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Original article

Lecithin and anionic lipids as an imitation of the lipid membrane in Parallel Artificial Membrane Permeation Assay (PAMPA) blood –brain barrier Models

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Summary. *Background/Aim:* One of the biggest challenges today is producing pharmaceutical forms of drugs for a successful treatment of pathological disorders of the central nervous system (CNS), bearing in mind that the brain tissue is highly selective when it comes to permeation of all substances, particularly drugs. *In vitro* Parallel Artificial Membrane Permeation Assay (PAMPA) drug permeability assay is an extremely important experimental model in the process of drug development. The aim of this study is to modify this model in order to test drug permeability through the blood-brain barrier. *Methods:* The substances with pharmacological activity tested in this study are the following: theophylline, sulfasalzine, risperidone, haloperidol, lidocaine and propranolol. We studied some of the key parameters which are important for good prediction of permeability – lipid composition and buffer composition. We also investigated the effect of co-solvents in the donor compartment and surfactants in the acceptor compartment, as well as the influence of the anionic lipid composition on drug permeability through the blood-brain barrier. As co-solvents, we used polyethylene glycol 200 (PEG 200) at concentrations of 1% and 0.24%, and as surfacants we applied sodim lauryl sulfate (SLS) at concentrations of 2% and 0.5%. In addition, the influence of lecithin and the anionic lipid composition was investigated and the following lipids were used for this purpose: 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (PS18: 1), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC18: 1), and cholesterol. *Results:* The use of PEG 200 at concentrations of 1% and 0.24% proved to be good and allowed for a proper classification of the substances into those of high and low permeability. SLS at concentrations of 2 and 0.5% impaired the integrity of the lipid membrane, with the solution penetrating from the acceptor into the donor compartment. A brief experiment with methanol and SLS combined, showed that the membrane integrity remains preserved. The anionic lipid composition also proved favourable and the substances tested were properly classified into those of high and low permeability. *Conclusion:* The lipids that were used as an imitation of lipid membrane such as a 10% lecithin and combination of 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (PS18: 1), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC18: 1) and cholesterol in the anionic-pampa model proved to be good and allowed for a proper classification of the substances into those of high and low permeability.

Keywords: Pampa; Blood-brain barrier; Lecithin; Anionic Lipids

Introduction

Although biological activity is a key problem in the development of potent drugs, other factors such as solubility, absorption, distribution, or biodegradation are equally important. Unfortunately, the optimization process of these important properties is often carried out late in the process of drug discovery and development. Therefore, early testing of pharmacologically relevant physicochemical and biophysical properties of compounds is an important issue which needs to be addressed [1]. On the one hand, advances in bioanalytical methodologies and technologies applied at the level of cell, have enabled early evaluation of the

desired pharmacokinetic properties, which is applied in the selection of new drugs. On the other hand, the Pharmacokinetics/Pharmacodynamics relationship has become an integral part of many clinical drug development programmes [2,3]. The group of drugs acting at the level of the central nervous system (CNS) has a particularly difficult task, as they must cross a highly selective, anatomically and physiologically specific blood-brain barrier (BBB) in order to act pharmacologically. On the other hand, for drugs which exert their effects on the periphery, going through the BBB is not desirable due to a possible manifestation of side effects [4]. *In vitro* assays, as well as tools based on physicochemical properties, are used to predict the characteristics and outcomes of the *in vivo* absorption processes. Examples of such *in vitro* methods include simple artificial systems such as the Parallel Artificial Membrane Permeability Assay (PAMPA) model, and biological membrane systems or assays based on biological cell layers, for example the Caco-2 cells. In particular, the well-established Caco-2 cell line is widely used as it generates reproducible and biorelevant permeability results based on high permeability [5]. This permeability model enables the investigation of carrier-mediated transport due to the expression of influx and efflux transporters in the intestine [6,7]. Cellular permeation systems also show relative incompatibility with food components and certain pharmaceutical excipients, the absence of cytochrome P450 3A40 (CYP3A4) and the lack of mucosal layer [8]. Major advantages of the *in vitro* models such as PAMPA, especially when compared to *in vivo*, include higher throughput, lower cost and lower amount of the compounds required to conduct the experiments, ability to quantify the compounds directly in the saline buffer, and fewer animals required to perform the experiment [9]. It is also imporant that the *in vitro* BBB models also provide mechanistic information by detecting complex molecular interactions which regulate the cerebral endothelial cells (CEC) throughput under normal and pathological conditions [10]. Bearing ind mind that efflux transporters are more expressed in the brain compared to the other absorption sites in the body, this deficiency has a more significant effect in the PAMPA-BBB models as it may overestimate the *in vivo* BBB permeability of drugs which would otherwise be actively pumped out the brain [11]. In order to obtain the most accurate results using the PAMPA model and to overcome these limitations, cell-based models are often integrated additionally and they need to meet certain criteria which will be used for screening potential drugs [12]. The PAMPA model was first introduced in 1998 by Kansy et al., as a high-throughput permeability assay which can predict gastrointestinal drug absorption by transcellular passive diffusion. Kansy and co-workers used a solution of lecithin (1-20%) in an organic solvent (dodecane, hexane and 1.9 decadiene), in order to mimic the lipid composition of mammalian membranes [13].

As mentioned previously, various studies have been published in order to optimize the PAMPA model and increase its predictive power. One of the aims of this experimental work was to investigate the influence of co-solvents in the donor, surfactants in the acceptor compartment and the influence of the anionic lipid compositions in order to test drug permeability through the blood-brain barrier. The permeability of six pharmacological active compounds was examined.

Materials and Methods

In the initial experiments, 20% lecithin was used as an imitation of the lipid membrane. Due to its high viscosity, difficult application of lecithin to the filter at this concentration at a volume of 4 μL was observed. In the following experiment, lecithin concentrations of 5% and 10% were examined. Based on the obtained results, no significant differences were observed between the two concentrations, and the application of lipid on the filter membrane was significantly facilitated, so it was decided to continue with a concentration of 10% lecithin in the following experiments. Also, the anionic lipids were examined as an imitation of the lipid membrane because it was found that membranes of neuron cells are more negatively charged compared to peripheral cells [11]. The donor and acceptor compartments contained phosphate buffer solution at pH 7.4. In the first experiment, these buffers did not contain co-solvent and surfactant, whereas in the following experiments they contained these substances. After an incubation for 4 h, the quantification of substances in

the donor and acceptor compartments was performed using the UV/VIS method. Using obtained absorption values, the concentrations of the substances in the donor (Cd) and in the acceptor compartment (Ca) were calculated and later included in the equation for the calculation of the Pe. Based on the obtained Pe values and the literature data, the substances were classified either as good or poorly permeable.

Materials

The substances with pharmacological activity tested in this study are the following: Theophylline (anhydride) (Sigma, Canada), Sulfasalazine (Fluka Analytical, China), Risperidone (Sequia Research Products Ltd, United Kingdom), Haloperidol (Sigma, China), Lidocaine chloride (AppliChem, Germany), Propranolol chloride (Fluka, USA).

Following chemicals were also used: dimethyl sulfoxide (DMSO) (Sigma, France), lecithin, dodecane (Sigma-Aldrich, China,) hexane (Honeywell, Germany), sodium lauryl sulfate (SLS) (Sigma-Aldrich, China), polyethylene glycol 200 (PEG200) (Serva Feinbiochemica, Germany), 1,2-dioleoyl-sn-glycero-3- [phospho-L-serine] (PS18: 1) (Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC18: 1) (Avanti Polar Lipids), Cholesterol, (Lex Slovenija) Ph.Eur. 8th Ed. Pampa test was performed with Multiscreen Filter Plate (96 well tray with lid, 0.4 μm PCTE membrane), Cat.No. MPC4NTR10, Millipore; Multiscreen Transport Receiver plate (96 well, Polystyrene), Cat.No. MATRNPS50, Millipore; UV-VIS spectrophotometer; UV panels.

Methods

First it was planned to test the permeability of substances with 10% lecithin as an artificial lipid membrane. The pH was 7.4 in both the donor and acceptor compartments without any addition of co-solvents and surfactants (Table 1A). Second test consisted of two parts. In the first part, only the influence of the 1% co-solvent PEG 200 was examined, and in the second part, the common effect of the co-solvent and surfactant SLS was examined. The surfactant was at a concentration of 2%. pH was 7.4 in both the donor and acceptor regions (Table 1B). Furthermore, the effect of lower concentrations of co-solvent and surfactant on substance permeability was examined. In the first part of the experiment, only the influence of 0.24% co-solvent PEG 200 was examined, and in the second part was examined the common influence of the 0.24% PEG200 in the donor and the 0.5% surfactant SLS in the acceptor compartment (Table 1C). The experiment was performed under the same pH conditions as in the previous. Also, there was an idea to change the lipid composition. It was recently discovered that cerebral endothelial cells (CECs) have a more negatively charged membrane than other cells, making anionicity another determinant factor of BBB permeation [11]. Therefore, the aim of this experiment was to examine the effect of anionic lipids on drug permeability. Such a PAMPA model is called the anion-PAMPA model (Table 1D) [14]. Therefore, the following lipids were used: 1,2-dioleoyl-sn-glycero-3- [phospho-L-serine] (PS18: 1) - anionic lipid; 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC18: 1) - neutral lipid; Cholesterol - a neutral lipid. PC18: 1 (2.6% w/v), PS18: 1 (0.9% w/v) and cholesterol (1.5% w / v) were dissolved in n-dodecane. In the donor and the acceptor solution there was only PBS, without the addition of co-solvent and surfactant and the pH was 7.4. In all the experiments, samples were analyzed using a UV-VIS spectrometer. 150 μL of the donor and the acceptor solution was transferred to UV-VIS plates for analysis at the 230 nm to 370 nm wavelength range. The concentrations in the donor and acceptor solutions were obtained by the following equations:

$$Cd = Co * \frac{(Ad\text{-}Abuffer)}{(Ao\text{-}Abuffer)}$$

$$Ca = Co * \frac{(Aa\text{-}Abuffer)}{(Ao\text{-}Abuffer)}$$

Ao- Absorbance of the donor solution before incubation, Ad- Absorbance of the donor solution after incubation, Aa- Absorbance of acceptor solution after incubation, Abuffer- Buffer absorbance. The following

Table 1 (A) 10 % Lecithin as an imitation of the lipid membrane; **(B)** a) 1% PEG 200 as a co-solvent b) 1% PEG 200 as a coslovent and 2% SLS as a surfactant; **(C)** a) 0.24% PEG 200 as a co-solvent b) 0.24% PEG 200 as a coslovent and 0.5% SLS as a surfactant; **(D)** Lipids used as an imitation of lipid membrane (Anionic-Pampa): 1,2-dioleoyl-sn-glycero-3- [phospho-L-serine] (PS18: 1) 1,2-dioleoyl-sn-glycero-3 phosphocholine (PC18: 1) and cholesterol

A	Plate	Multiscreen Transport Receiver plate in Filter Plate		
	Lipid	10% Lecithin		4 µl
	Donor compartment	200 µM, PBS		300 µL
	Acceptor compartment	PBS		200 µl
	Conditions	4h, 37°C, without stirring		
B	Plate	Multiscreen Transport Receiver plate in Filter Plate		
	Lipid	10%Lecithin		4 µL
	Donor compartment	200 µM, PBS + 1%PEG200		300 µL
	Acceptor compartment	**a)**PBS	**b)**PBS + 2%SLS	200 µL
	Conditions	4h, 37°C, without stirring		
C	Plate	Multiscreen Transport Receiver plate in Filter Plate		
	Lipid	10%Lecithin		4 µl
	Donor compartment	40 µM, PBS + 0,24%PEG200		300 µL
	Acceptor compartment	**a)**PBS	**b)**PBS + 0,5%SLS	200 µL
	Conditions	4h, 37°C, without stirring		
D	Plate	Multiscreen Transport Receiver plate in Filter Plate		
	Lipid	Anionic-PAMPA		5 µl
	Donor compartment	200 µM, PBS		300 µL
	Acceptor compartment	PBS		200 µL
	Conditions	4h, 37°C, without stirring		

equation [15] was used to calculate the permeability coefficient:

$$P_e = -\frac{2{,}303 V_d}{A \times D_A (t - \tau_{ss})} \times \left(\frac{1}{1 + r_v}\right)$$

$$\times \log\left[1 - \left(\frac{1 + r_v^{-1}}{1 - R_M}\right) \times \frac{C_A(t)}{C_D(0)}\right]$$

Pe…. effective permeability [cm/s], Vd… volume of donor solution [cm3], Va…. volume of acceptor solution [cm3], A…. membrane surface [cm^2] - Millipore plate: 0.3 cm^2, D_A… membrane porosity A - Millipore plate: 0.76, t… incubation time (s), τss…. membrane saturation time (s) – negligible, rv = Vd/Va …. the ratio of the volume of the donor and acceptor solution, R_M…. retention of substances on the membrane, C_R…concentration of reference solution at the beginning = C_D concentration of donor solution at baseline (t = 0) (C_D (0)), C_D…. concentration of donor solution at time t (C_D (t)), C_A…. concentration of acceptor solution at time t (C_A (t)). The classification of substances was empirically performed according to the following framework [4]: a) CNS + (high BBB permeability); Pe () > 4.0; b) CNS- (low BBB permeability); Pe () < 2.0; c) CNS +/- (uncertain BBB permeability); Pe () from 4.0 to 2.0.

Results

CNS classification of model compounds is presented in Table 2[16]. Later, this data were used for

Table 2 Model compounds and their CNS classification

Substances	**CNS classification**	
Theophylline	(CNS–)	Pe () < 2.0
Sulfasalazine	(CNS-)	Pe () < 2.0
Risperidone	(CNS+)	Pe () > 4.0
Haloperidol	(CNS+)	Pe () > 4.0
Lidocaine	(CNS+)	Pe () > 4.0
Propranolol	(CNS+)	Pe () > 4.0

comparison with obtained results from the experiments.

In the first test, where lecithin was used as an imitation of lipid membrane, the obtained results allowed clear classification of model compounds as good or poorly permeable, according to the literature data (Table 3A).

In the course of the second and third experiment it was noticed that SLS at both concentration 2% and 0.5% had a big influence on the integrity of membrane, causing the leakage of the acceptor solution into the donor solution. Due to this, there was not enough volume of the acceptor solution for UV/VIS analysis.

In order to confirm this observation, the influence of SLS was examined with different combinations of lipid and organic solvents in different ratios, which are used to mimic lipid membrane: dodecane and hexane: 1:3; dodecane and hexane: 3:1; 10% lecithin in dodecane; only dodecane. Chen and al. [17] used 10% methanol solution as an additional compound in the acceptor compartment. Following this idea, a brief experiment has been done with the addition of 10% methanol and 0,5% SLS. As a result of this, filter membrane was not affected and there was not leakage of the acceptor solution into the donor solution. Results which were obtained with only cosolvent (PEG200) at 1% and 0.24% concentration in the donor compartment can correctly classify compounds into the CNS+ (good permeability trough blood brain barrier) and CNS- (low permeability trough blood brain barrier) according to the literature data [16]. Both concentrations of PEG 200 gave results corresponding to the limits of the permeability coefficient (Table 3B,3C).

Results obtained in the fourth test have also shown that the permeability of model compounds can be correctly predicted using anionic lipids as an imitation of a lipid membrane (Table 3D). This is especially important because CECs have a more negatively charged membrane than the other cells.

Table 3 (A) Pe values of model compounds when lecithin is used as an imitation of lipid membrane; **(B)** Pe values obtained with 1% PEG 200 as a co-solvent; **(C)** Pe values obtained with 0.24% PEG 200 as a co-solvent; **(D)** Pe values obtained with anionic lipids

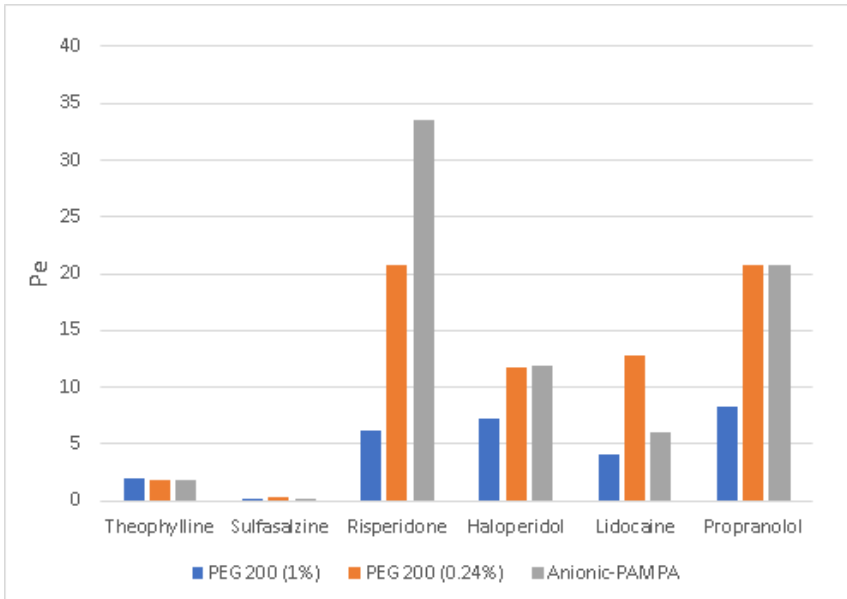
	A	**B**	**C**	**D**
Substances	**Pe ()**	**Pe ()**	**Pe ()**	**Pe ()**
Theophylline	1.48	1.90	1.83	1.76
Sulfasalazine	0.37	0.21	0.23	0.10
Risperidone	41.50	6.21	20.81	33.47
Haloperidol	34.51	7.29	11.74	11.95
Lidocaine	16.23	4.08	12.77	6.04
Propranolol	44.71	8.31	20.72	20.80

Graph 1. shows and compares the permeability coefficients of model substances obtained in the experiments where PEG 200 was used as a co-solvent at concentrations of 1% and 0.24% and where anionic lipids were used as an imitation of the lipid membrane.

Discussion

It was confirmed that SLS had a big influence on integrity of the filter membrane, therefore SLS cannot be present alone in the acceptor solution.

Both concentrations of PEG 200 gave results corresponding to the limits of the permeability coefficient[16]. In the case where 0.24% PEG 200 was used, higher Pe values were obtained compared to the experiment where 1% PEG 200 was used, except in the case of theophylline and sulfasalazine where the values were approximately similar (Table 3B,3C). Results obtained in the Anionic-Pampa test have also shown that the permeability of model compounds can be correctly predicted using these lipids as an imitation of a lipid membrane. The lipid composition made of anionic lipids may be promising tool for adequately predicting permeability of drugs trough blood-brain barrier. Since parameters of the lipid composition and the buffer solutions were different, the variations in Pe



Graph 1 Compared Pe values where 1% and 0,24% PEG 200 and anionic lipids were used

values were consequently present. What can be concluded is that the modification of these parameters allowed for the proper classification of substances into those with good permeability (CNS+) and those with poor permeability trough BBB (CNS-).

Conclusions

Various modifications of the PAMPA model could serve as a very useful tool in the pharmaceutical industry for testing drug permeability in the early stages of development and research, not only through the intestinal mucosa but also other barriers, including the blood-brain barrier. In this study, some of the key parameters for testing drug permeability across the blood-brain barrier were examined, namely the lipid composition and buffer composition. It was concluded that SLS cannot be used alone in the acceptor buffer due to the impairment of lipid membrane integrity and penetration of the acceptor solution into the donor. The combination of SLS and 10% methanol in the acceptor solution did not affect the membrane integrity indicating that an organic solvent such as methanol protects the membrane from the negative influence of SLS. The addition of co-solvent PEG 200 (0.24% and 1%) in the donor compartment allowed the proper classification of the substances on CNS+ and CNS-. This indicates the possibility that this co-solvent can be used to test the permeability of low solubility substances. The lipids that were used as an imitation of lipid membrane such as a 10% lecithin and combination of 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (PS18: 1), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC18: 1) and cholesterol in the anionic-pampa model proved to be good and allowed for a proper classification of the substances into those of high and low permeability. The anionic pampa model is especially significant in testing drug permeability trough BBB due to the fact that CECs have a more negatively charged membrane than the other cells. This kind of model is promising one and can help for closer imitation of a cell membrane in blood-brain barrier of humans.

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