AAPS PharmSciTech (© 2018)
DOI: 10.1208/s12249-018-0965-8

Research Article

Artificial Lipid Membrane Permeability Method for Predicting Intestinal Drug Transport: Probing the Determining Step in the Oral Absorption of Sulfadiazine; Influence of the Formation of Binary and Ternary Complexes with Cyclodextrins

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Received 10 November 2017; accepted 24 January 2018

Abstract. We propose an *in vitro* permeability assay by using a modified lipid membrane to predict the *in vivo* intestinal passive permeability of drugs. Two conditions were tested, one with a gradient pH (pH 5.5 donor/pH 7.4 receptor) and the other with an iso-pH 7.4. The predictability of the method was established by correlating the obtained apparent intestinal permeability coefficients (P_{app}) and the oral dose fraction absorbed in humans (f_a) of 16 drugs with different absorption properties. The P_{app} values correlated well with the absorption rates under the two conditions, and the method showed high predictability and good reproducibility. On the other hand, with this method, we successfully predicted the transport characteristics of oral sulfadiazine (SDZ). Also, the tradeoff between the increase in the solubility of SDZ by its complex formation with cyclodextrins and/or aminoacids and its oral permeability was assessed. Results suggest that SDZ is transported through the gastrointestinal epithelium by passive diffusion in a pH-dependent manner. These results support the classification of SDZ as a high/low borderline permeability compound and are in agreement with the Biopharmaceutics Classification Systems (BCS). This conclusion is consistent with the *in vivo* pharmacokinetic properties of SDZ.

KEY WORDS: sulfadiazine; β-cyclodextrin; aminoacids; binary and ternary complexes; *in vitro* permeability method; intestinal absorption of sulfadiazine.

INTRODUCTION

Lipophilicity and permeability are two of the key physicochemical properties of new compounds determined during early stages of drug discovery. Traditionally, lipophilicity is expressed as the logarithm of the partition coefficient of a compound between *n*-octanol and water (log $K_{o/w}$) or between *n*-octanol and an aqueous buffer solution. The coefficient determines that a compound prefers interactions with other organic molecules to hydrogen bonding and dipole interactions with water. The most common pathway for drug absorption is passive transcellular diffusion. Currently, it is estimated that over 80% of orally administered drugs are absorbed *via* a passive absorption mechanism at the level of epithelial mucosae [1].

The parallel artificial membrane permeability assay (PAMPA) is a technique used in early screening during drug discovery to study the passive diffusion transport of drugs through epithelial tissues. PAMPA was first introduced by Kansy *et al.* as an *in vitro* transcellular passive permeation method to predict drug gastrointestinal absorption [2]. The proposed technique involves the use of microfilters impregnated with a lecithin solution in *n*-dodecane.

Since the PAMPA method was introduced by Kansy *et al.*, several modifications in membrane compositions have been proposed to make this technique able to predict passive permeability of drugs through the intestinal membrane [3–7], the blood-brain barrier [8, 9], or the human dermal layer [10]. Although the PAMPA technique has the disadvantage of predicting only the passive transport of drugs due to the lack of transport proteins, which makes it impossible to evaluate actively transported drugs and drugs that interact with efflux proteins [11], this method is a widely used tool for passive permeability screening in the early phase of discovery in the pharmaceutical industry.

The PAMPA assays are performed in a 96-well filter plate impregnated with a liquid artificial membrane that separates two compartments, a donor spiked with a test analyte and a receptor that contains an aqueous acceptor solution. In this system, a filter support such as polyvinylidene difluoride (PVDF), polytetrafluorethylene (PTFE), polycarbonate, or cellulose ester is coated with

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Published online: 15 February 2018

natural phospholipids dissolved in an organic solvent (usually *n*-dodecane) [12].

Therefore, one of the main objectives of this work was the optimization of the PAMPA setup, in particular its barrier properties, to improve the prediction accuracy of the absorption potential of drugs and formulations. To achieve this purpose, the *n*-dodecane solution was replaced by an *n*-octanol solution of phospholipids (Lipoid 75) to assess the influence of the organic solvent on the barrier properties of the artificial lipid membrane. The structural characteristics of *n*-octanol (i.e., lipophilic carbon chains and hydrophilic hydroxyl groups), its ability to form hydrogen bonds and take up water molecules, and its solubility parameter render *n*-octanol properties that are very close to those of the phospholipid-based biological membranes. For this reason, *n*-octanol is used in high-throughput screenings of lipophilicity [13].

Based on this concept, a biomimetic artificial membrane (BAM) was prepared by dissolving Lipoid 75 in *n*-octanol. Note that it is critical to obtain a suitable BAM simulating the behavior of the natural gastrointestinal membrane, since the availability of reliable high-throughput screening methods is required for the rapid assessment and prediction of the biopharmaceutical properties of drugs for the early identification of potential bioavailability problems.

On the other hand, the experimental limitations of the classical PAMPA assays, where stirring is neglected or nonexistent, include the influence of the aqueous boundary layer (ABL) adjacent to the membrane surface, called the unstirred water layer (UWL), which results from inefficient stirring during the permeability assay. This hydrodynamic barrier might lead to the underestimation of the intrinsic permeability for hydrophobic drugs, resulting in poor correlations between the absorbed fraction (f_a) and the apparent permeability (P_{app}). Therefore, in this work, the experiments were carried out in Franz horizontal diffusion cells with magnetic stirring in both chambers to minimize the ABL effect on the drug transport through the lipid membrane and to overcome the negative effects of the UWL of the classical PAMPA system [14].

Cyclodextrins (CD) are natural cyclic oligosaccharides that have a chemical structure associated with a high molecular weight (from 972 to 2000 Da) and a low octanol/water partition coefficient (log $P_{o/w}$ from −3 to 0) which predicts that CD will not easily penetrate the artificial membrane [15]. The CD complexation of a poorly soluble lipophile can improve its aqueous solubility, but the complex itself is, in general, unable to permeate biological membranes *per se*. Consequently, CD can both enhance and hamper drug permeation through biological membranes [16].

Some investigations carried out in our laboratory have shown the feasibility of complex formation between sulfadiazine (SDZ), CD, and/or aminoacids (AA) and have demonstrated that complexation improved the aqueous solubility of the drug [17–19]. On the basis of the previous considerations, another important objective of this study was to examine the apparent permeability (P_{app}) of SDZ and its complexes using the developed BAM in Franz diffusion cells, as well as to investigate the potential advantages of the combined use of βCD and AA, which were chosen because they have demonstrated a synergistic effect on the solubilizing properties of the βCD on the SDZ's solubility [17–19] to better exploit their favorable carrier properties and evaluate their possible synergistic effects on the absorption behavior of SDZ.

MATERIALS AND METHODS

Materials

SDZ was obtained from Parafarm (Buenos Aires, Argentina); βCD was a gift from Ferromet S.A. (agent of Roquette in Buenos Aires, Argentina); L-Leucine (LEU) and L-Arginine (ARG) were purchased from SIGMA-ALDRICH (Saint Louis, USA); L-Aspartic Acid (ASP) and L (+) - Glutamic Acid (GLU) were obtained from Anedra (Buenos Aires, Argentina) and Glycine (GLY) from Todo Droga (Córdoba, Argentina); Lipoid 75 was a gift from DROMEX S.A. Argentina (Buenos Aires, Argentina).

Theophylline, amoxicillin, carbamazepine, and caffeine were purchased from Todo Droga (Córdoba, Argentina); propranolol, verapamil, ketoprofen, chloramphenicol, atenolol, furosemide, norfloxacin, hydrochlorothiazide, and antipyrine were supplied by Parafarm (Buenos Aires, Argentina); allopurinol was obtained from Unifarma (Buenos Aires, Argentina); metoprolol was purchased from SIGMA-ALDRICH (Saint Louis, USA) and acyclovir from ELEA (Buenos Aires, Argentina).

Apparatus

The apparatuses used in this work were a Cary 60 UV–VIS spectrophotometer (Agilent, Santa Clara, CA, USA); a MM6 multi-position magnetic stirrer (Auto Science, Channahon, IL, USA); a LC-30H Ultrasonic bath (Elma, Germany); an OHAUS analytical balance (0.0001 g), (Parsippany, NJ, USA); in-house side-by-side diffusion chambers (Figmay, Córdoba, Argentina); a circulation pump; and a HAAKE F3 Water bath (Eindhoven, Netherlands).

Methods

Preparation of the Biomimetic Artificial Membrane

The BAM was obtained by impregnating a cellulose ester support (GS MS membrane, 0.22 μm pore size, 25 mm of diameter, Millipore® Billerica, MA, USA) with 10% (*w*/*v*) an *n*-octanol solution containing a mixture of Lipoid 75 (fat free soybean lecithin with 70% phosphatidylcholine) (Table I).

Table I. Composition of Lipoid 75

Phospholipids	Composition (g/100 g)
Phosphatidylcholine	68.0–73.0
Phosphatidylethanolamine	7.0–10.0
Lysophosphatidylcholine	< 3

Permeability Studies

The permeability experiments were conducted using in-house-built side-by-side diffusion cells with donor and receptor compartments separated by the BAM (Fig. 1). Both the donor and the receptor chambers were agitated by magnetic stirring (Fig. 1). The BAM was previously equilibrated in its respective buffer solution and mounted between the half-cells of the receptor and donor compartments. Transport studies were performed in two set of experiments. In the first step, the donor and receptor chambers contained 2.2 ml of a pH 7.4 phosphate-buffered saline (PBS) solution. In other sets of experiments, the donor and receptor compartments had different pH values: the donor contained a pH 5.5 buffer solution and the receiver was filled with a pH 7.4 buffer solution, respectively. The temperature for all the experiments was maintained at 37 °C by circulating water from a thermostatic bath.

A rank order relationship was established between the apparent permeability coefficient values (P_{app}) obtained with our *in vitro* permeability technique, and the extent of intestinal absorption in humans (f_a) by using a set of 16 structural diverse drugs to assess the functional suitability and permeability of the assay of our proposed permeability method for the prediction of human drug absorption.

The effectiveness of CD complexes in improving the permeation of SDZ was investigated. For this purpose, the proposed permeability assays were carried out by placing in the donor compartment either SDZ (0.4 mg /ml), binary complexes with equivalent amounts of 0.4 mg/ml of SDZ (SDZ:βCD, SDZ:MβCD, and SDZ:HPβCD, molar relation 1:1) or the ternary complexes SDZ:βCD:AA (1:1:1) (Delrivo *et al.*, 2016) with equivalent amounts of 0.4 mg/ml of SDZ. Also, aliquots of 1-ml samples were withdrawn from the receptor compartment at established intervals, and the concentration of the solution was determined by a UV–Vis spectrophotometer. These aliquots were replaced with same volume of PBS at 37 °C. The cumulative amount of drug permeated per unit of area (exposed surface of the artificial membrane, 0.502 cm^2) (mg/cm^2) was plotted as a function of time (s). Then, the apparent coefficient permeability (P_{app}) was calculated using the following equation based on the Fick's Law (Eq. 1):

$$P_{app} = dQ/dt \times 1/ACo \quad (1)$$

where P_{app} is the apparent permeability coefficient ($cm.s^{-1}$), dQ/dt is the amount of drug permeated per unit of time (mgs^{-1}), A is the area (cm^2), and Co ($mg.ml^{-1}$) is the initial drug concentration in the donor compartment. Each experiment was repeated at least three times, and the results reported were the mean values.

At the end of each experiment, the amount of drug retained by the BAM was determined according to Eq. 2:

$$\%R = \left(C_{r,end}Vr + C_{d,end}.Vd\right)/(Co.Vd) \quad (2)$$

where $C_{r,end}$ and $C_{d,end}$ are the drug concentration measured in the receptor and donor compartment, respectively, at the end of the assay; Co is the initial concentration; and Vr and Vd are the volumes of the receptor and donor compartment, respectively.

The estimated fraction dose of SDZ and its CD complexes absorbed in humans (f_a) was assessed by calculating the P_{app} coefficients obtained with the proposed *in vitro* permeability model [20] as follows:

$$Fa = (1–\exp(a \times P)) \times 100 \quad (3)$$

where a is a specific constant of this correlation.

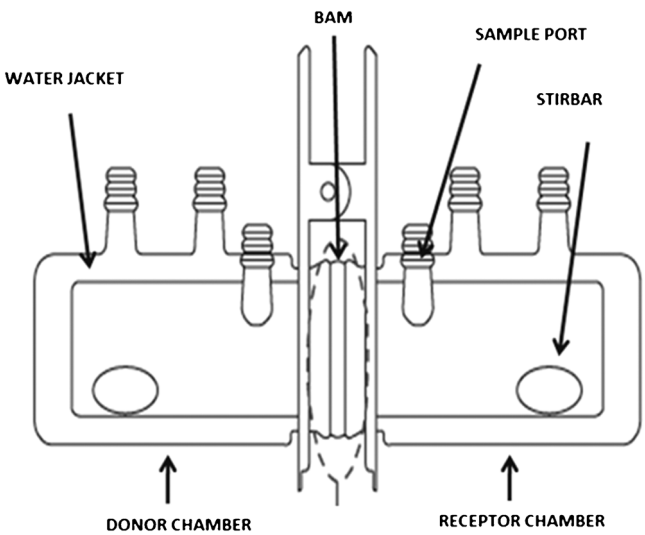

Fig. 1. In-house horizontal diffusion chambers

RESULTS

In Vitro Apparent Intestinal Permeability of the Validation Set

The P_{app} coefficient values of the 16 validated drugs determined by the modified *in vitro* permeability model were calculated to demonstrate the suitability of the proposed permeability assay for BCS permeability classification according to the BCS/FDA biowaiver guidance [21].

The validation set of known human absorption values represents a range of low (e.g. < 50%), moderate (e.g. 50–84%), and high (≥ 85%) absorption. Some of these were selected from the list of drugs approved by the FDA (e.g. antipyrine, caffeine, carbamazepine, ketoprofen, metoprolol, naproxen, propranolol, theophylline, verapamil, amoxicillin, atenolol, furosemide, and hydrochlorothiazide) and the others were chosen taking into account the information available on mechanism of absorption and reliable estimates of the extent of drug absorption in humans. Also, all compounds covered a broad range of lipophilicity.

The maximum dose strength, pKa, dose absorbed fraction (f_a), the P_{app} obtained under both pH-donor conditions (5.5 and 7.4), and $\%R$ are summarized in Table II. The accumulated amount transported *versus* time plots obtained from the validation set of drugs in the modified PAMPA assay were lineal. The P_{app} values obtained under a pH condition of 5.5 in the donor compartment and a pH condition of 7.4 in the receiver compartment ranged from

Table II. Physicochemical and Biopharmaceutical Properties of the Validation Set Compounds

Compound	[a]Max. dose strength (g)	[b]pKa	[c]Log D (7.4)	[d]Fraction dose absorbed in humans (f_a)	Apparent permeability coefficient (P_{app}) $\times 10^{-5}$ cm/s		Amount of drug retained by the BAM (%R)	
					pH values of 5.5 donor/7.4 receptor	pH values of 7.4 donor/7.4 receptor	pH values of 5.5 donor/7.4 receptor	pH values of 7.4 donor/7.4 receptor
Acyclovir	800	2.3, 9.2	−1.76	0.21	0.16 ± 0.04	0.18 ± 0.02	103 ± 4	78 ± 9
Allopurinol	300	10.2	−0.55	0.70	0.76 ± 0.08	0.9 ± 0.1	91 ± 3	97 ± 9
Amoxicillin	875	2.4, 9.6, 7.4	−1.52	0.93	0.10 ± 0.01	1.1 ± 0.3	67 ± 5	82 ± 8
Antipyrine	500	0.65	0.202	1.00	4.4 ± 0.1	3.0 ± 0.2	95 ± 7	102 ± 11
Atenolol	100	9.6	−1.03	0.52	0.33 ± 0.02	0.40 ± 0.03	99.7 ± 0.4	86 ± 3
Caffeine	65	10.4	−0.07	1.00	1.78 ± 0.06	1.6 ± 0.3	N.D.	89 ± 2
Carbamazepine	300	9.3	0.25	1.00	1.6 ± 0.1	1.7 ± 0.3	94 ± 8	N.D.
Chloramphenicol	125	11.0	1.00	0.90	3.4 ± 0.4	2.9 ± 0.5	99 ± 1	98 ± 13
Furosemide	80	3.9	−1.54	0.60	2.7 ± 0.4	0.68 ± 0.02	96 ± 5	85 ± 5
Hydrochlorothiazide	50	7.92	−0.07	0.70	1.5 ± 0.5	1.0 ± 0.2	92 ± 7	81 ± 2
Ketoprofen	75	4.8	−0.01	1.00	7.2 ± 0.2	1.9 ± 0.2	90 ± 12	110 ± 17
Metoprolol	100	9.7	0.16	0.95	1.6 ± 0.1	3.1 ± 0.5	102 ± 4	106 ± 8
Norfloxacin	800	8.7, 4.4	−2.00	0.35	0.53 ± 0.03	0.16 ± 0.03	N.D.	N.D.
Propranolol	80	9.45	1.72	0.93	0.72 ± 0.03	1.1 ± 0.2	82 ± 1	70 ± 4
Theophylline	600	8.4	−0.02	0.97	2.7 ± 0.6	2.79 ± 0.08	93 ± 8	103 ± 4
Verapamil	120	8.92	1.81	0.98	0.54 ± 0.09	2.9 ± 0.2	105 ± 3	94 ± 19

N.D. not determined
[a] Values obtained from Kim JS *et al.* [22]; Varma *et al.* [23]
[b] Values obtained from Kotecha *et al.* [24]; Sjögren *et al.* [5]; Bicker *et al.* [9]
[c] Values obtained from Larregieu and Benet [25]
[d] f_a values obtained from Sugano *et al.* [26] and Corti *et al.* [27]; Tam *et al.* [28]

0.10×10^{-5} to 7.2×10^{-5} cm/s, with amoxicillin showing the least P_{app} value and ketoprofen the maximum permeability, while the P_{app} coefficient values under a pH condition of 7.4 in the donor compartment and a pH condition of 7.4 in the receiver compartment ranged from 0.18×10^{-5} to 3.0×10^{-5} cm/s for acyclovir and antipyrine, respectively (Table II). The correlation curve between the human f_a values and the P_{app} coefficients was generated by fitting Eq. 3 to the correlation data shown in Fig. 2.

The best correlation between the P_{app} coefficient values and human f_a values was observed when the pH of the donor buffer solution was 7.4 ($R^2 = 0.96$). The regression fit between the P_{app} values and human f_a values suggested that f_a can be predicted from Eq. 3, with a correlation scalar "a value" of −168,541 ± 12,274. In contrast, a poor correlation was obtained when the pH of the donor medium was 5.5 ($R^2 = 0.77$). A similar a value (a value = − 163,909 ± 22,324) with improved fit to the data ($R^2 = 0.88$) was obtained when amoxicillin, furosemide, and verapamil were excluded from the plot (Fig. 2a, b).

Figure 3a, b shows the correlation between the estimated f_a values by applying Eq.3 and the f_a values of the tested set of compounds obtained from the literature under both pH conditions in the donor solution. The best correlation was obtained under a pH condition of 7.4 in the donor solution and a pH condition of 7.4 in the receptor solution, yielding an R^2 of 0.92, which might indicate a good ability of the permeability model to predict the oral absorption of compounds with different permeability characteristics. The graph in Fig. 3b shows that all data points fall within the limits of 95% confidence/prediction intervals (pH 7.4 donor/pH 7.4 receptor), while the graph in Fig. 3a (pH 5.5 donor/pH 7.4 receptor) shows a poor correlation ($R^2 = 0.77$) and three compounds (amoxicillin, furosemide, and verapamil) that fall out of the range of 95% confidence/prediction intervals.

Because the best correlation was obtained with the experimental setting at a pH value of 7.4 in both donor and receptor solutions, we determined the P_{app} of three compounds (acetylsalicylic acid, piroxicam, and trimethoprim) using our proposed permeability model to validate the absorption predictive capacity of our system. Then, these values were input into Eq. 3. As seen in Table III, the estimated f_a values from Eq. 3 were close to those reported from the literature.

The correlation curve analysis of the 16 data set, for which human f_a values were available, and the P_{app} coefficient values were obtained under a pH condition of 5.5 in the donor solution and a pH condition of 7.4 in the receiver solution (excluding the training compounds amoxicillin, furosemide, and verapamil) and under a pH condition of 7.4 in both donor and receptor solutions, suggested a P_{app} cutoff of 1.16×10^{-5} cm/s and 1.13×10^{-5} cm/s, respectively, which could be used to predict the fraction dose absorbed in humans which should be equal to or greater than 0.85.

The recovery of only two tested validation drugs was less than 80% using the pH 7.4 donor condition (Table II). One drug (propranolol) was a BCS high-permeability drug (class I), with a mass balance of 70% and the other (acyclovir) was a BCS low-permeability drug (class III), with a mass balance of 78%, which could indicate that the intrinsic permeability values for these drugs can be underestimated. However, only

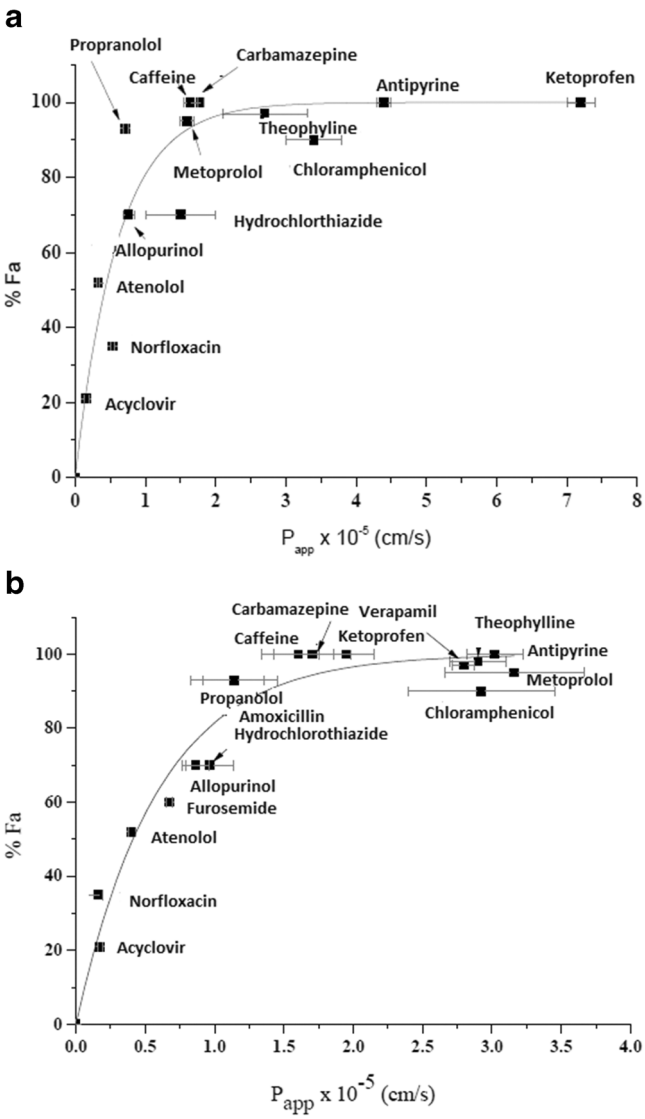

Fig. 2. Method suitability from P_{app} values of a 16-validation set across the *in vitro* permeability assay *versus* the extent of human intestinal absorption (f_a) under pH 5.5 donor (**a**) and pH 7.4 donor (**b**) conditions

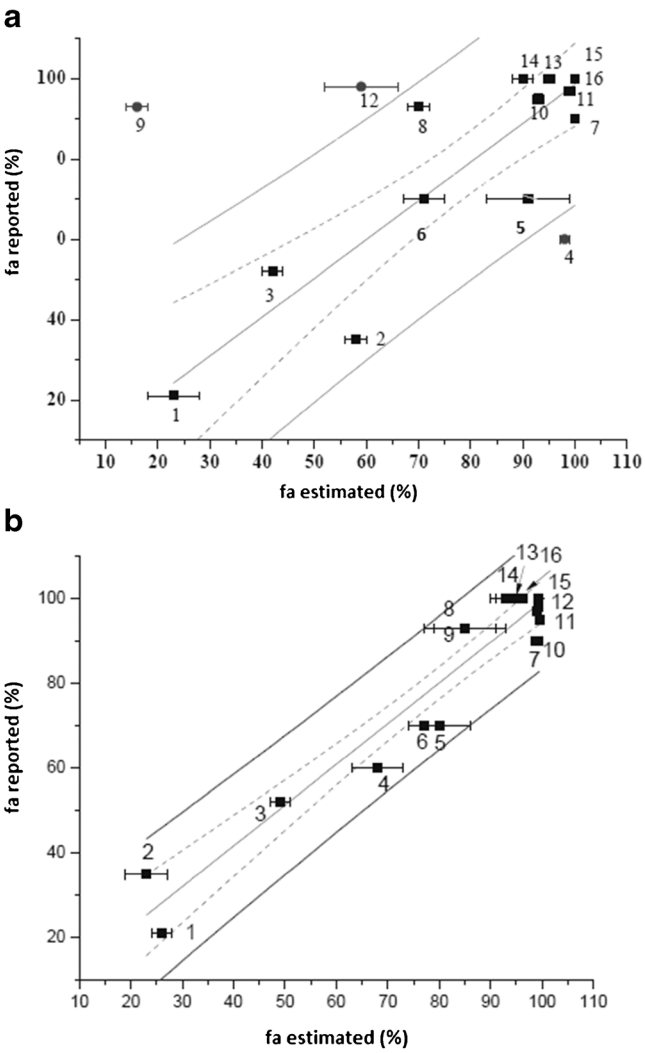

Fig. 3. Correlation between known and predicted human fraction dose absorbed in humans (based on Eq.3) for the validation set; **a** pH 5.5donor, **b** pH 7.4 donor. 1, acyclovir; 2, norfloxacin; 3, atenolol; 4, furosemide; 5, hydrochlorothiazide; 6, allopurinol; 7, chloramphenicol; 8, propranolol; 9, amoxicillin; 10, metoprolol; 11, theophylline; 12, verapamil; 13, carbamazepine; 14, caffeine; 15, antipyrine; and 16, ketoprofen

one drug (amoxicillin, a BCS class compound III with poor permeability) yielded a recovery lower than 80% under pH 5.5 donor/pH 7.4 receptor conditions. On another hand, this parameter for norfloxacin and carbamazepine could not be determined because these drugs were like suspensions in the donor chamber.

It is important to highlight that the *in vitro* permeability of metoprolol was pH-dependent, with the highest P_{app} coefficient value being under pH 7.4 donor condition (Fig. 3b, Table II).

Correlation Between *In Vitro* Apparent Intestinal Permeability (Log P_{app}) *Versus* Coefficient of Distribution n-Octanol/Buffer pH 7.4 (Log *D* (7.4) of the 16 Compounds

Due to the lipophilicity of a molecule is the main physicochemical property determining the permeation of substances through cell phospholipid membranes, in order to assess limitations of the proposed setup of our *in vitro* permeability assay for the prediction of *in vivo* membrane permeability and evaluate properties of applied lipid membrane mimicking cell membranes, we determined the relationship between logarithm of the apparent permeability coefficient (P_{app}) values, by applying the *in vitro* permeability

Table III. Apparent Permeability Coefficients Obtained with the *In Vitro* Permeability Method Proposed and Fractions of Dose Absorbed in Humans or Predicted by Applying Eq.3

Compound	$P_{app} \times 10^{-5}$ ± DS cm/s	% f_a reported in human	% f_a predicted
Acetylsalicylic acid	1.5 ± 0.5	0.98	0.90 ± 0.07
Trimethoprim	2.89 ± 0.08	0.98	0.992 ± 0.001
Piroxicam	3.0 ± 0.3	1.0	0.996 ± 0.004

assays proposed herein, carried out at the setup of 7.4/7.4 pH donor-receptor compartments, respectively, and logarithm of distribution coefficients in n-octanol/buffer pH 7.4 (Table II) of the 16 tested compounds. It was found a good linear correlation between log P_{app} and log D (7.4) values (Fig. 4). Although, cationic drugs (propranolol and verapamil) showed higher log P_{app} (pH 7.4) values than non-charged or anionic compounds with a similar log D (7.4), indicating their higher affinity to the PAMPA-lipid solution as compared to octanol (log P_{app} > log D (7.4)). These results suggest that n-octanol did not induce the formation of reverse micelles, which were observed with the dodecane–lecithin system, because the measured permeabilities are consistent with the change in log D (7.4) distribution coefficients, which demonstrates that Overton's rule applies for the permeation setup proposed herein, and are in line with those results obtained by Meadows *et al.* [29], who found a strong linear trend between logarithms of permeability coefficients obtained from a planar bilayer lipid membrane (BLM) formed from soy phosphatidylcholine (PC) lipids and logarithms of water/octanol partition coefficients.

In Vitro Apparent Intestinal Permeability of Sulfadiazine and Its Binary and Ternary Complexes with Cyclodextrins and/or Aminoacids

Table IV shows the P_{app} coefficient values of SDZ and the binary and ternary CD complexes of SDZ using both conditions: pH 5.5 donor/pH 7.4 receptor and pH 7.4 donor/pH 7.4 receptor compartments, respectively.

SDZ is an important antibacterial compound member of the large family of sulfonamides. SDZ is an ampholyte, which has an amide moiety ($pK_{a1} = 2.2$) that is able to release a proton and a basic amine moiety ($pK_{a2} = 6.4$) that is suitable for gaining a proton under specific pH conditions. Thus, the ionization of SDZ followed a two-step protolysis process (Fig. 5). The permeation of SDZ was investigated by applying our proposed *in vitro* permeability model using two pH donor solutions, one with a pH of 5.5, at which most of the SDZ molecules are non-ionized, and the other with a pH of 7.4, at which SDZ is mainly anionic. In this work, SDZ showed low apparent intestinal permeability values (P_{app}) under both pH donor conditions (Table IV), although at a pH of 5.5, the P_{app} of SDZ was slightly higher, probably due to a higher fraction of the non-ionized drug. The P_{app} decreased with increasing ionization of SDZ at a pH of 7.4, where it was almost completely ionized (99%), indicating that the transport of SDZ across the lipid membrane was pH-dependent (Table IV).

To assess the impact of the low aqueous solubility of SDZ on its permeability behavior, the solubility-enabling formulations of SDZ with CD, with or without AA, were assayed using our *in vitro*-modified permeability method under two pH donor conditions (pH values of 5.5 and 7.4). It was found that the apparent permeability of SDZ greatly increased with all the formulations assayed. A greater than twofold increase was observed for the permeability of SDZ in the presence of CD, with or without AA, and in the presence of the above mentioned excipients under the two pH donor conditions. However, the highest apparent permeability values of SDZ were obtained under a pH condition of 5.5 in the donor solution.

On the other hand, to determine the effect of the concentration of βCD on a combined approach of pH adjustment and complexation, the *in vitro* permeability profiles of SDZ were carried out in the presence of different concentrations of βCD and SDZ:βCD with molar ratios of 1:0.5; 1:1; 1:2, respectively, at a pH value of 7.4 in the donor solution. Figure 6 shows that βCD increased the flux of SDZ in a concentration-dependent manner and that an increasing amount of βCD resulted in a continuous increase in the flux of SDZ through the lipid membrane. At the beginning, a 60-min lag time was observed, during which the apparent permeability of SDZ remained constant. After that, the transport of SDZ through the lipid membrane started to increase.

DISCUSSION

In Vitro Apparent Intestinal Permeability of the Validation Compound Set

The best correlation between the apparent permeability coefficients (P_{app}) and the corresponding human absorption data (f_a) was obtained with the validation compound set under a pH 7.4 donor condition. The $R^2 = 0.96$ indicated a high predictive absorption power of the proposed method (Fig. 3a). However, when the pH of the donor chamber was 5.5, the permeability model was less predictive because amoxicillin and verapamil were above and furosemide below the regression line, respectively (Fig. 3b), showing a slightly weaker correlation between the P_{app} data for the validation set and the corresponding data of absorption in humans ($R^2 = 0.77$). The recovery (%R) of amoxicillin under a pH condition of 5.5 for the donor and a pH condition of 7.4 for the receiver was less than 80%, which might be one of the causes that contributed to underestimate its P_{app} value (false negative). Amoxicillin is an amphiphilic compound, which carries both acidic and basic functions in the same molecule, and according to its pKa values (2.4 and 9.6), most of the amoxicillin molecules at a pH of 5.5 exist as net neutral species, including both the uncharged and zwitterionic species. Therefore, the higher lipophilicity of these species might slow down the drug permeation due to the partition of the membrane, which may become rate-limiting of the permeability process.

Regarding the high permeability of verapamil, its P_{app} value was also above the regression line of the correlation plot. However, it is a weak base and at a pH of 5.5, most molecules are protonated. Assuming that the net neutral species are the preferred species for permeation, the determined P_{app} obtained under a condition of pH 5.5 in the donor chamber would be underestimated (false negative), as the fraction of neutral species of total solutes is less than pH 7.4. However, the acidic drug (furosemide) was below the correlation line in the correlation plot obtained under a condition of pH 5.5 in the donor chamber (Fig. 3b).

Furosemide showed a high P_{app} value, which might result in an overestimation of fraction absorbed (false positive). In this case, this result could be explained because the permeation of furosemide through the intestinal wall depends not only on its permeant hydrophobicity, but also on its efflux transporters in the intestinal membrane. These are the possible factors causing its poor oral absorption [30, 31].

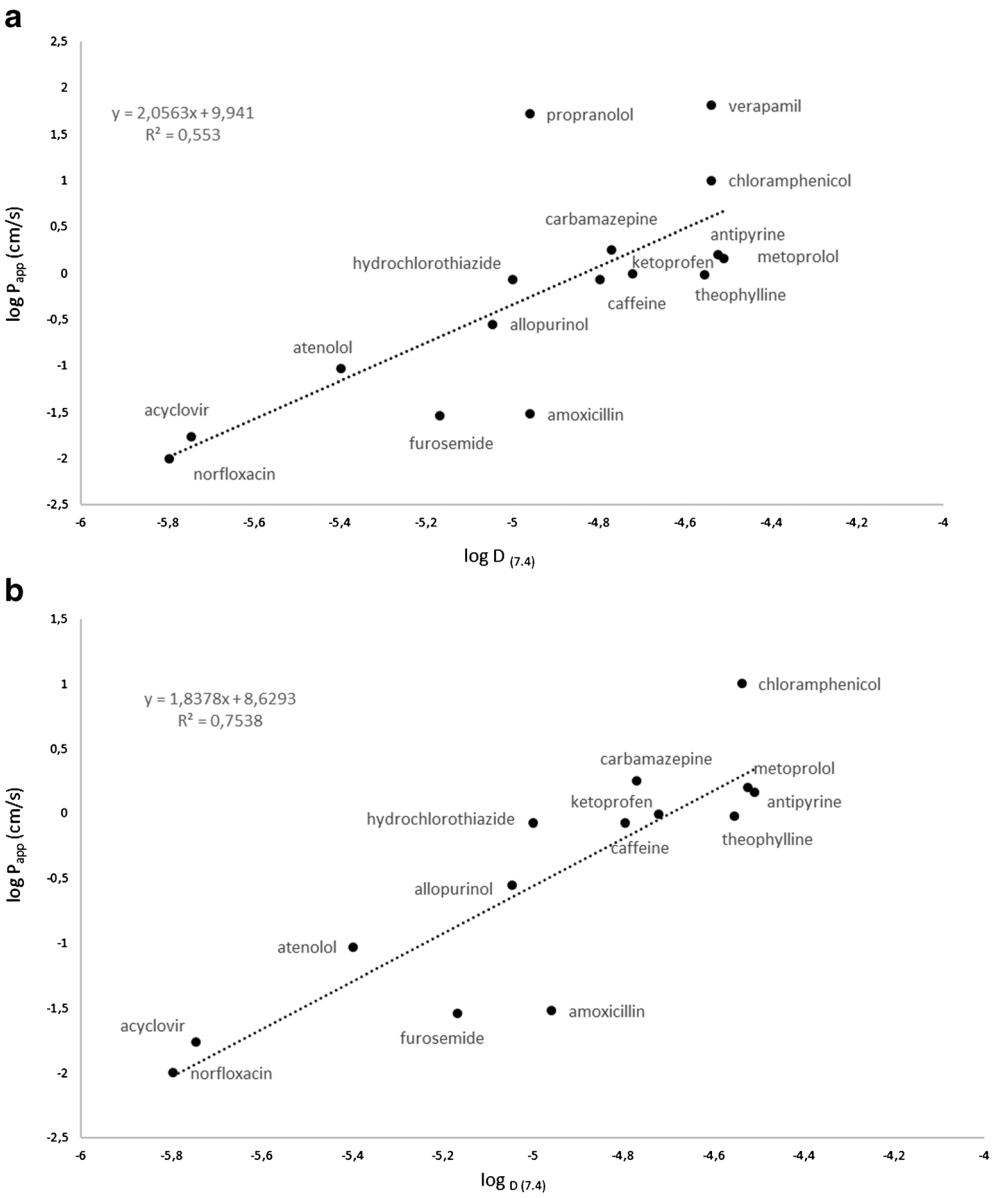

Fig. 4. Logarithm dependence of apparent permeability coefficients (log P_{app}) *versus* distribution coefficients (log D (7.4)) in n-octanol/buffer pH 7.4 of (**a**) 16 tested compounds and (**b**) tested compounds excluding propranolol and verapamil

Table IV. Apparent Permeability Coefficients (P_{app}) and Estimated f_a Percentage of Different Formulations of SDZ

Formulation	$P_{app} \times 10^{-5} \pm$ DS cm/s; 5.5 pH donor	$P_{app} \times 10^{-5} \pm$ DS cm/s; 7.4 pH donor	%R	% estimated f_a by applying Eq.3	
				5.5 pH donor	7.4 pH donor
SDZ	0.55 ± 0.07	0.27 ± 0.02	81 ± 5	59	37
SDZ:βCD	1.1 ± 0.1	0.46 ± 0.02	78 ± 3	82	54
SDZ:HPβCD	0.7 ± 0.1	0.44 ± 0.06	98 ± 3	69	52
SDZ:MβCD	1.10 ± 0.03	0.47 ± 0.06	90 ± 4	84	55
SDZ:βCD:GLY	1.0 ± 0.2	0.86 ± 0.04	102 ± 3	81	77
SDZ:βCD:LEU	0.9 ± 0.2	0.46 ± 0.05	103 ± 3	77	54
SDZ:βCD:GLU	0.96 ± 0.05	0.61 ± 0.09	114 ± 11	79	64
SDZ:βCD:ASP	1.1 ± 0.2	0.65 ± 0.1	99 ± 1	84	67
SDZ:βCD:ARG	0.81 ± 0.09	0.43 ± 0.04	102 ± 2	73	52

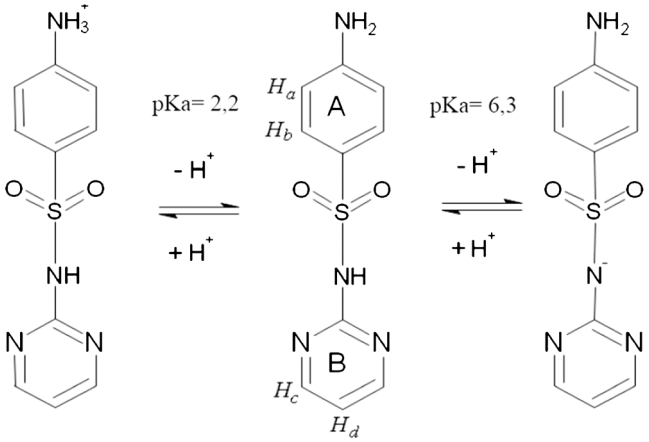

Fig. 5. Protolytic equilibria of SDZ

Even though transport proteins are missing in the non-cellular model, it represents a suitable model for permeation studies, as passive diffusion is the main route for drug uptake *in vivo*.

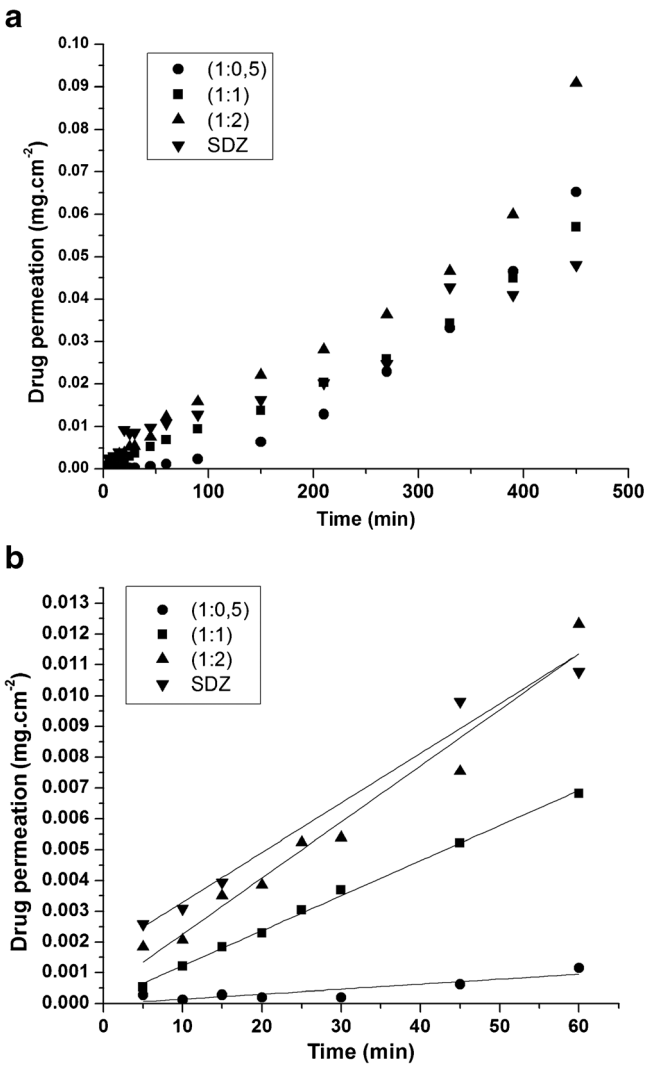

Fig. 6. *In vitro* permeability profiles of SDZ, in the presence of different molar concentrations of βCD under iso-pH 7.4 conditions. **a** up to 500 min and **b** up to 60 min

On the other hand, Fig. 3a, b shows that a clear pH-dependent permeability was found for metoprolol, with the highest P_{app} coefficient value being under a pH 7.4 donor condition. Metoprolol can be regarded as a low-/high-permeability class boundary standard, because it has been demonstrated to have almost complete absorption (~ 100%), and its intestinal permeability is passive and does not involve carrier-mediated absorption [32]. However, a constant intestinal permeability for the low-/high-permeability class boundary standard is required to be considered an optimal boundary marker. On the other hand, the pH of the donor medium could have a very important effect on the membrane permeability of drugs, because it depends upon both the dissociation condition of the permeating drug in the solution and its intrinsic permeability. In this study, metoprolol showed a pH-dependent permeability. Similar results were reported by Dahan *et al.* [33], by applying *in situ* rat permeability studies, finding that metoprolol has a pH-dependent permeability, with the highest intestinal permeability being at the distal regions where pH values are higher. This phenomenon might be explained because metoprolol has a secondary amine with a pK_a of 9.51 and a log *P* of 2.2 at pH 5.5, which is ~ 4 pH units away from its pK_a, so the drug is almost completely positively charged and its log *P*, transport under these conditions, is lower than pH 7.4. At a pH of 7.4 in the donor cell, a significant fraction of the drug becomes neutral and thus its permeability increases.

In Vitro Apparent Intestinal Permeability of Sulfadiazine and Its Binary and Ternary Complexes with Cyclodextrins and Aminoacids

It is important to note that the P_{app} values of SDZ obtained with the proposed *in vitro* permeability assays did not match the adequate *in vivo* physiological oral availability of SDZ in humans. The intestinal oral bioavailability of SDZ is obtained by determining the extent of urinary excretion of SDZ in humans after oral administration [34–37], and also in other animal species [38, 39] was reported to be 81–100%.

We speculated that a possible reason for the disparity between the *in vitro* P_{app} values of SDZ obtained in this study and the almost complete absorption of SDZ after oral administration in humans or animals could be due to the extremely low water solubility of this drug. This compound has a pH-dependent solubility because its aqueous solubility increases with increasing pH due to a shift from neutral-to-anionic species. In this context, the measurement of the %*R* of SDZ in the *in vitro* permeability assay in a donor solution at pH 5.5 presented an analytical problem due to the extremely low aqueous solubility of SDZ, where its precipitation was in fact observed in the donor chamber.

Therefore, to investigate if the low aqueous solubility of SDZ had a relationship with the *in vitro* apparent low permeability (P_{app}) values obtained in the current study and to determine if it is the rate-determining step for the transport of SDZ across the lipid membrane, we performed *in vitro* permeability assays with binary and ternary complexes of SDZ with CD and/or AA to improve the solubility of SDZ. In addition, we have previously reported that these complexes have solubilizing properties for SDZ [17–19].

Table V. Data of the Phase Solubility Studies of SDZ and Its Formulations. Data Obtained from Delrivo *et al.* [18], Delrivo *et al.* [19]

Formulation	pH_1	Solubility (mg/ml)	Kc M^{-1}	pH_2	Solubility (mg/ml)	Kc M^{-1}
SDZ	5.3	0.073 ± 0.009		7.4	0.90	
SDZ:βCD	5.96	0.33 ± 0.02	282 ± 15	7.65	1.54 ± 0.03	55 ± 2
SDZ:HPβCD	5.66	0.288 ± 0.03	123 ± 2	7.80	1.90 ± 0.03	28.2 ± 0.5
SDZ:MβCD	5.63	1.90 ± 0.03	243 ± 6	7.62	3.566 ± 0.008	44 ± 2
SDZ:βCD:GLI	5.78	0.400 ± 0.009	258.4 ± 0.3	5.78	0.4 ± 0.09	258.4 ± 0.3
SDZ:βCD:LEU	6.14	0.32 ± 0.01	389 ± 7	6.14	0.32 ± 0.01	389 ± 7
SDZ:βCD:GLU	3.45	0.68 ± 0.04	122 ± 7	5.22	0.488 ± 0.02	136 ± 25
SDZ:βCD:ASP	3.19	0.62 ± 0.01	105 ± 5	3.19	0.28 ± 0.01	476 ± 24
SDZ:βCD:ARG	8.49	13.18 ± 0.03	1156 ± 13	8.47	13.5 ± 0.5	1156 ± 94

Tables IV and V show that, under a pH condition of 5.5 in the donor solution, there was an interplay between the apparent solubility of SDZ increased by complex formation and the apparent intestinal membrane permeability of the drug because binary and ternary complexes of CD allowed increasing twofold the P_{app} of SDZ. Table IV shows a very good correlation between the estimated dose fraction absorbed in humans (f_a) for SDZ formulations and their corresponding P_{app} values by applying Eq. 3, at pH 5.5 of the donor solution.

These results indicate that the aqueous solubility of SDZ has an important impact on its *in vitro* permeability across the intestinal epithelium.

On the other hand, a significant enhancement of the solubility of SDZ upon complexation with CD and/or AA was reported at pH ~ 7.4 (see Table V). It was observed that the highest solubility of SDZ occurred at a pH value of 7.4 in comparison with that observed at a pH value of 5.5; however, there was not a substantial increase in the P_{app} value of SDZ compared with that obtained by complexation of SDZ with CD and/or AA under the most acidic pH condition (Table IV). Among ternary complexes of SDZ with βCD and the different AA tested, ARG has better solubilizing properties for SDZ, but the measured pH of the resulting solution was alkaline (~ 8.5), and therefore, at this pH value, SDZ is almost completely ionized, with species with negative net charge being the predominant form and with less affinity for the lipid membrane than neutral species (Table V). On the other hand, the equilibrium constant for the ternary inclusion complex of SDZ with βCD and ARG (Kc), at this pH value (Table V), was also the highest among those obtained for the ternary complexes of SDZ with βCD and the remaining AA tested, despite the predominance of SDZ-species at the pH value of the solution. This was reflected in the P_{app} value of the SDZ obtained with this formulation when the *in vitro* permeability assays were carried out at the pH of 7.4 in the donor compartment, which was the lowest one among those obtained for the ternary formulations with the other AA studied. Whereas, there was no increase in the P_{app} value of the SDZ with the ternary complex whose AA was LEU compared to the binary complex of SDZ with βCD. This could be attributed to the fact that there is no increase in the solubility of SDZ in relation to the increase in the solubility of this drug obtained with the binary complex with βCD.

It was found that the transport of SDZ across the lipid membrane increased exponentially with increasing amounts of βCD (Fig. 6a). However, within the first 60 min (Fig. 6b), the flow of SDZ was linear, with molar ratios of 1:0.5 and 1:1, respectively, for SDZ: βCD, and lower than that obtained with the drug alone. After 60 min, the flow of SDZ formulations of SDZ with βCD increased exponentially, while the flow of the free SDZ remained constant. These results could be explained by the displacement of SDZ from the CD cavity because SDZ passes through the lipid membrane, and thus the donor compartment increases the CD concentration required to reach the complete solubilization of the drug because the CD does not permeate the lipid membrane. This phenomenon was greater, as the molar ratio between SDZ and βCD increased. These results showed a very good correlation between the substantial increase in the solubility of SDZ by using a combination of pH adjustment and complexation with CD and the increase in the apparent permeability values of SDZ obtained. However, the anionic species of SDZ were less permeable than those of the neutral form.

It has sometimes been reported that although a solubility enhancement may be reached by the formulation of a poorly soluble drug with CD, a simultaneous decrease is associated with permeability [40, 41]. In this work, however, an enhancement in the solubility of SDZ by complexation with CD or by combination of CD with AA or pH adjustment was also associated with a rise in its permeability. The moderate stability constants obtained with binary and ternary complexes of SDZ and the sufficient CD levels to solubilize the dose of the drug could probably lead to an increase in the transport of SDZ through the lipid membrane [42].

CONCLUSIONS

In the current study, we propose an *in vitro* permeability method by using a modified lipid membrane supported on a hydrophilic filter and placed in horizontal diffusion cells, with agitation in both chambers (donor and receptor) to predict the *in vivo* intestinal passive permeability of drugs.

The proposed permeability method involved a pH of 7.4 in the acceptor compartment, two set of assays, one with a gradient pH (pH 5.5 donor/pH 7.4 receptor) and the other with an iso-pH 7.4, covering compounds with a variety of acid–basic characteristics. This method is an interesting tool to distinguish passive diffusion from active transport, since the P_{app} values correlate well with *in vivo* absorption rates for

drugs that cross the intestinal epithelium by passive diffusion under both pH donor conditions. In summary, the proposed modified PAMPA assay performed under different pH donor conditions showed high predictability and good reproducibility.

On the other hand, by applying the proposed *in vitro* permeability method, we were able to successfully predict the disposition characteristics of oral sulfadiazine (SDZ), finding that the solubility of SDZ in the intestinal lumen plays a key role in the oral absorption of this compound. The results obtained in this study suggest that SDZ is transported through the gastrointestinal epithelium by passive diffusion in a pH-dependent permeability way.

Taken together, these results support the classification of SDZ as a high/low borderline permeability compound and thus are in agreement with the criteria of the Biopharmaceutics Classification Systems [43]. This conclusion is consistent with the *in vivo* pharmacokinetic data of SDZ.

In addition, the results suggest that the use of suitable combinations of SDZ, CD, and AA could be exploited to develop appropriate oral pharmaceutical forms of SDZ capable of simultaneously improving the solubility and permeability of the drug and thus enhancing its bioavailability and reducing the dose-related side effects.

Finally, based on these findings, the proposed carrier combination could serve as a general enhancer of drug intestinal absorption.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the assistance of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba (SECyT-UNC), and Fondo para la Investigación Científica y Tecnológica (FONCYT) which provided support and facilities for this investigation. We also thank Ferromet S.A. (agent of Roquette in Argentina) for their donation of cyclodextrins and DROMEX SA Argentina for the gift of Lipoid 75.

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