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High Throughput Prediction of Oral Absorption: Improvement of the Composition of the Lipid Solution Used in Parallel Artificial Membrane Permeation Assay

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ABSTRACT

The purpose of the present study was to improve the composition of the lipid solution used in parallel artificial membrane permeation assay for the precise prediction of oral absorption. We modified the composition of lipid solution, which was used to make a lipid membrane on the filter support. First, we changed the chain length of organic solvent (PC/alkyldienes [C7–C10]). A negative charge was then added to the membrane to mimic the intestinal membrane (PC/stearic acid/1,7-octadiene and PC/PE/PS/PI/cholesterol/1,7-octadiene). Finally, we examined the predictability of the PC/PE/PS/PI/CHO/1,7-octadiene membrane using structurally diverse compounds. Permeability coefficients of tested compounds were increased as the chain length of alkyldiene became shorter. The addition of a negative charge to the membrane increased the permeability of the basic compounds. However, the negatively charged membrane with stearic acid showed different permeability profiles from PC/PE/PS/PI/CHO. The predictability of the PC/PE/PS/PI/CHO/1,7-octadiene membrane was adequate ($r = 0.858$, $n = 31$) for use during the early stages of the drug discovery/development process.

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 as a result of inadequate pharmacokinetics.[1] Among many pharmacokinetic factors, gastrointestinal absorption often becomes a key problem. Therefore, incorporation of absorption assay during the early stages of the drug discovery/development process has been anticipated. There are three pathways of drug absorption through the intestinal membrane: the passive transcellular pathway, the passive paracellular pathway, and the active transport pathway. The vast majority of drugs are absorbed through the passive transcellular pathway. Assays based on biological cell layers (e.g., Caco-2) are often used as an *in vitro* method.[2,3] One of the advantages of this method is that they contain all the pathways of absorption. Unfortunately, these methods often fail to predict the extent of oral absorption because of the possible quantitative under- or overexpression of transport proteins.[4] In addition, these methods are laboratory intensive and therefore currently not suited for high throughput screening (HTS).

Octanol buffer distribution coefficient (logD) has been extensively used as a surrogate parameter of passive absorption via the transcellular pathway.[5,6] However, a good correlation was only found within a homologous series of compounds. 1-Octanol cannot mimic the interfacial character of the bilayer structure of biomembranes and ionic interactions between membrane phospholipids and solutes. To overcome this weak point of logD, the immobilized artificial membrane (IAM) column[7] and liposome partitioning[8] have been introduced. However, the throughput of these methods is low.

Parallel artificial membrane permeation assay (PAMPA) was first introduced by Kansy et al.[9] PAMPA is an application of the filter-supported lipid membrane system.[10] In this system, to make lipid membranes, phospholipids and other membrane constituents are added to the filter support as a solution in an organic solvent. PAMPA is an excellent assay method, especially from the HTS point of view. However, previous PAMPA conditions, which used phosphatidylcholine and alkane or alkyldiene (>C9), had difficulties in the precise classification of oral absorption probability, especially in the case of lower permeability compounds. In addition, the classification scheme required two measurements, at pH 6.5 and pH 7.4. The measurement at pH 7.4, which appears not to be a typical pH of the small intestine,[11] helps to avoid the underestimation of the

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permeability of some basic compounds. For example, metoprolol, propranolol, and guanabenz, whose human absorption was classified as "high" in the classification scale, were estimated as "intermediate" at pH 6.5 and "high" at pH 7.4. At pH 7.4, the undissociated form of the basic compound was increased; therefore, the permeability of the basic compound through the lipid membrane was increased.[12] However, the use of pH 7.4 seems unreasonable. It was suggested that these disadvantages arose from the inadequate composition of the lipid solution used to make membranes on the filter support.

In the present study, we improved the composition of the lipid solution used in PAMPA for more precise prediction of oral absorption.

MATERIALS AND METHODS

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-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylserine (PS), L-α-phosphatidylinositol (PI), and cholesterol (CHO) were purchased from Sigma Chemical Co. (St. Louis, MO). Quinidine, stearic acid (SA), 1,6-heptadiene, 1,7-octadien, 1,8-nonadiene, and 1,9-decadiene were purchased from Tokyo Kasei (Tokyo, Japan). Mordant yellow 5 (olsalazine) was purchased from Aldrich (Milwaukee, WI). Octanol was purchased from Wako Pure Chemicals (Tokyo, Japan). Practolol was purchased from Tocris (Northpoint, UK). Chlorothiazide was purchased from Alexis (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 (Bedford, MA).

Permeability studies

Permeability studies were performed in the same manner as described previously.[9] A 96-well microplate (acceptor compartment) was completely filled with 50 mM sodium phosphate buffer containing 5% DMSO. A hydrophobic filter plate (donor compartment) was fixed on the buffer-filled plate. The filter surface was impregnated with 5 μl lipid solution. The composition of the lipid solutions are described in the *Results and Discussion* section. (Note that alkyldienes are irritants 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 ultraviolet (UV) spectroscopy, using the SPECTRAmax® 190 microtiter plate reader (Molecular Devices, Sunnyvale, CA) at 250–450 nm at intervals of 10 or 20 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 Equation 1:

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

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

where:

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

Distribution coefficient measurement

Distribution coefficient at pH 6.5 was determined by the shaking flask method in the 1-octanol–50 mM sodium phosphate buffer system. Before use, the 1-octanol was saturated with phosphate buffer. A known concentration of the compound in phosphate buffer was shaken for 30 min at room temperature with a suitable volume of 1-octanol. After shaking, the phases were separated by centrifugation at 2,000g for 1 min. The concentrations in the buffer phase before and after partitioning were determined by UV spectroscopy as described under *Permeability Study*. The volume ratio of 1-octanol:buffer was adjusted to make the concentration ratio in the buffer before/after shaking between 0.1 and 0.9.

Statistical Analysis

Tukey's test was used to evaluate the significance of difference in all cases with each compound (SAS™, ver. 6.12, SAS Institute Japan, Ltd., Tokyo, Japan). A minimum p value of 0.05 was used as the significance level for all tests. Curve fitting and correlation coefficients were calculated by the least-squares method (Delta Graph™, ver. 4.0J, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Effect of chain length of organic solvents (alkyldienes)

As described in the *Introduction*, one of the disadvantages of previous PAMPA conditions was its limitation for evaluation of lower permeability compounds. One reason for this limitation was the low concentration of permeated compounds in the acceptor compartment. UV spectrometry was used as a detection method in PAMPA, even though its detection limit is low, because a simple analytical method is one precondition for HTS. Other analytical methods are inferior to UV spectrometry with respect to assay speed. There are some solutions to overcome inaccuracy, other than changing the detection method: (A) increase the sample concentration in the donor compartment, (B) prolong the incubation time, or (C) modify the composition of the lipid solution. Solution A is not appropriate for HTS because a high concentration often cannot be achieved in low-solubility compounds. Solution B is also not appropriate because this increases the risk of decomposition of the compounds during incubation; in addition, a long incubation time lowers the throughput. Therefore, we thought that so-

lution C, modification of the composition of the lipid solution, was the most appropriate.

The overall membrane resistance is the sum of the contributions from the interfacial (head group) region and the hydrocarbon chain region.[13] As described in the *Introduction*, to make lipid membranes, phospholipids and other membrane constituents are added to the filter support as a solution in an organic solvent. In such a membrane system, the organic solvent remains in the hydrocarbon chain region.[14,15] Therefore, we first modified the organic solvent.

Among the organic solvents reported previously,[9] 1,9-decadiene showed the largest permeability and the best predictability in our study (data not shown), but the permeability of some compounds still could not be measured (Table 1). Therefore, we tested shorter chain alkyldienes (C7 to C9). As typical model compounds, we selected six drugs that have different lipophilicities and charges: hydrocortisone (high lipophilicity, neutral), propranolol (high lipophilicity, base), ketoprofen (high lipophilicity, acid), hydrochlorothiazide (low lipophilicity, neutral), procainamide (low lipophilicity, base), and furosemide (low lipophilicity, acid). PC alone was used as the lipid, to enable a simple comparison of the effect of the alkyldienes. The pH of the donor and acceptor solutions was set at a typical pH of the small intestine, namely pH 6.5. We performed the per-

Table 1. Pam, Fa%, and Molecular Properties

				P_{am} ($\times 10^{-6}$ cm/sec)[f]		
Compound	*MW*[a]	*Charge*[b]	*logD*[c] *(pH 6.5)*	*BML*[g] */1.7-octadiene*	*PC (2%) /1,9-decadiene*	*Fa%*[h]
Acebutolol	336	+	−1.0	3.68 ± 0.31	0.41 ± 0.23	90
Acyclovir	225	0	−1.7	0.09 ± 0.01	<0.04[i]	20
Amiloride	230	0	−2.2	0.68 ± 0.09	0.27 ± 0.21	50
Atenolol	266	+	<−2.3	0.86 ± 0.03	<0.42[i]	50
Ceftriaxone	555	−	−1.7	0.17 ± 0.06	<0.02[i]	1
Cefuroxime	424	−	−1.2	0.04 ± 0.01	<0.03[i]	5
Chlorothiazide	296	0	−0.1	0.21 ± 0.06	<0.05[i]	13
Cytarabine	243	0	−2.1	<0.04[i]	<0.04[i]	<20
Doxycycline	444	−	0.1	213 ± 2.1	1.53 ± 0.19	95 (90–100)
Enalapril	476	−	−0.9	1.38 ± 0.28	0.52 ± 0.59	65 (55–75)
Furosemide	331	−	−0.5[d]	0.73 ± 0.10[j]	0.24 ± 0.06[j]	61
Guanabenz	231	+	0.7	10.4 ± 0.2	4.63 ± 1.64	75
Hydrochlorothiazide	298	0	−0.2[d]	2.01 ± 0.11[j]	0.75 ± 0.32[j]	67
Hydrocortisone	362	0	1.6	23.0 ± 0.3[j,k]	1.94 ± 0.41[j,k]	91
Ketoprofen	254	−	0.8[k]	18.6 ± 1.5[j,k]	10.2 ± 3.0[j,k]	100
Metoprolol	267	+	−0.9	6.97 ± 0.70	0.75 ± 0.08	95
Nadolol	231	+	−1.9	1.15 ± 0.24	<0.53[i]	35
Naltrexone	341	+	−0.2	4.52 ± 0.32	14.4 ± 5.2	96
Olsalazine	302	−	2.1[e]	<0.07[i]	<0.07[i]	2
Oxytetracycline	460	−	−0.8	6.11 ± 1.17	1.30 ± 0.47	60
Penicillin V	366	−	0.5	0.56 ± 0.01	<0.45[i]	45
Pindolol	248	+	−0.9	7.94 ± 0.27	0.71 ± 0.44	90
Practolol	266	+	−2.3	1.55 ± 0.26	0.30 ± 0.15	100
Pravastatin	424	−	0.9	0.61 ± 0.10	<0.07[i]	34
Procainamide	235	+	−1.7	7.26 ± 0.40[j]	0.31 ± 0.11[j]	85 (75–95)
Propranolol	259	+	0.9[d]	28.5 ± 0.9[j,k]	13.9 ± 0.7[j,k]	90
Quinidine	324	+	1.4	15.0 ± 1.3	4.56 ± 0.53	80
Ranitidine	314	+	−1.4	2.19 ± 0.02	0.14 ± 0.03	50
Sulfasalazine	398	−	2.2	1.75 ± 0.72	0.23 ± 0.09	65
Sulpiride	341	+	−1.4	2.24 ± 0.11	<0.08[i]	35
Tetracycline	444	−	−0.9	7.63 ± 0.15	1.42 ± 0.35	78 (75–80)
Timolol	316	+	−0.7	11.9 ± 0.6	3.11 ± 2.06	90

[a]Molecular weight.
[b]Net charge at pH 6.5.
[c]Octanol/buffer distribution coefficient. Mean of three experiments. In all cases, standard deviations were less than ±0.2.
[d]Data from Winiwarter et al.[16]
[e]This value is larger than the expected value from logD (pH 7.4) as previously reported.[2]
[f]Artificial membrane permeability coefficient. Values are represented as mean ± SE. The assays were performed in triplicate, or as otherwise noted. The incubation time was 15 h, or as otherwise noted.
[g]PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%).
[h]Fa% (the fraction of a dose absorbed in humans) values were obtained from previously reported values.[3,9,17–19] When the Fa% value was reported as a range, the median was used (parentheses indicate range).
[i]Less than the detection limit. Detection limit was set at $OD_{ac} = 0.005$.
[j]$n = 6$.
[k]The incubation time was 2 h.

meation assay at 30°C because permeability increases as temperature increases. However, the filter was sometimes peeled from the plate at higher temperatures.

Shorter chain alkyldienes showed larger permeability of compounds than 1,9-decadiene (Fig. 1). However, the order was not related to chain length, except for hydrocortisone. One reason for enhanced permeability was thought to be the lower viscosity of the shorter chain alkyldienes, because this affects the diffusion rate of compounds through the hydrocarbon chain region. We are presently investigating why the influence of chain length differs among these compounds.

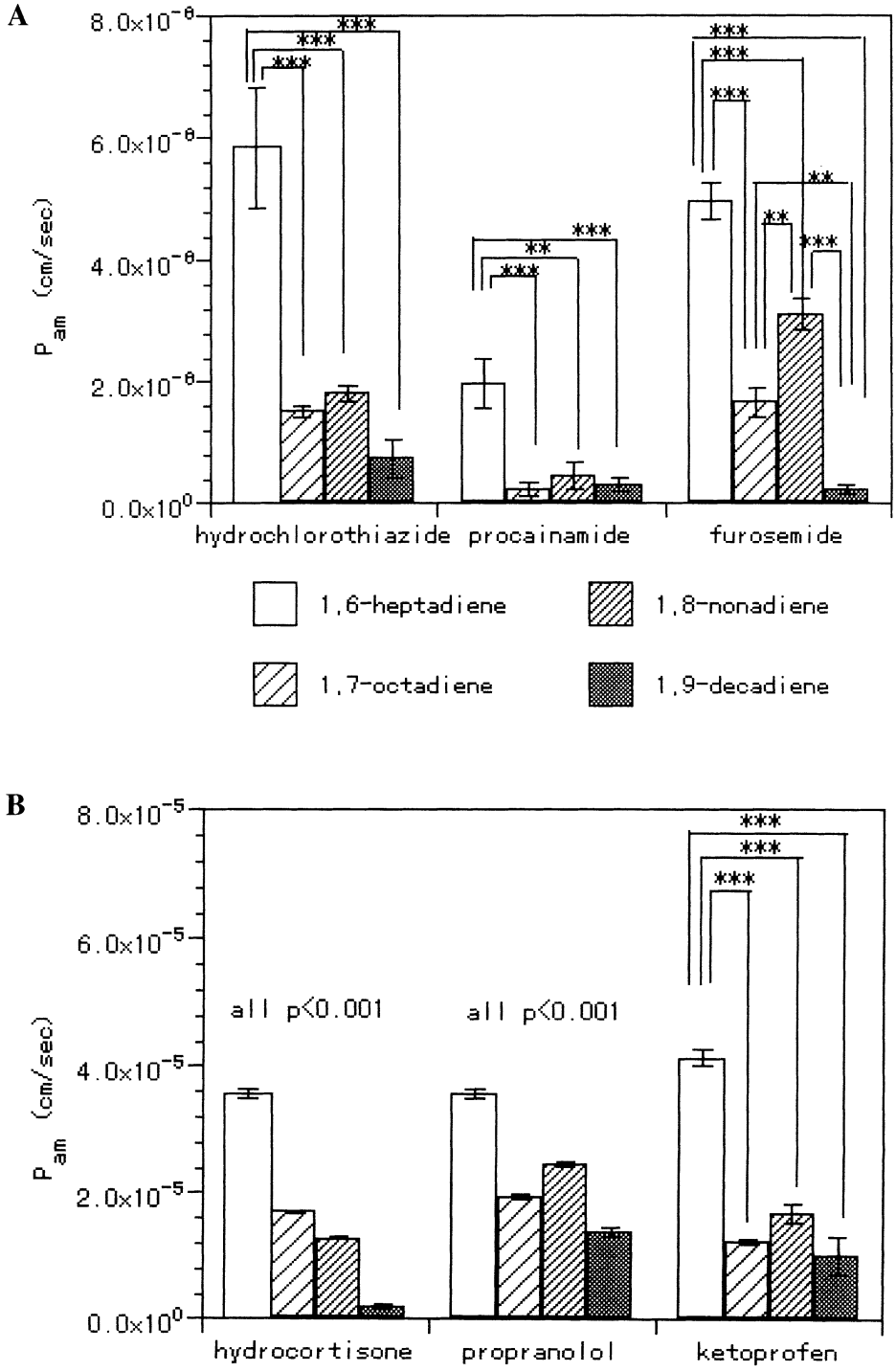

FIG. 1. Effect of organic solvent (alkyldienes) on permeability. In all permeation assays, PC (2%) was used as the lipid. (**A**) Low lipophilicity compounds (incubation time, 15 h). (**B**) High lipophilicity compounds (incubation time, 2 h). Values represent the mean ± SE of six experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

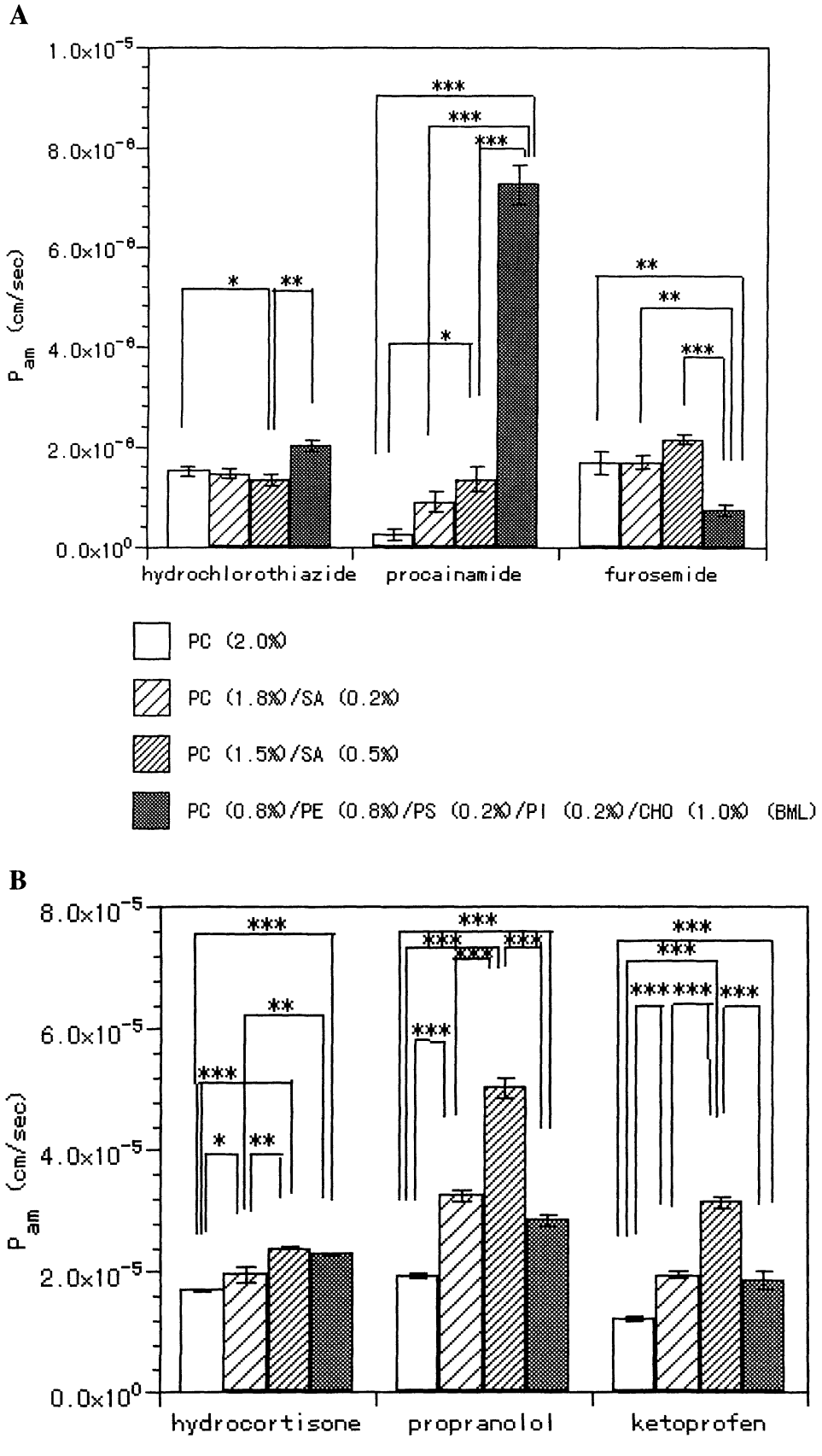

FIG. 2. Effect of lipid on permeability. In all permeation assays, lipids were added to the filter in a 1,7–octadiene solution. (**A**) Low lipophilicity compounds (incubation time, 15 h). (**B**) High lipophilicity compounds (incubation time, 2 h). Values represent the mean ± SE of six experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Effect of modification of lipids

PAMPA, with PC alone as a lipid, was reported to underestimate the permeability of some basic compounds at pH 6.5.[9] In the present study, the permeability of basic compounds (procainamide) was also underestimated. Procainamide is orally absorbed to a greater extent than hydrochlorothiazide and furosemide; the fraction of a dose absorbed in humans (Fa%) is 85% (range, 75%–95%) for procainamide, 67% for hydrochlorothiazide, and 61% for furosemide.[17,18] However, Inui et al.[20] reported that the permeability of acidic compounds through the intestinal lipid membrane, which was made with lipids extracted from the rat small intestine, was larger than that through the PC membrane. These findings suggest that the lipid solution with PC alone is not suitable for either basic or acidic compounds. One of the characteristic attributes of intestinal membrane is that it is negatively charged. A negative charge increases the affinity of basic compounds for the membrane.[21] In addition, the negative charge at the surface of the membrane lowers the pH close to the surface of the membrane.[22] According to the pH partition theory,[12] reduction of pH increases the permeability of acidic compounds. Therefore, we added an anionic lipid to the lipid composition. We provided a lipid composition that mimics the small intestine, that is, a bio-(intestinal)-mimetic lipid (BML) system with PC (0.8%), PE (0.8%), PS (0.2%), PI (0.2%), and CHO (1.0%).[23] In this system, PS and PI are the source of the negative charge. 1,7-Octadiene was used as organic solvent in this study. Sphingomyelin, which is one of the main constituents of the intestinal membrane, could not be added because of its low solubility in 1,7-octadiene. In addition, we provided a simple negatively charged membrane in which SA was added as a simple negatively charge source: PC (1.8%)/SA (0.2%) and PC (1.5%)/SA (0.5%). The negative charge densities of PC (1.8%)/SA (0.2%) and BML were similar. Comparing these membranes, it was possible to clarify whether the negative charge alone affected permeability.

The permeability of basic compounds was increased by the addition of a negative charge to the membrane, whereas the permeability of neutral compounds was little changed (Fig. 2). However, the negatively charged membrane with SA showed different permeability profiles than BML. In the case of furosemide, permeability through the BML membrane was lower than that through the PC membrane and PC/SA membranes. Also, in the case of procainamide, permeability through the BML membrane was 8-fold larger than that through the PC (1.8%)/SA (0.2%) membrane, even though these two membranes had similar anion densities and showed permeability similar to propranolol and ketoprofen. Furosemide and procainamide have more hydrogen bond donors and acceptors than propranolol and ketoprofen. Hydrogen bonds between compounds and the head group of the lipid are thought to have an effect on membrane permeability.[24,25] These findings suggest that not only anionic charge but also some other characteristics of the head group (e.g., hydrogen bonds) are important factors that affect the permeability. Therefore, simple addition of a negative charge, as with SA, is insufficient to mimic the intestinal membrane.

Examination of performance of the BML/1,7-octadiene system

The artificial membrane permeability of 33 structurally diverse compounds, with molecular weights (MW) in the range of 225–555 and different net charges, were measured using BML/1,7-octadiene and PC/1,9-decadiene. PC/1,9-decadiene is one of the lipid compositions reported by Kansy et al.[9] We mainly selected compounds whose Fa% was less than 90%, because we wanted to examine the predictability for lower permeability compounds.[26] In addition, compounds smaller than MW 200 were excluded to eliminate the absorption via the paracellular pathway.[27] Compounds that are absorbed via the active transport pathway were also excluded. In the case of the PC/1,9-decadiene membrane, the permeability of hydrophilic compounds was often below the detection limit (Table 1). In addition, the permeability of some basic compounds was underestimated. In the case of the BML/1,7-octadiene membrane, the permeability of almost all the compounds was increased, especially that of basic compounds. Therefore, the predictable range of oral absorption was expanded and the permeability of basic compounds was measured adequately.

The permeability through the BML/1,7-octadiene membrane was plotted against Fa% in humans (Fig. 3). The curved line and correlation coefficient (r) in the figure was obtained by fitting the following equation:

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

where $a = 6.21 \times 10^5$, $r = 0.858$, and $n = 31$.[28] In comparison with the prediction method described by Kansy et al.,[9] which requires additional permeability measurement at pH 7.4 for adequate prediction of oral absorption of basic compounds, our method (1) does not require measurement at pH 7.4, (2) can be used to predict a wider range of oral absorption, and (3) is reasonable from the perspective of similarity to intestinal con-

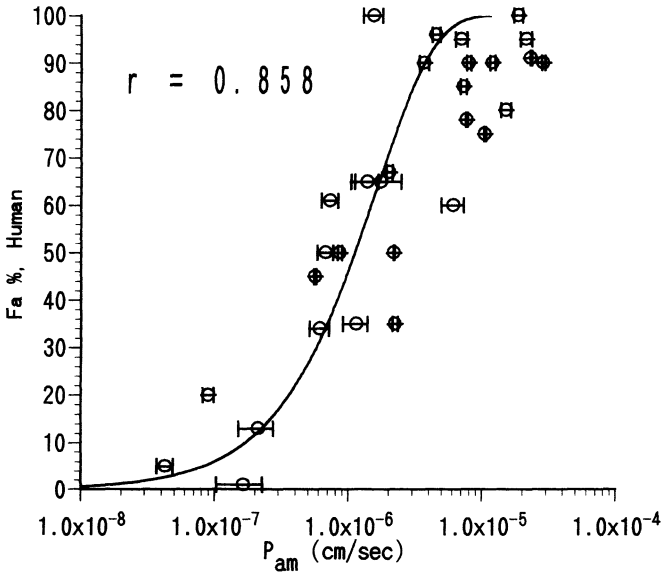

FIG. 3. The fraction of a dose absorbed in humans (Fa%) versus P_{am} measured with PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%) in 1,7-octadiene at pH 6.5. Olsalazine and cytarabin were excluded because their P_{am} values were less than the detection limit. The curved line and correlation coefficient (r) in the figure were obtained by fitting the equation, $Fa\% = (1 - \exp(a \times P_{am})) \times 100$ ($a = -6.21 \times 10^5$, $r = 0.858$, $n = 31$). Values represent the mean ± SE of three or six experiments.

ditions, such as pH and lipid composition. In some compounds, the permeability was still under- or overestimated to some extent. Further investigations are required to clarify the details of the BML/1,7-octadiene membrane and the adaptable physicochemical and structural range of compounds. However, the BML/1,7-octadiene membrane showed adequate predictability to be used during the early stages of the drug discovery/development process.

Relationship between logD and P_{am}

To clarify the characteristics of the BML/1,7-octadiene membrane, P_{am} through the BML/1,7-octadiene membrane was plotted against logD (pH 6.5) (Fig. 4). We found only a slight correlation. The permeability of the basic compound was 1- to 100-fold larger than the neutral compound with a similar logD value. The larger permeability of the basic compound was one reason for the good predictability of the BML/1,7-octadiene membrane. This phenomenon is thought to come from the negative charge of the membrane. However, there are several other factors that account for the differences between logD and P_{am} through the BML/1,7-octadiene membrane. With more knowledge of the P_{am} values of widely diverse compounds, a prediction scheme for P_{am} from structural information and physicochemical properties of compounds can be made.

Consideration from the HTS point of view

It appears that alkyldienes with shorter chains (≤C7) may increase the permeability of compounds (Fig. 1). However, as the chain length becomes shorter, alkyldienes become increasingly volatile and show lower viscosity, which is unsuitable for HTS from the handling point of view (boiling points of 1,5-hexadiene, 1,6-heptadiene, and 1,7-octadiene are 60°, 89–90°, and 114–121°C, respectively). Therefore, 1,6-heptadiene and/or 1,7-octadiene are thought to be adequate.

The permeability of some compounds was found to be sensitive to the composition of the lipid (Fig. 2). It is promising to use lipid extractions from the small intestine, if costs and labor are not limiting factors. However, the running costs of the assay are an important practical problem in the use of HTS.[29] The cost of materials of the improved PAMPA format (BML membrane) is about 1.2-fold more expensive than that of the previous format (about 50 cents US/well; the filter plate is the most expensive material in PAMPA).

PAMPA can be operated almost automatically by a typical liquid-handling instrument. At present, we manually fix the filter plate on the acceptor plate, and also manually remove the filter plate. However, this semiautomatic operation allows hundreds of assays a day. Our preliminary results, using the MultiPROBE® II_{EX} (Packard Instrument Company, Meriden, CT), suggested that the accuracy is similar between manual and semiautomatic operation. We are now investigating a fully automatic version.

In general, the method of membrane permeability assay should be selected according to the aim of the assay. The previous PAMPA format, with the simple lipid composition (PC alone) and two pH conditions (pH 6.5 and pH 7.4), is thought to put some emphasis on the aspect of physicochemical characterization. Therefore, its findings can be adapted to various purposes, but at the same time the predictability for oral absorption is complicated. However, the BML membrane focuses on the precise prediction of human oral absorption probabilities.

CONCLUSIONS

We modified organic solvents and lipids used in PAMPA. Shorter chain alkyldienes showed larger permeability of compounds than did 1,9-decadiene. Modification of the lipid com-

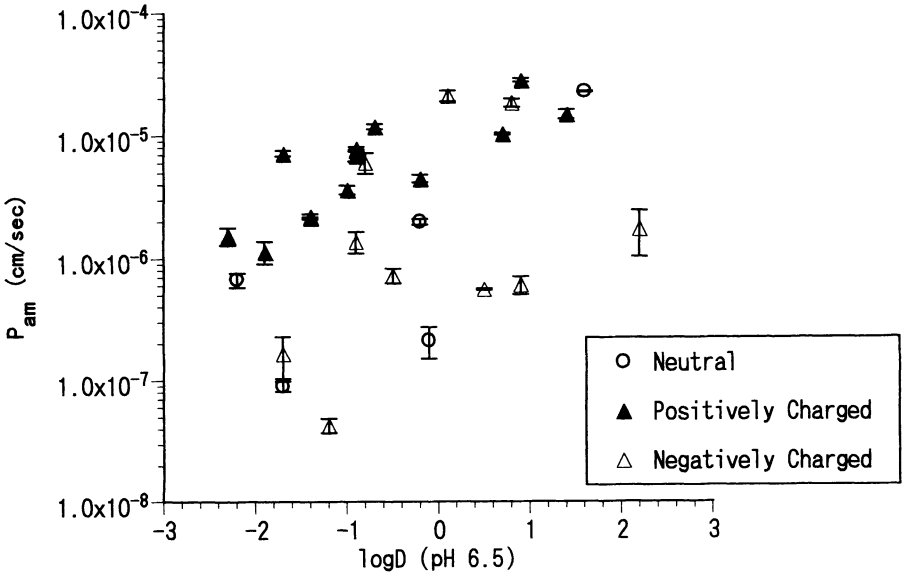

FIG. 4. LogD (pH 6.5) versus P_{am} measured with PC (0.8%)/PE (0.8%)/PS (0.2%)/PI (0.2%)/CHO (1.0%) in 1,7-octadiene at pH 6.5. Olsalazine, cytarabin, and atenolol were excluded because their P_{am} or logD values were less than the detection limit. Values of P_{am} represent the mean ± SE of three or six experiments.

position to mimic the intestinal membrane had a significant effect on the permeability of charged drugs. After these modifications, the BML/1,7-octadiene membrane was shown to be able to predict the oral absorption more precisely than the PC/1,9-decadiene membrane.

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