

## Research Article

# Evaluation of the Membrane Permeability (PAMPA and Skin) of Benzimidazoles with Potential Cannabinoid Activity and their Relation with the Biopharmaceutics Classification System (BCS)

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**Abstract.** The permeability of five benzimidazole derivatives with potential cannabinoid activity was determined in two models of membranes, parallel artificial membrane permeability assay (PAMPA) and skin, in order to study the relationship of the physicochemical properties of the molecules and characteristics of the membranes with the permeability defined by the Biopharmaceutics Classification System. It was established that the PAMPA intestinal absorption method is a good predictor for classifying these molecules as very permeable, independent of their thermodynamic solubility, if and only if these have a Log  $P_{\text{oct}}$  value  $<3.0$ . In contrast, transdermal permeability is conditioned on the solubility of the molecule so that it can only serve as a model for classifying the permeability of molecules that possess high solubility (class I: high solubility, high permeability; class III: high solubility, low permeability).

**KEY WORDS:** BCS; benzimidazole; PAMPA; transdermal penetration.

## INTRODUCTION

Biopharmaceutical Drug Classification (BCS) arose in 1995 and established in which cases an *in vivo* bioavailability study can be replaced by an *in vitro* dissolution study (1). It is determined that this correlation depends basically on two parameters of the molecule: solubility in water and permeability. Thus, molecules can be classified into four classes: class I (high solubility, high permeability); class II (low solubility, high permeability); class III (high solubility, low permeability); and class IV (low solubility, low permeability) (1,2).

A provisional classification of the solubility of molecules can be done using values described in the bibliography (e.g., Merck Index, USP, etc.) (3). However, BCS expresses solubility by means of the dimensionless dose figure,  $D_o$ . This  $D_o$  corresponds to the ratio of drug concentration in the administered volume (250 mL) to the saturation solubility of the drug in water [ $D_o = (\text{dose}/250)/\text{solubility}$ ] (1,4). In general, drugs with  $D_o \leq 1$  were classified as high-solubility drugs, and drugs with  $D_o > 1$  were classified as low-solubility drugs (4). Ideally, in order to calculate the permeability of a molecule, experimental studies should be performed that allow relating data between the permeability of the jejunum and a reference intravenous dosage. However, transitory permeability can be

calculated through Log  $P_{\text{oct}}$ , which is defined as the ratio between the equilibrium concentrations of a substance dissolved in a system of two immiscible solvents: *n*-octanol and water (5,6).  $P_{\text{oct}}$  is a dimensionless figure usually expressed as a base 10 logarithm (Log  $P_{\text{oct}}$ ) (7). Some authors sustain that an ideal Log  $P_{\text{oct}}$  value is within 1 and 4 because in this range, the molecule has enough lipid affinity to cross membranes and enough water affinity to diffuse and dissolve in body fluids (6,8–10). The value of Log  $P_{\text{oct}}$  is only an estimate of the permeability since the said parameter does not consider absorption through transporters, which is an important absorption mechanism of drugs (3).

One of the *in vitro* methods that can be used is the “parallel artificial membrane permeability assay” (PAMPA). This method consists of a hydrophobic filter material that is coated with a mixture of lecithin and inert organic solvent creating an artificial lipid membrane (11–15). The extent of permeation through the membrane is measured and compared with a known degree of drug absorption in humans. An excellent correlation was demonstrated between the flux across PAMPA systems and the extent of absorption of a diverse set of well-characterized drugs in humans (16–21). No good correlation is observed when the drug is transported by active transport mechanisms.

It would be interesting to study the potential of other membranes with characteristics that, albeit different from the intestinal epithelium (*i.e.*, the skin), could serve as a model to classify the permeability of compounds as defined by the BCS.

In this paper, we study a series of benzimidazoles synthesized in our laboratories as modulators of CB1 cannabinoid receptors. We analyzed their permeability in

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two models of membranes—PAMPA and skin—in order to study the relationship of the physicochemical properties of the molecules and characteristics of the membrane with the permeability defined by the BCS.

## MATERIALS AND METHODS

### Reagents

Acetonitrile and methanol (high-resolution liquid chromatography (HPLC) quality) were bought from Merck S.A. Lecithin from egg yolk, verapamil, and dodecane were bought from Sigma Aldrich. All the other reagents used ( $\text{Na}_2\text{HPO}_4$ , aniline, benzene, toluene, bromobenzene, naphthalene, *etc.*) were of analytic grade. The molecules 2-pyridin-3-yl-1H-benzimidazole (PB), 2-(1H-benzimidazole-2-yl)aniline (AB), 2-(6-fluor-1H-benzimidazole-2-yl) aniline (ABF), 2-(6-cloro-1H-benzimidazole-2-yl)aniline (ABCl), and *N,N*-dimetyl-4-((2-fenyl-1H-benzoimidazole-1-yl) carbonyl) aniline (DMAB) were synthesized in the synthesis laboratory headed by Dr. David Pessoa-Mahana (Pontificia Universidad Católica de Chile, Faculty of Chemistry, Pharmacy Department). The filters for the PAMPA were Millipore MultiScreen® IP 0.45  $\mu\text{m}$  hydrophob.

### Determination of Log $P_{\text{oct}}$ of Benzimidazole Compounds

High-resolution liquid chromatography was used (22–24). To do so, a Shimadzu C-R6A liquid chromatograph was employed, with a model SPD-6A UV detector, a Shimadzu model LC-9A pump, and a Shimadzu C-R6A integrator. The analysis was done under the following conditions: LiChospher® 100 RP-18 (5  $\mu\text{m}$ ) column; mobile phase: For molecules found in the neutral state at pH 5.5, a mixture of methanol/water in a proportion of 65:35 was used, while for the determination of the Log  $P_{\text{oct}}$  of molecules that present an ionized form at this pH, the pH was adjusted to 7.0; modifying the mobile phase to methanol/buffer  $\text{Na}_2\text{HPO}_4$  0.02 M, pH 7.0, in equal proportion; flow, 0.8 mL/min. The reference compounds used were aniline, benzene, toluene, bromobenzene, naphthalene, and benicilic alcohol. For the quantification of each molecule, their maximum longitude of absorption was used (24). In this way, the correlation graph of Log  $k$  vs. Log  $P_{\text{oct}}$  was obtained,  $k$  being the capacity factor.

### Gastrointestinal Permeability Studies (PAMPA)

The permeability method used in these studies was carried out in a 96-well format. A 96-well microtiter plate and a 96-well filter plate (Millipore MultiScreen® IP 0.45  $\mu\text{m}$  hydrophob, USA) were assembled into a “sandwich” such that each composite well was separated by a 125- $\mu\text{m}$  micro-filter disc. The hydrophobic filter was coated with 4  $\mu\text{L}$  of 2% lecithin dissolved in dodecane. Subsequently, the filter plate was placed on the microtiter plate containing 330  $\mu\text{L}$  of the compound in the range of concentration 20–200  $\mu\text{M}$  dissolved in buffer  $\text{KH}_2\text{PO}_4$  0.2 M, pH 7.4, with no more than 1% of DMSO. This constituted the donor solution. The acceptor wells (in the top of the wells) of the sandwich were hydrated with 330  $\mu\text{L}$  of buffer  $\text{KH}_2\text{PO}_4$  0.2 M, pH 7.4. The system was incubated for 4 h at 37°C and stirred at 200–300 rpm in an

orbital well plate agitator throughout this time (Thermo Micromixer Mxi4t, Finepcr). To prevent loss by evaporation, the system was first covered with a wet paper and then with a plastic film.

### Quantification of Benzimidazole Compounds in the PAMPA Studies

The receptor solutions were diluted in such a way as to allow quantification by fluorescence (Fluorimeter Perkin Elmer LS 55) using a calibrated line that was built for each compound under study by the corresponding longitude of absorption and excitation. The apparent permeability ( $P$ ) was estimated using the equation (Eq. 1) (17):

$$Pe = \frac{V \cdot dC}{A \cdot C_0 \cdot dT} \quad (1)$$

where  $P$  is the permeability in centimeters per second,  $V$  is the volume of the receiver compartment,  $A$  is the surface area (0.3  $\text{cm}^2$ ),  $C_0$  is the starting concentration in the donor compartment in micromolar or nanograms per milliliter, and  $dC/dT$  is the rate of change of compound concentration, in micromolar per second or nanograms per milliliter, in the receiver compartment at 4 h.

### Data Analysis by Permeability Studies (PAMPA)

Three replicates of each experiment were used. Results are presented in the text as the means+SDs. The data were analyzed by variance analysis and Dunn’s tests or Student–Newman–Keuls tests for comparisons of multiple means. Statistical significance was fixed at  $P < 0.05$  (24,25).

### Skin Absorption of Benzimidazole Compounds

The skin used was from 2- to 3-day-old pigs (25,26). This skin was frozen ( $-20^\circ\text{C}$ ) before being used. Around 1 h before each experiment, the skin was thawed, shaved, and cut to an adequate size. The epidermis was put into contact with the donor solution. Vertical diffusion cells of 4.15  $\text{cm}^2$  were used (Laboratory Glass Apparatus Inc., USA) and thermostated to 37°C using a water bath. The donor solution contained the benzimidazole compounds in a concentration of 0.5 mg/mL dissolved in PEG 20 oleyl ether (6%, *v/v*) (27). For each cell, 3 mL of each of the donor solutions was used. The receptor solution corresponded to PEG 20 oleyl ether (6%, *v/v*) using around 5 mL of these solutions for each cell.

### Quantification of Benzimidazole Compounds in the Transdermal Studies

Quantification of benzimidazole compounds in receptor solutions was as follows: First, all of the receptor solution was evaporated to dryness in an oven (Fisher Isotemp Oven Senior Model) at 50°C. The dry residue was then dissolved in 0.8 mL of the HPLC mobile phase (see below), and the solution was then centrifuged (Universal 32R Hettich Zentrifugen) for 15 min at 11,000 rpm. The supernatant was filtered (Sartorius AG-37070, Germany, 0.45  $\mu\text{m}$ ) and the compounds quantified in the filtrate by HPLC, as below.

The compounds were quantified by HPLC (mobile phase: acetonitrile/buffer Na<sub>2</sub>HPO<sub>4</sub> 0.05 M, pH 6.5; 50:50; LiChospher®100 RP, 18.5- $\mu$ m column; and flow rate 0.8 mL/min) using a calibrated line that was built for each compound under study by the corresponding maximum longitude of absorption.

### Data Analysis by Transdermal Experiments

At least five or six replicates of each experiment were used. Results are presented in the text as the means+SDs. The data were analyzed by Dunn's tests for comparisons of multiple means. Statistical significance was fixed at  $P < 0.05$  (24,25).

## RESULTS AND DISCUSSION

### Determination of Log $P_{oct}$ of Benzimidazole Compounds

The calibrated line equation obtained with the reference compounds was  $\text{Log } K = -0.78678 + 0.50661 \text{ Log } P_{oct}$  ( $R^2 = 0.99$ ). The experimental Log  $P_{oct}$  of the benzimidazole compounds were in the range of 1.8–3.5 (Table I). The theoretical method of the Log  $P_{oct}$  determination considers 0.4 logarithmic units as the margin of error for low-molecular-weight and structurally non-complex molecules. Therefore, the values obtained experimentally are considered reliable (see Table I).

### Gastrointestinal Permeability Studies (PAMPA)

Initially, we study the absorption of verapamil (50 mM) by PAMPA. The verapamil permeability described in the literature is about 14.0 ( $10^{-6}$  cm/s) (28,29); meanwhile, the experimental permeability determined in our studies was  $11.5 \pm 3.21$  ( $10^{-6}$  cm/s), so it can be assumed that the conditions of the PAMPA experiment are correct. The five compounds studied are shown in Fig. 1. All of them are compounds derived from benzimidazole, as well as omeprazole, and have potential cannabinoid biological activity. Due to their physical chemical properties (Log  $P_{oct}$  and solubility in water; Table I), PB and AB belong to class I according to biopharmaceutic classification: drugs with high solubility and high permeability. Likewise, the ABF, ABCI, and DMAB molecules would belong to class II according to this classification: drugs with low solubility and high permeability (1). Since our purpose was to provisionally classify according to the BCS a set of investigational compounds with unknown clinical potency (dose), we were unable to calculate the dose number as in BCS.

However, we found it convenient to use the classification strategy by Dahan *et al.* (3). This strategy considered the analysis of a large number of drugs and concluded that in 65–68% of cases, solubility of  $< 0.1$  mg/mL implies that the drug may be classified as high solubility. Drugs that cannot be categorized in this way are high potency, such as digoxin (1,4). On the other hand, we will use the coefficient of partition as a criterion of permeability. Molecules exhibiting *n*-octanol/water partition coefficient value greater than metoprolol (Log  $P_{oct} = 1.72$ ) were categorized as high permeability since metoprolol is known to be absorbed from the GI and hence may be used as a reference standard for low/high class boundary (3). The fundamental difference between class I and class II drugs is the feasibility of establishing correlations among the dissolution studies *in vitro* and the absorption studies *in vivo*. However, all of them, once soluble, should pass through membranes without difficulty (2). Consequently, *in vitro* intestinal absorption was studied for these five compounds that theoretically possess high permeability and a varied range of solubility in order to establish whether the PAMPA method is adequate for predicting the permeability of all these molecules since it is known that Log  $P_{oct}$  is only one of the parameters that determine absorption through membranes. The results of the different PAMPA studies are shown in Table II, where the influence of the concentration of each compound in the donor solution was studied. Not all studies were performed with the same range of concentrations, basically due to the different solubilities the compounds have. Although in all cases a higher concentration of each compound in the donor implied a greater absorption of the respective compound, there were no statistically significant differences among the different concentrations used in each compound. Consequently, in order to compare permeabilities among the compounds under study, the average value among the permeabilities found for each compound was used (Fig. 2).

The compound that shows the greatest permeability is AB ( $19.03 \pm 3.56$  ( $10^{-6}$  cm/s)), followed by ABF ( $12.77 \pm 1.33$  ( $10^{-6}$  cm/s)), then ABCI ( $5.49 \pm 0.91$  ( $10^{-6}$  cm/s)), and finally, the last that can be calculated is PB ( $2.73 \pm 0.15$  ( $10^{-6}$  cm/s)). The compound DMAB was not quantified as a receptor because its permeability is considered null in these conditions.

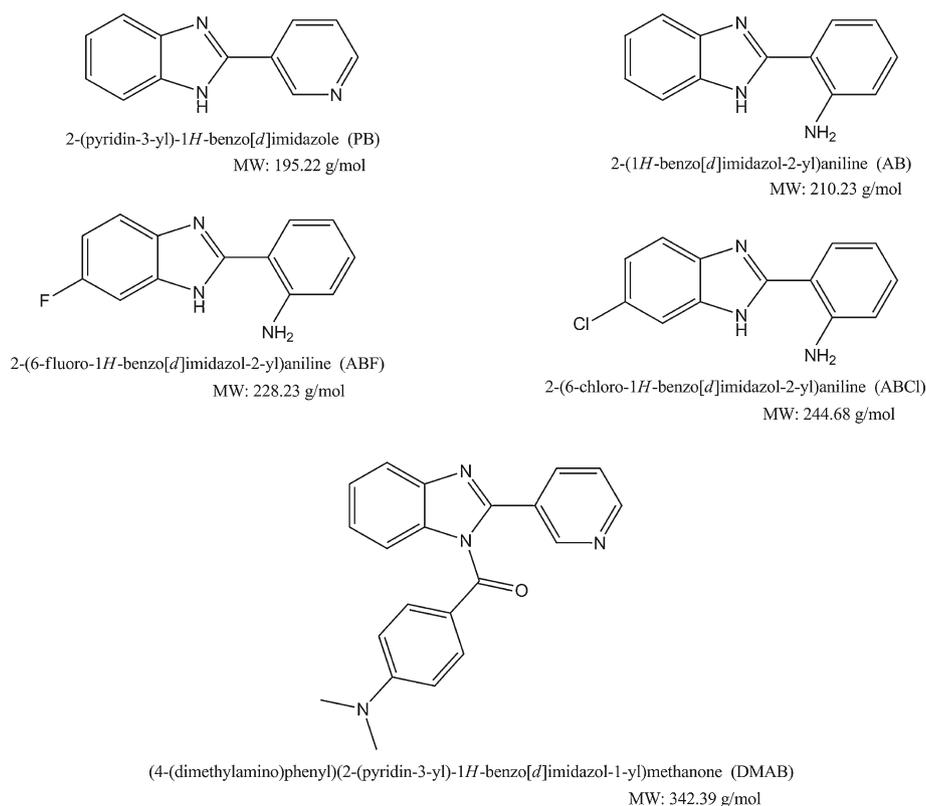
In the analysis of the class I compounds AB and PB, the permeability values that are apparently very different are observed. However, both present a  $Pe > 1$  ( $10^{-6}$  cm/s) (30). That is to say, the Log  $P_{oct}$  values as well as the  $Pe$  values classify them as very permeable drugs, such that it is expected that if they were administered orally they would have an absorption  $> 50\%$ .

**Table I.** Log  $P_{oct}$  and Solubility of the Benzimidazole Compounds

Compounds	Log $P_{oct}$ (experimental)	Log $P_{oct}$ (theoretical) <sup>a</sup>	Solubility (mg/mL) <sup>b</sup>
PB	1.84	1.53	0.2730
AB	1.86	2.06	0.0970
ABF	2.18	2.22	0.0076
ABCI	2.81	2.62	0.0063
DMAB	3.54	3.66	0.0094

<sup>a</sup> Using Chem Draw. computational program

<sup>b</sup> Theoretical value calculation by ADME-Tox. computational program



**Fig. 1.** Chemical structures of the benzimidazole compounds

In the analysis of the class II compounds ABF, ABCI, and DMAB, we observe that the first two show a permeability value that classifies them as very permeable. In contrast, DMAB is not absorbed. It should be noted that the BCS does not establish upper limits on the Log  $P_{oct}$  value

**Table II.** Gastrointestinal Permeability of the Benzimidazole Compounds Using PAMPA

Compound	Concentration ( $\mu\text{M}$ )	Permeability ( $10^{-6}$ cm/s)
PB	100	2.568 $\pm$ 0.244
	200	2.768 $\pm$ 0.297
	250	2.862 $\pm$ 0.410
AB	50	15.063 $\pm$ 0.763
	75	20.088 $\pm$ 0.799
	90	21.940 $\pm$ 2.274
ABF	50	11.398 $\pm$ 1.328
	75	12.872 $\pm$ 1.125
	90	14.048 $\pm$ 0.120
ABCI	50	4.874 $\pm$ 1.069
	75	5.049 $\pm$ 0.941
	90	6.533 $\pm$ 0.881
DMAB	60	– <sup>a</sup>
	50	– <sup>a</sup>
	20	– <sup>a</sup>

Each data point represent the mean  $\pm$  SD of three repeats. No significantly different between the different concentrations studied (Student–Newman–Keuls test,  $p < 0.05$ )

<sup>a</sup> Could not be quantified

to classify a drug as very permeable. However, it is known that the optimal Log  $P_{oct}$  value to cross membranes is between 1 and 4 (5,7–9), such that the null permeability observed for DMAB could be attributed to its elevated Log  $P_{oct}$  (3.7). The high lipophilia of DMAB makes one think that it was not absorbed because it possibly was retained in the membrane, a situation which is described in a PAMPA study using compounds with a similar structure by Brain-Isasi *et al.* (31).

Therefore, it can be noted that the PAMPA method of intestinal absorption is a good predictor for classifying molecules derived from benzimidazoles, independent of their degree of solubility, as long as they have a Log  $P_{oct} < 3.0$ .

### Skin Absorption of Benzimidazole Compounds

Transdermal analyses of the five compounds under study were performed with the aim of determining their respective permeability in this membrane (Fig. 3). It is known that optimal Log  $P_{oct}$  values for transdermal absorption are between 2 and 3 (32), which are values a little higher than the ideal for oral absorption, the ideal molecular weight being under 500 g/mol (33,34).

As is well known, the skin is not a reference membrane to oral absorption, and there is not a model drug for transdermal permeability equivalent to metoprolol for the oral route (3). In addition, the permeability and potency of the drug must be known for using transdermal administration. There is general agreement that the maximum daily dose that can be absorbed is no more than 10 mg (35,36). Analyzing the

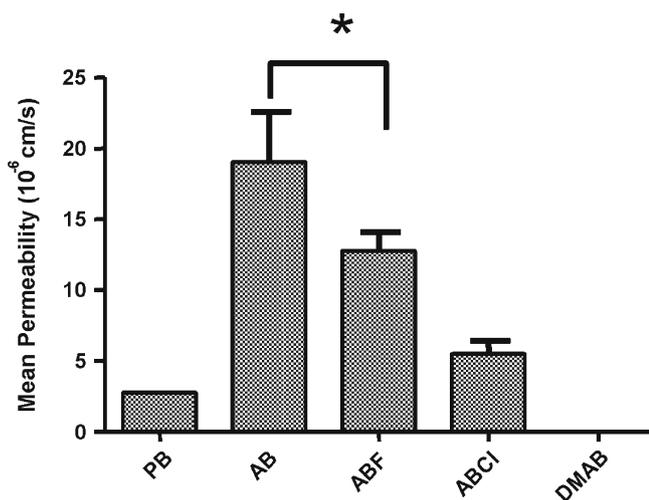


Fig. 2. Gastrointestinal mean permeability of benzimidazole compounds using PAMPA. Each data point represents the mean  $\pm$  SD of three repeats. \*No significantly different (Dunn's test,  $p < 0.05$ )

results of this study, it can be affirmed that all the compounds are absorbed through skin.

The compound that is most absorbed is PB ( $17.05 \pm 4.26 \mu\text{g}/\text{cm}^2$  at 24 h), followed by the compound AB ( $6.74 \pm 1.07 \mu\text{g}/\text{cm}^2$  at 24 h), with the transdermal absorption of PB 2.5 times greater than that of AB, with both values presenting a statistically significant difference at 24 h of study. Both compounds also have practically equal lipophilia (Log  $P_{\text{oct}}$  of 1.8 and 1.9, respectively). However, the solubility of PB is around three times greater than that of AB (see Fig. 1), although both compounds are classified as very soluble according to the BCS. This latter parameter can be attributed to the difference in transdermal absorption that was found. Nevertheless, as the ideal value of permeability is unknown, it cannot be concluded that they would be classified as very permeable. It can only be affirmed that the two are the most permeable of the series.

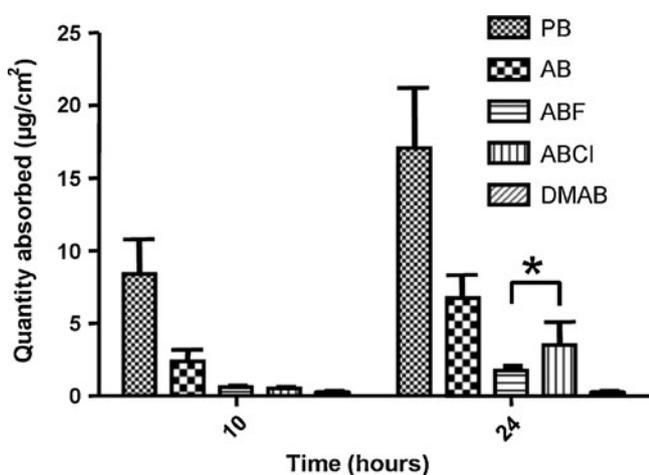


Fig. 3. Transdermal absorption of benzimidazole compounds. Each data point represents the mean  $\pm$  SD of five or six repeats. \*No significantly different (Dunn's test,  $p < 0.05$ )

For the other compounds under study, ABF, ABCI, and DMAB, statistically significant differences are only found between ABF and ABCI at 24 h of study ( $1.73 \pm 0.30$  and  $3.50 \pm 1.58 \mu\text{g}/\text{cm}^2$ , respectively). This is fundamentally attributable to the reliability of the quantification technique for finding low transdermal absorption values since both compounds have very similar solubility and Log  $P_{\text{oct}}$  values. This low permeability of ABF and ABCI is in comparison to PB and AB, but does not necessarily imply that they should be categorized as low permeable in a model that uses skin as a membrane. The compound that was least absorbed, DMAB ( $0.22 \pm 0.08 \mu\text{g}/\text{cm}^2$  at 24 h), which also was not possible to quantify between 10 and 24 h of study, possesses the greatest lipophilia, with a Log  $P_{\text{oct}}$  value greater than the ideal described for the skin. It is possible that DMAB gets trapped in the skin, the same as that observed in previous transdermal studies performed by our research group in which microemulsions were used as the administration vehicle (24). It would be inappropriate to assume this low permeability as a consequence of its higher molecular weight because it is under 500 g/mol (see Fig. 1).

It could be inferred then that for benzimidazole derivatives that have low solubility in water ( $< 0.001 \text{ mg}/\text{mL}$ ), what determines their higher or lower transdermal permeability is their lipophilia, with the optimal Log  $P_{\text{oct}}$  range for these types of compounds being 2.2–2.8. Log  $P_{\text{oct}}$  values greater than this make the adequate penetration of the molecule difficult (31).

The transdermal permeation results for benzimidazole derivatives suggest that the high aqueous solubility enhances the experimental precision of the permeability measure, thus increasing the reliability of the skin model in classifying derivatives as per BCS. This could be explained by the tortuous path a water-soluble compound must diffuse through when crossing the stratum corneum, the rate-limiting step for transdermal absorption. On the other hand, models of oral absorption such as PAMPA can be visualized as two aqueous compartments separated by a lipid barrier, the latter being the rate-limiting step for mass transport.

## CONCLUSIONS

It was established that the PAMPA intestinal absorption method is a good predictor for classifying benzimidazole derivatives as very permeable, independent of their degree of thermodynamic solubility, if and only if these have a Log  $P_{\text{oct}}$  value  $< 3.0$ . However, skin can only serve as a model for classifying benzimidazole derivatives with high solubility (class I: high solubility, high permeability; class III: high solubility, low permeability). The main difference between these two studied models resides on the hydrophilicity requirements for compounds to be delivered by each administration route. In the case of the transdermal delivery, the molecule must cross hydrophilic regions, so water solubility takes more relevance as a physicochemical property.

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