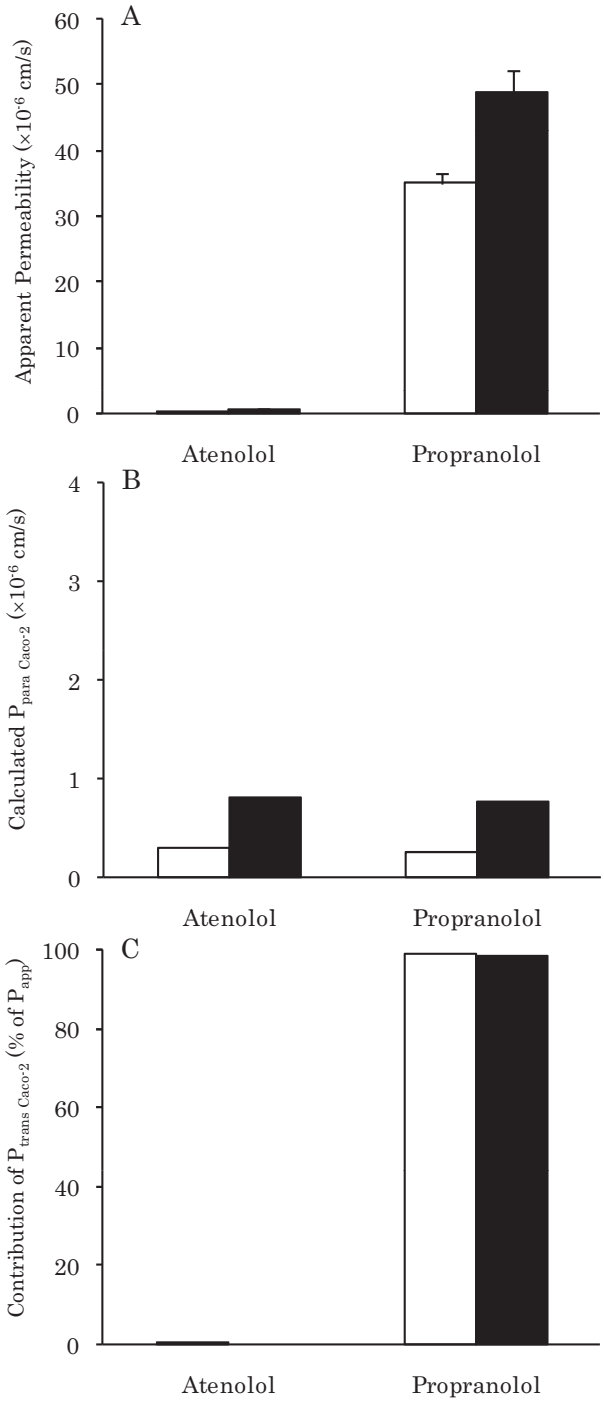

Fig. 2. (A) Permeability of atenolol and propranolol to various Caco-2 cell monolayers, (B) paracellular permeability of atenolol and propranolol and (C) contribution of transcellular permeation to total permeation across various monolayers. Open and closed bars express the values obtained with 21-day and 5-day Caco-2 cell monolayers, respectively.

form atenolol permeability. The ion selectivity (κ) was estimated to be 0.994 from the atenolol permeability (0.2×10^{-4} cm/s) in the human intestine (Kasim et al., 2004) from Eq. (3) with 3.79 cm^{-1} for the value of ε/δ. Physicochemical properties of compounds were estimated and were shown in Table 4. The paracellular and transcellular permeation to human intestine was divided from by subtracting reported permeability to calculated paracellular permeability on the basis of Renkin function with properties of the pore (Table 4). The contribution ratio of transcellular perme-

Table 4
Physicochemical properties and intestinal permeability of various compounds.

Compound	M.W.	Charge	Molecular volume[a] (cm^3/mol)	r (Å)	D ($\times10^{-6}$ cm^2/s)	$P_{eff\ human}$ [b] ($\times10^{-4}$ cm/s)	$P_{para\ human}$ [c] ($\times10^{-4}$ cm/s)	$P_{trans\ human}$ [d] ($\times10^{-4}$ cm/s)	Contribution ratio [e] (%)
Atenolol	266	+	301	4.92	6.67	0.20	0.20	0.00	0.19
Carbam azepine	236	+	203	4.32	7.60	4.30	0.38	3.92	91.3
Cimetidine	252	+	245	4.60	7.14	0.35	0.28	0.07	19.5
Furosemide	331	–	270	4.75	6.91	0.30	0.09	0.21	70.2
Ketoprofen	254	–	217	4.41	7.43	8.70	0.13	8.57	98.6
Metoprolol	267	+	322	5.04	6.52	1.34	0.18	1.16	86.8
Piroxicam	331	–	287	4.85	6.77	10.4	0.08	10.3	99.2
Propranolol	259	+	308	4.96	6.61	2.72	0.19	2.53	93.0
Ranitidine	314	+	322	5.04	6.52	0.43	0.18	0.25	58.8

[a] Predicted with QMPRPlus™ (SimulationPlus, Inc.).
[b] Data taken from Kasim et al. (2004).
[c] $P_{para\ human}$ was calculated on the basis of Renkin function with properties of the pore.
[d] $P_{trans\ human}$ was obtained by subtracting $P_{para\ human}$ from $P_{eff\ human}$.
[e] Contribution ratio of transcellular permeation to total permeation of compounds.

Table 5
Scale-up of *in vitro* permeation data with 21-day Caco-2 cell monolayer to human intestinal permeability.

Compound	$P_{app\ Caco\text{-}2}$ [a] ($\times10^{-6}$ cm/s)	$P_{para\ Caco\text{-}2}$ [b] ($\times10^{-6}$ cm/s)	$P_{trans\ Caco\text{-}2}$ [c] ($\times10^{-6}$ cm/s)	$P_{predicted\ trans\ human}$ [d] ($\times10^{-4}$ cm/s)	P_{human} [d] ($\times10^{-4}$ cm/s)	$P_{predicted}$ [e] ($\times10^{-4}$ cm/s)
Atenolol	0.24	0.19	0.05	0.01	0.20	0.21
Carbam azepine	50.1	0.90	49.2	6.56	0.38	6.93
Cimetidine	0.39	0.34	0.05	0.01	0.28	0.29
Furosemide	0.09	0.02	0.07	0.01	0.09	0.10
Ketoprofen	42.5	0.00	42.5	5.66	0.13	5.79
Metoprolol	11.1	0.20	10.9	1.45	0.18	1.63
Piroxicam	90.9	0.00	90.9	12.1	0.08	12.2
Propranolol	18.5	0.20	18.3	2.44	0.19	2.63
Ranitidine	0.36	0.18	0.18	0.02	0.18	0.20

[a] Permeability of compounds to 21-day Caco-2 cell monolayer.
[b] $P_{para\ Caco\text{-}2}$ was calculated on the basis of Renkin function with properties of the pore.
[c] $P_{trans\ Caco\text{-}2}$ was calculated by subtracting $P_{para\ Caco\text{-}2}$ from $P_{app\ Caco\text{-}2}$.
[d] $P_{predicted\ trans\ human}$ was calculated by product of area factor (13.3) and $P_{trans\ Caco\text{-}2}$.
[e] $P_{predicted}$ was the sum of product of $P_{predicted\ trans\ human}$ and $P_{para\ human}$.

ation on total permeation ranged from 0.19% (atenolol) to 99.2% (piroxicam).

4.4. Scale-up of in vitro membrane permeability to human intestinal permeability

4.4.1. Apparent permeability to Caco-2 cell monolayers

Apparent permeabilities of atenolol, carbamazepine, cimethidine, furosemide, ketoprofen, metoprolol, piroxicam, propranolol and ranitidine to 21-day and 5-day Caco-2 cell monolayers were measured under pH gradient conditions (Tables 5 and 6). Fig. 3 shows the relationships between the fraction of absorbed dose in humans and the apparent permeability to Caco-2 cell monolayers, together with reported human intestinal permeability (Kasim et al., 2004). As shown in Fig. 3, 10–1000-fold differences were observed between the human intestinal permeability and the apparent permeability to Caco-2 cell monolayers.

4.4.2. Analysis of in vitro membrane permeability of various compounds

The paracellular permeabilities of 9 compounds to each Caco-2 cell monolayers were calculated on the basis of Renkin function

Table 6
Scale-up of *in vitro* permeation data with 5-day Caco-2 cell monolayer to human intestinal permeability.

Compound	$P_{app\ Caco\text{-}2}$ [a] ($\times10^{-6}$ cm/s)	$P_{para\ Caco\text{-}2}$ [b] ($\times10^{-6}$ cm/s)	$P_{trans\ Caco\text{-}2}$ [c] ($\times10^{-6}$ cm/s)	$P_{predicted\ trans\ human}$ [d] ($\times10^{-4}$ cm/s)	$P_{para\ human}$ ($\times10^{-4}$ cm/s)	$P_{predicted}$ [e] ($\times10^{-4}$ cm/s)
Atenolol	1.11	0.83	0.28	0.02	0.20	0.22
Carbam azepine	54.4	1.80	52.6	4.59	0.38	4.97
Cimetidine	1.00	0.92	0.08	0.01	0.28	0.29
Furosemide	0.60	0.21	0.40	0.03	0.09	0.12
Ketoprofen	77.4	0.30	77.1	6.73	0.13	6.85
Metoprolol	16.5	0.00	16.5	1.44	0.18	1.62
Piroxicam	197	0.00	197	17.2	0.08	17.3
Propranolol	23.4	0.60	22.8	1.99	0.19	2.18
Ranitidine	1.36	0.82	0.54	0.05	0.18	0.22

[a] Permeability of compounds to 5-day Caco-2 cell monolayer.
[b] $P_{para\ Caco\text{-}2}$ was calculated on the basis of Renkin function with properties of the pore.
[c] $P_{trans\ Caco\text{-}2}$ was calculated by subtracting $P_{para\ Caco\text{-}2}$ from $P_{app\ Caco\text{-}2}$.
[d] $P_{predicted}$ trans human was calculated by product of area factor (8.7) and $P_{trans\ Caco\text{-}2}$.
[e] $P_{predicted}$ was the sum of product of $P_{predicted\ trans\ human}$ and $P_{para\ human}$.

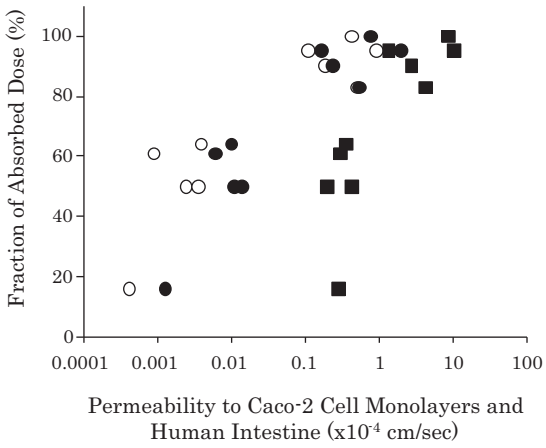

Fig. 3. Correlation of the fraction of absorbed dose in humans with the apparent permeability to the human intestine (■) and 21-day (○) and 5-day (●) Caco-2 cell monolayers. The data are expressed as the mean of at least three independent experiments. Human intestinal permeabilities of compounds are cited from a report by Kasim et al. (2004).

with properties of the pore with physicochemical properties of each compound (Tables 5 and 6). Then, the transcellular permeabilities to each monolayer were obtained by subtracting calculated paracellular permeability from apparent permeability (Tables 5 and 6). Paracellular permeabilities of carbamazepine, ketoprofen, metoprolol, piroxicam and propranolol were significantly low compared to transcellular permeabilities.

4.4.3. Prediction of human intestinal permeability

The difference in the effective surface area between the human intestine and Caco-2 cell monolayers could be defined as the ratio of the transcellular permeability of lipophilic compounds, assuming that lipophilic compounds show over 90% of contribution of transcellular permeation on human intestinal permeability. Area factors (S_f) were obtained as 13.3 for 21-day Caco-2 cell monolayer and 8.7 for 5-day Caco-2 cell monolayer from the average ratio of transcellular permeabilities of carbamazepine, ketoprofen, piroxicam and propranolol between the human intestine and Caco-2 monolayers. The paracellular permeability of each compound to human intestine was estimated on the basis of the Renkin function using the characteristics of the paracellular pathway. Then, the permeability to the human intestine was calculated using Eq. (9). The estimated human intestinal permeabilities of compounds to both Caco-2 cell monolayers indicate significant correlations with observed permeabilities to the human intestine (Fig. 4).

5. Discussion

The traditional method of Caco-2 cell culture (21-day Caco-2 cell monolayer), which needs 21 days for the differentiation period, might be labor-intensive, time-consuming and incompatible with the high-throughput screening of numerous compounds. That is why many researchers have tried to develop short-term and simplified culture methods of Caco-2 cells. A short-term culture method (3-day Caco-2 cell monolayer) was developed by Chong et al. (1997). Yamashita et al. (2002) established a 5-day culture method (5-day Caco-2 cell monolayer) by modifying the original short-term culture method. Although the same Caco-2 cell line is used, a significant difference in the permeabilities of mannitol and urea among 21-day, 5-day and 3-day Caco-2 cell monolayers is observed (Fig. 1). This difference could be due to the structure of monolayers prepared with each culture method.

It is well known that the tight junction plays an important role in restricting the movement of compounds through the paracellular pathway. Adson et al. (1995) described that the paracellular permeability could be estimated using pore theory on the basis of the Renkin function. Thus, we performed characterization of the paracellular pathway in three Caco-2 cell monolayers with the Renkin function. Pore radii and the relationships between the porosity and length of the pore of the paracellular pathway in various membranes can be calculated from the ratio of permeabilities between mannitol and urea, since both compounds are considered to permeate the membrane predominantly through the paracellular pathway (Gan et al., 1993; Knipp et al., 1997). Because the porosity and pore length could not be determined individually, ε/δ was obtained and used as a hybrid of both parameters. The ion selectivity was different between membranes, which could affect the permeation of ionic compounds through the paracellular pathway. Adson et al. (1995) estimated that the pore radius, κ and ε/δ in the paracellular pathway of their Caco-2 cell monolayers were 12 ± 1.9 Å, 0.63–0.78 and 1.21 cm^{-1}, respectively. These properties for the paracellular pathway in their Caco-2 cell monolayers are similar to those in the 3-day Caco-2 cell monolayer used in our study. These differences in the properties of the paracellular pathway in various membranes indicated that the difference not only in culture conditions but also in laboratories affected the formation of the tight junction, although all monolayers originated from the same cell line.

When the paracellular permeation of cationic drugs such as atenolol was estimated, the contribution rate of paracellular pathway in 5-day Caco-2 cell monolayer (79.2%) was much higher than that in 21-day Caco-2 cell monolayer (74.8%), whereas the pore radius of 5-day Caco-2 cell monolayer was lesser than that of 21-day Caco-2 cell monolayer. This discrepancy is due to the fact that not only the pore radius but also the ion selectivity of the pore is the important factor to determine the paracellular permeation of ionic drugs. The ion selectivity of 5-day Caco-2 cell monolayer is much lower than that of 21-day Caco-2 cell monolayer, indicating that paracellular permeability of cationic drugs in 21-day Caco-2 cell monolayer might be relatively large compared to that in 5-day Caco-2 cell monolayer. Therefore, the contribution rate of paracellular pathway of drugs does not comply with the size of pore radii of the monolayers.

It was assumed that the permeation of compounds through the transcellular route indicates a similar permeability for all membranes used because the permeation of compounds via the transcellular route basically depends only on their lipophilicity. In contrast, the permeation of compounds through the paracellular pathway is often changed because it is affected by its own properties, such as pore radius, κ and ε/δ. Thus, the permeability of compounds to various membranes could be corrected by estimation of the paracellular permeability on the basis of the Renkin function. As paracellular properties of standard Caco-2 cell monolayer, it was assumed that mannitol permeability was 0.5×10^{-6} cm/s and values of ε/δ and κ were obtained from 21-day Caco-2 cell monolayer. The pore size of standard Caco-2 cell monolayer was calculated as 6.11 Å. The paracellular permeability of each compound to standard paracellular pathway was estimated by Renkin function using these properties and therefore the permeability to standard Caco-2 cell monolayer was obtained from sum of transcellular permeability obtained from 21-day and 5-day Caco-2 cell monolayers and estimated paracellular permeability. In Fig. 5, our theory to standardize the membrane permeability of compounds clearly indicates better correlation among standardized permeabilities obtained from both membranes. Caco-2 cell monolayer should be validated by measurement of reference compounds such as mannitol, atenolol and propranolol across monolayers obtained at same time. The per-

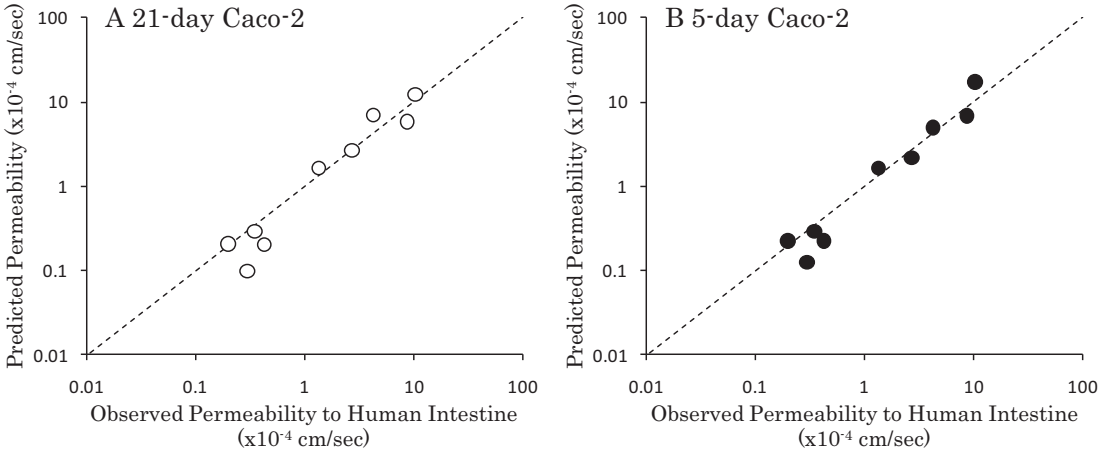

Fig. 4. Relationship of the human intestinal permeability predicted from *in vitro* permeation assay with the observed human intestinal permeability. (A) Predicted permeability from 21-day Caco-2 cell monolayer vs. observed permeability to the human intestine and (B) predicted permeability from 5-day Caco-2 cell monolayer vs. observed permeability to the human intestine. The dotted line represents agreement of the two parameters.

meability of drug candidates could be evaluated relative to that of reference drugs. The established procedure to standardize the permeability enables to directly compare the permeability of compounds with other results obtained with monolayers of different batches.

Lennernäs et al. (1992) developed an *in vivo* single-pass perfusion technique to measure the intestinal permeability in humans. Using this perfusion technique, the permeabilities of about thirty compounds have been reported (Kasim et al., 2004). However, although this technique is very valuable to estimate human intestinal permeability, this assay could not be performed on all compounds, especially on those under development, owing to a lack of safety evidence for human use. Recently, *in silico* techniques have been developed to predict the oral absorption of drugs in humans (Agoram et al., 2001; Lennernäs, 2007). These methods often require knowledge of the permeability to the human intestine to provide precise prediction as an output. Therefore, it is very important to estimate the human intestinal permeability of compounds from *in vitro* permeation study. Significant difference in the permeability of compounds between the human intestine and Caco-2 monolayers was observed (Fig. 3). This difference is likely to be due to the difference in the effective surface area and the tight junction between the human intestine and Caco-2 cell monolayers. The intestinal epithelium forms a villous structure to enlarge the surface area for efficient absorption of nutrients. Thus, the effective surface area of the intestine could be much larger than that calculated assuming that the intestine is just a cylinder. In the case of cell-based membranes, the effective surface area is the same as the culture area of inserts. The ratio of effective surface area between human intestine and Caco-2 cell monolayer (human intestine/Caco-2) enables to correct the effective permeation area. In the case of the paracellular pathway, the structure of the pore could also be different between epithelial membranes in the intestine and Caco-2 cell monolayers. The contribution ratio of transcellular permeation of atenolol was calculated to be quite low compared with that of propranolol (Fig. 2C), suggesting that transcellular permeability of atenolol could be ignorable. Assuming that atenolol permeates only through the paracellular pathway, the ion selectivity values in cell-based membranes were calculated as 2.22 for 21-day Caco-2 cell monolayer and 1.15 for 5-day Caco-2 cell monolayer. Since the value of each compound is similar to

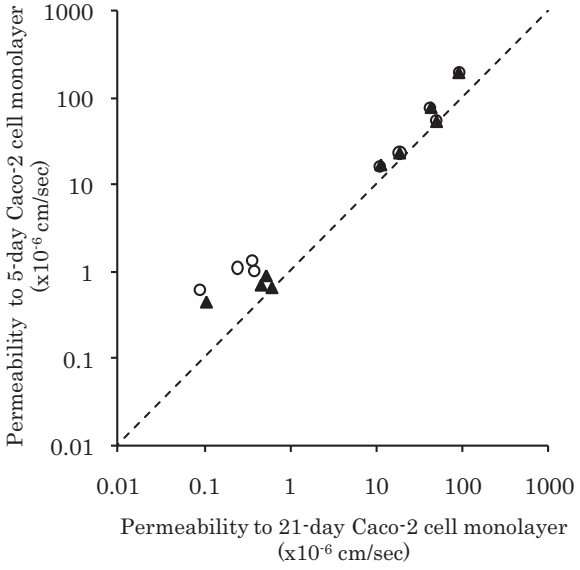

Fig. 5. Intermembrane relationship of the observed (○) and standardized (▲) permeability of compounds.

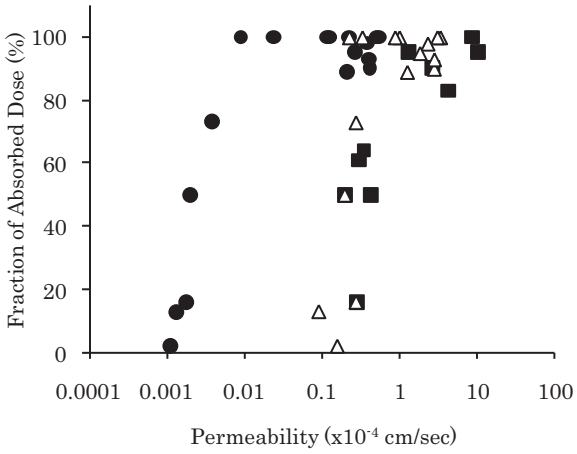

Fig. 6. Correlation between the fraction of absorbed dose in humans and the apparent permeability to the human intestine and Caco-2 cell monolayers. The permeabilities of compounds to Caco-2 cell monolayers (●) and the human intestine (■) were obtained from reports by Artursson and Karlsson (1991) and Kasim et al. (2004), respectively. Open triangles indicate the predicted human intestinal permeability from reported apparent permeability to Caco-2 cell monolayers by Artursson and Karlsson (1991).

that obtained from the diffusion potential study, the ion selectivity in the human intestine could be estimated from the permeability of atenolol. The contribution rate of transcellular permeation of atenolol in the human intestine was calculated to be more or less on naught (0.19%), indicating that relevance of the procedure to obtain the ion selectivity could be demonstrated. Because the paracellular permeability of compounds can be estimated on the basis of the Renkin function using the parameters characterizing the pore and compounds, the difference in the paracellular permeability between the human intestine and Caco-2 cell monolayers can be corrected. Thus, the transcellular and paracellular permeabilities in the human intestine were predicted with *in vitro* permeation assay and *in silico* estimation, respectively. Using this theory, we successfully predicted the human intestinal permeability of compounds (Fig. 4). The data set of this study might be few (nine drugs). However, nine drugs could be enough to demonstrate our idea to estimate human intestinal drug permeability from *in vitro* data, because the properties of model drugs used in this study are variety. In order to validate our theory, the same prediction was performed on the permeability to Caco-2 cell monolayers previously reported by another group (Artursson and Karlsson, 1991). As a result of the prediction, the relationship between the predicted intestinal permeability and the fraction of absorbed dose was found to be the same as that observed in human intestinal permeability (Fig. 6). This agreement suggested that our theory could be useful for estimation of the intestinal permeability in humans from *in vitro* permeation assay using cell-based membranes.

6. Conclusions

In this study, we have established a theoretical method to enable precise prediction of the permeability to the human intestine from *in vitro* permeation assay.

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