

DRUG PERMEABILITY ACROSS A PHOSPHOLIPID VESICLE BASED BARRIER:

A NOVEL APPROACH FOR STUDYING PASSIVE DIFFUSION

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Abstract

The aim of this study was to develop a novel predictive medium-throughput screening method for drug permeability, with use of a tight barrier of liposomes on a filter support. To our knowledge no one has succeeded in depositing membrane barriers without the use of inert solvent such as hexadecane. The first part of the study involved development of a protocol for preparation of these barriers, which were made of liposomes from egg phosphatidylcholin in phosphate buffer pH 7.4 with 10 % (v/v) ethanol. The liposomes were deposited into the pores and onto the surface of a filter support (mixed cellulose ester) by use of centrifugation. Solvent evaporation and freeze-thaw cycling were then used to promote fusion of liposomes and a tight barrier could be obtained as shown with calcein permeability and electrical resistance. In the second part of the study the model was validated using 21 drug compounds, which cover a wide range of physicochemical properties and absorption (F_a) in humans (13-100%). The drug permeation studies were carried out at room temperature with phosphate buffer (pH 7.4) in both acceptor and donor chambers. The apparent permeability coefficients obtained from the phospholipid vesicle based model correlated well with literature data on human absorption *in vivo*, which suggests that its performance is adequate and that the method is suitable for rapid screening of passive transport of new chemical entities. The results obtained from our model were compared with polar surface area (PSA) and experimental log D and results obtained by established permeability screening methods such as immobilised liposome chromatography (ILC), the PAMPA models and the Caco-2 model. Our approach seems to model the *in vivo* absorption better than PSA, experimental log D, the ILC and PAMPA models, when similar conditions are used as in our assay, and equally well as the Caco-2 model and the Double Sink PAMPA (DS-PAMPA) model.

Keywords: Artificial membrane, prediction, oral absorption, PAMPA, Caco-2 model, immobilized liposome chromatography (ILC), PSA, passive diffusion, permeability classification, liposome, intestinal absorption, phospholipid

1 Introduction

The increasing awareness of the importance of ADME-screening (absorption distribution metabolism excretion) of potential drug candidates already in early drug discovery has encouraged strong efforts in the development of rapid and reliable screening tools (Hidalgo, 2001; Kariv et al., 2002; Kerns and Di, 2003; Lipinski et al., 1997; Smith and van de Waterbeemd, 1999; van de Waterbeemd et al., 2001). Both *in silico* and experimental methods are available today for prediction and/or screening of properties related to oral drug absorption. Many of the experimental cell-based techniques, e.g. the Caco-2 monolayer model, give reliable results, but are too complicated and time-consuming to fit in a true high-throughput format. Among the most used techniques today for rapid screening are the PAMPA (parallel artificial membrane permeation assay)(Avdeef, 2003; Kansy et al., 2004; Kansy et al., 1998; Sugano et al., 2001; Wohnsland and Faller, 2001) and the ILC (immobilized liposome chromatography) methods (Beigi et al., 1995; Liu et al., 2002; Lundahl and Beigi, 1997). PAMPA uses a simple phospholipid/organic solvent-coated filter as the permeability barrier, which allows for medium- to high-throughput screening of permeability properties. ILC is basically an HPLC-method by which the drug-membrane interaction is studied as differences in retention times on columns covered with immobilized liposomes. The throughput of this method is mainly limited by the analytical method used (HPLC). Such simplified permeability approaches do not model paracellular or active transport pathways. However, recent reports conclude that 80-95% of commercial drugs are absorbed primarily by passive diffusion (Mandagere et al., 2002). Therefore a model merely

monitoring passive diffusion appears useful as a first step in biopharmaceutical characterisation of new chemical entities.

We have developed a novel approach for measuring steady-state drug permeability using a permeation barrier made of a tight layer of phospholipid vesicles. This barrier is advantageous to the permeation barrier used in PAMPA and ILC as it appears to mimic the *in vivo* situation better or at least equally well in terms of predicting fraction absorbed in humans after oral administration (F_a). In addition, the method is easy to use and gives highly reproducible results. It is presently designed for a medium-throughput format but can easily be extended for use in rapid screening of large number of compounds. The method has been validated using a series of drugs covering a broad range of physicochemical properties and absorption properties upon oral administration in humans.

2 Experimental section

2.1 Materials

Egg phosphatidyl choline, Lipoid E-80 was obtained from Lipoid, Germany. Alprenolol hydrochloride, atenolol, acebutolol hydrochloride, caffeine, chloramphenicol, chlorothiazide, enalapril maleate, hydrochlorothiazide, metoprolol tartrate, nadolol, ranitidine hydrochloride, sulphasalazine, sulphiride, testosterone, terbutaline hemisulfate, timolol maleate, tranexamic acid, calcein and 2',7'-dichlorofluorescein were purchased from Sigma-Aldrich Co, St. Louis, USA. Amiloride hydrochloride and fluorescein sodium were supplied by Merck KGaA, Darmstadt, Germany. Salicylic acid and acetyl salicylic acid were obtained from NMD, Oslo, Norway. Cimetidine and 5(6)-carboxyfluorescein were purchased from Allphamed Pharma GmbH, Göttingen, Germany, and Fluka Chemie AG, Buchs, Switzerland, respectively.

Culture inserts (Transwell-Clear, d = 6.5 mm) and plates were purchased from Corning Inc, Corning, USA, the existing filter removed and the mixed cellulose ester filters from Millipore, Billerica, USA fused on.

2.2 Preparation of vesicular phospholipid barriers¹

The present technique essentially involves deposition of liposomes, prepared by film hydration/filter-extrusion, on filter supports in culture inserts, to obtain a barrier for transport studies. Egg phosphatidyl choline (1.8 g) was dissolved in a mixture of chloroform and methanol (2:1) (6 mL) in a round bottom flask. The organic solvent was removed under vacuum at 55 °C. The deposited lipid film was exposed to vacuum of 55 hPa at room temperature for an additional period of 3 hr to remove traces of solvent, before hydration with phosphate buffer [KH_2PO_4 (0.3 g); $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$ (3.2g); NaCl (3.62g); NaN_3 (0.1 g); water to 500 mL; pH adjusted to 7.4 by HCl/NaOH] containing 10 % (v/v) ethanol to obtain a 6 % (w/v) liposomal dispersion.

The liposome dispersion was then filter-extruded through 0.8 and 0.4 μm polycarbonate membrane filters (Millipore) at room temperature. The extrusion was done by hand with a syringe through the filters in a 25 mm Swinnex[®] Filter Holder from Millipore. To obtain liposomes of two different sizes one portion was extruded five times through the 0.8 μm polycarbonate membrane filter while another portion was extruded five additional passages through filters with 0.4 μm pore size. The liposome layer for permeation experiments was produced according to the preparation process shown schematically in Figure 1. Culture inserts bearing the 0.65 μm mixed cellulose ester filter were prepared in-house by fusion of

¹ The development of the method is described in detail in the Supplementary material

such a filter to the base of the culture insert at moderate pressure for about 5 min at 130 °C.² An aliquot of the liposomal suspension (100 µL), which had been finally extruded through 0.4 µm polycarbonate membrane filters, was added to each culture insert. The inserts were placed into wells of 24-well culture plates, covered with a lid and centrifuged at 600 g (2000 rpm) for 4 min at 25 °C in a Biofuge Stratos from Heraeus instrument, Germany, to force the suspension through the filter. Then inserts were moved to fresh wells and an additional amount of the 0.4 µm membrane-extruded liposome suspension (100 µL) was applied. The plates were rotated 180° horizontally, followed by centrifugation under similar conditions for 10 min. Thereafter, inserts were transferred to fresh dry well plates, covered with lids and maintained at 50 ± 1 °C at 11 % relative humidity for 45 min. This step was performed partly to evaporate the ethanol-water mixture and to increase the viscosity of the suspension as well as to promote coating of the filter with liposomes. After inserts had attained room temperature another portion of the liposome suspension (100 µL) that had been extruded through 0.8 µm filters only, was added to each insert and centrifuged at 600 g (2000 rpm) for 30 min. Upon centrifugation, only a small volume of the suspension was forced into the lipid coated pores, while the excess of the supernatant was removed by inverting the inserts and allowing the suspension to flow out under gravity. If any insert did not manage to hold up the given volume of suspension during this step it was excluded from the batch. Subsequently, the inserts were centrifuged in an upside down position at 25 g (300 rpm) for 5 min, followed by a freeze-thaw protocol, where inserts were frozen at –80 °C for 60 min and thawed at +65 °C for 30 min (relative humidity 6 %). The inserts were stored in the refrigerator (2-8 °C) overnight (16-18 hr) until permeation studies were conducted.

Figure 1

² Scanning electron microscope (Jsm-6300 from JEOL, Japan) pictures of the mixed cellulose ester filter, magnified 10000 times is given in Figure C in the Supplementary material.

2.3 Test compounds

The drug substances chosen to validate the phospholipid vesicle based membrane model cover a wide range of physicochemical properties (molecular weight, pKa, log D and hydrogen bonding properties, see Table 1) and a broad range of fraction absorbed after oral administration in humans ($F_a = 13-100\%$). The 21 chosen drugs along with the concentration of stock solutions and the wavelengths used for UV analysis are listed in Table A in the Supplementary material. Four fluorescent dyes, i.e. calcein, 5(6)-carboxyfluorescein, fluorescein sodium and 2',7'-dichlorofluorescein, were also included as reference compounds. These four dyes were selected, as they are hydrophilic and relatively low in molecular mass (~350-650 Da) and hence would give an indication of potential aqueous pathways in the lipid barrier.

Table 1

Stock solutions of the test compounds were prepared by dissolving the drug in phosphate buffer pH 7.4 on a sonication bath (Branson 1510, Branson Ultrasonic B.V., The Netherlands) followed by filtration through 0.22 μm filter (Millex-GS, Millipore, USA) and pH adjustment with HCl/NaOH to 7.4 if necessary. The concentrations of the different drug solutions (see Table A, Supplementary material) were chosen by analytical considerations as well as to obtain the sink conditions during the permeation studies. The concentration of the different drug solutions had to be high enough that the amount of drug in the acceptor chambers during permeation studies could be quantified by means of UV-absorbance, and still be below the solubility limits.

2.4 Permeation studies

Permeation studies were performed after loading the inserts with drug solution (100 μL) and placing them in separate acceptor compartments containing phosphate buffer (600 μL) (pH 7.4) (see Figure 2). The permeation experiment was carried out at room temperature without agitation and inserts were moved to wells containing an equal quantity of fresh buffer at 1 hr intervals for the first 3 hr and 0.5 hr interval for the next 2 hr of study. For cimetidine, nadolol, chlorothiazide and hydrochlorothiazide inserts were moved every second hour for 12 hr.* At the end of the permeation experiment, samples (200 μL) from each acceptor compartment were transferred into 96-well UV transparent plates (Costar) and the concentration of drug was measured spectrophotometrically (Spectramax 190; Molecular devices, Molecular Device Corporation, California, USA) at the wavelength most appropriate for each drug (see Table A, Supplementary material) equilibrated at 28.5 °C. Fluorescence measurements of the dyes were performed by use of 96-well black plates (Costar) in a Polarstar (Fluostar, BMG Technologies) fluorimeter with excitation and emission wavelengths at 485 and 520nm, respectively, at 28.5°C. A blank consisting of pure phosphate buffer pH 7.4 was subtracted from the absorbance/fluorescence values. The standard curves were made of 11 different points and every point represented the mean of 6 parallels. The r^2 -values were always higher than 0.99. The repeatability was between 3.9 % and 20.1 %, and the intermediate precision between 2.7 % and 29.4 % for the different drugs using this novel method. The electrical resistance of the lipid barriers was measured (Millicell-ERS, Millipore, USA) immediately after completion of permeation studies. A value of 119 ohms resulting from the filters was subtracted from the observed resistance to account for that resulting from

* The prolongation of permeation time for the four drug compounds mentioned was done because the amount of the different drugs in the acceptor chambers after the original permeation time was too low to be detected by the analytical method used. And the concentration of the stock solutions could not be increased due to solubility considerations.

the lipid barrier alone. The obtained value was then multiplied by the surface area (0.33 cm²) to normalize for the dimensions of the insert. The experiments were performed at least in triplicate with six inserts in each parallel for every compound. The mean values and standard deviations are reported.

Figure 2

2.5 Permeability calculations

The cumulative amount of permeated drug was plotted against time and the slope of the linear part of the curve represented the steady-state flux rate. Since a lag time was observed before steady state conditions were attained (see Figure 3), only the last four time points of the cumulative plot were used for calculation of the slope. This means that the permeability calculation is based on steady-state flux. The r^2 -values were better than or equal to 0.99 for all of the drugs. The obtained flux rates were then used to calculate the apparent permeability coefficient (P_{app}) by use of the following equation:

$$P_{app} = J/(A(C_d - C_a)) \text{ [cm/sec]} \quad (\text{Eq 1})$$

where J is the observed flux rate at steady state [nmol/sec], A is the surface area of the insert [cm²] and C_d and C_a are concentrations of solution in donor and acceptor chambers [nmol/mL], respectively. The experiments were performed under sink conditions, i.e. the drug concentration in the acceptor chamber did not exceed 10 % of the drug concentration in the donor chamber at any time (Artursson, 1990). By using sink conditions, the influence of drug diffusing back from the acceptor to the donor chamber could be minimized and the concentration of solution in the acceptor chamber was considered small and neglected for calculation purposes. The equation could thus be simplified to:

$$P_{app} = J/(AC_d) \text{ [cm/s]} \quad (\text{Eq 2})$$

Figure 3

3 Results and discussion

3.1 The structure of the liposome membrane

So far no attempts have been made to elucidate the molecular arrangement and morphology of the structures formed by the phospholipids during barrier preparation, but in analogy with literature data it may be concluded that liposomal or closed vesicular structures are present in the pores of the filter. Extrusion of hydrated phospholipids has earlier been shown to give liposomes of a size corresponding to the pore size of the filters used (Berger et al., 2001). The use of repeated extrusion cycles through the same filter pore size has also been shown to reduce the liposome size (Hope et al., 1993). The presence of ethanol in the hydrated phospholipids, in the concentrations used in this study, has also been shown to result in liposome formation (Polozova et al., 2005). Aqueous phospholipid dispersions of equally high concentration as that of the deposited layer in the current studies, have previously been described as “vesicular phospholipid gels” (Brandl et al., 1997). Such gels have been shown to consist of uni- to oligolamellar bilayer-vesicles in tight packing and to act as a diffusion barrier for incorporated drug compounds (Tardi et al., 1998). In contrast, in the PAMPA-model, the phospholipid is applied as a solution in organic solvent (Kansy et al., 1998), which eventually forms monolayers, bilayers or multiple layers at the interface to the water phase. Thompson et al (Thompson et al., 1980) have done studies that strongly indicate that a single bilayer is present per pore. In contrast Avdeef et al (Avdeef et al., 2001) reports multilamellar bilayers forming inside the filter channels. Wohnsland and Faller (Wohnsland and Faller, 2001) have been working with a system with only organic solvent in a filter support which strongly indicate solvent filled filter pores. A recent observation by Nielsen and Avdeef, that excess inert solvent with phospholipid increases the apparent porosity of the PAMPA model (Nielsen and Avdeef, 2004), also seems to point towards a coherent solvent phase both on top of the filter and in the pores. Despite all uncertainty on the morphologies there appears to be a

distinct difference between the two models, which may have implications in terms of partitioning and permeation of drug compounds.

3.2 Choice of drug compounds for the validation of the model

The drug compounds used to validate the present model were chosen from several different therapeutic areas. The diversity in chemical structures was large, which made the drug compounds cover a wide range of physicochemical properties (the structures of the compounds are given in Figure 1 in the Supplementary material and some of their physicochemical properties are given in Table 1). There are acidic and basic, as well as ampholytic and non-proteolytic drugs among the chosen compounds, to encompass both negatively, positively and non-charged compounds at pH 7.4. The compounds differ in size (mw 138 - 398) and log D values (-3.00 - 1.38), and were chosen to cover a wide range of absorbed fraction in humans after oral administration (F_a 13 - 100 %). The majority of the compounds has been reported to cross the intestinal epithelia mainly by passive diffusion, and as they are commonly studied and their permeability values in different models are available in the literature they were considered useful for comparison. However, some of the compounds are known to be substrates for carrier or efflux proteins; e.g. enalapril has been shown to be a substrate for the intestinal oligopeptide carrier PEPT1 (Amidon and Lee, 1994) and salicylic acid and acetylsalicylic acid are substrates for the monocarboxylic acid carrier (Stenberg et al., 2000). Cimetidine and ranitidine are known to be substrates for the efflux protein p-glycoprotein (Chan et al., 2004).

3.3 Permeation studies

3.3.1 Permeation studies with fluorescent markers

In order to have meaningful results from permeability studies, a validation of the transport barrier was performed which included tests to assess the integrity of the barriers both by measuring the electrical resistance across the barriers and the flux of four different hydrophilic fluorescent markers (Table 2). None of the tested markers showed a rate greater than 0.04 %/h.

Table 2:

Calcein and carboxyfluorescein show permeability values about four times lower than fluorescein sodium and 2',7'-dichlorofluorescein. The reason for this is that calcein and carboxyfluorescein are charged at pH 7.4, whereas fluorescein sodium and 2',7'-dichlorofluorescein are uncharged.

By increasing the centrifugation time from 30 to 40 min after the heating step in the barrier preparation process the mean permeability of calcein was reduced from $0.061 \pm 0.005 \times 10^{-6}$ cm/sec to $0.015 \pm 0.001 \times 10^{-6}$ cm/sec and the mean resistance was increased from 525 to 819 $\Omega \times \text{cm}^2$ (n=3). This confirmed our expectation that the resistance was increasing with increasing centrifugation time and that the permeability of compounds like calcein is decreasing with increasing resistance.

3.3.2 Permeation studies with drug compounds

Permeation studies were performed for 5 hr for most compounds, but for some compounds (see 2.4) the permeation time was prolonged to 12 hr. Tests with both short and long permeation time periods were run for enalapril to verify that the obtained permeation value

was independent of the duration of the permeation experiment (results not shown). The prolonged permeation time for cimetidine, nadolol, chlorothiazide and hydrochlorothiazide was done because the amount of the different drugs in the acceptor chambers after the original permeation time was too low to be detected by the analytical method used. Another approach, in cases when drug candidates show too low or no UV absorbance, is to extend the method by using LC-MS or radio-labeled drug compounds, but such methods will probably not be as appropriate for high throughput screening, and use of radio labeling would in addition be more complicated due to the safety precautions. We therefore chose to extend the permeation time rather than to use other more sensitive techniques.

The experiments were performed at least in triplicate with six inserts in each parallel for every compound. By the approach presented here, it was possible to obtain steady-state permeation values for all the 21 drug compounds used. A mean value for the apparent permeability coefficient (P_{app}) was calculated for each drug (see Table 3). As can be seen from Figure 5A, for the vast majority of drugs studied the P_{app} obtained using the phospholipid vesicle based barrier showed a typical sigmoidal correlation with the fraction absorbed in humans after oral administration. The same shape of correlation curve had been observed also with other comparable models for permeation studies. Within our test set, there are two clear outliers, a) salicylic acid, which have been shown to be actively transported in vivo (Stenberg et al., 2000) and b) tranexamic acid, which deviates from the rest of the drug compounds by being an ampholyte (which also has low UV-absorbance). Also in other studies in the literature tranexamic acid deviates in permeability compared to other drugs with the same fraction absorbed in vivo (Sugano et al., 2002). The apparent permeability coefficients (P_{app}) obtained by studying the permeation of the 21 drug compounds were compared with PSA and

experimental log D, and literature data from alternative permeation models such as ILC, the Caco-2 model and the PAMPA models (Table 1 and 3).

Table 3

These models were chosen for comparison partly because of their popularity in industry and academia for screening of permeability properties of drug compounds, and partly because of their similarity to the present model. PAMPA and Caco-2 models have a similar experimental set-up and ILC and the PAMPA model make use of phospholipids to mimic the *in vivo* epithelial barrier. The correlation plots between the calculated properties and permeability values and percent absorbed *in vivo* in humans for the different models are given in Figure 4 and 5.

Figure 4

Figure 5

3.4 Permeability classification

Attempts were performed to use the P_{app} values obtained in the present model for classification of the test compounds in classes according to their permeability properties.

For the present model it could be stated that compounds with P_{app} -values $<0.1 \times 10^{-6}$ cm/sec are poorly absorbed (<30 % absorbed *in vivo*), compounds with P_{app} -values between $0.1-0.9 \times 10^{-6}$ cm/sec are moderately absorbed (30-70 % absorbed *in vivo*), while compounds with P_{app} -values of $>0.9 \times 10^{-6}$ cm/sec have excellent oral absorption (>70 % *in vivo* absorption). Using this classification scheme 17 out of 21 compounds (81 %) could be correctly predicted in their *in vivo* absorption ability. The four drugs, for which the P_{app} values did not lead to correct classification, were tranexamic acid, sulphasalazine, amiloride and salicylic acid (see Table 3 for P_{app} values). On the other hand the compounds that are not correctly classified are found in the neighbouring class to the correct one (see Figure 6), for instance amiloride that is reported

to be well absorbed *in vivo* in humans is classified as moderately absorbed according to the present model. None of the five compounds was classified completely wrong e.g. being poorly absorbed in humans and excellently absorbed according to our model or the opposite.

Figure 6

The P_{app} - values and classification obtained from our model were compared with the results from other models. Two physicochemical properties, PSA and experimental log D, were used as well as the permeability models ILC, PAMPA and Caco-2 cells

When plotting the experimental log D values against the percent absorbed in humans after oral administration (Figure 4), no clear correlation was observed. By using the suggested classification by Zhu et al. (Zhu et al., 2002) that compounds with experimentally determined log D values between -0.5 and 4.5 are likely to be well absorbed, 55% of the drugs would be correctly classified. Thus, there is no difference in percent of correctly classified compounds using either experimental log D values or the calculated ones (data not shown).

Values for polar surface area (PSA) (Zhu et al., 2002) were also plotted against fraction absorbed in humans after oral administration (Figure 4). By using the suggested classification from Zhu et al (Zhu et al., 2002) that PSA values less than 120 \AA^2 may suggest good absorption, 50 % of the drug would be correctly classified.

The results obtained by Österberg et al (Osterberg et al., 2001) using ILC showed poor correlation with the fraction absorbed in human after oral administration, and no attempts to divide the drugs into classes had been done.

The permeation values obtained from PAMPA models taken from the literature differ to a large extent between laboratories. It is also more difficult to categorize the permeability values obtained from PAMPA into classes of poor, moderately and well absorbed drugs, as can be done with the present model. Stronger correlation between permeability values and percent drug absorbed in human after oral administration has been achieved with PAMPA models using double sink conditions (DS-PAMPA), i.e. to control the sink conditions by the use of both a pH-gradient between the donor and acceptor chamber and a chemical scavenger system present in the acceptor compartment (Avdeef, 2004).

Yazdanian et al (Yazdanian et al., 1998) stated that compounds with a P_{app} -value in Caco-2 cells lower than 0.4×10^{-6} cm/sec exhibited poor oral absorption whereas compounds with P_{app} -value higher than 7×10^{-6} cm/sec had excellent oral absorption. But permeation values obtained from the Caco-2 model in different laboratories often differ from each other due to use of cell lines of different origin (Artursson et al., 1996). For a critical review of the Caco-2 model see Ungell (Ungell, 2004).

From Figure 5 it can be seen that the P_{app} values obtained by our model show good correlation with fraction absorbed in humans after oral administration. Using this set of drugs our approach appears to model the *in vivo* absorption better than the ILC and the PAMPA models, under similar conditions as used in the present method, and equally well as the Caco-2 model and DS-PAMPA models.* Our model therefore seems to be a promising tool for early screening of new drug candidates. The advantage of the phospholipid vesicle based membrane model is that it is explicitly simpler and a lot easier to handle compared to cell

* The correlations presented with log scales are to be found in the Supplementary material

based assays. The use of liposomes instead of living cells also should reduce the problem with inter-laboratory differences in permeability values. However, the disadvantage of the developed *in vitro* model is the lack of resemblance in terms of morphological features such as the villi that make up the brush border of epithelial cells and the availability of active transport proteins.

3.5 The unstirred water layer

Diffusion across an artificial membrane consists of two parts; diffusion through the membrane and diffusion through the so-called unstirred water layer (UWL). If the thickness of the UWL is much larger than the membrane, this part can become rate limiting for diffusion of lipophilic drugs (Kansy et al., 2004). If this phenomenon is not taken into account, absorption parameters can be underestimated. The absorption of highly permeable drugs, such as testosterone, in *in vitro* permeability assays has been shown to depend on the stirring conditions (Karlsson and Artursson, 1991). So, when not compensate for the UWL effect when using an unstirred permeability assay, the permeability of the lipophilic compounds in the UWL could significantly contribute to the resulting permeability values. The *in vivo* UWL is thought to be 30-100 μm thick, consistent with very efficient mixing at the surface of the gastrointestinal tract (Lennernas, 1998). In the Caco-2 model the thickness of the aqueous boundary layer for testosterone has been reported to be about 1550 μm without any agitation (Karlsson and Artursson, 1991) and in the PAMPA model the thickness of the UWL for desipramine has been reported to be 1227 μm with no agitation (Avdeef et al., 2004). The thickness of the UWL for testosterone was reported to be 220 μm in a Caco-2 model at a shaking speed of 150 rpm (Adson et al., 1995), and for desipramine the thickness was reported to be 73 μm in a PAMPA model with a stirring speed of 182 rpm (Avdeef et al., 2004). Our model has shown to withstand shaking at 200 rpm without disturbances of the

liposome membrane in terms of resistance (results not shown). More research has to be done to see if an extension with shaking of this first simple model would lead to a more efficient approach, better reproducibility or different permeability values for the highly permeable drugs controlled mainly by the membrane.

3.6 Mass balance

Mass balance determinations were performed for metoprolol, ranitidine and sulpiride. The membrane retention in a PAMPA model with 20 % (w/v) soy lecithin with sink conditions in the acceptor wells was reported to be 26 % for metoprolol and 14 % for ranitidine at pH 7.4 (Avdeef, 2004). With our model the membrane retention was found to be 11.4 % for metoprolol, while practically no retention was observed for ranitidine and sulpiride. Our membrane consists of tightly packed liposomes and hence the drug molecules have to overcome many interfaces/bilayers to reach the acceptor compartment. In contrast, in the PAMPA model the membrane consists of a much thicker, coherent lipophilic phase, which may lead to a higher degree of retention of drug molecules inside of the membrane. Some PAMPA models use surfactants in the acceptor phase to obtain sink conditions and to minimize the retention. Our assay is performed under steady-state conditions, so the data used to calculate the permeability are not affected by any membrane saturation. This taken together with the fact that since sink conditions already are obtained and the mass balance satisfactory, use of surfactants is not requested in our model.

4 Conclusions

We have managed to make a tight barrier of liposomes on a filter support suitable for measurement of passive drug permeability. This novel method is to our knowledge the first where one has succeeded in depositing membrane barriers without use of inert solvents such

as hexadecane. With the set of drug compounds used the model shows differentiation in permeability between drug compounds according to their different physical chemical properties. We have been able to correlate the permeability values from the developed phospholipid vesicle based model with human absorption in vivo, which suggests that the performance of the method is adequate and that it can be useful for rapid screening of passive transport of new chemical entities. By using the common classification system for permeability properties 17 (81%) of the set of 21 compounds could be correctly predicted according to their in vivo absorption abilities. Using the chosen set of drug compounds our approach appears to model the in vivo absorption better than both the ILC and the PAMPA model used under similar conditions as in the present method, and equally well as the Caco-2 model and DS-PAMPA. In addition, our model has the advantage of being lot easier to handle compared to cell based assays. Further improvement of the method could include the use of shaking to reduce the UWL. In conclusion, the phospholipid vesicle based membrane method described in this study is a very promising approach for future medium throughput screening of permeability properties of potential drug candidates early in the development process.

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Supplementary material available

The 21 drugs along with their concentrations of stock solutions and the wavelengths of UV analysis are listed in Table A and the molecular structures for the drug compounds used in this study are given in Figure A. The development of the method to produce membranes is also described in detail in this section. The correlations of the permeability values from the present model as well as the existing permeability models compared with presented with log scale is given in Figure B. Supplementary material is available at <http://www.sciencedirect.com>.

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Figure 1: Flow chart for the preparation of the liposome membranes.

Figure 2: Schematic drawing of experimental set up used in the permeation studies.

Figure 3: The cumulative amount of drug (nmol) plotted against time (hr) showing the lag time until steady state conditions are obtained in the permeation experiments. Sulpiride (top) and testosterone (bottom) were chosen as examples of a highly and poorly permeable drug, respectively.

Figure 4 A comparison of correlation plots for PSA (Zhu et al., 2002) and experimental log D (Zhu et al., 2002) and extent of absorption in humans of orally administered drugs.

Figure 5: A comparison of correlation plots between permeability values from the different models and extent of absorption in humans of orally administered drugs. A: the present model, B:(Sugano et al., 2001) and C:(Avdeef, 2004) the PAMPA model, D:(Yazdanian et al., 1998) Caco-2 monolayer and E:(Osterberg et al., 2001) immobilized lipid chromatography (ILC). All permeability values are given as 10^{-6} cm/sec. For C and E the standard deviations were not reported.

Figure 6: Diagram showing the correlation of permeability classes according to percent absorbed in human and the values obtained from the present model. White boxes indicate perfect classification, light grey indicate classification into the neighbour class to what was expected from percent absorbed in humans while dark grey indicate complete incorrect classification.

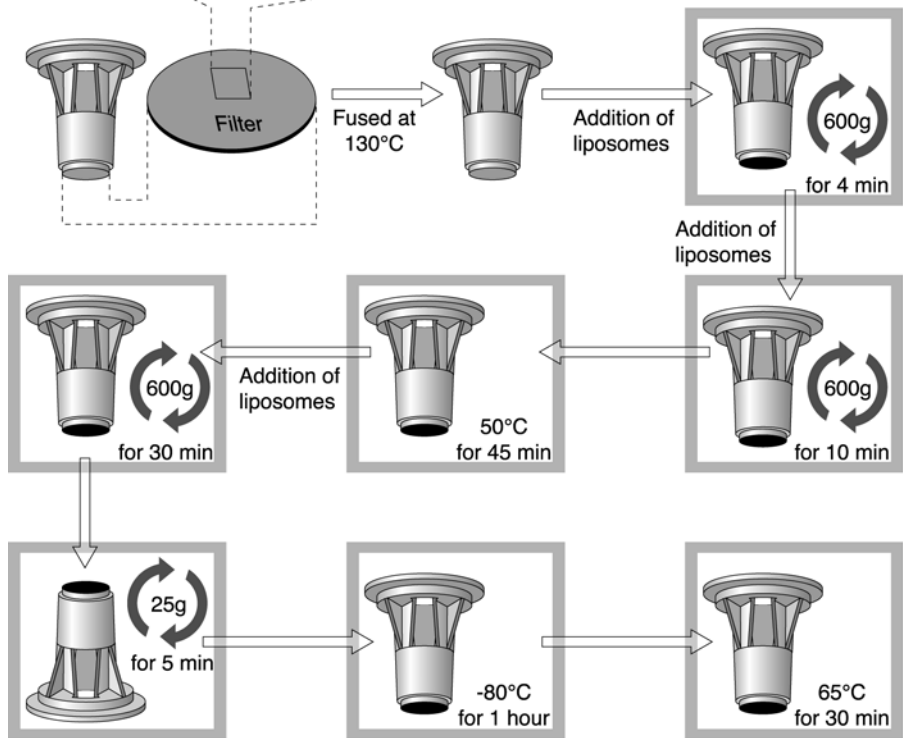
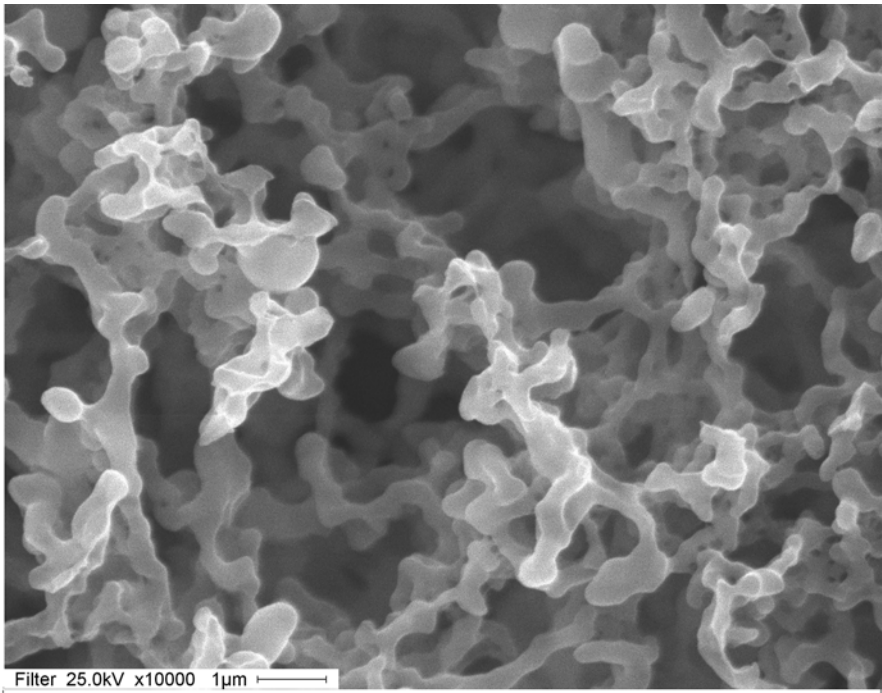


Figure 1

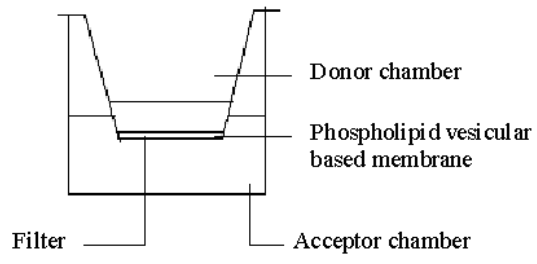


Figure 2

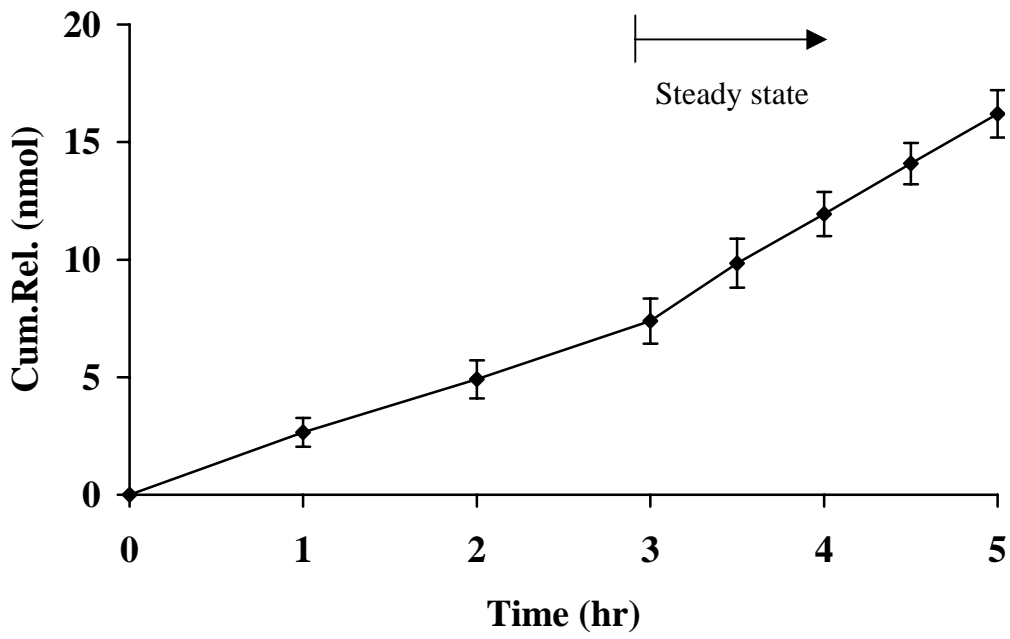
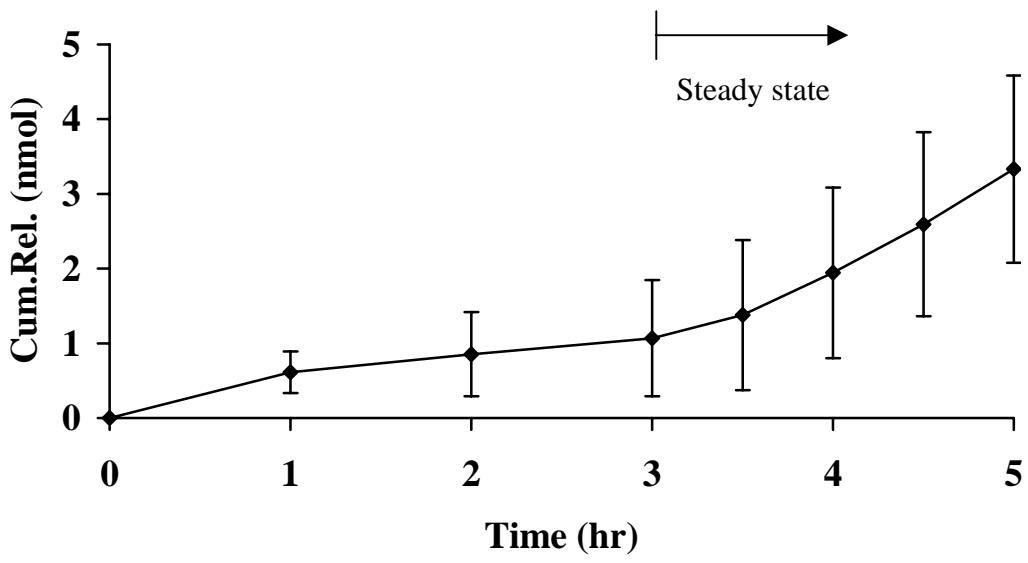


Figure 3

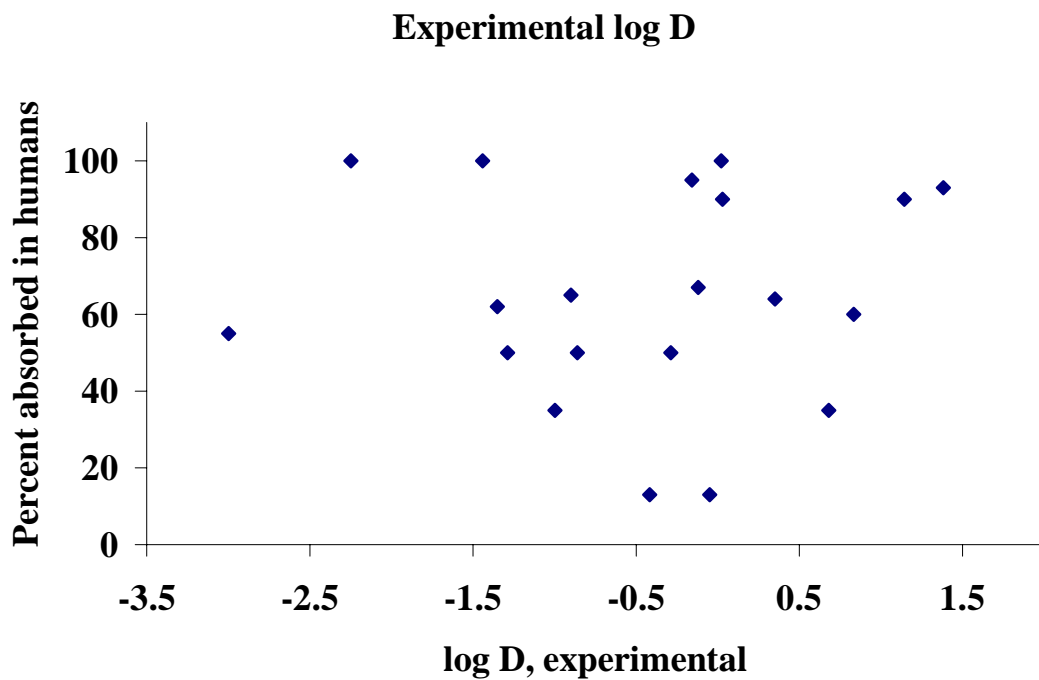
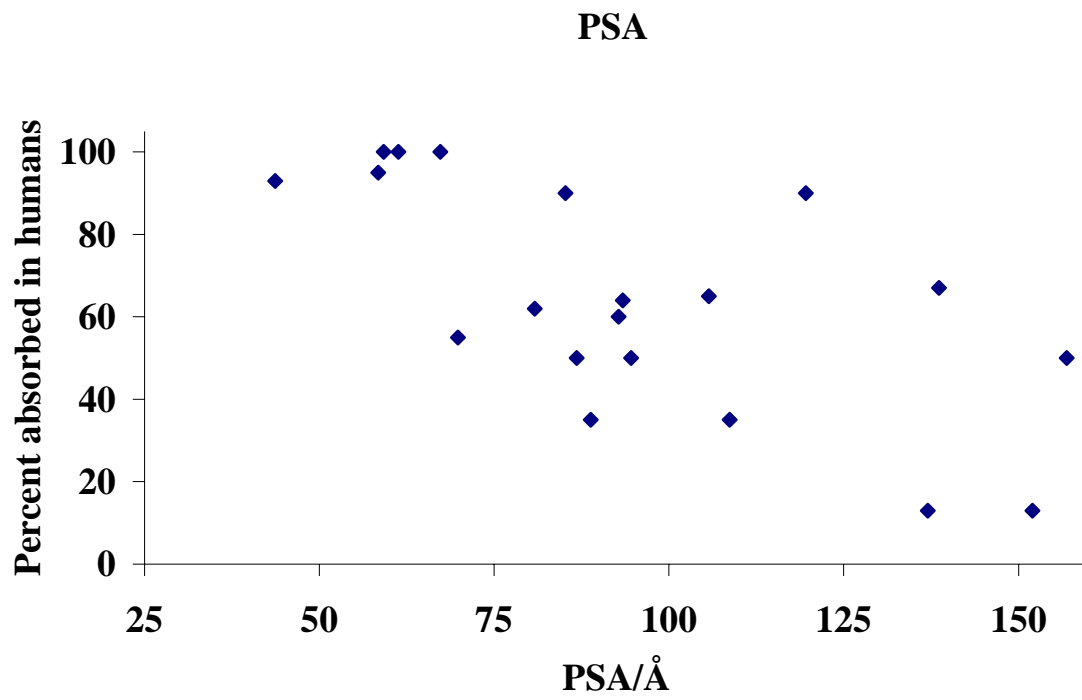
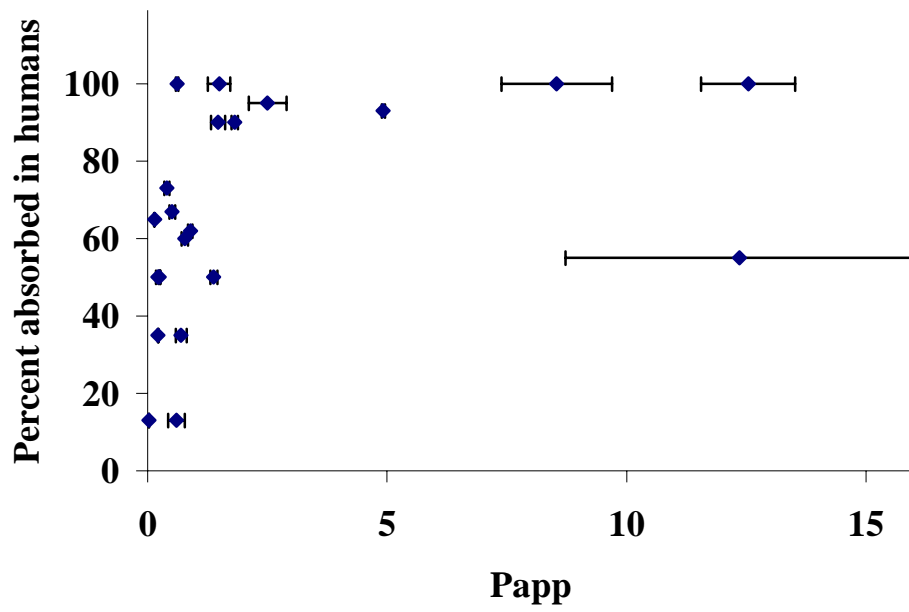
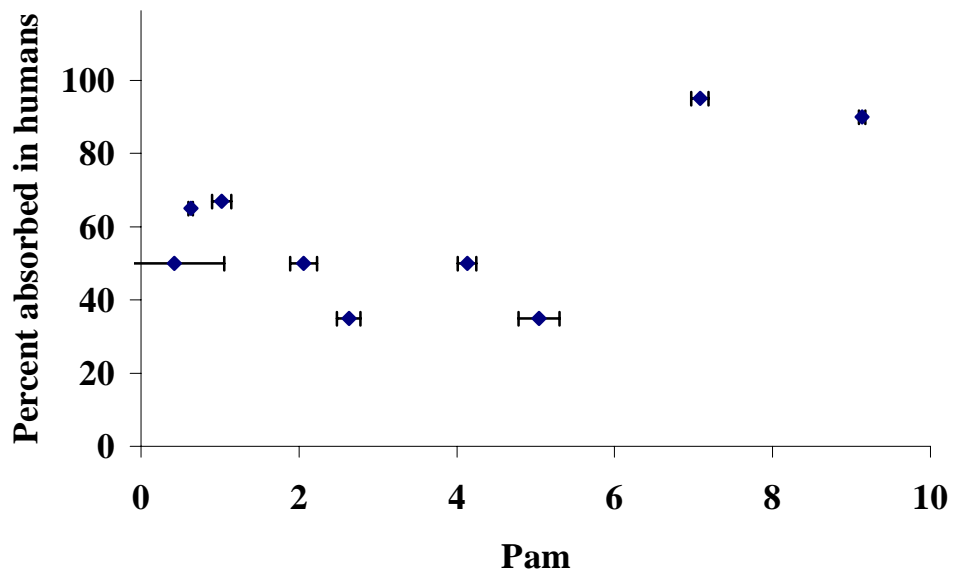


Figure 4

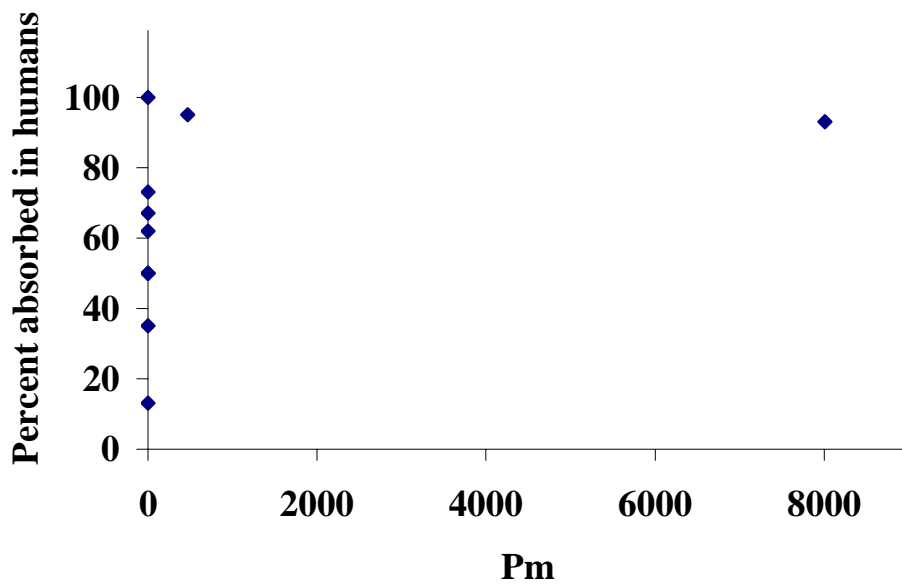
Liposome model (A)



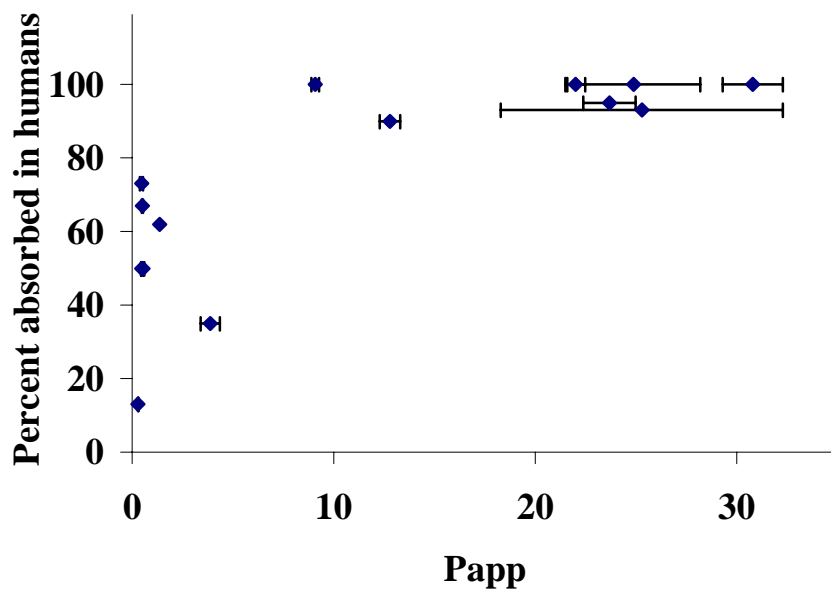
Bio-mimetic PAMPA (B)



Double-sink PAMPA (C)



Caco-2 cell model (D)



ILC (E)

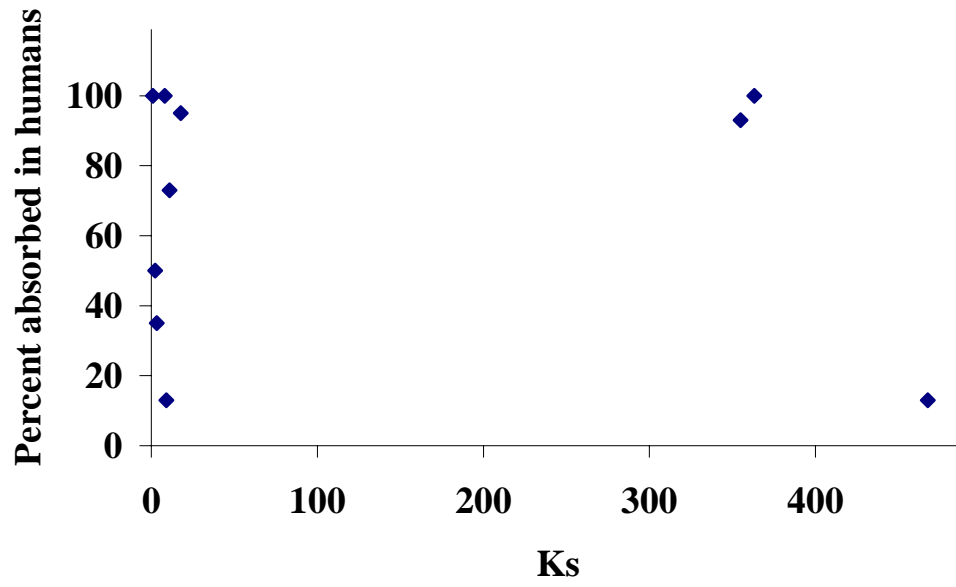


Figure 5

		Fraction absorption in humans after oral administration		
		Excellent	Moderately	Poorly
Permeability in the liposome model	Excellent	Timolol Chloramphenicol Alprenolol Metoprolol Testosterone Acetylsalicylic acid Caffeine		
	Moderately	Amiloride Tranexamic acid Salicylic acid	Sulpiride Nadolol Ranitidine Atenolol Acebutolol Cimetidine Enalapril Hydrochlorothiazide Terbutaline	Sulphasalazine
	Poorly			Chlorothiazide

Figure 6

Table 1: The physicochemical properties of the drugs used in the validation of the phospholipid vesicle membrane model

Drug ^a	MW ^a	Log D ^b	PSA ^b	PKa ^c	F _a % ^{d,e}	F _a % range in literature ^e
Chlorothiazide	295.7	-0.05	137	6.7*	13 ¹	13 ¹ -25 ⁸
Sulphasalazine	398.4	-0.42	152.3	2.8/8.3/11.0	13 ²	12 ⁷ -13 ¹
Sulpiride	341.4	-1.00	108.7	9.0*	35 ³	35 ³ -44 ⁹
Nadolol	309.4	0.68	88.8	9.7	35 ¹	20-57 ⁹
Ranitidine	350.9	-0.29	86.8	8.3/2.1	50 ²	39-88 ⁹
Atenolol	266.3	-1.29	94.6	9.5	50 ²	49 ⁴ -54 ⁷
Amiloride	229.6	-0.86	156.9	8.7	50 ¹	-
Tranexamic acid	157.2	-3.00	69.8	4.5/10.7*	55 ⁴	-
Acebutolol	336.4	0.83	92.8	9.5	60 ¹	>50 ⁷ -90 ⁴
Terbutaline	225.3	-1.35	80.8	8.7/10.0/11.0	62 ⁴	60 ⁷ -68 ¹⁰
Cimetidine	252.3	0.35	93.4	6.9	64 ⁴	64 ⁴ -95 ⁶
Enalapril	376.5	-0.90	105.7	2.9/5.2	65 ¹	55-75 ¹
Hydrochlorothiazide	297.7	-0.12	138.6	8.9/10.3	67 ¹	65-72 ⁹
Timolol	316.4	0.03	85.2	9.5	90 ¹	81 ¹⁰ -95 ⁹
Chloramphenicol	323.1	1.14	119.6	5.5*	90 ⁴	-
Alprenolol	249.4	1.38	43.7	9.2	93 ²	93 ² -96 ⁷
Metoprolol	267.3	-0.16	58.4	9.6	95 ²	95 ² -102 ⁷
Salicylic acid	138.1	-1.44	61.3	3.0	100 ²	-
Testosterone	288.0	-	-	-*	100 ²	98 ³ -100 ²
Acetylsalicylic acid	180.2	-2.25	67.3	3.5	100 ³	98 ¹⁰ -100 ³
Caffeine	194.2	0.02	59.2	-	100 ⁵	-

^a For structures see Figure A, Supporting Information.

^b The log D and PSA values were taken from (Zhu et al., 2002)

^c The pKa values were obtained from the reference Absorption and Drug Development; Solubility, Permeability and Charge State(Avdeef, 2004) except those marked with *. They were taken from (Sugano et al., 2002)

^d Fraction absorbed in humans after oral administration. For data are taken from(Sugano et al., 2001)¹; (Yazdanian et al., 1998)²; (Kansy et al., 1998)³; (Sugano et al., 2002)⁴; (Yee, 1997)⁵;

(Yazdanian et al., 1998)⁶; (Osterberg et al., 2001)⁷; (van de Waterbeemd H et al., 2003)⁸; (Zhao et al., 2003)⁹ and (Zhu et al., 2002)¹⁰

^e The range of values reported in literature for fraction absorbed in humans is quite broad for some of the compounds. The Fa value chosen to use were mostly taken from the same articles as the permeability values for the models to compare with

Table 2: Permeability coefficients and resistance of the phospholipid vesicle based membrane barrier to various hydrophilic fluorescent markers

Marker	MW	pKa ^a	Concentration (mM)	Permeability ^b (10 ⁻⁶ cm/sec)	Mean resistance ^c (Ω×cm ²)
Calcein	622.5	1.8/9.2	95.5	0.061 (0.005)	525
5(6)-Carboxy- fluorescein	376.3	3.8 (3.5)	81.4	0.054 (0.011)	667
Fluorescein sodium	376.3	8.6	86.2	0.227 (0.045)	439
2',7'-Dichloro- fluorescein	401.2	3.8	28.2	0.258 (0.046)	450

^a The values are calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V4.67 (1994-2005 ACD/Labs)

^b All values are mean of three parallels with six inserts each. Standard deviations are shown in parentheses.

^c Values represent the resistance of the lipid barrier at the end of the transport study.

Table 3: The results from the permeation study using the present model together with permeability data from ILC, PAMPA and Caco-2-cell models

Drug	Liposome model ^a	ILC ^b			Caco-2 model ^d	F _a ^g
		P_{app} ^f	$\log K_s$	Bio-mimetic P_{am} ^e		
					P_{app} ^{e,f}	%
Chlorothiazide	0.03 (0.003)	0.96	< 0.05		0.19(0.02)	13
Sulphasalazine	0.60 (0.178)	2.67		0.001 ⁴	0.30(0.02)	13
Sulpiride	0.22 (0.006)	0.52	5.04(0.26)			35
Nadolol	0.70 (0.113)		2.63(0.15)	0.24 ²	3.88(0.48)	35
Ranitidine	0.24 (0.010)		4.13(0.12)	0.061 ¹	0.49(0.06)	50
Atenolol	0.22 (0.048)	0.37	2.06(0.17)	0.06 ²	0.53(0.07)	50
Amiloride	1.38 (0.071)		0.37-1.48	0.002 ¹		50
Tranexamic acid	12.36(3.641)					55
Acebutolol	0.78 (0.070)		3.91(0.30)		0.51(0.02)	60
Terbutaline	0.40 (0.053)	1.04		0.003 ²	0.47(0.08)	62
Cimetidine	0.89 (0.046)			0.47 ²	1.37(0.34)	64
Enalapril	0.14 (0.012)	<0	0.63(0.03)			65
Hydrochlorothiazid	0.51 (0.058)		1.02(0.12)	0.005 ¹	0.51(0.02)	67
Timolol	1.47 (0.145)		9.13(0.04)		12.8(0.5)	90
Chloramphenicol	1.81 (0.072)					90
Alprenolol	4.92 (0.340)	2.55		8010 ⁴	25.3(7.0)	93
Metoprolol	3.23 (0.778)	1.25	7.08(0.11)	466 ⁴	23.7(1.3)	95
Salicylic acid	0.59 (0.025)	0.91			22.0 (0.5)	100
Testosterone	8.54 (1.159)	2.56			24.9 (3.3)	100
Acetylsalicylic acid	1.49 (0.233)	-0.01			9.09 (0.19)	100
Caffeine	12.54 (0.98)			2.8 ³	30.8 (1.5)	100

^a The permeation values are the mean of three parallels with six insert in each. SD is given in paranthese

^b ILC data were taken from (Osterberg et al., 2001) Standard deviations were not reported there

^c For comparison with the PAMPA model literature data from two different approaches have been used, Bio-mimetic PAMPA(Sugano et al., 2001) and DS-PAMPA (Avdeef, 2004)¹(Avdeef et al., 2005)² (Nielsen and Avdeef, 2004)³ (Ruell et al., 2004)⁴. Standard deviations were not reported for the last one.

^d Caco-2-cell permeation data were taken from (Yazdanian et al., 1998)

^e The P_{app} values are given in 10^{-6} cm/sec

^f Yazdanian et al used radiochemical method of analysis while present and PAMPA studies were based on UV spectrophotometry.

^g For references see Table 1

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