Development of a biomimetic phospholipid vesicle-based permeation assay (PVPA) for the estimation of intestinal drug permeability

Elenaz Naderkhani[a], Johan Isaksson[b], Alexey Ryzhakov[a,c], Gøril Eide Flaten*[a]

[a]Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø, Norway

[b]Drug Discovery and Design, Department of Chemistry, University of Tromsø, Norway

[c]Institute of Solution Chemistry, Russian Academy of Sciences, Ivanovo, Russia

*To whom correspondence should be addressed: Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø, N-9037 Tromsø, Norway.

Phone: +47 77646169. Fax: +47 77646151. E-mail: Goril.Flaten@uit.no

ABSTRACT

Permeability is a crucial property of orally administered drugs. Therefore, in drug discovery, it is important to employ methods suitable for rapidly and reliably screening of the permeability of large numbers of new drug candidates.

The PVPA, a model consisting of a tight layer of liposomes immobilized on a filter, offers potential advantages unmet by other methods and has been successfully used in permeability testing of novel active substances as well as formulations. In this study, the PVPA was developed into a more robust, biomimetic model by employing a lipid composition matching that of the intestinal permeation barrier and performing the experiments at the more biologically relevant pH 6.2.

As expected, positively charged basic compounds demonstrated increased permeability through the negatively charged biomimetic barriers, and the degree of correct classification according to *in vivo* absorption was comparable between the original PVPA and the biomimetic PVPA.

The biomimetic PVPA further proved to be tremendously more robust towards the presence of tensides compared to the original PVPA; this is a promising finding that renders the biomimetic PVPA an enhanced ability to estimate the permeability of poorly soluble compounds. Hence, the PVPA model developed in this study has evolved an important step forward.

Keywords: *in vitro* model, liposome, lipid, artificial membrane, intestinal absorption, screening, tenside, co-solvent, absorption, surfactants

List of abbreviations

Chol, cholesterol; E-80, Egg phospholipids; EPE, Egg phospatidylethanolamine; PE, phospatidylethanolamine; PI, phosphatidylinositol; EPS, Egg phospatidylserine; PS, phospatidylserine; PAMPA, parallel artificial membrane permeability assays; PCS, photon correlation spectroscopy; ft, freeze-thaw; PBS, phosphate-buffered saline; PVPA, phospholipid vesicle-based permeation assay: $PVPA_o$, E80, $PVPA_{biomimetic}$, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, cholesterol

INTRODUCTION

Assessment of the oral bioavailability of new drug candidates in the early stages of drug development is of special interest since modern drug discovery regimens produce hundreds of thousands of potential drug candidates for high-throughput screening for activity or potency related characteristics. There is thus a rapidly increasing demand for *in vitro* models that can reliably assess drug absorption and disposition, preferably in a high-throughput test format.

Both *in silico* and experimental methods are currently available for the prediction and/or screening of properties related to oral drug absorption. The gold standard in *in vitro* permeability screening is currently the cell-based Caco-2 model, which is widely established in both academia and industry.[1-3] This model is of special value in estimating the impact of active transport proteins on the permeability of orally administered drugs. However, cell-based models, such as the Caco-2 model or the novel method of co-culturing three different cell lines to obtain a more complete picture of oral absorption,[4] might be too complicated and time-consuming in regard to modern high-throughput requirements. One of the most commonly used current techniques for the rapid screening of drug permeability through the intestinal epithelia is the parallel artificial membrane permeation assay (PAMPA).[5-10] PAMPA uses a simple phospholipid/organic solvent-coated filter as the permeability barrier, which allows for medium- to high-throughput screening of permeability properties. Such simplified permeability approaches model only the passive pathway, not paracellular or active transport pathways. However, reports conclude that 80-95% of the commercial drugs are absorbed primarily by passive diffusion.[11] Therefore, a robust model focused on passive diffusion, not relying on cells and thus more compatible with high-throughput applications appears to be a useful first step in the biopharmaceutical characterization of new chemical entities.

We originally reported the PVPA, a predictive medium- to high-throughput screening method for passive drug permeability based on a tight barrier of liposomes on a filter support.[12] To the best of our knowledge, this was the first successful attempt to deposit a lipid-based membrane barrier without the use of an inert solvent, such as hexadecane. The PVPA barriers were originally prepared from egg phosphatidylcholine liposomes placed onto a filter support by centrifugation. Solvent evaporation and freeze-thaw cycling were then used to promote liposome fusion, resulting in a tight barrier consisting of layers of liposomes mimicking a cell-layer/tissue. The PVPA was validated using a selection of compounds and the apparent permeability coefficients obtained correlated well with literature data on human absorption *in vivo*, which suggested that the method is suitable for rapidly screening of the passive transport of drugs and new chemical entities.[12] The PVPA approach appears to, under comparable

settings, mimic *in vivo* absorption better than the biomimetic PAMPA model[8] and performs equally well in comparison with the Caco-2 cell model[13] and the double sink PAMPA (DS-PAMPA) model[5] in predicting the passive diffusion of drug compounds.[12] The model has further been shown to be adaptable to automation using a laboratory robotic system[14] and is stable at −80 °C for up to two weeks,[15] drastically increasing throughput. The functionality of the barriers has also been demonstrated to be stable within a pH range of 2.0 to 8.0, which makes the barriers suitable for further studies to provide, for example, insight into segmental absorption in the human gastrointestinal tract or to mimic other absorption barriers with a pH environment different from pH 7.4.[15] The PVPA has further been used to test the permeability of new active substances as well as drugs in formulations. The oral permeability of antibacterial peptides has been predicted[16-18] and different formulations, *i.e.,* micelles, solid solutions and liposomes, have been tested in our model.[19,20] Recently, a modified PVPA mimicking healthy and compromised skin barriers has been introduced.[21]

The goal of an *in vitro* permeability model is the ability to predict permeability values that correlate well with *in vivo* permeability data. To accomplish this, it is important that the model mimics the *in vivo* situation as closely as possible. Factors receiving much attention in this respect include the pH profile of the gastrointestinal tract and the lipid composition of the artificial barriers. It has been shown that membranes consisting of only phosphatidylcholine (PC) underestimate the permeability of basic and acidic drugs and a better *in vivo-in vitro* correlation can be obtained using a negatively charged barrier with a biomimetic lipid composition.[8,22]

Furthermore, an increasing problem in drug development is that a large number of new drug candidates demonstrate low water solubility, which may result in problems pertaining to permeability and bioavailability. To overcome this challenge, solubility-increasing agents can be added to the donor phase to increase drug solubility. It is therefore also important that a permeability model maintains its integrity in the presence of the solubility-enhancing agents of interest.

The aim of this study was thus to improve the biomimetic properties of the PVPA model by first investigating if the use of more biologically relevant pH conditions in the permeability experiments would lead to improved prediction of *in vivo* absorption. Second, a biomimetic lipid composition was designed to better mimic the intestinal barrier in an effort to significantly influence the permeation of drugs. A change in the lipid composition could potentially provide

a more robust barrier capable of handling harsher conditions, which in turn would enable an estimation of the permeability of poorly soluble compounds. Finally, the stability of the biomimetic PVPA in the presence of tensides and co-solvents was investigated.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine, Lipoid E-80, egg phosphatidylethanolamine (EPE) and egg phosphatidylserine (EPS) were obtained from Lipoid, Ludwigshafen, Germany. Cholesterol, phosphatidylinositol (PI), methanol, alprenolol hydrochloride, atenolol, acebutolol hydrochloride, caffeine, chloramphenichol, chlorothiazide, enalapril maleate, hydrochlorothiazide, metoprolol tartrate, nadolol, ranitidine hydrochloride, sulphasalazine, sulpiride, testosterone, terbutaline hemisulfate, timolol maleate, tranexamic acid, calcein, ibuprofen, flufenamic acid, flubiprofen and metronidazole were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Amiloride hydrochloride and chloroform were supplied by Merck KGaA, Darmstadt, Germany. Salicylic acid and acetyl salicylic acid were obtained from NMD, Oslo, Norway. Cimetidine was purchased from Allphamed Pharma GmbH, Göttingen, Germany.

Propranolol hydrochloride was purchased from TCI Europe N.V., Zwijndrecht, Belgium. Diclofenac sodium was purchased from AWD Pharma GmbH & Co., KG, Dresden Germany.

Filter inserts (Transwell, d=6.5 mm) and plates were purchased from Corning Inc., Corning, NY, USA, and the mixed cellulose ester filters (0.65 μm pore size) were obtained from Millipore, Billerica, MA, USA.

Liposome preparation

Liposomes were prepared using the film hydration method. Lipids were dissolved in a mixture of chloroform and methanol (2:1) in a round bottom flask. The organic solvent was removed under vacuum at 45 °C. The deposited lipid film was exposed to a vacuum of 55 hPa at room temperature for an additional 3 h period to remove all traces of solvent prior to hydration with phosphate buffer containing 10% (v/v) ethanol, thereby yielding a 6% (w/v) liposomal

dispersion as previously described.[12] The liposomes were then extruded by hand using either syringe filter holders or Lipofast (Avestin Europe GmbH, Mannheim, Germany).

Liposome size distributions were measured using photon correlation spectroscopy (PCS) in a Submicron Particle Sizer 370 (PSS Nicomp Particle Sizing Systems, Santa Barbara, CA, USA). Sample preparation and measuring conditions were as previously described.[23] Three cycles of 15-min measurements were performed.

Zeta potential measurements were performed on liposome dispersions diluted in PBS pH 7.4 using a Malvern Zetasizer nano Z (Malvern, Worcestershire, UK).

The original preparation method for the PVPA barriers

The PVPA barriers were prepared according to the procedure previously described[12] and were stored for up to two weeks at − 80 °C, in accordance with previous stability studies.[15] In brief, liposome dispersions with a mean size of 300 nm (volume weight) and 600 nm (number weight) were deposited on a filter support by centrifugation. The liposomes were added in consecutive steps from smallest to largest. Freeze-thaw cycling was then used to promote fusion of the liposomes and hence produce a tight barrier.[12]

Permeation studies

Permeation studies were performed using solutions of drugs or hydrophilic markers in phosphate buffer (pH 7.4 or 6.2), according to the procedure previously described.[12] Briefly, inserts were loaded with drug solution (100 µL) and placed in separate acceptor compartments containing phosphate buffer (600 µL). During the first 3 h of the study, the loaded inserts were moved to wells containing equal quantities of fresh buffer at 1 h intervals; over the subsequent 2 h, the inserts were moved at 0.5 h intervals. For cimetidine, nadolol, chlorothiazide and hydrochlorothiazide, the inserts were moved every second hour for 12 h. At the end of the permeation experiment, samples (200 µL) from each acceptor compartment (5 wells for each insert) were transferred into 96-well titer plates for analysis. Samples from the permeation experiments with drugs were measured spectrophotometrically (Spectramax 190; Molecular Devices, Sunnyvale, CA, USA) at the wavelength most appropriate for each drug (Table A, Supporting Information). Fluorescence measurements of the hydrophilic markers were

performed using a Polarstar fluorimeter (Fluostar, BMG Technologies, Offenburg, Germany) with excitation and emission wavelengths at 485 and 520 nm, respectively. A blank consisting of the same phosphate buffer as that used to prepare the drug solutions was subtracted from the absorbance/fluorescence values. The standard curves were derived from 11 different points (for the concentration range, see Table A, Supporting Information), and each point represented the mean of six parallels. The resistance of the lipid barriers was measured (Millicell-ERS, Millipore, Billerica, MA, USA) immediately after completing the permeation studies. A value of 119 ohms was subtracted from the observed resistance of the filters to account for the background resistance of the lipid barrier alone. The mean values and standard deviations are reported.

Calculations of physicochemical properties

QikProp, version 3.4, (2011)(Schrödinger, LLC, New York, NY, USA) was used with Maestro, version 9.2.112, to calculate the absorption percentage in the gastrointestinal tract as well as M_w and log P of the drugs. All substances were within the structural limits of the program.

Modifications of the PVPA barrier lipid composition to better mimic the intestinal epithelia

To produce barriers that could potentially mimic the lipid composition found *in vivo* in intestinal epithelia to a greater extent as well as to provide a more robust system capable of withstanding harsher procedural conditions[8,22,24], permeation barriers were prepared from liposomes containing the following: 26% phosphatidylcholine (PC), 26.5% phosphatidylethanolamine (PE), 7% phosphatidylserine (PS), 7% phosphatidylinositol (PI) and 33% cholesterol.

To prepare tight permeation barriers with the new lipid compositions, the original preparation process presented above required alterations. The different parameters investigated in the preparation process were as follows: i) the centrifugation time and speed after the addition of the small and large liposomes, respectively, ii) the temperature and duration of heating during the freeze-thaw cycling, iii) the number of freeze-thaw cycles, iv) the concentrations of the liposome dispersion, and v) the use of evaporation instead of centrifugation to settle the larger liposomes on the filter support. The permeability of the hydrophilic marker calcein as well as

the electrical resistance across the barriers were used to monitor the effects of changing the different parameters on the barrier properties.[12]

Stability of the PVPA in the presence of tensides and co-solvents

The stability of the PVPA barriers in the presence of tensides and co-solvents was examined to determine the feasibility of using these models in estimating the permeability of poorly soluble compounds of interest. The permeability of calcein and the electrical resistance across the barriers, markers previously used to indicate barrier integrity, were used to monitor the effect of the presence of additives in different concentrations.[12,25] The surfactants were each used at concentrations greater than their critical micelle concentration (CMC). PBS was included as a control and Triton X, which highly solubilizes liposomes, was included as a reference.

Statistical analysis

To determine if the changes in the preparation procedure provided significant changes in the permeability values, Student's *t*-tests for the comparison of two means were performed. A significance level of $p < 0.05$ was consistently used. The hypotheses determined the choice of a one- or two-sided *t*-test.

RESULTS AND DISCUSSION

Comparison of the experimental permeation values obtained from the PVPA with the calculated values of absorption and lipophilicity

The original PVPA (PVPA$_o$) was first compared with an *in silico* model for the estimation of permeability. A selection of 25 drugs, chosen to cover both acidic, basic and neutral drugs as well as a wide range of fractions absorbed in humans, was made. The results from the permeability values experimentally obtained from the PVPA$_o$ together with the calculated values for absorption as well as the molecular descriptors for the selection using QikProp are presented in Table 1.

The PVPA provided a means through which to classify the compounds into poor, moderate and excellent absorption categories according to their permeability values in the assay. At pH 7.4, compounds with P_{app}-values $< 0.1 \times 10^{-6}$ cm/s are poorly absorbed ($< 30\%$ fraction absorbed *in*

vivo), compounds with P_{app}-values between 0.1 and 0.9×10^{-6} cm/s are moderately absorbed (30–70% fraction absorbed *in vivo*), and compounds with P_{app}-values of $>0.9 \times 10^{-6}$ cm/s have excellent oral absorption (>70% fraction absorbed *in vivo*).[12] Based on this classification, 22 of the 25 drugs, or approximately 90%, were correctly classified with the $PVPA_o$ (salicylic acid, sulphasalazine and tranexamic acid were incorrectly classified). In comparison, only 19 of the 25 were correctly classified based on the QikProp-calculated absorbed fractions. When comparing the calculated fraction absorbed with the *in vivo* data (Figure A, Supporting Information), the calculated values appeared elevated relative to the *in vivo* data for the drugs with low absorption, while the opposite was seen for the drugs with high absorption *in vivo*. The experimentally determined log D and calculated log P did not demonstrate a correlation with *in vivo* fraction absorbed for this selection of drugs, as has been observed for other selections of drugs.[12]

The $PVPA_o$ thus appears capable of predicting intestinal absorption for marketed drugs with a degree of accuracy that is significantly better than theoretical models. The next step was thus to investigate if the use of more biologically relevant pH conditions and a biomimetic lipid barrier composition could further improve the model.

Investigation of various pH conditions representative of the different intestinal regions

The goal of an *in vitro* permeability model is to generate permeability values that correlate significantly with *in vivo* permeability data. To reach this ambition, it is important that the model mimics the *in vivo* situation as closely as possible. A factor receiving much attention in this respect is the pH profile of the gastrointestinal tract.[26]

The pH in the gastrointestinal tract increases from the acidic environment of the stomach to the more basic environment of the large intestine. In addition, the pH differs under fasting and fed conditions. The median pH in the duodenum has been reported to be 6.1 in the fasted state, with a decrease to pH 5.4 during a meal.[27] Between the proximal jejunum and the distal ileum, the pH gradually increases from approximately 6 to approximately 8.[26]

This provides a challenge when considering an estimation of permeability. To achieve a better understanding of drug permeability *in vivo*, the models must maintain their integrity across the pH range normally found in the intestine, and investigations into how changes in pH influence drug permeability are of high interest.

The integrity of the original PVPA barriers across a pH range from 2.0 to 8.0 was previously reported, and the barriers were found to maintain their integrity in terms of unchanged permeability of the hydrophilic marker fluorescein as well as electrical resistance.[25] The permeabilities of metoprolol and naproxen have also been shown to decrease with increasing ionization, according to the pH partition hypothesis. The model has thus shown useful potential in monitoring pH as an influencing factor in respect to the permeability of drugs and providing information about segmental absorption in the gastrointestinal tract.[15]

The permeability values of the 25 selected drug compounds, containing acidic, basic and neutral compounds, were evaluated by comparing those with pH 6.2 in the donor compartment with those obtained with pH 7.4 in the donor chamber (see Figure 1 and Table 1). It is apparent that some drugs showed the same permeation, independent of pH, while the basic drugs were mostly found in the dark grey triangle and the acidic drugs are mostly found in the light gray triangle, as expected according to the pH partition hypothesis. The degree of correct classification according to the *in vivo* absorption determined using the previously suggested classification system[12] were, however, not significantly different for the two pH conditions. However, this classification was set based on data obtained at pH 7.4, so the fact that the more biorelevant pH 6.2 did not lead to a higher degree of correct classification is not necessary surprising. One could suggest narrowing the range of the moderate absorption category to compounds with P_{app}-values between 0.1 and 0.7×10^{-6} cm/s. This would lead to no difference in the degree of correct classification for the drugs at pH 6.2 but would lead to the misleading classification of cimetidine (Fa=64%) at pH 7.4 into the class of excellent absorbed drugs.

Biomimetic PVPA (PVPA_biomimetic)

Lipoid E-80 with 82% (w/w) phosphatidylcholine (PC), 9% phosphatidylethanolamine (PE), 3% lysophosphatidylcholine, 2% sphingomyelin, 3% triglycerides, and 1% cholesterol was previously used in the development of the assay.[12] In this study, we aimed to adapt the model to better resemble *in vivo* conditions by changing the lipid composition of the barrier to one that more closely mimics the lipid composition of intestinal cells. It has been shown that membranes consisting of only PC are underestimating the permeability of basic and acidic drugs and that a better *in vivo-in vitro* correlation is obtained by using a negatively charged barrier with a biomimetic lipid composition of 26% PC, 26.5% phosphatidylethanolamine (PE), 7% phosphatidylserine (PS), 7% phosphatidylinositol (PI) and 33% cholesterol.[8,22] Utilizing this biomimetic lipid barrier composition in the PVPA, we investigated whether the alterations

significantly affected the permeation of drugs and if this new composition could improve the accuracy of the assay.

Development of preparation procedure

The basis for the development of a suitable preparation procedure for the new biomimetic PVPA barriers containing PC/PE/PS/PI/Chol stemmed from the original preparation method for PVPA barriers composed of E-80.

Liposomes composed of PC/PE/PS/PI/Chol were extruded through filters with pore sizes of 600 and 1200 nm to yield populations with mean sizes of 367 nm and 901 nm, which were entered into the pores of the filter and layered on top of the filter support, respectively. The zeta potentials were found to be -1.13 ± 0.25 mV and -20.95 ± 0.52 mV for the E-80 liposomes and the liposomes composed of PC/PE/PS/PI/Chol, respectively. This demonstrates that the chosen lipid composition results in liposomes with a negative surface charge, which are expected to produce barriers with the same property.

By applying the original preparation procedure described above to the liposomes composed of PC/PE/PS/PI/Chol, highly leaky barriers, characterized by high calcein permeability, were obtained. The follow parameters were thus investigated to obtain tighter barriers: i) the centrifugation time and speed after addition of small and large liposomes, respectively, ii) the temperature and duration of heating during freeze-thaw cycling and iii) the number of freeze-thaw cycles. Unfortunately, these approaches did not result in tight barriers (see Supporting Information for details), indicating that the original preparation procedure using centrifugation to immobilize the liposomes was not a promising approach. This can most likely be attributed to less efficient packing of the liposomes in the pores and on top of the barrier than in the original barriers.

An attempt was therefore made to use the original method of using small liposomes of E-80 to fill the pores. This was followed by the addition of larger liposomes with the biomimetic lipid composition, centrifugation at 2500 rpm for 30 min to settle the liposomes on top of the filter and invert centrifugation at low speed to remove the buffer. Freeze thaw cycles were performed as in the original procedure, with thawing at 60 °C for 60 min. The obtained barriers exhibited a permeation value for calcein of 2.307×10^{-6} cm/s (procedure 1, Figure 2), which was an improvement; however, the assay still lacked satisfactory integrity.

The next step was to omit the invert centrifugation step after allowing the larger liposomes consisting of PC/PE/PS/PI/Chol to settle and instead remove the buffer by evaporation at 50 °C for 60 min: a strategy that has been successfully used for the preparation of PVPA barriers composed of skin lipids.[21] This approach (procedure 2, Figure 2) resulted in tighter barriers with a mean permeation of calcein of 0.063 x 10^{-6} cm/s. Furthermore, the addition of the larger liposomes without centrifugation before the evaporation step was tested. The results (procedure 3, Figure 2) show a mean permeation of calcein of 0.073 x 10^{-6} cm/s, indicating that centrifugation does not provide significantly tighter packing for the larger liposomes and could thus be skipped to simplify the procedure.

The influence of using a 6% liposome dispersion, as in the original preparation procedure, instead of a 3% liposome dispersion on the tightness of the barriers was also investigated. The resulting calcein permeability and electrical resistance across the different barriers (procedure 3 vs. 4 in Figure 2) surprisingly showed no significant differences; therefore, 3% was kept as the concentration for the liposome dispersion in the final preparation procedure. This avoids the use of extensive amounts of lipids and reduces the total cost of the model.

Furthermore, an attempt to use a lower thawing temperature over a prolonged time period was evaluated. Both 50 °C for 105 min (procedure 5, Figure 2) and 30 °C for 270 min (procedure 6, Figure 2) were analyzed to elucidate their impact on barrier tightness. Procedure 5 resulted in barriers with a mean calcein permeability of 0.052 x 10^{-6} cm/s, while procedure 6 resulted in barriers with a mean calcein permeability of 0.026 x 10^{-6} cm/s. Both procedures showed high reproducibility, as reflected in their low standard deviations. However, even though procedure 6 resulted in slightly tighter barriers, the faster and simpler preparation protocol of 50 °C for 105 min was chosen for the final preparation process.

Based on the obtained results and an evaluation of feasibility with respect to cost and time consumption, the final preparation procedure for PVPA$_{biomimetic}$ barriers is summarized as follows:

- Addition of small (extruded through 400 nm pore filter) liposomes of E-80 (6%; w/v) to fill the pores of the filter: 100 µl of liposomes was added and centrifuged at 2000 rpm (950 *g*) for 5 min; the procedure was repeated with centrifugation extended to 10 min
- Heating at 50 °C for 45 min
- Addition of large (extruded through 1200 nm pore filter) liposomes of PC/PE/PS/PI/Chol (3%; w/v) to settle on top of the filter support:

- o 50 µl of liposomes was added, the solvent was allowed to evaporate in 50 °C incubator for 30 min, and the procedure was performed twice.
- Freezing at -70 °C for minimum 60 min
- Heating at 50 °C for 105 min

The barriers prepared by this procedure exhibited calcein permeability of 0.052 x 10^{-6} ± 0.016 x 10^{-6} cm/s. In summary, the major changes from the original PVPA were i) the exchange of centrifugation for evaporation as the method to settle the large liposomes on top of the filter support and ii) the performance of thawing in the freeze-thaw step at a lower temperature over a longer duration of time.

Evaluation of the biomimetic PVPA

From the zeta potential measurements, it was evident that the $PVPA_{biomimetic}$ barrier had a net negative charge. This is in contrast to the $PVPA_o$ barrier made from egg PC that exhibited no net charge. It was thus expected that the permeability of the charged compounds would be more affected than that of the neutral compounds in the new $PVPA_{biomimetic}$ compared to $PVPA_o$. A negatively charged barrier is expected to increase the affinity of basic compounds for the barrier. Moreover, the negative charges on the surface of the barrier can reduce the pH close to the surface and thus increase the permeability of acidic drugs.[8,22] On the other hand, the surface potential that arises from charged lipid head groups at the barrier surface can also attract counter ions from the buffer to the interface, giving rise to a so-called electrical double layer and the establishment of an electric potential profile at the barrier surface. The surface potential would not have an effect on the permeation of neutral species through the bilayer; however, positively charged compounds would be attracted, thus increasing the surface concentration and giving rise to a higher probability of permeation. The opposite should be the case for negatively charged compounds, and the probability of permeation should decrease.[28]

The newly developed $PVPA_{biomimetic}$ was evaluated by performing permeability experiments at pH values of 6.2 and 7.4 for a selection of 19 drugs chosen to cover acidic, basic and neutral drugs as well as a wide range of fractions absorbed in humans. The results are displayed in Table 2. The drugs salicylic acid and sulphasalazine, which were incorrectly classified in the $PVPA_o$, were included in this selection of compounds to challenge the new model.

As expected, at pH 7.4, the positively charged, basic compounds showed a general increase in permeability through the biomimetic barriers compared to the barriers made from PC only. No

clear trend was evident for the negatively charged, acidic compounds; the permeabilities increased, decreased and were unchanged relative to the $PVPA_o$. This was also the case for a selection of acids tested in the PAMPA model using a biorelevant lipid composition, as reported by Sugano and colleagues.[22] For the neutral compound, a decrease in permeability was observed; however, because the permeability values observed in both models were very high, there was no significant difference regarding the drug classification.

When comparing the $PVPA_{biomimetic}$ with the $PVPA_o$, the same drugs that were incorrectly classified in the $PVPA_o$ were also incorrectly classified in the $PVPA_{biomimetic}$. In addition, nadolol, a positively charged compound that, as expected, exhibited increased permeability through the negatively charged barriers, showed a P_{app}-value that indicated *excellent* absorption *in vivo* while the correct class should be *moderate* absorption. This was the only drug for which the $PVPA_o$ performed better than the $PVPA_{biomimetic}$.

The $PVPA_{biomimetic}$ also proved to be stable at pH 6.2, based on the permeability of calcein and the electrical resistance across the barriers (Table 2). The permeation of the drugs at different pH conditions was consistent with the pH partition hypothesis, with increased permeability of acidic drugs and decreased permeability of basic drugs at pH 6.2 compared to pH 7.4. Both pH conditions elicited the same degree of correct classification into the *in vivo* absorption categories, with the exception of timolol, which demonstrated the expected decrease in permeability at pH 6.2 compared to that at pH 7.4 but resulted in a P_{app} value of 0.8×10^{-6} cm/s, which falls below the limit of 0.9×10^{-6} cm/s originally required to be correctly classified into the *excellent* absorption category *in vivo*. However, when using the narrowed range of permeability values giving the moderate absorption category as discussed above, timolol would also be correctly classified as having *excellent* absorption at pH 6.2. However, this would additionally classify the moderately absorbed drugs atenolol and sulpiride into the *excellent* category at pH 7.4.

Stability of the biomimetic PVPA in the presence of tensides and co-solvents

Investigation into the biomimetic barriers' integrity in the presence of tensides and co-solvents was performed to determine the feasibility of estimating the permeability of poorly soluble compounds. Calcein permeability and electrical resistance, both indicators of barrier integrity, were measured in the presence of three co-solvents and six tensides, as reported in Table 3.

The presence of the co-solvents ethanol, DMSO and PEG 400 did not induce any significant change in calcein permeability in all concentrations tested, indicating the retained integrity of the permeation barriers under these conditions. Similar results were previously shown for the PVPA$_o$.[25] However, in respect to the effect of the tensides, the PVPA$_{biomimetic}$ appears to be significantly more robust than the PVPA$_o$, which is a promising and important finding carrying the model forward. The presence of Poloxamer 188 evoked no significant change in the permeability of calcein up to 60 mg/ml and the presence of Span 20 resulted in no significant change at a concentration of 20 mg/ml. The presence of Tween 80, Brij 35 and Cremophor EL induced statistically significant increases in the permeability of calcein. However, this increase was small and the electrical resistances remained within the limits set for the barriers to indicate a maintenance of integrity (1000 ohm/insert).[12] It has also been previously stated that the PVPA$_o$ barriers are considered to be stable up to a calcein permeability of approximately 0.15×10^{-6} cm/s.[29] Even Triton X, included as a reference due to its known efficiency in solubilizing phospholipids, showed a permeability value of calcein below this limit. This is a tremendous increase in barrier integrity stability compared to the PVPA$_o$, where even the lowest examined concentrations of tensides resulted in a 10-20-fold increase in calcein permeability as well as electrical resistance values below 500 ohm.[19,25,29] The reason for this increased stability is most likely the presence of cholesterol, which is known to increase the rigidity of the bilayer and hence the stability towards solubilization by tensides.[30]

It is, however, important to consider the solubility-permeability interplay when estimating the permeability of poorly water-soluble drugs in solubility-enhancing formulations.[31] Apparent permeability has been shown to decrease with an increase in solubility through the use of both surfactants and co-solvents: the first due to a reduced fraction of free drug and the latter due to a change in the distribution coefficient.[31,32] The reduction in the drugs thermodynamic activity, and thereby permeability, by formulating poorly water-soluble drugs using tensides has also been demonstrated with the PVPA.[19]

The highly increased stability in the presence of tensides is, however, a very promising finding that renders the PVPA$_{biomimetic}$ much more suitable to also estimating the permeability of poorly soluble compounds of interest and hence carrying the model an important step forward.

CONCLUSIONS

To improve the PVPA to more closely mimic the *in vivo* situation in the GI tract, the pH was changed to a more biologically relevant pH of 6.2 and the barrier lipids altered to a biomimetic lipid composition. The lipid composition as well as the barrier preparation procedure has been optimized, resulting in the successful development of permeation barriers with an intestinal-mimicking lipid composition that exhibit low permeability of hydrophilic markers and the ability to distinguish between substances with varying degrees of intestinal absorption. Positively charged basic compounds showed increased permeability through the negatively charged biomimetic barriers, as expected. The degree of correct classification according to *in vivo* absorption was comparable for the $PVPA_o$ and the $PVPA_{biomimetic.}$ Importantly, the $PVPA_{biomimetic}$ appears to be significantly more robust towards the presence of tensides compared to the $PVPA_o$, which is a very promising and important finding rendering the biomimetic PVPA much more suitable to estimating the permeability of poorly soluble compounds of interest and thus carrying the model an important step forward.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to disclose.

REFERENCES

1.   Artursson P 1990. Epithelial Transport of Drugs in Cell-Culture .1. A Model for Studying the Passive Diffusion of Drugs over Intestinal Absorptive (Caco-2) Cells. J Pharm Sci 79(6):476-482.
2.   Artursson P 1991. Cell cultures as models for drug absorption across the intestinal mucosa. Critical Reviews in Therapeutic Drug Carrier Systems 8(4):305-330.
3.   Artursson P, Palm K, Luthman K 1996. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Delivery Rev 22(1-2):67-84.
4.   Araujo F, Sarmento B 2013. Towards the characterization of an in vitro triple co-culture intestine cell model for permeability studies. Int J Pharm in press.
5.   Avdeef A 2003. High-throughput permeability and membrane retention measurement using artificial phospholipid membranes. Chimia 57(1-2):61-61.

6.	Wohnsland F, Faller B 2001. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. J Med Chem 44(6):923-930.
7.	Kansy M, Senner F, Gubernator K 1998. Physicochemical high throughput screening: Parallel artificial membrane permeation assay in the description of passive absorption processes. J Med Chem 41(7):1007-1010.
8.	Sugano K, Hamada H, Machida M, Ushio H, Saitoh K, Terada K 2001. Optimized conditions of bio-mimetic artificial membrane permeation assay. Int J Pharm 228(1-2):181-188.
9.	Kansy M, Avdeef A, Fischer H 2004. Advances in screening for membrane permeability: high-resolution PAMPA for medicinal chemists. Drug Discovery Today: Technologies 1(4):349-355.
10.	Avdeef A, Tsinman O 2006. PAMPA--a drug absorption in vitro model 13. Chemical selectivity due to membrane hydrogen bonding: in combo comparisons of HDM-, DOPC-, and DS-PAMPA models. Eur J Pharm Sci 28(1-2):43-50.
11.	Mandagere AK, Thompson TN, Hwang KK 2002. Graphical model for estimating oral bioavailability of drugs in humans and other species from their caco-2 permeability and in vitro liver enzyme metabolic stability rates. J Med Chem 45(2):304-311.
12.	Flaten GE, Dhanikula AB, Luthman K, Brandl M 2006. Drug permeability across a phospholipid vesicle based barrier: A novel approach for studying passive diffusion. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 27(1):80-90.
13.	Yazdanian M, Glynn SL, Wright JL, Hawi A 1998. Correlating partitioning and Caco-2 cell permeability of structurally diverse small molecular weight compounds. Pharm Res 15(9):1490-1494.
14.	Flaten GE, Awoyemi O, Luthman K, Brandl M, Massing U 2009. The phospholipid vesicle-based permeability assay: 5. Development towards an automated procedure for high throughput permeability screening. JALA (14):12-21.
15.	Flaten GE, Bunjes H, Luthman K, Brandl M 2006. Drug permeability across a phospholipid vesicle-based barrier 2. Characterization of barrier structure, storage stability and stability towards pH changes. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 28(4):336-343.
16.	Flaten GE, Kottra G, Stensen W, Isaksen G, Karstad R, Svendsen JS, Daniel H, Svenson J 2011. In vitro characterisation of human peptide transporter hPEPT1 interactions and passive permeation studies of short cationic antimicrobial peptides. Journal of Medicinal Chemistry 54:2422–2432.
17.	Hansen T, Ausbacher D, Flaten GE, Havelkova M, Strom MB 2011. Synthesis of cationic antimicrobial beta(2,2)-amino acid derivatives with potential for oral administration. Journal of Medicinal Chemistry 54(3):858-868.
18.	Svenson J, Karstad R, Flaten GE, Brandsdal B-O, Brandl M, Svendsen JS 2009. Altered Activity and Physicochemical Properties of Short Cationic Antimicrobial Peptides by Incorporation of Arginine Analogues. Mol Pharm 6(3):996-1005.
19.	Fischer SM, Flaten GE, Hagesæther E, Fricker G, Brandl M 2011. In Vitro Permeability of Poorly Water Soluble Drugs in the Phospholipid Vesicle-Based Permeation Assay (PVPA): The Influence of Non-Ionic Surfactants. J Pharm Pharmacol accepted.
20.	Kanzer J, Tho I, Flaten GE, Magerlein M, Holig P, Fricker G, Brandl M 2010. In-vitro permeability screening of melt extrudate formulations containing poorly water-soluble drug compounds using the phospholipid vesicle-based barrier. Journal of Pharmacy and Pharmacology 62(11):1591-1598.
21.	Engesland A, Skar M, Hansen T, Skalko-Basnet N, Flaten GE 2013. New applications of phospholipid vesicle-based permeation assay: permeation model mimicking skin barrier. J Pharm Sci 102(5):1588-1600.
22.	Sugano K, Hamada H, Machida M, Ushio H 2001. High throughput prediction of oral absorption: Improvement of the composition of the lipid solution used in parallel artificial membrane permeation assay. Journal of Biomolecular Screening 6(3):189-196.
23.	Ingebrigtsen L, Brandl M 2002. Determination of the size distribution of liposomes by SEC fractionation, and PCS analysis and enzymatic assay of lipid content. AAPS PharmSciTech 3(2):9-15.

24.   Proulx P 1991. Structure-Function-Relationships in Intestinal Brush-Border Membranes. Biochim Biophys Acta 1071(3):255-271.

25.   Flaten GE, Luthman K, Vasskog T, Brandl M 2008. Drug permeability across a phospholipid vesicle-based barrier: 4. The effect of tensides, co-solvents and pH changes on barrier integrity and on drug permeability. Eur J Pharm Sci 34(2-3):173-180.

26.   Avdeef A. 2003. Absorption and Drug Development; Solubility, Permeability and Charge State. 1. ed., New Jersey: Wiley-Interscience. p 116-246.

27.   Dressman JB, Berardi RR, Dermentzoglou LC, Russell TL, Schmaltz SP, Barnett JL, Jarvenpaa KM 1990. Upper Gastrointestinal (Gi) Ph in Young, Healthy-Men and Women. Pharm Res 7(7):756-761.

28.   Malkia A, Murtomaki L, Urtti A, Kontturi K 2004. Drug permeation in biomembranes; In vitro and in silico prediction and influence of physicochemical properties. Eur J Pharm Sci 23(1):13-47.

29.   Fischer SM, Buckley ST, Kirchmeyer W, Fricker G, Brandl M 2012. Application of simulated intestinal fluid on the phospholipid vesicle-based drug permeation assay. Int J Pharm 422(1-2):52-58.

30.   Brandl M 2001. Liposomes as drug carriers: a technological approach. Biotechnol Annu Rev 7:59-85.

31.   Dahan A, Miller JM 2012. The solubility-permeability interplay and its implications in formulation design and development for poorly soluble drugs. The AAPS journal 14(2):244-251.

32.   Miller JM, Beig A, Krieg BJ, Carr RA, Borchardt TB, Amidon GE, Amidon GL, Dahan A 2011. The solubility-permeability interplay: mechanistic modeling and predictive application of the impact of micellar solubilization on intestinal permeation. Mol Pharm 8(5):1848-1856.
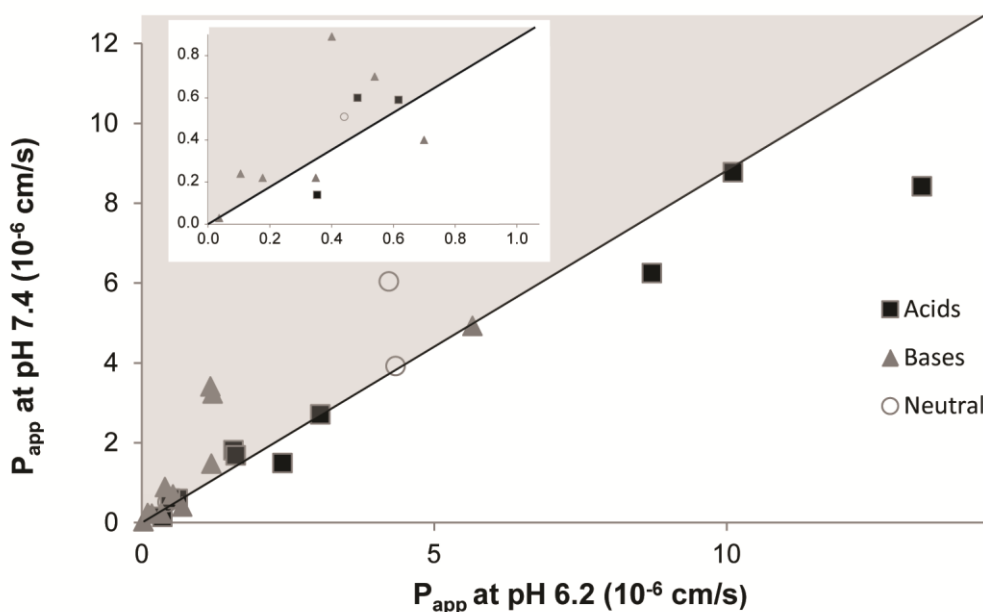
Figure 1. Correlation of the permeability values obtained at pH 6.2 vs. 7.4 in the donor compartment. The pH in the acceptor compartments was always 7.4.
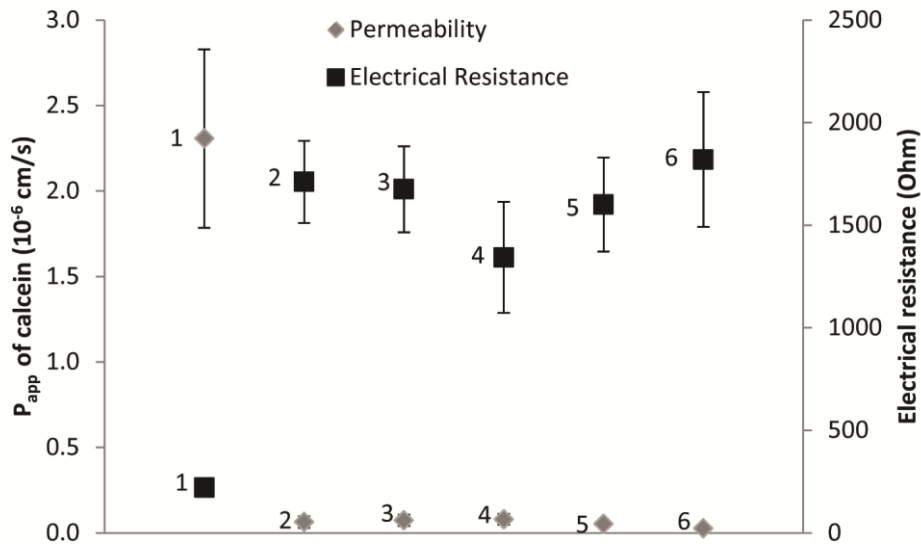
Figure 2. Apparent permeability (P_{app}) of calcein and electrical resistance across barriers prepared with different parameters. The different procedures represent changes in the following parameters:

1) 2500 rpm with large liposomes (3%) x 2, invert centrifugation, thawing at 65 °C for 30 min

2) 2500 rpm with large liposomes (3%) x 2, heating at 50 °C for 60 min, thawing at 60 °C for 60 min

3) Addition of large liposomes (3%), heating at 50 °C for 30 min x 2, thawing at 60 °C for 60 min

4) Addition of large liposomes (6%), heating at 50 °C for 30 min x 2, thawing at 60 °C for 60 min

5) Addition of large liposomes (3%), heating at 50 °C for 30 min x 2, thawing at 50 °C for 105 min

6) Addition of large liposomes (6%), heating at 50 °C for 30 min x 2, thawing at 30 °C for 270 min

Table 1. Experimental P_{app}-values from the $PVPA_o$ at pH 6.2 and 7.4, molecular properties and calculated and experimental literature values of percent absorption in humans (Fa)

Compound	PVPA$_o$ $P_{app}\pm SD$ 10^{-6} cm/s		Mw	logP calc	Fa calc %	logD[a]	pKa[b]	Fa[c] %
	pH 6.2	pH 7.4[d]						
Acetylsalicylic acid	1.95±0.65	1.49±0.23	180.2	1.19	72	-2.25	3.5[1]	100[6]
Alprenolol	1.57±0.09	4.92±0.34	249.4	3.07	97	1.38	9.2[1]	93[5]
Atenolol	0.18±0.01	0.22±0.05	266.3	-0.42	55	-1.29	9.5[1]	50[5]
Caffeine	6.24±0.17	6.04±0.57[11]	194.2	0.16	81	0.02	-	100[7]
Chlorothiazide	0.04±0.01	0.03±0.00	295.7	-0.07	52	-0.05	6.7[2]	13[4]
Chlor-amphenicol	1.57±0.25	1.81±0.07	323.1	1.07	64	1.14	5.5[2]	90[2]
Cimetidine	0.40±0.05	0.89±0.05	252.3	-0.25	72	0.35	6.9[1]	64[2]
Diclofenac	3.05±0.14	2.71±0.16[13]	296.2	0.42	100	1.15	4.0[2]	100[2]
Enalapril	0.35±0.03	0.14±0.01	492.5	4.50	52	-0.90	2.9/5.2[1]	65[4]
Flurbiprofen	8.72±0.34	6.24±0.30[13]	244.3	0.26	96	0.91[1]	4.03[1]	95[8]
Flufenamic acid	1.61±0.14	1.68±0.59[10]	281.2	5.23	89	2.45[1]	4.09[1]	-[8]
Hydro-chlorothiazide	0.44±0.06	0.51±0.06	297.7	4.16	53	-0.12	8.9/10.3[1]	67[4]
Ibuprofen	13.34±0.97	8.42±0.86[10]	206.3	-0.09	92	0.68	4.45[1]	95[8]
Metoprolol	1.26±0.33	3.23±0.78	267.3	3.50	89	-0.16	9.6[1]	95[5]
Metronidazol	4.34±0.12	3.92±0.25[13]	171.2	1.89	92	-0.02[1]	-	100
Nadolol	0.54±0.10	0.70±0.11	309.4	-0.02	67	0.68	9.7[1]	35[4]
Naproxen	10.17±0.40	8.73±0.61[9]	230.3	0.80	90	0.23	4.15[3]	99[2]
Propranolol	1.18±0.17	3.41±0.41[13]	259.3	3.09	97	1.41[1]	9.52[2]	90[4]
Ranitidine	0.11±0.02	0.24±0.01	350.9	3.18	70	-0.29	8.3/2.1[1]	50[5]
Salicylic acid	0.45±0.04	0.59±0.03	138.1	3.09	76	-1.44	3.0[1]	100[5]
Sulphasalazine	0.48±0.16	0.60±0.18	398.4	0.78	50	-0.42	2.8/8.3/ 11.0[1]	13[5]
Sulpiride	0.35±0.07	0.22±0.01	341.4	2.26	61	-1.00	9.0[2]	35[6]
Terbutaline	0.80±0.09	0.40±0.05	274.3	1.67	62	-1.35	8.7/10.0/ 11.0[1]	62[2]
Timolol	1.65±0.08	1.70±0.12	316.4	0.09	84	0.03	9.5[1]	90[4]
Tranexamic acid	5.34±0.46	12.36±3.64	157.2	3.31	43	-3.00	4.5/10.7[2]	55[2]
Calcein	-	0.061±0.005	622.5	-1.71	-	-	1.8/9.2[12]	-

[a] The log D values are from (Zhu *et al.*, 2002). (Avdeef, 2004)[1]

[b] The pKa values are from (Avdeef, 2004)[1], (Sugano *et al.*, 2002)[2], (Li *et al.*, 2012)[3], (Flaten *et al.*, 2006)[12]

[c] (Sugano *et al.*, 2001)[4], (Yazdanian *et al.*, 1998)[5], (Kansy *et al.*, 1998)[6], (Sugano *et al.*, 2002)[2], (Yee, 1997)[7], (Osterberg *et al.*, 2001)[8]

[d] The P_{app} values are taken from (Flaten *et al.*, 2006) unless stated otherwise;(Flaten *et al.*, 2008)[9], (Engesland *et al.*, 2013)[10], (Flaten *et al.*, 2009)[11], originally measured for this study[13]

Avdeef, A. 2004. Absorption and Drug Development; Solubility, Permeability and Charge State. New Jersey: Wiley-Interscience, 116-246.

Engesland, A., Skar, M., Hansen, T., Skalko-Basnet, N. & Flaten, G. E. (2013). New applications of phospholipid vesicle-based permeation assay: permeation model mimicking skin barrier. *J. Pharm. Sci.,* 102: 1588-600.

Flaten, G. E., Awoyemi, O., Luthman, K., Brandl, M. & Massing, U. (2009). The phospholipid vesicle-based permeability assay: 5. Development towards an automated procedure for high throughput permeability screening. *JALA*: 12-21.

Flaten, G. E., Dhanikula, A. B., Luthman, K. & Brandl, M. (2006). Drug permeability across a phospholipid vesicle based barrier: A novel approach for studying passive diffusion. *Eur. J. Pharm. Sci.,* 27: 80-90.

Flaten, G. E., Luthman, K., Vasskog, T. & Brandl, M. (2008). Drug permeability across a phospholipid vesicle-based barrier: 4. The effect of tensides, co-solvents and pH changes on barrier integrity and on drug permeability. *Eur. J. Pharm. Sci.,* 34: 173-180.

Kansy, M., Senner, F. & Gubernator, K. (1998). Physicochemical high throughput screening: Parallel artificial membrane permeation assay in the description of passive absorption processes. *J. Med. Chem.,* 41: 1007-1010.

Li, X. & Cooper, M. A. (2012). Measurement of Drug Lipophilicity and pKa Using Acoustics. *Anal. Chem. (Washington, DC, U. S.),* 84: 2609-2613.

Osterberg, T., Svensson, M. & Lundahl, P. (2001). Chromatographic retention of drug molecules on immobilised liposomes prepared from egg phospholipids and from chemically pure phospholipids. *Eur. J. Pharm. Sci.,* 12: 427-439.

Sugano, K., Hamada, H., Machida, M. & Ushio, H. (2001). High throughput prediction of oral absorption: Improvement of the composition of the lipid solution used in parallel artificial membrane permeation assay. *Journal of Biomolecular Screening,* 6: 189-196.

Sugano, K., Takata, N., Machida, M., Saitoh, K. & Terada, K. (2002). Prediction of passive intestinal absorption using bio-mimetic artificial membrane permeation assay and the paracellular pathway model. *Int. J. Pharm.,* 241: 241-251.

Yazdanian, M., Glynn, S. L., Wright, J. L. & Hawi, A. (1998). Correlating partitioning and Caco-2 cell permeability of structurally diverse small molecular weight compounds. *Pharm. Res.,* 15: 1490-1494.

Yee, S. (1997). In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man-fact or myth. *Pharm. Res.,* 14: 763-766.

Zhu, C., Jiang, L., Chen, T.-M. & Hwang, K.-K. (2002). A comparative study of artificial membrane permeability assay for high throughput profiling of drug absorption potential. *Eur. J. Med. Chem.,* 37: 399-407.

Table 2. Experimental P_{app} values from the PVPA$_{biomimetic}$ at pH 6.2 and 7.4, molecular properties and fraction absorbed (F%) in humans.

Compound	PVPA$_{biomimetic}$ $P_{app} \pm SD$ 10^{-6} cm/s		M_w	Charge at pH 6.2	Charge at pH 7.4	F[a] %
	pH 6.2	pH 7.4				
Alprenolol	1.07±0.04	1.59±0.07	249.4	+	+	93
Atenolol	0.49±0.06	0.76±0.11	266.3	+	+	50
Caffeine	4.04±0.08	4.32±0.28	194.2	0	0	100
Chlorothiazide	0.07±0.01	0.05±0.01	295.7	0	-	13
Diclofenac	4.25±1.00	2.23±0.39	296.2	-	-	100
Enalapril	0.42±0.04	0.43±0.05	492.5	-	-	65
Flurbiprofen	5.77±0.57	3.26±0.57	244.3	-	-	95
Ibuprofen	12.11±1.98	9.01±1.45	206.3	-	-	95
Metoprolol	1.23±0.18	1.61±0.45	267.3	+	+	95
Metronidazol	3.31±0.36	3.15±0.27	171.2	0	0	100
Nadolol	1.47±0.09	1.55±0.13	309.4	+	+	35
Naproxen	5.63±0.33	3.79±0.30	230.3	-	-	95
Propranolol	0.89±0.08	1.76±0.11	259.3	+	+	90
Ranitidine	0.25±0.02	0.40±0.07	350.9	+	+	50
Salicylic acid	0.69±0.02	0.54±0.01	138.1	-	-	100
Sulphasalazine	0.46±0.02	0.20±0.02	398.4	-	-	13
Sulpiride	0.37±0.03	0.89±0.08	341.4	+	+	35
Terbutaline	0.50±0.13	0.64±0.11	274.3	+	+	62
Timolol	0.79±0.03	1.57±0.02	316.4	+	+	90
Calcein	0.08±0.003	0.05±0.002	622.5	-	-	-

a See Table 1 for references

Table 3. The permeability (P_{app}) values for calcein $\pm$ SD (10^{-6} cm/s), together with the electrical resistances across the PVPA barriers (ohm) in the presence of the tensides and co-solvents

[*] The permeability values that were significantly ($p \leq 0.05$) different from control.

Additive in donor compartment	0 mg/ml	0.5 mg/ml	1 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml	40 mg/ml	60 mg/ml
Control (buffer)	0.053 ±0.013 [1532]	-	-	-	-	-	-	-
Triton X 100	-	0.131* ±0.024 [1257]	-	-	-	-	-	-
Tween 80	-	0.102* ±0.021 [1377]	0.095* ±0.015 [1390]	-	-	-	-	-
Brij 35	-	0.109* ±0.026 [1429]	0.117* ±0.025 [1258]	-	-	-	-	-
Span 20	-	-	-	-	-	0.062 ±0.032 [1547]	-	-
Cremophor EL	-	-	-	0.106* ±0.026 [1323]	-	0.110* ±0.021 [1480]	0.119* ±0.028 [1322]	-
Poloxamer 188	-	-	-	-	-	0.062 ±0.012 [1509]	0.041 ±0.012 [1608]	0.051 ±0.019 [2033]
PEG 400	-	-	-	-	0.053 ±0.014 [1453]	-	0.062 ±0.023 [1328]	-
Ethanol	-	-	-	-	0.075 ±0.022 [1452]	-	0.054 ±0.012 [1705]	0.074 ±0.024 (1727)
DMSO	-	-	-	0.053 ±0.012 [1585]	0.094 ±0.022 [1448]	-	0.078 ±0.019 [1544]	-