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Optimized conditions of bio-mimetic artificial membrane permeation assay

Kiyohiko Sugano [a,*], Hirokazu Hamada [a], Minoru Machida [a], Hidetoshi Ushio [b], Kimitoshi Saitoh [a], Katsuhide Terada [c]

[a] *Drug Metabolism and Pharmakokinetics Laboratory, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan*

[b] *Quality Assurance Department, Chugai Pharmaceutical Co., Ltd., 5-5-1 Ukima, Kita-Ku, Tokyo 115-8543, Japan*

[c] *Department of Pharmaceutics, School of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan*

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Abstract

Effects of pH and co-solvents on the bio-mimetic artificial membrane permeation assay were investigated to determine the optimal conditions for the prediction of oral absorption. The permeability (P_{am}) of 33 structurally diverse drugs to the PC/PE/PS/PI/CHO/1,7-octadiene membrane system (bio-mimetic lipid (BML) membrane) was measured at pH 5.5, 6.5, and 7.4. The pH dependence of P_{am} was in accordance with the pH partition theory. The better prediction of oral absorption (fraction of a dose absorbed) was shown under the pH 5.5 condition ($r = 0.866$, $n = 25$) and/or pH 6.5 ($r = 0.865$, $n = 28$), rather than pH 7.4 ($r = 0.767$, $n = 24$). Then, the appropriate conditions for determining the permeability of poorly soluble compounds were examined. Dimethysulfoxide (DMSO), ethanol (EtOH) and polyoxyethyleneglycol 400 (PEG 400) were added up to 30% to the transport medium as solubilizers. DMSO, EtOH and PEG 400 decreased P_{am} of hydrocortisone and propranolol. For example, DMSO (30%) decreased P_{am} of hydrocortisone by 60% and by 70% in the case of propranolol. DMSO and PEG 400 also decreased P_{am} of ketoprofen. In contrast, EtOH produced an opposite effect on permeability, i.e. an increased P_{am} of ketoprofen. Therefore, the high concentration of these co-solvents could lead to the under- or overestimation of drug permeability.

Keywords: Permeability; pH; DMSO; EtOH; PEG; Intestinal absorption

1. Introduction

Pharmacokinetics are widely recognized as an important factor in the drug discovery/development process, because many candidate compounds have been eliminated after starting clinical studies, due to inadequate absorption, distribu-

* Corresponding author. Tel.: +81-550-87-6781; fax: +81-550-87-5397.

E-mail address: suganokyh@chugai-pharm.co.jp (K. Sugano).

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tion, metabolism, excretion and pharmacokinetics (ADME/PK). Therefore, the ADME/PK study is usually incorporated in the drug-discovery process (Venkatesh and Lipper, 2000). Recently, the drug-discovery/development process was accelerated following rapid progress in drug synthesis by combinatorial chemistry (CC) and high throughput screening (HTS) based on the in-vitro pharmacological assay. Therefore, the ADME/PK study has remained as the bottleneck of the drug discovery/development process. Among many ADME/PK factors, gastrointestinal absorption often becomes a key problem. Although oral absorption includes several different processes, drug permeability to the intestinal membrane is the most important factor in defining the oral absorption. Historically, cell-based systems (e.g. Caco-2, MDCK) have been the most widely used as permeability assay (Hidalgo et al., 1989; Artursson and Karlsson, 1991). However, they have limitations for some classes of compounds and are very labor-intensive (Chong et al., 1996).

Parallel artificial membrane permeation assay (PAMPA) was first introduced by Kansy et al., as a rapid, 96-well plate technology-based in-vitro system for the evaluation of passive transcellular permeability (Kansy et al., 1998). PAMPA is an application of the filter supported lipid membrane system (Thompson et al., 1980). As an in-vitro permeation assay during the early stages of the drug discovery process, PAMPA is used by many pharmaceutical companies. In the Kansy's PAMPA system, phosphatidylcholine (PC) was simply used as a membrane constituent. Recently, we reported that utilization of the PC (0.8%)/phosphatidylethanolamine (PE, 0.8%)/phosphatidylserine (PS, 0.2%)/phosphatidylinositol (PI, 0.2%)/cholesterol (CHO, 1.0%) system (bio-(intestinal)-mimetic lipid (BML)), which has a similar lipid composition to intestinal brush border membrane (Proulx, 1991), increased the predictability of oral absorption (Sugano et al., 2001).

In the case of PAMPA with Kansy's simple PC system, artificial membrane permeability values at two pH conditions, namely pH 6.5 and pH 7.4, were used to predict oral absorption, because, with one pH condition, predictability is insufficient (Kansy et al., 1998). The measurement at pH 7.4, which appears not to be a typical pH of the small intestine (Maxwell et al., 1968), helps to avoid the underestimation of the permeability of some basic compounds. In the case of the BML membrane, an adequate predictability of oral absorption could be obtained at a typical intestinal pH condition, namely pH 6.5 (Sugano et al., 2001). However, the effect of pH on the predictability of the BML membrane system has not been clarified in detail.

In addition, to measure the permeability by PAMPA, compounds have to be dissolved to specific concentrations in a water-based medium. However, some classes of compounds are poorly soluble. To evaluate the permeability of poorly soluble compounds, several co-solvents have to be added to the medium as solubilizers. Therefore, it is important to clarify the effect of co-solvents on PAMPA.

In the present study, the optimal condition of BML membrane permeation assay is discussed, focusing on the effect of pH condition and the co-solvents.

2. Materials and methods

2.1. Materials

Sulpiride, guanabenz, metoprolol, sulfasalazine, atenolol, ranitidine, nadolol, furosemide, acycloguanosine (acyclovir), acebutolol, cefuroxime, ceftriaxone, cytarabine, pindolol, doxycycline, tetracycline, naltrexone, practolol, timolol, propranolol, ketoprofen, hydrocortisone, hydrochlorothiazide, amiloride, enalapril, oxytetracycline, penicillin V, procainamide, L-α-phosphatidylserine (PS), L-α-phosphatidylinositol (PI), and cholesterol (CHO) were purchased from Sigma Chemical (St. Louis, MO). L-α-phosphatidylcholine (PC) and L-α-phosphatidylethanolamine (PE) were purchased from Nippon Oil & Fats corporation (Tokyo, Japan). Quinidine, stearic acid (SA), and 1,7-octadien were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Mordant yellow 5 (olsalazine) was purchased from Aldrich Chemical Company (Milwaukee, WI). Practolol

was purchased from Tocris Cookson (Northpoint, UK). Chlorothiazide was purchased from Alexis corporation (San Diego, CA). Pravastatin was extracted from marketed tablets. Other reagents were of analytical grade. The hydrophobic filter plate (Durapore, pore size 0.45 μm) was purchased from Millipore corporation (Bedford, MA).

2.2. Permeability studies

Permeability studies were performed in the same manner as described previously (Kansy et al., 1998; Sugano et al., 2001). A 96-well microplate (acceptor compartment) was completely filled with 50 mM sodium phosphate buffer. The pH and co-solvents of the buffer are described in Section 3. A hydrophobic filter plate (donor compartment) was fixed on the buffer-filled plate. The filter surface was impregnated with 5 μl of lipid solution, which is composed of PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%)/1,7-octadiene (97.0%). 1,7-Octadiene is an irritant, and inhalation should be avoided. A 0.5 mM sample stock solution (100 μl) of the same buffer was added to the filter plate and incubated at 30 °C for 2 or 15 h. The filter plate was carefully removed. The concentration of the solution in the acceptor compartment was determined by UV spectroscopy, using the microtiter plate reader Spectramax 190 (Molecular Devices) at 250–450 nm at intervals of 10 nm. Reference solutions were prepared by diluting the sample stock solution to the same concentration as that with no membrane barrier. The permeability coefficient through the artificial membrane (P_{am}) was calculated using Eq. (1).

$$P_{am} = -2.303 \times \frac{V_{dn}V_{ac}}{V_{dn}+V_{ac}} \frac{1}{St} \log\left(1 - \frac{\text{flux}\%}{100}\right) \quad (1)$$

$$\text{flux}\% = \frac{OD_{ac}}{OD_{ref}} 100 \quad (2)$$

V_{dn} (ml) = volume of the donor compartment (0.1 ml)
V_{ac} (ml) = volume of the acceptor compartment (0.38 ml)
OD_{ac} = optical density of the solution of the acceptor compartment
OD_{ref} = optical density of the reference solution
S (cm^2) = membrane area (0.266 cm^2)
t (s) = incubation time.

3. Results and discussion

3.1. Optimization of pH condition

According to the pH partition theory, permeability of weak electrolytes is affected by the pH condition, following the change in compound dissociation (Hogben et al., 1959). Therefore, it is necessary to utilize appropriate pH condition for the adequate prediction of oral absorption. In physiological conditions, an acidic microclimate is found just above the epithelial cell layer in the upper part of the small intestine. In previous studies, the pH value of microclimate region was estimated to be 5.3–6.5 (Lucas, 1983; Said et al., 1986). However, pH 7.4 was often used in various in-vitro assays, including Kansy's PAMPA method, even though this pH appears not to be a typical physiological pH of the small intestine (Artursson and Karlsson, 1991; Kansy et al., 1998; Österberg et al., 2001). In the present study, we attempted to investigate the effect of the medium's pH on the predictability of oral absorption by the BML membrane system. We have selected pH 5.5, pH 6.5, and pH 7.4 for the medium pH of the donor and acceptor compartments. To compare the predictability, the permeability coefficient of 33 structurally diverse compounds was obtained in each condition. We mainly selected compounds whose Fa% was less than 90%, because prediction of this range of Fa% is anticipated for the in-vitro method in the drug-discovery process. Since PAMPA is a method for the assessment of passive transcellular permeation, compounds smaller than MW 200 were excluded to neglect the absorption via the paracellular pathway (Lennernäs, 1995). In addition, compounds absorbed via the active transport pathway were also excluded (Döppenschmitt et al., 1998; Bretschneider et al., 1999).

Table 1
Permeability, Fa%, and molecular properties

No.	Compound	Charge[a]	P_{am} ($\times 10^{-6}$ cm/s)[b]			Fa%[c]
			pH 5.5[d]	pH 6.5[d,e]	pH 7.4[d]	
1	Acebutolol	+	0.86 (0.66–2.59, 18)[f]	3.68 ± 0.31	3.91 ± 0.30	90
2	Acyclovir	0	0.04 (0.04–1.69, 21)[f]	0.09 ± 0.01	0.05 ± 0.01	20
3	Amiloride	+	0.84 ± 0.07	0.68 ± 0.09	0.42 (0.37–1.48, 24)[f]	50
4	Atenolol	+	<0.39[h]	0.86 ± 0.03	2.06 ± 0.17	50
5	Ceftriaxone	−	0.08 ± 0.05	0.17 ± 0.06	0.03 ± 0.01	1
6	Cefuroxime	−	0.20 ± 0.15	0.04 ± 0.01	0.05 ± 0.01	5
7	Chlorothiazide	0	0.22 ± 0.08	0.21 ± 0.06	<0.05[h]	13
8	Cytarabine	0	<0.05[h]	<0.04[h]	<0.05[h]	<20
9	Doxycycline	−	27.9 ± 0.6[i]	21.0 ± 1.6[g,i]	19.9 ± 0.1[i]	95 (90–100)
10	Enalapril	−	1.35 ± 0.35	1.38 ± 0.28	0.63 ± 0.03	65 (55–75)
11	Furosemide	−	7.88 ± 0.50[j]	0.73 ± 0.10[j]	0.75 (0.03–5.35, 45)[f,j]	61
12	Guanabenz	+	7.52 ± 0.49[i]	12.7 ± 1.7[g,i]	15.7 ± 1.6[i]	75
13	Hydrochlorothiazide	0	1.67 ± 0.15[j]	2.01 ± 0.11[j]	1.02 ± 0.12[j]	67
14	Hydrocortisone	0	21.5 ± 1.3[i,j]	23.0 ± 0.3[i,j]	19.7 ± 0.8[i,j]	91
15	Ketoprofen	−	60.2 ± 2.4[i,j]	18.6 ± 1.5[i,j]	2.35 ± 0.07[i,j]	100
16	Metoprolol	+	6.29 ± 0.16	6.97 ± 0.70	7.80 ± 0.11	95
17	Nadolol	+	0.83 ± 0.23	1.15 ± 0.24	2.63 ± 0.15	35
18	Olsalazine	−	0.57 ± 0.05	<0.07[h]	<0.18[h]	2
19	Oxytetracycline	−	5.88 ± 0.64	6.11 ± 1.17	2.72 ± 0.03	60
20	Penicillin V	−	<0.49[h]	0.56 ± 0.01	<0.49[h]	45
21	Pindolol	+	9.66 ± 0.55 [g,i,j]	6.80 ± 0.42[g,i,j]	5.99 ± 0.24[g,i,j]	90
22	Practolol	+	0.51 (0.09–1.82, 9)[f]	1.55 ± 0.26	3.95 ± 0.07	100
23	Pravastatin	−	3.42 ± 0.16	0.61 ± 0.10	0.21 ± 0.07	34
24	Procainamide	+	2.60 ± 0.23[j]	7.26 ± 0.40[j]	7.64 ± 0.66[j]	85 (75–95)
25	Propranolol	+	8.68 ± 0.28[i,j]	28.5 ± 0.9[i,j]	36.1 ± 1.6[i,j]	90
26	Quinidine	+	9.86 ± 0.12[i]	11.5 ± 0.5[g,i]	31.3 ± 0.8[i]	80
27	Ranitidine	+	2.00 ± 0.55	2.19 ± 0.02	4.13 ± 0.12	50
28	Sulfasalazine	−	7.88 ± 0.61	0.67 (0.39–1.89, 21)[f,g]	0.09 (0.02–2.80, 39)[f]	65
29	Sulpiride	+	1.01 ± 0.04	2.24 ± 0.11	5.04 ± 0.26	35
30	Tetracycline	−	5.23 ± 0.26	7.63 ± 0.15	2.23 ± 0.17	78 (75–80)
31	Timolol	+	13.3 ± 0.3[i]	7.67 ± 0.12[g,i]	9.13 ± 0.04[i]	90

[a] Net charge at pH 6.5.

[b] Artificial membrane permeability coefficient measured with the PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%)/1,7-octadiene membrane. Values are represented as the mean ± S.E. The assays were performed in triplicate, unless noted otherwise. The incubation time was 15 h, unless noted otherwise.

[c] Fa% (the fraction of a dose absorbed in humans) values were obtained from previously reported values (McEvoy, 1998; Wessel et al., 1998; Winiwarter et al., 1998). When the Fa% value was reported as a range, the mid-value of the range was used (values in parentheses indicating range.)

[d] pH of both donor and acceptor compartment.

[e] Values from Sugano et al. (2001) unless noted otherwise.

[f] P_{am} value varied, therefore, values are presented as medians (values in parentheses indicating the range and experiment number).

[g] Measured in this study.

[h] Less than the detection limit. The detection limit was set at $OD_{ac} = 0.005$.

[i] The incubation time was 2 h.

[j] $n = 6$.

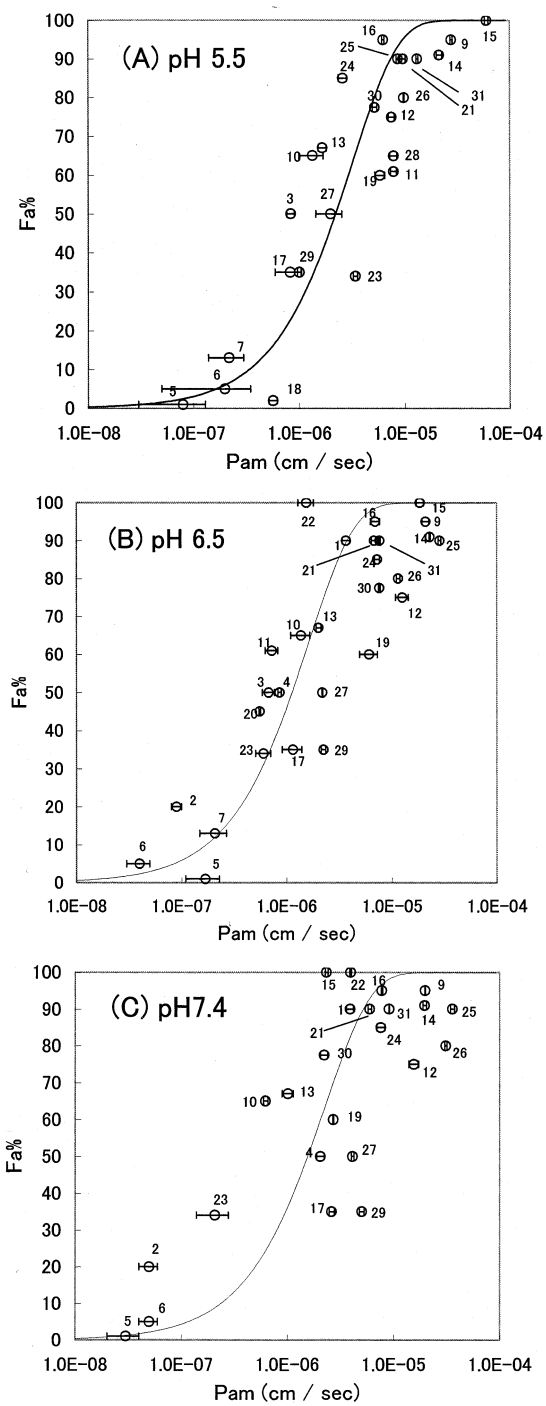

Fig. 1.

Table 2
Correlation coefficient (r) and coefficient (a) of Eq. (3)

pH condition	r	a ($\times 10^5$)	Number of compound
pH 5.5	0.866	3.11	25[a]
	0.878	3.52	20[b]
pH 6.5	0.865	6.20	28[a]
	0.919	3.18	20[b]
pH 7.4	0.767	4.48	24[a]
	0.738	4.08	20[b]

[a] All available permeability values at each pH were included.
[b] Compound for which permeability was available at all pH conditions employed.

P_{am} values under each pH condition are summarized in Table 1. The curved line in Fig. 1A–C was obtained by fitting Eq. (3) (Amidon et al., 1988). The correlation coefficient (r) and coefficient (a) obtained with all available permeability values at each pH condition are shown in Table 2. In addition, to compare the predictability, r and a values obtained with the same compound series at each pH condition were also indicated.

$$\text{Fa\%} = (1\text{-exp}(a \times P_{am})) \times 100 \quad (3)$$

Drugs with a negative charge showed a larger permeability in the acidic pH condition. Most drugs with a positive charge showed a larger permeability in alkaline pH conditions. These findings could be explained by the pH partition theory (Hogben et al., 1959). However, in some positive charge compounds, the effect of pH on the permeability is not in accordance with this theory. For example, pindolol showed lower permeability in alkaline pH conditions (P_{am} (pH); 9.66 ± 0.55 (pH 5.5), 6.80 ± 0.42 (pH 6.5), 5.99 ± 0.24 (pH 7.4), respectively). Comparison of the structure of pindolol and propranolol suggested that the indole moiety of pindolol might be the reason for this result.

Fig. 1. Fraction of a dose absorbed in humans (Fa%) versus P_{am} measured with PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%)/1,7-octadiene membrane at (A) pH 5.5, (B) pH 6.5, (C) pH 7.4. Compounds whose P_{am} value was varied or less than the detection limit were excluded. The curved line and correlation coefficient (r) in the figure was obtained by fitting the equation, $\text{Fa\%} = (1 - \exp(a \times P_{am})) \times 100$. Values represent the mean $\pm$ S.E. of three or six experiments.

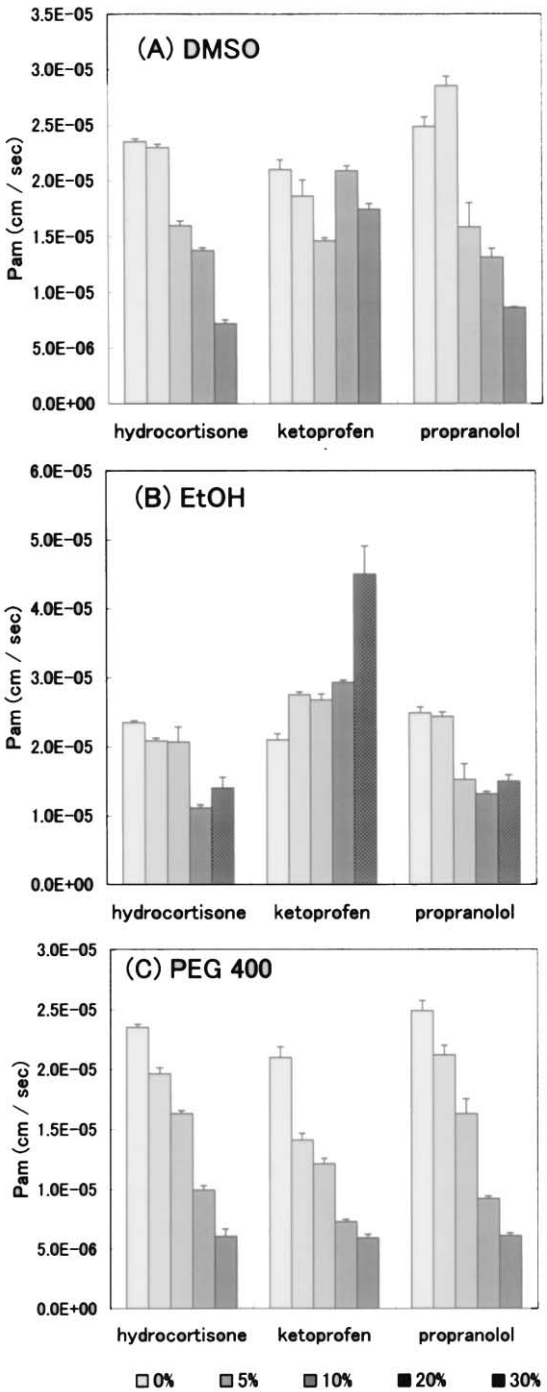

Fig. 2.

From the findings shown in Fig. 1A–C and Table 2, it is suggested that acidic pH conditions (pH 5.5 and/or pH 6.5) are more appropriate for the medium's pH conditions for the prediction of oral absorption. At pH 7.4, the permeability of some positive charge compounds was overestimated. Similar findings were also observed in the case of the Caco-2 cell assay (Yamashita et al., 2000). In Kansy's simple PC system, the measurement at pH 7.4, which appears not to be a typical pH of the small intestine, helps to avoid the underestimation of the permeability of some positively charged compounds. However, in the BML membrane system, a negative charge was added to the membrane as PS and PI to mimic the intestinal brush-border membrane, leading to an increase in the permeability of positively charged compounds. Therefore, the BML membrane system does not require the measurement at pH 7.4.

3.2. Effect of co-solvents on the permeability

During the drug-discovery/development process, we often encounter poorly water soluble compounds (Lipinski, 2000). These compounds often cause several problems when determining their permeability, especially in the in-vitro permeability assay. Usually, some co-solvents, e.g. DMSO, EtOH, PEG, are used to increase solubility. In the case of the cell-based system, the required solubility often could not be achieved, because of the limitation of the concentration of the co-solvents, due to their cell toxicity. However, because PAMPA is a completely artificial system, a higher concentration of co-solvents is expected to be adaptable. In the present study, we report on an investigation of the effects of various co-solvents on the permeability of the BML membrane. DMSO, EtOH and PEG400 were selected because these are the most used co-solvents. Usu-

Fig. 2. Effect of (A) DMSO, (B) EtOH, and (C) PEG 400 on the P_{am} of hydrocortisone, ketoprofen, and propranolol. P_{am} of each compound was measured with PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%)/1,7-octadiene membrane. Each concentration of DMSO, EtOH, and PEG 400 was added to the medium of both the donor and acceptor. The incubation time was 2 h. The values are expressed as the mean $\pm$ S.E of six experiments.

ally, poorly soluble compounds have a high lipophilicity (Jain and Yalkowsky, 2001). Therefore, hydrocortisone, propranolol and ketoprofen were selected as typical neutral, basic and acidic compounds. The octanol–water distribution coefficients (log D) of these compounds at pH 6.5 are 1.6, 0.9, and 0.8, respectively (Winiwarter et al., 1998; Sugano et al., 2001). In addition, Trypan Blue was used as a non-permeable marker to investigate the decomposition of the membrane.

In all the co-solvents tested here, leakage of Trypan Blue was not observed up to 30% after the incubation for 2 h (data not shown).

In the case of hydrocortisone and propranolol, the permeability was decreased by all co-solvents (Fig. 2). One reason is suggested to be the increased affinity of these hydrophobic compounds to the water phase by the addition of co-solvents, resulting in a decrease in the partitioning to the lipophilic part of the lipid membrane. In the case of ketoprofen, however, the effects of the co-solvents differed among these tested. In addition to an increased affinity of hydrophobic compounds to the water phase, co-solvents usually increase the pK_a of acid (Sarmini and Kenndler, 1999). Therefore, according to the pH partition theory, a co-solvent could increase the permeability of acid. An increase in the pK_a and an increase in the affinity to water phase might have an effect on the permeability of hydrophobic acid compounds in the opposite direction, resulting in the different effects among each co-solvent. Although more detailed studies are necessary to clarify the effect of these co-solvents more completely, use of these co-solvents could lead to under- or overestimation of drug permeability.

4. Conclusions

In this study, we have indicated the importance of the experimental conditions for PAMPA. Physiological pH, namely pH 5.5–6.5, should be used for PAMPA with the BML membrane. Although up to 30% of the additive concentration could be used, co-solvents can lead to the under- or overestimation of drug permeability. Consequently, it is most important to choose the most appropriate conditions according to the purpose of the study as well as the physicochemical property of the compounds to be tested.

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