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Pilot Study On Variation Of Dynamic Diffusion Cell Artificial Membrane Method For The Assessment Of Active Substances Permeability.

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ABSTRACT

In vitro methods for permeability assessment of active substances have important role in the development of new active substances, as screening methods, but also as supportive methods in biowaiver request submission. Variation of the published method of dynamic model of diffusion cells with artificial membrane was examined. Apparent permeability coefficients were determined for the substances representing each class of Biopharmaceutical Classification System: caffeine, naproxen sodium, metformin hydrochloride and hydrochlorothiazide. The influence of the membrane pore size and the origin of lecithin in solution for membrane impregnation were compared to the published method. Comparison of the obtained results for naproxen sodium with the published results indicate that the pore size of the supportive medium is not the determining factor for the apparent permeability coefficient. The results of the permeability determinations of the tested substances indicate that the lecithin composition in the solution for impregnation has certain influence. The correlation coefficient of the implemented method with the percent *in vivo* absorption in humans is higher than the literature accounts obtained with the other *in vitro* methods.

Keywords: *in vitro* permeability, diffusion cells, artificial membrane, method comparison

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INTRODUCTION

In order for the active substance to exert its action after oral application, it has to dissolve from the pharmaceutical form into the gastrointestinal fluids, pass through the mucous membrane of the gastrointestinal tract, enter the blood vessels and get to the site of action. All of this needs to happen at certain rate and extent in order to achieve therapeutic concentration at the site of action. Physicochemical characteristics of the substance, characteristics of pharmaceutical dosage form, physiological functions of gastrointestinal tract and biochemical and physical characteristics of epithelial barrier influence the complex process of absorption of the active substance. The success of oral therapy will depend, thus, on adequate intestinal absorption making the substance available at the site of action. Good oral bioavailability is enabled if the substance has the best permeability and solubility at the absorption site. Thus, *in vivo* extent of absorption can be estimated by solubility and permeability measurements. Fundamental relationship between the measured absorption rate, as the apparent permeability coefficient, and the extent of absorption led to the development of experimental models [1,2].

In vitro methods have important role in the development of new active substances, foremost as screening methods, but also as supportive methods in biowaiver request submission. They have advantages in these cases in terms of speed, simplicity and lower costs, even though they do not consider all transport mechanisms present in the intestinal epithelia. However, *in vitro* permeability model should be able to predict correctly *in vivo* intestinal drug absorption. If such models should be used in research of new substances, there is a need to establish correlation between experimental and *in vivo* absorption of standard methods of permeability data quantification [2]. The possibility of method variation of published dynamic diffusion cell artificial membrane method for determination of permeability of active substances [3,4] was tested in this study.

MATERIALS AND METHODS

Spectrophotometric assay was validated [5] for determination of concentrations in acceptor and donor solution during permeability studies of one representative of active substance from each BCS class: caffeine, naproxen sodium, metformin hydrochloride and hydrochlorothiazide (analytical standards were used).

Apparent permeability coefficients were determined for these substances by dynamic diffusion cell artificial membrane method on absorption simulator Sartorius Model SM 16750 (Sartorius Membranfilter GmbH, Germany) following apparatus Instruction manual and the published study [3,4] with some modifications. Modifications pertained to the membrane for permeability studies and solutions for impregnation of the artificial membrane. In this study the artificial membrane was mixed cellulose ester membrane with pore size of 0.45 m (Sartorius AG, Göttingen, Germany). Corti et al. [3] in method optimization study used membrane of the same material, but pore sizes were 0.22 m (Millipore) and also 0.025 m (Millipore), which was used in further work [4].

In order to obtain lipophilic characteristics typical of biological membranes, the membrane filter was impregnated for 10 minutes in 2 mL of the solution for impregnation that consisted of 2,10% cholesterol and 1,70% of egg lecithin (Lipoid® E 80) (Impregnation solution A), also used in the study Corti et al. [3]. Another variation of the method was that in Impregnation solution B instead of egg lecithin, soy lecithin (Lipoid® S 75) was used, thus monitoring the influence of origin/composition of lecithin on drug permeation.

After the impregnation, the excess of impregnation solution was removed, and the percent of lipid solution that impregnated the membrane was calculated by weighing the membrane before and after the impregnation.

This membrane was fitted to the diffusion cell (effective area of 12 cm^2) connected to the donor and acceptor compartment (Figure 1). The donor solution was 500 mol/L solution of the active substance in 100 mL phosphate buffer pH 7.4 [6], while the acceptor solution consisted of 100 mL phosphate buffer pH 7.4 [6]. Both compartments were thermostated at 37 ± 0.5°C and circulated on both

sides of the membrane in the diffusion cell via peristaltic pump (Figure 1). The samples were taken in 30 minute intervals in period of 2 hours and concentrations determined directly on UV/VIS spectrophotometer UV 1601 (Shimadzu, Japan) at the appropriate wavelength for each substance (Table 1).

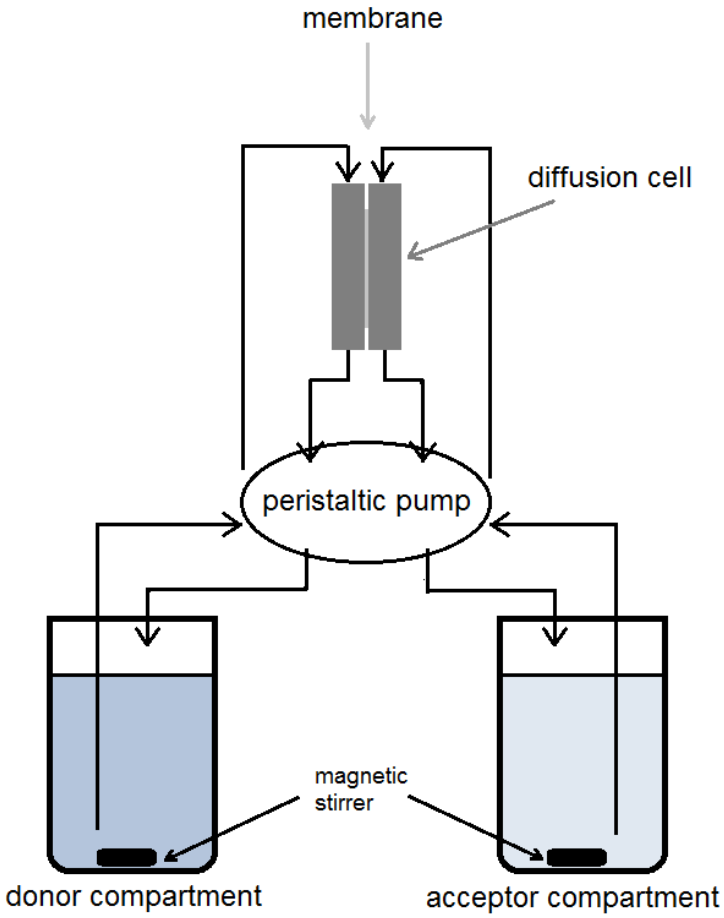

Figure 1. Absorption simulator Sartorius Model SM 16750

Table 1. Wavelengths of measurements

Active substance	Wavelength (nm)
Caffeine	273.5
Naproxen sodium	262.5
Metformin hydrochloride	233.0
Hydrochlorothiazide	272.0

The apparent permeability coefficient was determined using Equation (1):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0} \quad (1)$$

Where:
P_{app} – apparent permeability coefficient [cm/s]
dQ/dt – mass of substance permeated in time [mg/s]
A – effective area of artificial membrane [cm^2]

C_0 – concentration of substance in donor compartment at the beginning of the experiment [mg/mL].

At the end of each experiment the active substance concentration in the donor phase was determined, and mass balance calculated in order to determine the amount of the drug retained in the lipophilic membrane. Recovery of the drug was calculated using equation (2):

$$R\% = \frac{C_{a,end} \times V_a + C_{d,end} \times V_d}{C_{d,0} \times V_d} \times 100 \quad (2)$$

Where:
$C_{a,end}$ i $C_{d,end}$ – drug concentration measured at the end of experiment in acceptor and donor solutions, respectively [mg/mL],
$C_{d,0}$ – initial concentration in donor solution [mg/mL],
V_a i V_d – volumes of acceptor and donor solutions, respectively [mL].

Each determination was performed in triplicate and mean value with standard deviation were calculated.

RESULTS

Prior to the permeation experiments, the spectrophotometric assay was validated for each tested substance according to the ICH Guideline Q2-R1 [5]. The results of validation parameters are shown in Table 2.

Table 2. Validation parameters of spectrophotometric assay

Parameter		Substance			
		Caffeine	Naproxen sodium	Metformin hydrochloride	Hydrochlorothiazide
Linearity	Concentration range (μg/mL)	1.09 – 54.50	1.33 – 66.5	0.10 – 19.0	1.53 – 30.6
	Korrelation coefficient	0.9995	1.0000	0.9998	0.9998
	Limit of detection (μg/mL)	0.273	0.585	0.062	0.491
	Limit of quantification (μg/mL)	0.911	1.949	0.206	1.637
Precision/ Repeatability (RSD%)		0.394	0.269	0.246	0.183

The parameters shown in Table 2 comply with ICH Guideline on validation of analytical methods. However, interference (specificity) was tested by carrying out the permeation experiment with phosphate buffer pH 7.4 in both donor and acceptor solutions, and measuring the samples' absorbances at the wavelengths of interest listed in Table 1. These values were used to correct the absorbances measured in the subsequent permeation experiments of active substances.

Before each experiment, the membrane was weighted before and after the impregnation. After impregnation with Solution A, the membrane's mass increase was on average 156.96 ± 2.99%, while with Solution B it was 159.50 ± 2.82%.

The curves obtained by carrying out described method using either Impregnation solution A or B are shown in Figures 2 and 3, respectively, while the calculated apparent permeability coefficients are shown in Table 3.

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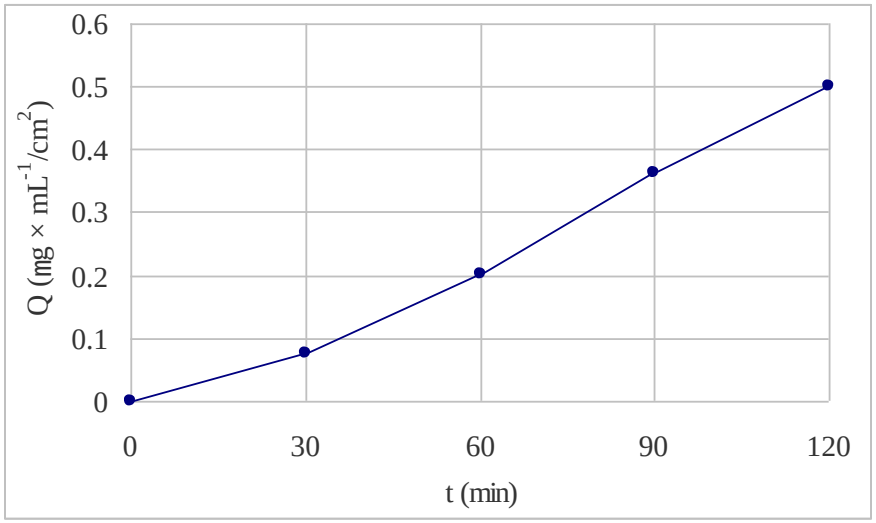

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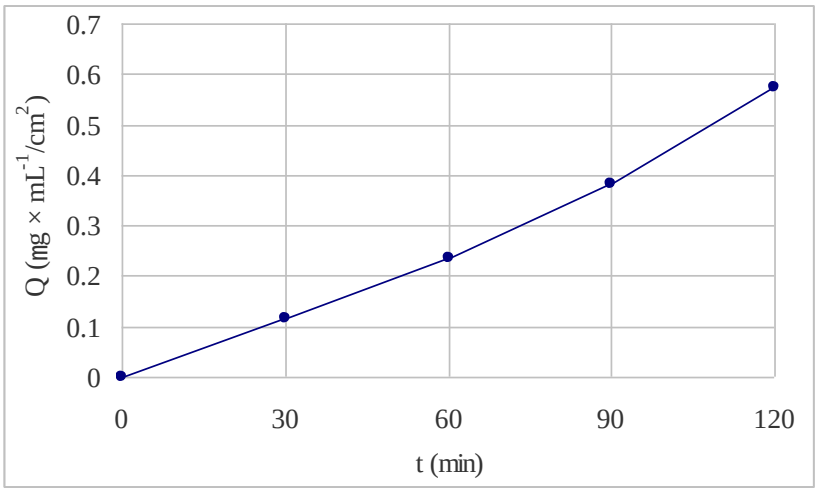

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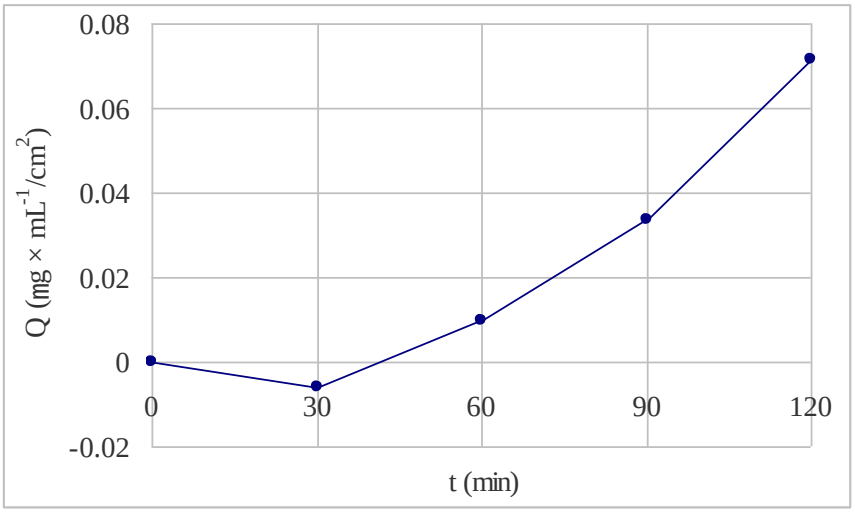

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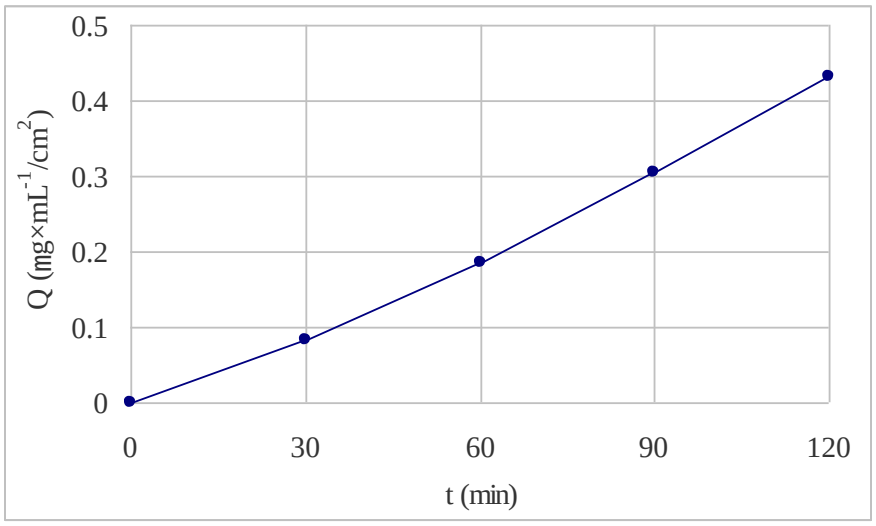
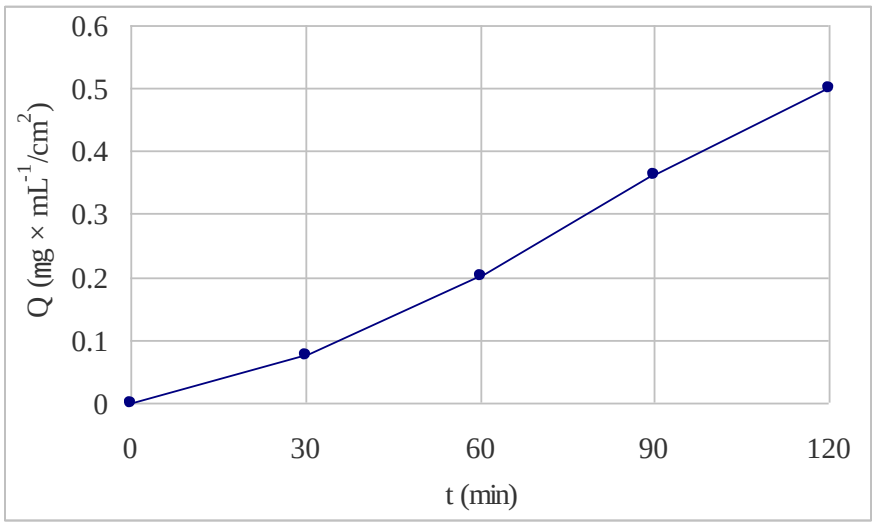


(d)

Figure 2. Mean permeability curves (n = 3) of caffeine (a), naproxen sodium (b), metformin hydrochloride (c) and hydrochlorothiazide (d) using Impregnation solution A



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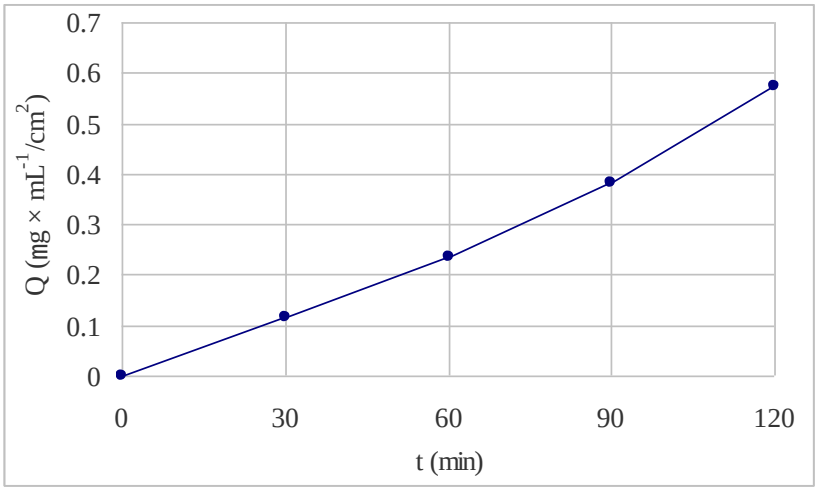

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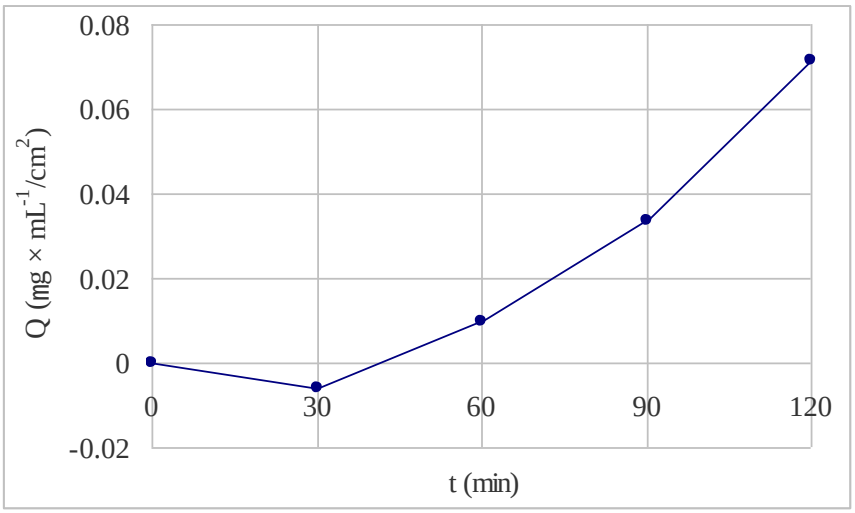

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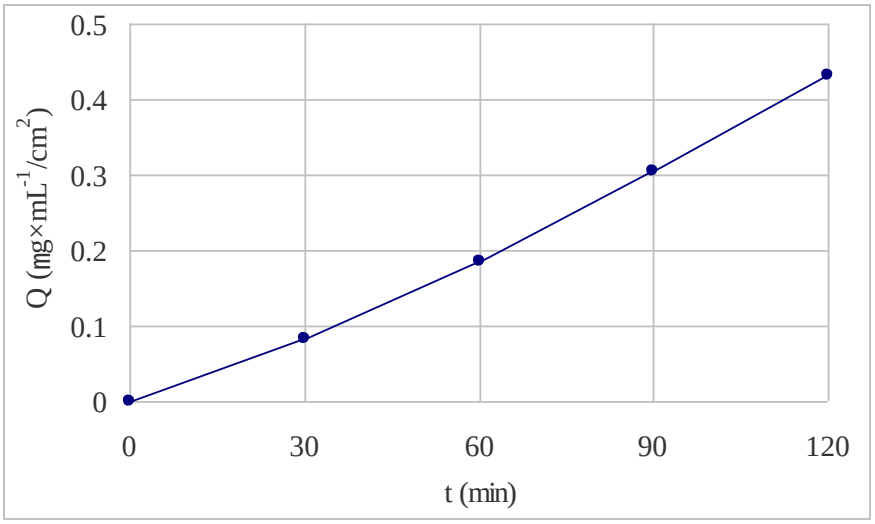

(d)

Figure 3. Mean permeability curves (n = 3) of caffeine (a), naproxen sodium (b), metformin hydrochloride (c) and hydrochlorothiazide (d) using Impregnation solution B

Table 3. Mean values (n = 3) of apparent permeability coefficients of active substances

Substance	Impregnation solution A P_{app} ± SD (× 10^{-5} cm/s)	Impregnation solution B P_{app} ± SD (× 10^{-5} cm/s)
Caffeine	7.33 ± 0.512	7.27 ± 0.510
Naproxen sodium	6.71 ± 0.319	6.20 ± 0.301
Metformin hydrochloride	0.86 ± 0.139	1.09 ± 0.194
Hydrochlorothiazide	4.01 ± 0.104	4.42 ± 0.138

After the permeability experiments, the percent of the recovered active substance was calculated (Equation (2)), and the results are shown in Table 4.

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Table 4. Mean values (n = 3) of the active substances recovered (R%) after the permeation experiments

Substance	Impregnation solution A R% ± SD (%)	Impregnation solution B R% ± SD (%)
Caffeine	91.88 ± 2.39	91.23 ± 0.31
Naproxen sodium	99.31 ± 1.20	91.60 ± 1.51
Metformin hydrochloride	100.85 ± 1.33	100.22 ± 2.26
Hydrochlorothiazide	93.45 ± 1.01	94.62 ± 1.36

The results of the apparent permeability coefficients of the tested substances obtained using either Impregnation solution A and B were correlated with each other (Figure 4).

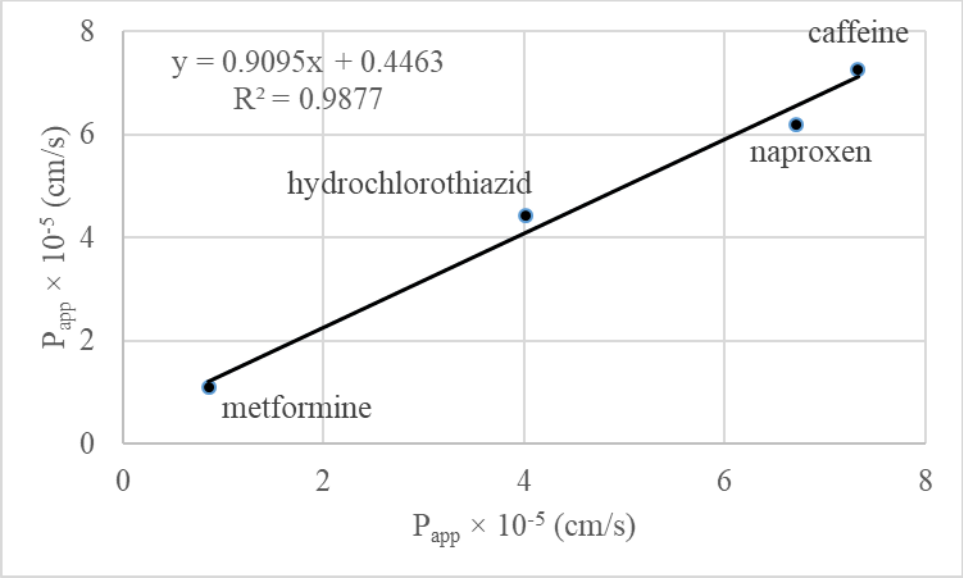

Figure 4. Correlation of the apparent permeability coefficients of the tested active substances through artificial membrane impregnated with Impregnation solution A versus those impregnated with Impregnation solution B

The results of the implemented method, as well as the other *in vitro* methods were correlated with the percent *in vivo* absorption in humans for the four tested substances (Table 5).

Table 5. Correlation coefficients (R^2) of *in vitro* methods with fractions absorbed in humans* for four tested substances

Method	R^2
Described method with Impregnation solution A	***0.9692***
Described method with Impregnation solution B	***0.9239***
PAMPA*	0.9144
Method described in Corti et al., 2006b*	0.9026
Caco-2*	0.8104
log $D_{7.4}$*	0.7539
log $P_{o/w}$*	0.4978

*Calculated based on data cited in Corti et al., 2006b

DISCUSSION

Drug solubility, as well as its dissolution rate from pharmaceutical dosage form are routinely tested *in vitro* [7-10], already at the discovery and characterization of novel active substances, and in the development of pharmaceutical forms or justification of the equivalence of generic with reference drugs.

On the other hand, taking into considerations all variables included in the permeation process through the physiological membranes, permeability assessment of active substances is officially primarily *in vivo* examination [8,10]. Other methods might be used only as supporting data for establishing bioequivalence and biowaiver requests. *In vivo* permeability studies on humans are exceptionally demanding and expensive with participation of a small number of respondents. The results of published *in vivo* perfusion are relatively rare, i.e. the number of the substances with such published results is less than one hundred [1,11,12]. These data for widely used active substances serve as sets for establishing suitability of the other methods in their developmental stages.

In vitro methods have several advantages in new drug candidate screening [2,13,14], as well as in the investigation of the influences of excipients on permeation of active substances [15-18]. These methods require less material resources and time and they are less labor intensive.

In parallel artificial membrane permeability assay (PAMPA) there is only one sampling. It is unknown if that one point, measured after certain time interval, is in linear part of the permeation curve or if it is already in the equilibrium part [19]. The curve goes through zero and that point, so that if the results were obtained in two time points, they don't necessarily have to give linear curve (e.g. substances with fast permeation rates). Thus, kinetics of permeation is not qualitatively monitored. Also, PAMPA demands commercial specialized equipment [19,20].

However, PAMPA method compared to the methods including tissues and cell monolayers is significantly shorter (results are obtained in one day), and it is carried out simultaneously on large number of replicate, thus obtaining statistically more confident results with lesser values of standard deviations and relative standard deviations [20].

Dynamic diffusion cell model with artificial membrane according to Corti et al. [3,4] has much less replicates compared to PAMPA model (3 vs. 48 in PAMPA). Every replicate is done individually and takes about two hours. The advantage of this method is that sink conditions are easier to obtain, since the volumes of donor and acceptor compartments are up to 100 mL, making it suitable for the substances with low solubility in water (PAMPA volumes are 10-200 □L [21]). Membrane area for permeation is larger (up to 40.8 cm^2), which is suitable for the substances with low permeability. These reasons are significant, since the assay is spectrophotometric. Sampling is multiple, so drug permeation kinetics could be monitored, since the apparent permeation coefficient is determined from the slope of the linear part of the curve, before reaching equilibrium state.

Validation of the spectrophotometric assay

The values of the validation parameters of UV-VIS spectrophotometric method are inside the required limits. However, there was some interference with components of the impregnation solution, that was pronounced in prolonged duration of method and at lower wavelengths. This represents a problem when testing the substances with low permeability whose concentration is monitored at lower wavelengths, such is metformin in this case (λ_{max} = 233 nm). The solution to this problem, however, is found in the subtraction of the interference values at the same wavelengths measured at certain time intervals from the measured sample absorbance during permeability experiments.

Permeation of the active substances through the artificial membrane

Filter impregnation was performed according to the instruction manual of the apparatus (Sartorius) and the literature accounts [3,4]. According to the instruction manual, after impregnation, the membrane mass should be 100 ± 5% of the dry membrane. After impregnation with either Solution A or B, the mass was 157-159.5%, which is at the same level as the results of Corti et al. [3]. This increase of mass is probably due to the membrane filter structure, which is not originally intended for this type of experiments, and

the original one is not produced any more. In the preliminary experiments, it was tried to remove the excess impregnation solution by applying increased pressure and longer exertion of pressure. However, this did not result in lower percentages of the mass increase. On the other hand, it influenced the inner structure of the filter (compaction of three-dimensional membrane filter), and in this instance resulted in lower permeation of the tested substances.

Corti et al. [3] described the optimization of conditions for the permeation experiments by dynamic diffusion cells with artificial membrane method using naproxen as the active substance. They optimized the method to give the most similar result to the one obtained by Caco-2 cell monolayers (4.88×10^{-5} cm/s). The chosen membrane filter was of mixed cellulose esters with pore size of 0.025 μm (Millipore) with the apparent permeability coefficient of naproxen of $5.59 \pm 0.24 \times 10^{-5}$ cm/s [4]. Filters of similar material that were rejected [3] were:

1. Mixed cellulose ester filter with 0.22 μm pores (Millipore) $P_{app} = 9.53 \pm 0.24 \times 10^{-5}$ cm/s;
2. Cellulose acetate filter with 0.2 μm pores (Sartorius) - $P_{app} = 4.09 \pm 0.68 \times 10^{-5}$ cm/s;
3. Cellulose nitrate filter with 0.2 μm pores (Whatman) - $P_{app} = 6.73 \pm 0.51 \times 10^{-5}$ cm/s;
4. Cellulose nitrate filter with 0.1 μm pores (Millipore) - $P_{app} = 5.08 \pm 2.20 \times 10^{-5}$ cm/s.

It is evident by comparison of two filters of the same material, but different pore sizes, that the apparent permeability coefficient does not increase or decrease proportionally to the pore size. The authors explain this due to the differences in porosity, and their results imply that with higher porosity the filter absorbs more impregnation solution, leading to the slower permeation of the substance through the membrane, i.e. lower apparent permeability coefficient [3]. It needs to be taken into consideration that in proving the method suitability, the authors listed that the apparent permeability coefficient of naproxen was 4.88×10^{-5} cm/s [4].

The results obtained using mixed cellulose ester filter with pore size of 0.45 mm were $6.71 \pm 0.319 \times 10^{-5}$ cm/s with impregnation with Solution A, and $6.20 \pm 0.301 \times 10^{-5}$ cm/s with impregnation with Solution B (Table 3). According to Corti et al. [3] the mass increase of the filters after impregnation here listed under 1, 2 and 3 (161.4 ± 0.1%, 160.5 ± 0.5%, and 158.8 ± 0.3%, respectively) are very similar to those obtained in this study (156.96 ± 2.99% for Impregnation solution A, and 159.50 ± 2.82% for Impregnation solution B). This would imply that the similar amounts of impregnation solutions were absorbed by membrane.

Based on these results and comparing the obtained apparent permeability coefficients of naproxen with the results of Corti et al. [3], it can be concluded that the pore size of filter, as supporting medium, does not influence permeability of the active substances through the artificial membrane.

However, the difference is evident in the percentage of the recovered substance, which is 99.5% - 98.7% for all tested substances, according to Corti et al. [3]. The results of this study indicate that the percentage of the recovered naproxen was 99.31% when using Impregnation solution A (Table 4), which is in agreement with the literature account, while with the Impregnation solution B only 91.60% naproxen was recovered. This difference in the amount of the substance retained by the membrane in this case could be explained by higher affinity of naproxen for the soy phospholipids, and consequently lower apparent permeation coefficient.

For the other tested substances, the recovered values were similar regardless of the impregnation solution used. Metformin hydrochloride is the only substance that did not show any affinity for the artificial membrane.

From the comparison of the permeation curves of the substances can be seen that they are in the part of the curve that is relevant for the determination of the apparent permeation coefficient, i.e. that equilibrium conditions were not reached during the experiments. The curves were relatively straight lines. After 30 minutes concentrations of metformin hydrochloride were negative values, which are actually the consequence of small variations in the interferences of the Impregnation solution released into the medium, and, on the other side, metformin's varying and low concentration in the beginning stages of the experiment. This is an analytical problem, since metformin absorbs in lower wavelength range, and, since it is a substance with low permeability, low concentrations were determined, therewithal the impregnation solution has the highest absorption in the

same range. Negative values (even though small) of metformin concentrations are the consequence of the calculation processing of the raw data, i.e. subtraction of the absorbance of medium from the absorbance of the sample.

For the substances with high permeability (caffeine and naproxen), the apparent permeability coefficients were somewhat higher with Impregnation solution A (egg lecithin). Conversely, for the substances with lower permeability the apparent permeability coefficients were higher with Impregnation solution B (soy lecithin). Only naproxen had significantly different percentage of the recovered substance, being lower with Impregnation solution A, compared to B.

Correlation coefficient of the results obtained with these different impregnation solutions was R^2 = 0.9877, with the slope of 0.9095, and x-axis intercept of 0.4463 (Figure 4). this suggests relatively good correlation. However, there are differences with regard to the solution used to impregnate the artificial membrane, particularly origin / composition of the lecithin used.

Lower corelation coefficients were obtained when the obtained permeation coefficients were correlated with the results cited in Corti et al. [4]. Thus, correlation of results of Corti et al. [4] and practically repeated method with only filter pore size variation, the correlation coefficient was R^2 = 0.8558, while when using soy lecithin instead of egg lecithin in the impregnation solution, correlation coefficient was R^2 = 0.7768.

The results of the apparent permeation coefficients were correlated with the fraction of the active substance absorbed in humans [4] for the four substances (Table 5). Higher correlation coefficient was obtained when the membrane was impregnated with Solution A (R^2 = 0.9692), then with Solution B (R^2 = 0.9239). The correlation coefficients of the other *in vitro* methods for the four substances are decreasing in the order PAMPA > method of Corti et al. [4] > Caco-2 > log $D_{7.4}$ > log $P_{u/v,}$ as can be seen in Table 5.

It is important to mention that, when comparing the other (*in vitro* and *in vivo*) methods, the permeability coefficients are generally logarithmically related to the fraction absorbed in humans (incuding the relationship of the effective permeation coefficient in humans). It is usually rank order relationship, i.e. it is usefull for ranking of substances as those of „low“ or „high“ permeability, with the range of permeability coefficients for the substances with high permeability being very broad, and, at the same time, very narrow for those with low permeability. In the method of Corti et al. [4], as well as in this study, there is a linear relationship when correlating that shows sharper differences in permeability of active substances.

Effective and apparent permeability coefficients obtained with different methods are different. It could be concluded that their absolute values do not say much, compared to their values within each individual method. In that case, the permeability coefficient of the substance of unknown permeability determined by a particular method, can be ranked as „low“, „middle“ or „high“ permeability substance, when compared to the substances of known permeability determined by the same method (in method suitability validation step).

CONCLUSIONS

The main goal of this study was to investigate the possibility of application of the dynamic model of diffusion cells with artificial membrane according to Corti et al. [4] while applying the supporting medium (filter) of larger pores, and varying the lecithin used for the impregnation solution. Thus, permeability of one of each BCS class substance was determined.

Based on the comparison of the permeation coefficients for naproxen presented here with those of Corti et al. [4], the filter pore size was not the factor determining the permeation through the artificial membrane. Increase in filter mass after impregnation was 157-159.5%, which is in accordance with the results of Corti et al. [4]. Affinity for the artificial membrane of caffeine, metformin and hydrochlorothiazide was not dependent on lecithin, which was the case with naproxen. Determined apparent permeability coefficients are of the order of those obtained with the other *in vitro* methods.

Correlation of the results obtained with permeability determination using the two impregnation solutions indicates there are the differences with regard to the solution, i.e. the composition/origin of the lecithin. Correlation coefficients of the apparent permeability coefficients obtained with this method with the fraction absorbed in humans were higher compared to the other *in vitro* methods.

Based on the examination of varying method conditions of dynamic model of diffusion cells with artificial membrane, further assessment of method suitability has grounds with the aim of method validation that could be used for permeability screening purposes of new substances intended to be incorporated in solid oral dosage forms.

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