Featured Article

A Novel Design of Artificial Membrane for Improving the PAMPA Model

Xiaoxi Chen,[1,3] **Anthony Murawski,**[1] **Karishma Patel,**[2] **Charles L. Crespi,**[1] **and Praveen V. Balimane**[2]

Purpose. Since the first demonstration of PAMPA, the artificial membrane has been traditionally prepared by impregnating a porous filter with a solution of lipid mixture. While the lipid solution-based method is simple and seems to provide good predictability for many compounds, it is challenged by several shortcomings including reproducibility, stability, mass retention and the incorrect prediction of a group of highly permeable compounds including caffeine and antipyrine. Here we present the validation of a novel artificial membrane formed by constructing a lipid/oil/lipid tri-layer in the porous filter.
Methods. Permeability values obtained from traditional and new artificial membrane were compared for their correlation with Caco-2 and human absorption values. Mass retention, stability and organic solvent compatibility of the new artificial membrane were studied.
Results. The new artificial membrane correctly predicts the permeability of the traditionally under-predicted compounds and improves the correlation with Caco-2 and human absorption values. Furthermore, the new artificial membrane reduces the mass retention of compounds that are highly retained by the traditional artificial membrane. The new artificial membrane is also found to be robust enough to sustain long term storage and has good compatibility with organic solvents.
Conclusions. The new artificial membrane provides an improved PAMPA model.

KEY WORDS: Caco-2; drug absorption; high throughput screening; parallel artificial membrane permeability assay (PAMPA); permeability.

INTRODUCTION

Drug candidates are screened for their oral-absorption potential early in the discovery and development phase, as a filter to remove poor performers and identify candidates that needs to be modified. Two permeability assays have become prevalent in recent years: cell based (especially Caco-2) permeability assay (1–3) and parallel artificial membrane permeability assay (PAMPA; 4–8). PAMPA is a quick, simple test to measure passive permeability in the absence of transporters or efflux systems. Caco-2 test alone measures the sum of passive and active permeabilities which cannot be decoupled easily without the information obtained from the PAMPA test of the same compound. Therefore PAMPA and Caco-2 tests can be complementary tests to determine both passive and active permeabilities. PAMPA has also been used to predict the passive permeabilities through the blood-brain barrier (9).

Since the first successful demonstration of PAMPA by Kansy *et al.* (4), the artificial membrane has been usually prepared by impregnating a porous filter with a solution of lipids and possibly other biological membrane constituents. In the original Kansy method, the lipid solution consists of 1–20%

lecithin in an organic solvent (4). In the bio-mimetic PAMPA developed by Sugano *et al.*, the lipid solution consists of a mixture of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cholesterol in an organic solvent (6). In the double-sink PAMPA developed by pION Inc., the lipid solution consists of 20% dodecane solution of a phospholipid mixture (7). While the lipid solution-based method—forming artificial membrane using lipids solutions—is simple and seems to provide good predictability for many compounds, it is challenged by the incorrect prediction of a group of drugs that are classified by the biopharmaceutical classification system (BCS) as high permeability compounds. Examples of these compounds include antipyrine and caffeine. These compounds have low PAMPA permeability values using the lipid solution-based methods (10–12). However these compounds have high human absorptions, high Caco-2 permeability values, and are not known to be mainly dependent on active transport.

The structure of the lipid solution-based artificial membrane is not known yet. We have hypothesized that the excess amount of solvents in the solution-based artificial membrane present an extra barrier for the permeating compounds, resulting in the under-prediction of some high permeability compounds. We have also hypothesized that the excess amount of solvent act like a trap for some compounds which have high retention in organic solvents. Therefore we have developed a novel procedure for preparation of artificial membrane that removes the excess amount of solvent by using lipids solution in volatile solvents that evaporates immediately after coating.

[1] BD Biosciences Discovery Labware, 2 Oak Park, Bedford, Massachusetts 01730, USA.
[2] Bristol-Myers Squibb, Route 206 and Provinceline Rd, Princeton, New Jersey 08543, USA.
[3] To whom correspondence should be addressed. (e-mail: Kevin_chen@bd.com)

Using a set of standard compounds with known human absorption values, we have compared the new artificial membrane with the solution-based artificial membrane for correlation with human absorption, for correlation with Caco-2 permeability values, and for the mass retention of several highly retained compounds.

In the conventional PAMPA methods, the artificial membrane is usually coated immediately before the assay (within the same day). This practice may result in poorer reproducibility due to day-to-day coating variability. We have studied a new approach where large batch of filter plates are pre-coated with the artificial membrane, validated, stored at low temperature and used when needed. The plate-to-plate reproducibility and long term stability are discussed in this study.

Low solubility compounds have been a challenge for permeability measurements. With low solubility compounds, the initial concentration on the donor side of the membrane barrier will be low, and the final concentration on the acceptor side of the membrane barrier will be even lower (this is especially the case for low permeability compounds). This means accurate measurement of the compound concentrations—especially of the acceptor solutions—is very difficult. Although some LC-MS instruments are very sensitive in detecting low concentrations, the concentration of low solubility compounds can still fall out of its detection limit. To assay the compounds with poor aqueous solubility, solubilizers, including organic solvents and excipients, have been added to the buffer used in PAMPA (13). A common approach is to increase the percentage of organic solvent in the PAMPA buffers. Therefore it is important to investigate the compatibility of the PAMPA artificial membrane with buffers containing solubilizers, especially buffers containing a higher percentage of organic solvents. In this study the organic solvent compatibility of the new artificial membrane is characterized by comparison of the permeability values of a set of standard compounds obtained with or without the addition of organic solvents in the buffer.

MATERIALS AND METHODS

Materials

Acyclovir, nadolol, sulpiride, famotidine, acebutolol, amiloride, atenolol, terbutaline, furosemide, ranitidine, hydrochlorothiazide, phenytoin, timolol, pindolol, metoprolol, theophyline, naproxen, antipyrine, caffeine, indomethacin, ketoprofen, sulfasalazine, alprenolol, desipramine, ibuprofen, verapamil, warfarin, diclofenac, diltiazem, imipramine, propranolol, carbamazepine, clonidine, nicotine, piroxicam were purchased from Sigma (St. Louis, MO). The pre-coated PAMPA plates were manufactured by BD Biosciences Discovery Labware (Bedford, MA) using Polyvinylidene fluoride (PVDF) 96-well filter plate with 0.45 μm pore size. The filter plates were coated with a lipid/oil/lipid tri-layer artificial membrane and then stored at –20°C until usage. The 96-well UV-transparent plates were also from BD Biosciences Discovery Labware (Bedford, MA).

Permeability Assay

In the artificial membrane permeability assay, the plate assembly consists of a 96-well filter plate pre-coated with the lipid/oil/lipid tri-layer membrane, and a 96-well receiver plate that matches with the filter plate. The pre-coated plate assembly, which was stored at −20°C, was taken to thaw for 30 min at room temperature. The permeability assay was carried out in a similar protocol as described in previous PAMPA studies (4,7,10–14). In summary, the 96-well filter plate was used as the permeation acceptor and the 96-well receiver plate was used as the permeation donor, as shown in Fig. 1. For simplicity, phosphate buffered saline (PBS, pH 7.4) has been used both as donor and acceptor buffer throughout this study. The initial donor solutions were prepared by diluting DMSO stock solutions in PBS. Since UV plate reader has been used to characterize compound concentration in our studies, we have used higher compound concentrations
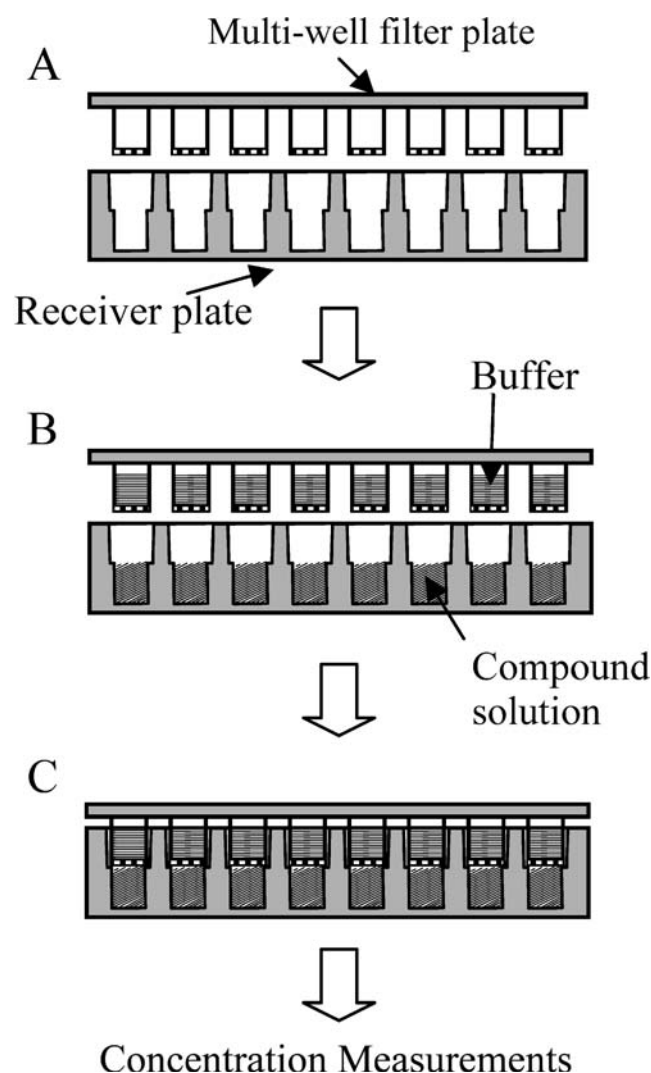
Fig. 1. Schematic illustration of a permeability assay: **A** Ninety-six-well filter plate pre-coated with an artificial membrane, and a matched 96-well receiver plate; **B** compound solutions are added in the wells of the receiver plate (permeation donor) and buffer is added in the wells of the filter plate (permeation acceptor); **C** The filter plate and the receiver plate are coupled together and incubated for a certain duration. Finally the plates are separated and the concentrations from both the donor and acceptor solutions are measured.

(>100 μM) to make sure that accurate UV readings can be achieved. In other studies using LC–MS detections, much lower compound concentrations are used. In this study, UV plate reader was used not LC–MS detections, the initial donor solution of most compounds was prepared by diluting 10 mM DMSO stock solution 1:50 in PBS, resulting in an initial donor concentration of C_0=200 μM. For some low UV absorption compounds, due to the detection limit of the UV plate reader, higher concentration is needed in order to obtain more accurate results. For low UV absorption compounds including nadolol, atenolol, phenytoin, ibuprofen, and clonidine, the initial donor solution was prepared by diluting 40 mM DMSO stock solution 1:50 in PBS, resulting in an initial donor concentration of C_0=800 μM.

The initial donor solutions were added to the wells (300 μL/well) of the receiver plate and PBS was added to the wells (200 μl/well) of the pre-coated filter plate. The filter plate was then coupled with the receiver plate and the plate assembly was incubated at room temperature without agitation for 4–5 h. Humidity chamber is not needed due to the negligible evaporation during this short incubation time. In the end of the incubation, the plates were separated and 150 μl solutions from each well of both the filter plate and the receiver plate were transferred to 96-well UV-transparent plates. The final concentrations of compounds in both donor wells and acceptor wells, as well as the concentrations of the initial donor solutions, were analyzed by a Molecular Devices SpectraMax Plus UV-plate reader. For each compound, the UV spectra (250–300 nm) of the donor solution, acceptor solution and dilutions of the initial donor solution in the buffer (50, 25, and 12.5% dilutions), as well as the buffer control, were obtained. The UV absorbance at various wavelength (λ=250, 260, …300 nm) of the dilutions and the buffer—$A_\lambda(C_0/2)$, $A_\lambda(C_0/4)$, $A_\lambda(C_0/8)$ and $A_\lambda(0)$—were used to generate a linear trendline in a spreadsheet. We selected a wavelength $\lambda1$ where the R^2 value of the trendline is higher than 99%, then used that trendline and the UV absorbance of donor and acceptor solutions at wavelength $\lambda1$–$A_{\lambda1}$(donor) and $A_{\lambda1}$(acceptor)—to calculate the concentrations of the donor and acceptor solutions C_D and C_A. We have found that for most of the compounds, a highly linear relationship (R^2> 0.99) between UV absorbance and concentration can be obtained at either 280 or 250 nm. Therefore usually the absorption at 280 nm was used for concentration calculations. If the absorption at 280 nm is low for a certain compound, such as acebutolol and sulpiride, then the absorption at 250 nm was used for concentration calculations.

Permeability Calculations

Permeability of the compounds was calculated using the following formula:

$$P_e = \frac{-\ln\left[1 - C_A(t)/C_{equilibrium}\right]}{A*(1/V_D + 1/V_A)*t} \quad (1)$$

where P_e is permeability in the unit of cm/s. A=effective filter area=f×0.3 cm^2, where f=apparent porosity of the filter (discussed below), V_D=donor well volume=0.3 ml, V_A= acceptor well volume=0.2 ml, t=incubation time (s), $C_A(t)$=

compound concentration in acceptor well at time t, $C_D(t)$= compound concentration in donor well at time t, and

$$C_{equilibrium} = [C_D(t)*V_D + C_A(t)*V_A]/(V_D + V_A) \quad (2)$$

Mass retention (R) was calculated using the following formula:

$$R = 1 - [C_D(t)*V_D + C_A(t)*V_A]/(C_0*V_D) \quad (3)$$

where C_0=initial compound concentration in donor well.

The permeability equation is deduced from the two-flux Equation (14):

$$C_A(t) = C_{equilibrium} \\ *\{1 - \exp[-P_e*A*(1/V_D + 1/V_A)*t]\} \quad (4)$$

For the apparent porosity of the PVDF filter, the assumption of f=1 for PVDF filter has been used in many PAMPA literatures (10,14,15). In some recent literatures (7,16,17) f=0.76 has been used so that the results obtained from PAMPA based on PVDF filter can be directly compared to the results obtained from PAMPA based on polycarbonate filter (5). By using f=0.76 instead of f=1, all of the permeability values are increased by a factor of 1/0.76. This simple scale factor does not affect the ranking of the compounds according to permeability. However in order to compare the absolute permeability values obtained from one PAMPA method to another PAMPA method, one needs to use the same porosity value. We have used f=1 in the calculations throughout this paper so that our results can be directly compared to the results cited from previous PAMPA literatures.

RESULTS AND DISCUSSION

Design of the Tri-Layer Artificial Membranes

We compared two types of artificial membranes used in PAMPA: the lipid solution-based membrane used in many previous studies and the tri-layer membrane used in this study. The lipid solution-based artificial membrane is prepared by coating a porous filter with a solution of lipids. The solvent used is usually dodecane. The lipids concentration is usually 1–20% (4,10,14). Figure 2 shows an image of part of a 96-well PVDF filter plate placed upside down to reveal the PVDF filters on the bottom of the wells. The PVDF filters in the first row (Fig. 2A) are not coated. The PVDF filters in the second row (Fig. 2B) are coated with 4 μl 2% DOPC in dodecane. The PVDF filters appeared wet and semi-transparent after coating, suggesting that the porous PVDF filter is entirely soaked with the lipid solution. The PVDF filters in the third row (Fig. 2C) are coated with the lipid/oil/lipid tri-layer artificial membrane used in this study. To prepare this artificial membrane, a volatile solvent—hexane—was used to disperse the contents of each layer of the tri-layer evenly inside the porous filter. The artificial membrane was formed in PVDF by three coating steps. In the first step, 1 μl of hexadecane is dispersed into PVDF using hexane as a carrier, forming the middle oil layer.

A Un-coated PVDF membrane

B PVDF membrane coated with lipid solution

C PVDF membrane coated with lipid/oil/lipid tri-layer
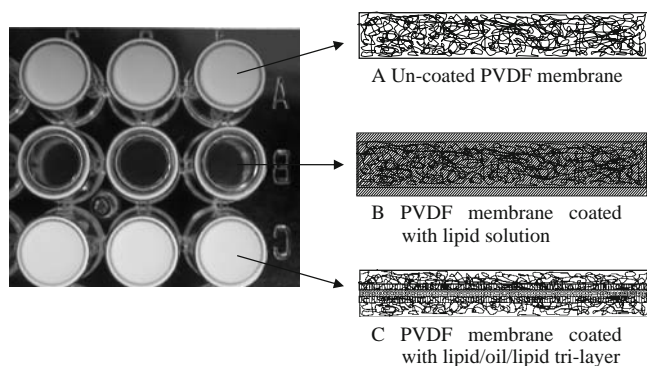
Fig. 2. Photo and schematic illustration of PVDF filters: **A** uncoated, **B** coated with 2% DOPC in dodecane, and **C** coated with lipid/oil/lipid tri-layer artificial membrane.

In the second step, 40 μg of a phospholipids mixture is dispersed into PVDF using hexane as a carrier, forming the top lipid layer. In the third step, the plate is turned up-side-down, and the phospholipids mixture is dispersed into PVDF using hexane as a carrier, forming the bottom lipid layer. After the hexane evaporates, the tri-layer forms without excess solvent present in the porous filter. The PVDF filter appeared dry after coating, suggesting that the lipid/oil/lipid tri-layer is embedded inside of the porous filter. In contrast with the lipid solution-based membrane, there is no excessive amount of solvent present in the tri-layer artificial membrane, as evident from the appearance of the coated porous filter.

The presence of the middle oil layer is critical for the robustness and stability the tri-layer artificial membrane. Although a true lipid bilayer is desired for mimicking the cell membrane, it has proven to be too fragile for high throughput screening. A method of forming a true bilayer lipid membrane at the interface of two liquid compartments, in which a small quantity of phospholipids is carefully placed over a small hole in a thin sheet of Teflon (the membrane formed is called black BLM), has been studied since 1962 (18). The black BLM has proven to be too fragile and too difficult to manufacture for high throughput screening. When the original PAMPA method was published by Kansy *et al.* (4), it has been speculated that the lipid bilayer forms inside the pores of the membrane since the lipid solution used to coat the porous filter is similar to the solution used to create black BLM. However, the formation of lipid bilayer in the lipid solution-based PAMPA artificial membrane is very unlikely since the porous filter is apparently entirely soaked in the lipid solution. A very different approach was used in designing the lipid/oil/lipid tri-layer artificial membrane: an oil layer is inserted in between two lipids layers to provide robustness and stability. The oil layer mimics the hydrophobic interior of the biological membrane and allows the amphiphilic lipids to anchor on it, and is crucial for maintaining a robust and stable artificial membrane.

Correlation with Caco-2 Data and Human Absorption Values

A set of structurally diverse compounds with known human absorption values was selected for validating the lipid/oil/lipid tri-layer artificial membrane. Table I lists 12 compounds tested at BMS, along with their human absorption values, Caco-2 permeability values, and PAMPA permeability values obtained from three different PAMPA methods. Table II lists 35 compounds tested at BD Biosciences, along with their human absorption values and PAMPA permeability values obtained from two different PAMPA methods.

Figure 3 compares the correlation plots of the Caco-2 and PAMPA permeability data of 12 compounds listed in Table I. Since none of these 12 compounds is known to be actively transported, the Caco-2 permeability values should correlate with the PAMPA permeability values as they both reflect the passive permeability of these compounds.

Table I. Human Absorption and Permeability Data of 12 Compounds

	Human Absorption (FA%)	Permeability (10⁻⁶ cm/s)			
		Caco-2	Solution-based PAMPA Model 1[a]	Solution-Based PAMPA Model 2[b]	Tri-Layer PAMPA Model[c]
Sulfasalazine	12[d]	0.4±0.1[d]	0.0±0.0	0.1	0.24±0.15
Nadalol	33[e]	1.7±0.4[e]	0.28±0.04	0.1	0.00±0.00
Norfloxacin	35[d]	1.9±0.3[d]		0.1	0.52±0.47
Etoposide	50[d]	1.8±1.0[d]		3.5	0.11±0.19
Acebutalol	55[d]	4±0.4[d]	0.03±0.01	1.1	0.18±0.08
Ketoprofen	90[d]	25.0±2.6[d]	0.05±0.01	1.0	4.60±0.29
Propranolol	90[d]	17.5±2.6[d]	14.3±0.1	62.8	8.08±1.02
Dexamethasone	95[d]	13.4±1.3[d]		27.8	2.71±0.41
Metoprolol	95[d]	12.0±1.0[d]	0.41±0.05	34.2	4.27±0.12
Antipyrine	100[e]	28.8±3.5[e]	0.74±0.06	1.4	8.42±0.32
Caffeine	100[e]	33.1±2.7[e]	1.2±0.1	1.9	9.58±0.63
Naproxen	100[d]	30.0±4.1[d]	0.34±0.01	3.3	6.77±0.49

[a] The solution-based PAMPA model 1 refers to the traditional PAMPA model in which the PVDF filter was coated with a solution of 2% DOPC in dodecane (10,14). The permeability values are cited from Ruell *et al.* (10).
[b] The solution-based PAMPA model 2 refers to the double-sink PAMPA method (7,11). The permeability values were obtained at BMS. The donor buffer was PBS, pH 7.4.
[c] The tri-layer PAMPA model refers to the model described in this paper. The permeability values were obtained at BMS.
[d] Values are cited from Balimane *et al.* (19) Table I.
[e] Values are cited from Marino *et al.* (20) Table I.

Table II. Human Absorption and Permeability Data of 35 Compounds

		Permeability (10^{-6} cm/s)	
	Human Absorption (FA%)[a]	Solution-Based PAMPA Model 1[b]	Tri-Layer PAMPA Model[c]
Sulfasalazine	13	0.00±0.00	0.20±0.04
Acyclovir	16	0.04±0.01	0.10±0.03
Nadolol	30	0.28±0.04	0.16±0.07
Sulpiride	35	0.03±0.00	0.18±0.02
Famotidine	40	0.06±0.00	0.04±0.02
Acebutolol	50	0.03±0.01	0.21±0.02
Amiloride	50	0.00±0.00	0.08±0.02
Atenolol	54	0.06±0.01	0.10±0.08
Terbutaline	60	0.05±0.05	0.46±0.15
Furosemide	61	0.01±0.01	0.46±0.06
Ranitidine	61	0.01±0.00	0.45±0.06
Hydrochlorothiazide	67	0.02±0.01	0.09±0.02
Phenytoin	90	0.38±0.06	5.73±0.53
Timolol	90	0.61±0.06	4.45±0.24
Pindolol	92	0.12±0.03	2.64±0.59
Alprenolol	93	11.81±0.25	9.71±0.25
Desipramine	95	16.59±0.28	8.67±0.14
Ibuprofen	95	2.40±0.38	10.73±1.62
Metoprolol	95	0.41±0.05	4.29±0.25
Verapamil	95	39.4±4.2	8.75±0.53
Theophyline	98	0.04±0.00	3.53±0.38
Warfarin	98	1.58±0.20	4.96±0.40
Diclofenac	99	1.37±0.20	6.95±0.31
Diltiazem	99	17.09±0.75	10.61±0.39
Imipramine	99	14.04±0.43	10.11±0.30
Naproxen	99	0.34±0.01	6.03±0.59
Propranolol	99	14.30±0.10	8.64±0.25
Antipyrine	100	0.74±0.06	7.51±1.43
Caffeine	100	1.20±0.10	9.89±1.52
Carbamazepine	100	6.40±0.20	9.44±1.11
Clonidine	100	1.50±0.20	6.45±1.07
Indomethacin	100	0.30±0.02	6.24±0.26
Ketoprofen	100	0.05±0.01	4.13±0.42
Nicotine	100	10.47±1.57	4.28±0.47
Piroxicam	100	2.64±0.31	4.96±0.68

[a] Values are cited from Ruell *et al.* (10).
[b] The solution-based PAMPA model 1 refers to the traditional PAMPA model in which the PVDF filter was coated with a solution of 2% DOPC in dodecane (10,14). The permeability values are cited from Ruell *et al.* (10).
[c] The tri-layer PAMPA model refers to the model described in this paper. The number of replicates per compound is four.

In Fig. 3A, the PAMPA data were obtained using a traditional PAMPA model, in which the PVDF filter was coated with a solution of 2% DOPC in dodecane (10,14). The PAMPA permeability values of several compounds do not correlate well with the Caco-2 values. Antipyrine, caffeine, ketoprofen, metoprolol and naproxen have high Caco-2 permeability values but low PAMPA permeability values. These compounds are under-predicted using this traditional PAMPA model.

In Fig. 3B, the PAMPA data were obtained using the double-sink PAMPA method, in which the PVDF filter was coated with a 20% dodecane solution of a phospholipid mixture (*p*ION, Woburn, MA) and the receiver solutions contained a surfactant mixture (7,11). Using this PAMPA model, the correlation with Caco-2 values has improved. Metoprolol is not under-predicted. However, antipyrine, caffeine, ketoprofen, and naproxen are still under-predicted.

In Fig. 3C, the PAMPA data were obtained using the lipid/oil/lipid tri-layer PAMPA model. Using this PAMPA model, the correlation with Caco-2 values has dramatically improved. Antipyrine, caffeine, ketoprofen, metoprolol and naproxen are not under-predicted.

Figure 4 compares the correlation plots of the human absorption and PAMPA permeability data of 12 compounds listed in Table I. The biopharmaceutical classification system (BCS) defines highly permeable compounds as those that have human oral absorption greater than 90%. A good PAMPA model should be able to accurately rank the compounds (with the exception of actively transported compounds) into a high permeability group and a low permeability group. Since none of these 12 compounds are known to be actively transported, it is expected that the low permeable compounds defined in BCS produced low PAMPA values and the high permeable compounds defined in BCS produced high PAMPA values and the two groups of compounds can be clearly separated.

In Fig. 4A, the PAMPA data were obtained using the traditional PAMPA model explained in Table I. The PAMPA permeability values of several compounds do not correlate well

A

B

C
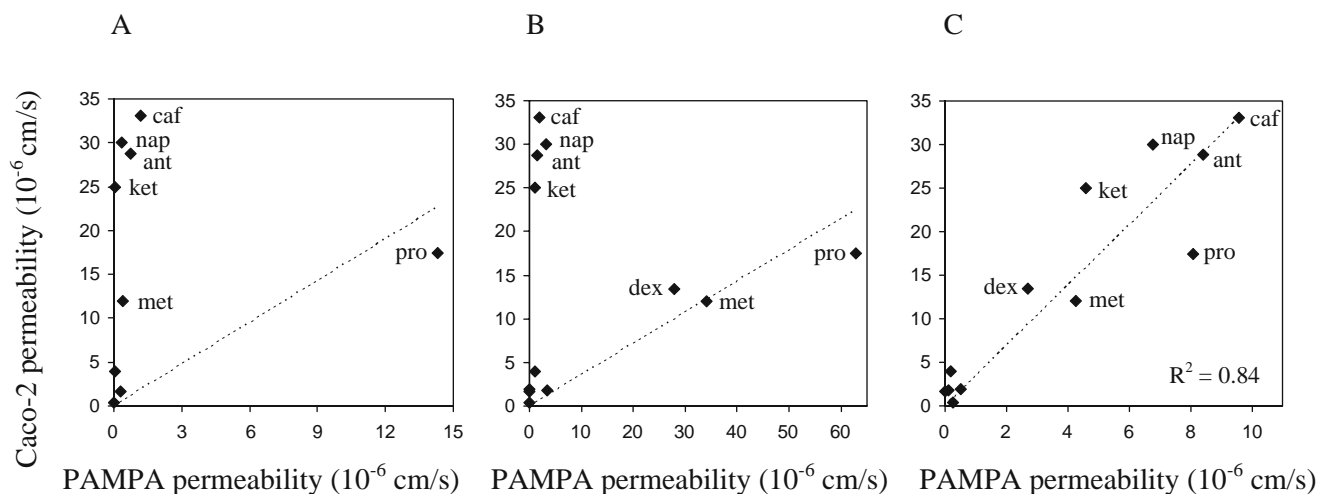
Fig. 3. Comparison of Caco-2/PAMPA correlation plots of 3 PAMPA models: **A** solution-based PAMPA model 1—PVDF filter coated with 2% DOPC in dodecane; **B** solution-based PAMPA model 2—double-sink method; **C** lipid/oil/lipid tri-layer PAMPA model. The compounds and their respective Caco-2 and PAMPA permeability values are listed in Table I. Some of the compounds are denoted in the plots: *caf* caffeine, *nap* naproxen, *ant* antipyrine, *ket* ketoprofen, *pro* propranolol, *met* metoprolol, *dex* dexamethasone. The *dashed line* of each plot is a linear trend line.

with the human absorption values. Antipyrine, caffeine, ketoprofen, metoprolol and naproxen have high human absorption values but low PAMPA permeability values. These compounds are under-predicted using this traditional PAMPA model.

In Fig. 4B, the PAMPA data were obtained using the double-sink PAMPA method. Using this PAMPA model, the correlation with human absorption values has improved. Metoprolol is not under-predicted. However, antipyrine, caffeine, ketoprofen, and naproxen are still under-predicted. It is noted that the values of some high permeability compounds have gotten very high—for example, 62.8×10^{-6} cm/s for propranolol—presumably due to the use of the double-sink acceptor solution. The presence of a surfactant mixture in the double-sink acceptor solution seems to have greatly enhanced the permeability values of some high

permeability compounds, possibly due to preferential binding of these compounds to the surfactant mixture.

In Fig. 4C, the PAMPA data were obtained using the lipid/oil/lipid tri-layer PAMPA model. Using this PAMPA model, the correlation with human absorption values has dramatically improved. Antipyrine, caffeine, ketoprofen, metoprolol and naproxen are not under-predicted. The PAMPA permeability values of the compounds with high human oral absorption are larger than 1.5×10^{-6} cm/s, while the PAMPA permeability values of the compounds with low human oral absorption are smaller than 1.5×10^{-6} cm/s. Therefore the permeability property of the compounds is corrected predicted using the lipid/oil/lipid tri-layer PAMPA model.

Figure 5 further compares the performance of a solution-based PAMPA model and the tri-layer PAMPA model by

A

B

C
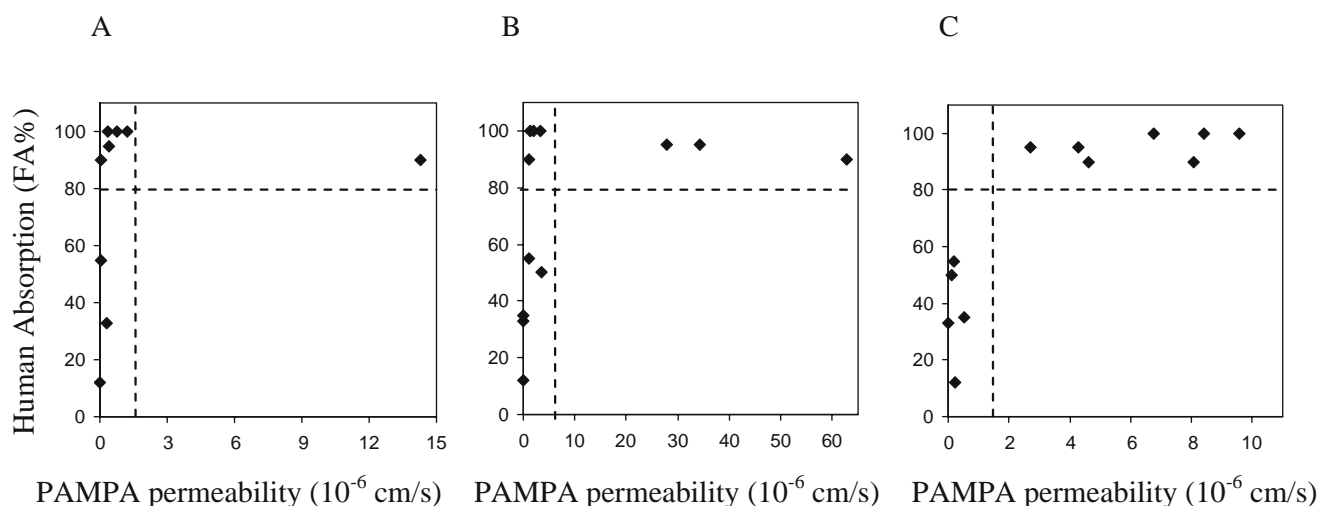
Fig. 4. Comparison of human absorption/PAMPA correlation plots of three PAMPA models: **A** solution-based PAMPA model 1—PVDF filter coated with 2% DOPC in dodecane; **B** solution-based PAMPA model 2—double-sink method; **C** lipid/oil/lipid tri-layer PAMPA model. The compounds and their respective human absorption values and PAMPA permeability values are listed in Table I. The *horizontal dashed line* separates high human absorption and low human absorption compounds. The *vertical dashed line* separates high PAMPA permeability and low PAMPA permeability compounds.
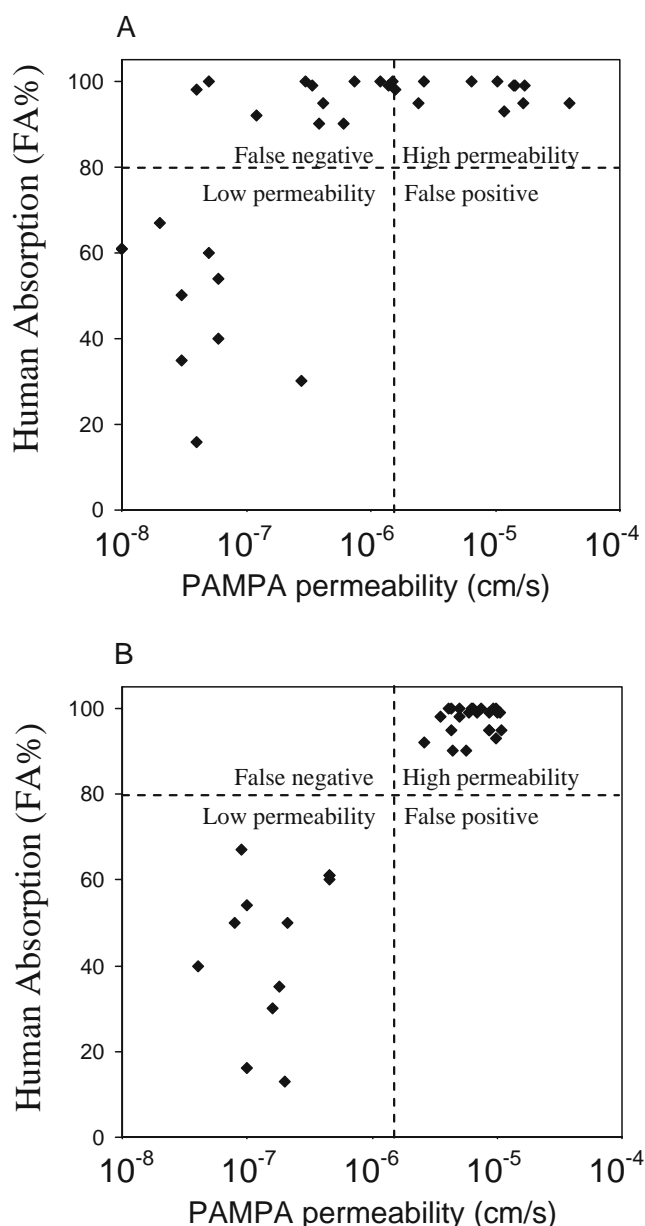
Fig. 5. Comparison of human absorption/PAMPA correlation plots of two PAMPA models: **A** solution-based PAMPA model 1—PVDF filter coated with 2% DOPC in dodecane; **B** lipid/oil/lipid tri-layer PAMPA model. The compounds and their respective human absorption values and PAMPA permeability values are listed in Table II. The *horizontal dashed line* separates high human absorption and low human absorption compounds. The *vertical dashed line* separates high PAMPA permeability and low PAMPA permeability compounds.

analyzing the correlation of the permeability data with the human absorption data for a set of 35 compounds listed in Table II. The permeability data of the lipid solution-based PAMPA membrane were cited from Ruell *et al.* (10). The permeability data of the tri-layer PAMPA membrane were obtained using similar conditions as those used by Ruell *et al.* (10): Both donor and acceptor buffers were PBS, pH 7.4; and the PAMPA assembly was incubated at room temperature without agitation. Because similar buffer and incubation conditions have been used, the significant differences in

permeability data are results from the difference in the artificial membrane. In Fig. 5, a vertical line at 1.5×10^{-6} cm/s separates the low and high permeability compounds determined by PAMPA. Using the lipid solution-based artificial membrane, there is a group of compounds with high human absorption values that are under-predicted. None of these compounds are known to be actively transported. Remarkably, this group of compounds is correctly predicted using the tri-layer PAMPA membrane. This is a strong indication that the cause of under-prediction is the presence of excess amount of solvents in the lipid solution-based PAMPA membrane, since the only major difference between our PAMPA study and the PAMPA study conducted by Ruell *et al.* (10) is the structure of the artificial membrane. The experimental conditions used in the two studies are similar—the filter plate materials, donor and acceptor buffers, pH, temperature, no stirring and the formula used for calculating permeability are the same; the incubation time is different but is factored out in the permeability calculations.

We have not compared to some other PAMPA models (21), as it is hard to find the same set of compounds being used in multiple literatures reporting different PAMPA methods. We have noticed that some PAMPA methods have also improved the prediction of some of the traditional under-predicted compounds. For example, the use of octanol as the phospholipid solvent in PAMPA has yielded higher permeability value for antipyrine (22). This is another piece of evidence that the solvent of the phospholipids in the solution-based PAMPA methods plays an important role in the permeability of some compounds.

Mass Retention

Mass retention is the percentage of the total mass of the compound lost during the permeability measurement as a result of binding to the plastic surface and/or retaining in the artificial membrane. The mass retention values of most of the commercial compounds we studied are small (lower than 20%). High mass retention may affect the accuracy of permeability measurement. However, the permeability of high mass retention compound can still be calculated according to Eq. 1, which has already taking the mass retention into account by using $C_{equilibrium}$ instead of C_0 for permeability calculations (14). By using $C_{equilibrium}$ instead of C_0, the part of compound mass that is bound to the plastic surface and/or retained in the artificial membrane is considered to have no contribution to the permeability.

The loss of compound mass due to retention in the artificial membrane is expected to reduce for the tri-layer PAMPA membrane compared to the lipid solution-based PAMPA membrane, because the excess amount of solvent in the lipid solution-based PAMPA membrane is not present in the tri-layer PAMPA membrane. Table III compares the mass retention of amitriptyline, ketoconazole and phenazopyridine in PAMPA using the solution-based membrane and the tri-layer membrane. These three compounds have been reported to have high mass retention with the solution-based PAMPA membrane (14). Since the only major difference between our PAMPA study and the PAMPA study conducted by Avdeef *et al.* (14) is the structure of the artificial membrane and the experimental conditions used in the two studies are essential-

**Table III.** Mass Retention Values of Three Compounds

	Solution-Based PAMPA Model 1[a] (%)	Tri-Layer PAMPA Model (%)
Amitriptyline	53	27
Ketoconazole	62	32
Phenazopyridine	69	39

[a] The solution-based PAMPA model 1 refers to the traditional PAMPA model in which the PVDF filter was coated with a solution of 2% DOPC in dodecane (10,14). The mass retention values are cited from Avdeef *et al.* (14).

ly the same, the reduction of mass retention must be due to the tri-layer membrane structure, which apparently retain less of these compounds. The reduction of mass retention can also be achieved by other methods, such as using the special receiver buffer in the double-sink PAMPA method (7,11).

Membrane Stability

A stable artificial membrane offers the capability of pre-coating the filter plate in large batches, which can be validated and used over an extended period of time. This practice reduces uncertainties due to the potential day-to-day variability of the membrane preparation process. To study the stability of the lipid/oil/lipid tri-layer PAMPA membrane, filter plates pre-coated with the tri-layer PAMPA membrane
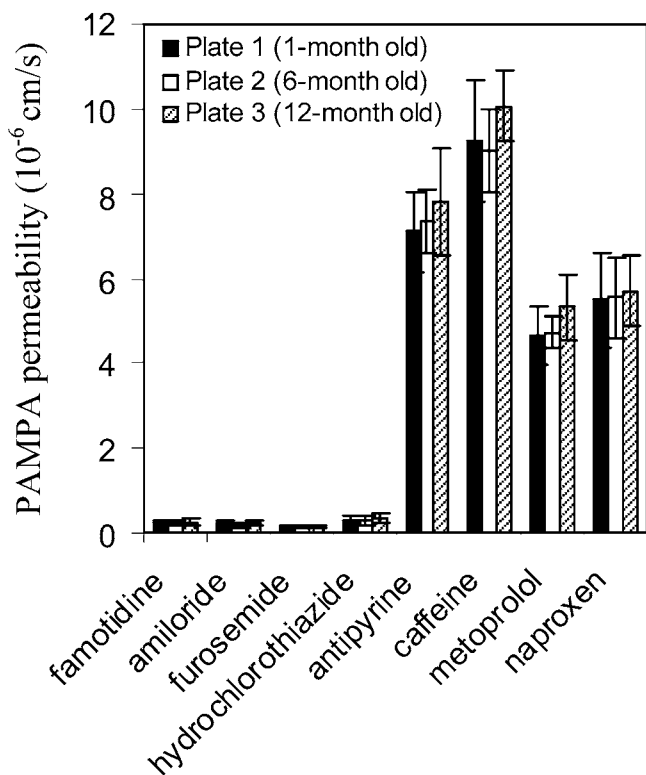
were stored at −20°C for a long period of time and tested periodically. In order to exclude the variability caused by reagents and other experimental conditions, we have tested plates pre-coated on different days with the same set of reagents and same experimental conditions at the same time. Figure 6 shows the results of such a test involving three plates: plate #1 was coated one month before the test date, plate #2 was coated 6 months before the test date, and plate #3 was coated 12 months before the test date. Eight compounds were tested, with each compound having 11 replicates on each plate. Four compounds—famotidine, amiloride, furosemide, hydrochlorothiazide—have low permeability; the other four compounds—antipyrine, caffeine, metoprolol, naproxen—have high permeability. The results show that the permeability values obtained from three plates are the same within the standard deviation. This not only confirms that the results obtained from different batches of pre-coated plates are highly reproducible when the same reagent set and same experimental conditions are used, but also confirms that the tri-layer PAMPA membrane is stable for at least 1 year when stored at −20°C.

Membrane Compatibility with Organic Solvents

Low solubility compounds have been a challenge for PAMPA measurement, since their maximum concentrations in
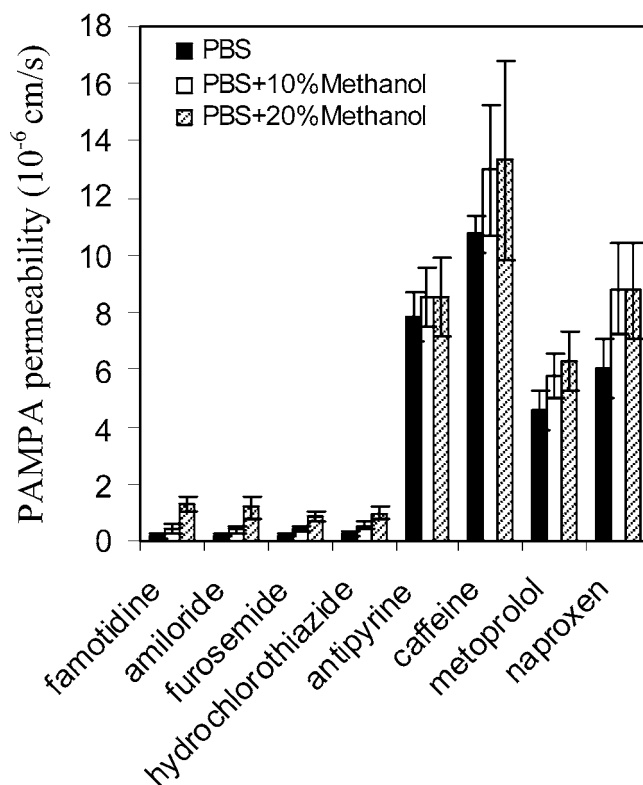

Fig. 6. Comparison of lipid/oil/lipid tri-layer PAMPA permeability values of eight compounds obtained using three pre-coated plates prepared at different times: Plate 1 was prepared one month before the day of assay, plate 2 was prepared 6 months before the day of assay, and plate 3 was prepared one year before the day of assay. The number of replicates per compound on each plate is 11.

Fig. 7. Comparison of lipid/oil/lipid tri-layer PAMPA permeability values of eight compounds obtained using three buffer conditions: Condition 1 used PBS as the buffer for both donor and acceptor solutions, condition 2 used PBS + 10% methanol as the buffer for both donor and acceptor solutions, and condition 3 used PBS + 20% methanol as the buffer for both donor and acceptor solutions. The number of replicates per compound on each plate is 11.

aqueous buffer are too low for accurate concentration measurement. In order to improve solubility, a common practice has been to increase the percentage of organic solvents in the aqueous buffer. In order to do this, the PAMPA artificial membrane needs to be proved to be compatible with the higher percentage of organic solvents.

Since the compound stock solutions are usually prepared in DMSO, it has been a common practice to use higher percentage of DMSO in aqueous buffer to improve solubility. However due to the UV absorption of DMSO at low wavelengths (<280 nm), measurements of compounds with UV absorption at low wavelength can be affected by the use of higher percentage DMSO (>5%), especially when UV plate reader is used to measure concentrations. Therefore, it is advantageous to use a more UV transparent solvent, such as methanol, to help increasing the compound solubility.

Figure 7 compares the permeability measurements of 8 compounds in 3 conditions. In the first condition, the donor solutions are prepared by diluting (1:50 dilution) the DMSO stock solutions of the compounds into PBS, and the acceptor solution is PBS. In the second condition, the donor solutions are prepared by diluting (1:50 dilution) the DMSO stock solutions of the compounds into PBS + 10% methanol, and the acceptor solution is PBS + 10% methanol. In the third condition, the donor solutions are prepared by diluting (1:50 dilution) the DMSO stock solutions of the compounds into PBS + 20% methanol, and the acceptor solution is PBS + 20% methanol. With 10% methanol, there are slight increases of the permeability values of the high permeability compounds, while the permeability values of the low permeability compounds stay low. This indicates that the artificial membrane has maintained its integrity and the correct ranking of the compounds can be obtained with 10% methanol. With 20% methanol, there are noticeable increases in the permeability values of the low permeability compounds, indicating that the artificial membrane barrier has started to get weakened by the presence of methanol. However, the correct ranking of the compounds can still be obtained with 20% methanol. With 30% methanol (data not shown), the permeability values of the low permeability compounds increased dramatically, indicating that the artificial membrane has broken down with 30% methanol. Therefore, the lipid/oil/lipid tri-layer membrane can tolerate up to 20% methanol in PBS buffer.

CONCLUSION

The lipid solution-based artificial membrane models predominantly used today have under-predicted some compounds classified by BCS as highly permeable, presumably due to the presence of excess amount of solvents in the artificial membrane. The lipid/oil/lipid tri-layer artificial membrane improved the predictability, as demonstrated by the improved correlation with Caco-2 permeability values and the improved correlation with human absorption values.

The lipid/oil/lipid tri-layer PAMPA model also reduces the mass retention of the highly retained compounds reported in previous studies. This can also be contributed to the reduction of the amount of compounds being trapped in the excess amount of solvents in the artificial membrane.

The filter plates pre-coated with the lipid/oil/lipid tri-layer artificial membrane can be stored at −20°C for at least 1 year, as confirmed by conducting permeability assay of a same set of compounds using plates with different ages and comparing the results obtained from different plates. This good stability provides a means for reducing the uncertainties due to day-to-day coating variability and improving PAMPA reproducibility.

The lipid/oil/lipid tri-layer artificial membrane is found to be compatible with 10–20% methanol, as confirmed by conducting permeability assay of a same set of compounds using buffers with different concentrations of methanol. This compatibility provides a means for improving the permeability measurements for low solubility compounds.

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