

# Frog intestinal sac as an *in vitro* method for the assessment of intestinal permeability in humans: Application to carrier transported drugs

Massimo Franco<sup>a</sup>, Angela Lopodota<sup>a</sup>, Adriana Trapani<sup>a</sup>, Annalisa Cutrignelli<sup>a</sup>,  
Daniela Meleleo<sup>b</sup>, Silvia Micelli<sup>b</sup>, Giuseppe Trapani<sup>a,\*</sup>

<sup>a</sup> Dipartimento Farmaco-Chimico, Facoltà di Farmacia, Università degli Studi di Bari, Via Orabona 4, 70125 Bari, Italy

<sup>b</sup> Dipartimento Farmaco-Biologico, Facoltà di Farmacia, Università degli Studi di Bari, Via Orabona 4, 70125 Bari, Italy

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This work is dedicated to the memory of our colleague Prof. Gaetano Liso, recently deceased.

## Abstract

The aim of this study was to investigate the presence of pharmaceutically relevant drug transporters in frog intestine which has been proposed as model for intestinal permeability screening assays of passively absorbed drugs in humans [Trapani, G., Franco, M., Trapani, A., Lopodota, A., Latrofa, A., Gallucci, E., Micelli, S., Liso, G., 2004. Frog intestinal sac: a new *in vitro* method for the assessment of intestinal permeability. *J. Pharm. Sci.* 93, 2909–2919]. The expression of transporters in frog intestine was supported by the following observations: (i) the involvement of purine nucleobase transport system was deduced by inhibition of acyclovir transport in the presence of adenine; (ii) baclofen or L-dopa transport was inhibited by the digitalis steroid ouabain and it may be related to the Na<sup>+</sup> electrochemical potential difference, presumably involving amino acid transporters; (iii) the presence of proton-dependent peptide transporters was argued evaluating the effect of the pH change (from pH 5.9 to pH 7.4) on the transport of glutathione; (iv) the possible expression in the frog intestine of an efflux system distinct from P-glycoprotein (Pgp) in the benzylpenicillin transport was deduced using a glucose enriched frog Ringer with or without the known Pgp inhibitor verapamil; (v) the contribution of Pgp-mediated efflux system in determining the frog intestinal absorption of drugs was supported by the specific inhibition of cimetidine or nadolol transport in the presence of verapamil. These results indicate that pharmaceutically relevant drug transporters should be also expressed in frog intestine.

In this work, an attempt was also made to compare the measured  $P_{app}$  values in the frog intestinal model for the aforementioned series of actively/effluxed transported drugs in humans to the corresponding literature values for the fraction absorbed. The  $P_{app}$  values used in these comparisons were obtained at high concentrations of drugs at which probably saturation of the carrier occurs. Interestingly, it was found that drugs that are completely absorbed had  $P_{app}$  values  $>3 \times 10^{-6}$  cm/s, while drugs absorbed  $<90\%$  had  $P_{app}$  values lower than  $1 \times 10^{-6}$  cm/s. In these cases, indeed, a borderline region characterized by the apparent permeability coefficient  $P_{app}$  value between  $1 \times 10^{-6}$  and  $3 \times 10^{-6}$  cm/s should be considered for which the prediction of the absorbed fraction after oral administration in humans become more uncertain by the frog intestinal sac system.

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**Keywords:** Frog intestinal sac; Permeability; Carrier transported drugs; Transporter systems

## 1. Introduction

The rate-limiting barriers for the absorption of orally administered drugs are aqueous solubility and intestinal permeability (Lipinski et al., 1997). Thus, assessment of intestinal permeability represents one essential part in the prediction of oral

bioavailability of new drug candidates. For the assessment of intestinal drug permeability, different *in vitro* methods are available such as the use of excised tissues, epithelial cell culture models, artificial membranes and these techniques have been recently reviewed (Tukker, 2000). Among these different approaches, the methods based on drug transport across intestinal epithelial monolayers, such as Caco-2 cells, are at present most utilized. In fact, they show features similar to the human small intestine giving rise to good correlations with the fraction absorbed in humans for a variety of compounds. However,

\* Corresponding author. Tel.: +39 080 5442764; fax: +39 080 5442754.

E-mail address: [trapani@farmchim.uniba.it](mailto:trapani@farmchim.uniba.it) (G. Trapani).

some important drawbacks, such as time consuming, high cost per assay, possibility of microbial contamination, and wide inter-laboratory variations, are considered the weakness of the Caco-2 cell culture model as screening tool. Moreover, Caco-2 cells as other cell lines are generally lacking mucus layer which covers the intestinal epithelium. Therefore, alternative procedures to the use of cultured cells are currently pursued (Castella et al., 2006).

Recently, we reported a new experimental protocol utilizing isolated frog intestinal sac as a new *in vitro* screening tool for assessing human intestinal permeability (Trapani et al., 2004). We showed that the *in vitro* permeability coefficient deduced from isolated frog intestinal experiments is a reasonable predictor of *in vivo* peroral absorption in humans for compounds that are passively absorbed. We also demonstrated that the frog intestinal permeability model could be used to classify these drugs into the Biopharmaceutics Classification System (BCS) (Trapani et al., 2004). Following our interest in this field, we decided to investigate the applicability of the frog intestinal sac to carrier transported drugs. It should be noted that while for active transport, the majority of studies reported in the literature are aimed at determining the expression of drug transporter systems in the examined experimental model for assessing human intestinal permeability, for passive transport attention has been focused mainly to prediction of the fraction absorbed (FA) in humans. In this last case, *in silico* models that predict drug permeability from molecular descriptors has also been developed. About the prediction of FA for actively transported drugs in humans, the existing methods for the assessment of *in vitro* intestinal permeability showed some limitations and generally unsatisfactory results were obtained. Thus, it has been demonstrated that both the Caco-2 and the small-intestine like 2/4/A1 cell models are not able to predict FA for compounds exhibiting significant active transport mechanisms *in vivo* (Chong et al., 1996; Matsson et al., 2005).

To account for these limitations it should be also considered that for actively absorbed drugs the transport is concentration dependent and hence it could change considerably with different donor concentrations.

In this work, we explored the possible presence of pharmaceutically relevant drug transporters in frog intestine. In this regard, although there have been only a few studies report-

ing the expression of glucose (GLUT) and amino acid (AAT) transporters as well as metabolising enzymes (CYP1A) in frog intestine (Csáky and Gallucci, 1977; Huang et al., 2001), to the best of our knowledge, a deeper study on the expression of pharmaceutically important transporter systems was not reported in literature. In addition, an attempt was made to compare the measured  $P_{app}$  values in the frog intestinal model for a series of actively/effluxed transported drugs in humans to the corresponding literature values of FA.

## 2. Materials and methods

### 2.1. Chemicals

Baclofen, benzylpenicillin, cimetidine, L-dopa, enalapril maleate, phloridzin dihydrate (1-[2-(β-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone, glutathione, nadolol, (±)-verapamil hydrochloride and (–)ouabain hydrate were purchased from Sigma-Aldrich Chemical Co. (USA). Acyclovir was extracted from tablets of Acyclovir EG<sup>®</sup> purchased from a local drugstore. Identity and purity of the latter compound was checked by means of spectroscopic methods (IR, <sup>1</sup>H NMR, and mass spectroscopy), thin layer chromatography and HPLC and resulted greater than 98%. All other reagents and materials were of the highest purity commercially available.

### 2.2. Quantitative analysis by HPLC

The high-performance liquid chromatography (HPLC) system consisted of a Waters (Waters Corp., Milford, MA) Model 600 pump equipped with a Waters 990 variable wavelength UV detector, and an injection valve with a 20 μl loop. A Symmetry (Waters) C<sub>18</sub> reversed-phase column (25 cm × 4.6 mm; 5 μm particles) or a Synergy Hydro-RP (25 cm × 4.6 mm; 4 μm particles; Phenomenex, Torrance, CA) column in conjunction with a guard column insert (μBondapak C<sub>18</sub>) were used for the separations. Further technical details about the HPLC analysis are listed in Table 1. Quantification of the compounds was carried out by measuring the peak areas in relation to those of standards chromatographed under the same conditions. Standard curves were linear ( $R^2 > 0.98$ ) over the range of concentrations of interest.

Table 1  
HPLC conditions for the determination of tested compounds<sup>a</sup>

Drug	Column	Flux (ml/min)	Detection λ (nm)	Retention (min)	Mobile phase <sup>b</sup>
Baclofen	Symmetry	0.7	270	6.5	A
Benzylpenicillin	Symmetry	0.8	254	9.8	B
Cimetidine	Symmetry	0.6	220	7.0	C
L-dopa	Synergy Hydro	0.7	280	8.0	D
Enalapril maleate	Synergy Hydro	0.7	215	7.9	D
Glutathione	Synergy Hydro	0.7	220	7.8	D
Nadolol	Spherisorb C <sub>8</sub>	0.7	278	5.37	E

<sup>a</sup> The HPLC conditions for the determination of acyclovir were previously reported (Trapani et al., 2004).

<sup>b</sup> A: 30% methanol in 70% (v/v) Milli-Q-water + CF<sub>3</sub>COOH; B: 35% acetonitrile, 65% (v/v) 0.025 M phosphate buffer pH 2.76 containing 2 mM Na<sub>2</sub>EDTA. C: 55% methanol in 45% (v/v) Milli-Q-water; D: acetonitrile 1%, 0.025 M phosphate buffer pH 2.76; E: 35% methanol in 65% (v/v) Milli-Q-water, HCl 0.005 M, NaCl 0.05 M.

Table 2  
pH and composition of the frog Ringer used in transport experiments

Frog Ringer	Composition <sup>a</sup>	pH <sup>b</sup>
A	KCl 5.0 mM, CaCl <sub>2</sub> 1.0 mM, NaHCO <sub>3</sub> 2.5 mM NaCl q.s.	8.2 (with NaOH)
B	Frog Ringer A and D-glucose 6 mM	8.2 (with NaOH)
C	CaCl <sub>2</sub> × 2H <sub>2</sub> O, 0.97 mM, TRIS 0.17 M	8.2 (with HCl)
D	CaCl <sub>2</sub> × 2H <sub>2</sub> O, 0.97 mM, KCl 2.61 mM, K <sub>2</sub> HPO <sub>4</sub> 0.98 mM, KH <sub>2</sub> PO <sub>4</sub> 5.14 mM, NaCl 0.105 mM	5.9 (with H <sub>3</sub> PO <sub>4</sub> 75%)
E	CaCl <sub>2</sub> × 2H <sub>2</sub> O, 0.97 mM, KCl 2.61 mM, K <sub>2</sub> HPO <sub>4</sub> 38 mM, KH <sub>2</sub> PO <sub>4</sub> 12 mM, NaCl 42 mM	7.4 (with H <sub>3</sub> PO <sub>4</sub> 75%)
F	CaCl <sub>2</sub> × 2H <sub>2</sub> O, 0.98 mM, KCl 2.58 mM, Na <sub>2</sub> HPO <sub>4</sub> 0.66 mM, NaH <sub>2</sub> PO <sub>4</sub> 5.1 mM, NaCl 84 mM, D-glucose 3.0 mM	6.8 (with NaOH)

<sup>a</sup> The osmolality of all Ringers used was 230 ± 10 mOsm/kg isotonic for amphibian.

<sup>b</sup> In parentheses are shown the strong acids or strong bases to be added for obtaining the pH indicated.

### 2.3. Transport experiments

Transport experiments were carried out according to previous work (Trapani et al., 2004) and in particular as described under Method B. Animal care and handling throughout the experimental procedure were performed in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). Briefly, segments of approximately 5.0 cm in length (starting immediately after the stomach) of healthy green frogs, were used for the permeability experiments. Once the intestinal segment was filled with 300 µl of the appropriate sample solution, the intestinal sac was immersed at 26 ± 0.1 °C in a vial containing 1500 µl of frog Ringer solution (FR) pH 8.2, with osmolality of 230 ± 10 mOsm/kg and resulting isotonic for amphibian (Conway, 1957). At predetermined times 100 µl aliquots of receiving mixture were taken and replaced with 100 µl of fresh drug-free FR. The transport experiments were monitored for about 3 h and the integrity of membranes was controlled by using phloridzin as internal standard. The drug concentration of each aliquot was determined by HPLC. The cumulative amount of drug permeated was plotted vs. time for each experiment. The composition of FR was: KCl 5.0 mmol, CaCl<sub>2</sub> 1.0 mmol, NaHCO<sub>3</sub> 2.5 mmol, and the amount required of NaCl (q.s.) to 230 ± 10 mOsm/kg (Micro-Osmometer Automatic Type 13 RS, Hermann Roebling Messtechnik Berlin, D). All the other Ringer solutions used in transport experiments had the same osmolality. In Table 2 the pH values and composition of all the Frog Ringer used in the transport experiments are reported in detail. It should be noted that in most cases the drugs were examined in unbuffered FR and therefore we measured the

pH values of the sample solutions just after dissolution in FR and at the end of the assay. Hence, these last values should be considered as the pHs of the experiments (Table 3).

### 2.4. Calculations and statistics

Three experiments, each in triplicate, were performed for all the drugs tested. The apparent permeability coefficient ( $P_{app}$ ) of the drug was calculated from the linear portion of a plot of the total amount of drug transported versus time, and determining the slope of the straight line. The mean  $P_{app}$  values and standard errors of replicates are shown in Table 4. The apparent permeability coefficient  $P_{app}$  of the drugs across the intestinal mucosa, expressed as cm/s, were calculated according to the relationship  $P_{app} = \Delta Q / \Delta t \times 1/60 \times 1/A \times 1/C_0$ , where  $\Delta Q / \Delta t$  is the permeability rate (µmol/min) namely the amount of a compound traversing the tissue in time  $t$  (min),  $A$  is the area of exposed tissue (about 2.5 cm<sup>2</sup>) and  $C_0$  represents the initial concentration of drug inside the intestinal sac. The statistical comparisons were made by utilizing analysis of variance (ANOVA) followed by the Tukey post hoc tests (GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, CA). Differences were considered statistically significant at  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Carrier-mediated transport systems in frog intestine

To evaluate whether important transporters are expressed in frog intestine, a series of actively transported drugs in humans

Table 3  
pK<sub>a</sub> of drugs investigated in transport experiments and pH of the sample solution measured just after dissolution in FR and at the end of the assay

Drug	pK <sub>a</sub> <sup>a</sup>	pH just after dissolution <sup>b</sup>	pH at the end of the assay <sup>b</sup>
Acyclovir	2.3, 9.3 <sup>c</sup>	8.1	8.1
Baclofen	3.9, 9.3	7.2	7.1
Benzylpenicillin	2.8	7.9	7.9
L-dopa	2.3, 8.7, 9.7, 13.4	7.6	7.6
Enalapril Maleate	3.0, 5.4	3.7	3.7
Glutathione	2.1, 3.4, 8.7, 9.5 <sup>d</sup>	5.9 (7.4)	5.9 (7.4)
Cimetidine	6.8 <sup>c</sup>	6.8	6.8
Nadolol	9.7	10.2	10.2

<sup>a</sup> Unless otherwise stated, pK<sub>a</sub> values were taken from Dollery (1991).

<sup>b</sup> pH values were measured at 26 ± 0.1 °C.

<sup>c</sup> Taken from Florence and Attwood (2006).

<sup>d</sup> Taken from Bieri and Burgi (2005).

Table 4  
Experimentally determined permeability values across the frog intestinal sac, and corresponding human fraction (Fa) absorbed

Drug	Active transport mechanism <sup>a</sup>	Type of frog Ringer <sup>b</sup>	Applied conc. ( $\times 10^{-3}$ M)	Frog intestinal $P_{app}$ ( $\times 10^{-6}$ cm/s) <sup>c</sup>	Human intestinal $P_{eff}$ ( $\times 10^{-4}$ cm/s) <sup>d</sup>	Fa <sup>e</sup> (%)
Acyclovir	ENT	A	1.37 <sup>e</sup>	0.44 $\pm$ 0.08 <sup>*e</sup>		19
		A	0.41	4.37 $\pm$ 1.7 <sup>*</sup>		
		A	0.22	9.31 $\pm$ 0.08 <sup>*</sup>		
Baclofen	AAT	A	2.64	3.38 $\pm$ 0.54		>95
Baclofen + ouabain		A	2.64	0.56 $\pm$ 0.32		
Benzylpenicillin	PEPT	A	5.00	70.08 $\pm$ 21.36		30
Benzylpenicillin		B	5.00	0.78 $\pm$ 0.28		30
Benzylpenicillin + verapamil		B	5.4	1.19 $\pm$ 0.11		
L-dopa	AAT	A	5.07	33.07 $\pm$ 3.79	3.4 $\pm$ 2.6	100
L-dopa		C	5.07	1.83 $\pm$ 0.32		
L-dopa + ouabain		A	5.07	0.32 $\pm$ 0.060		
Enalapril maleate	PEPT	A	4.1	2.56 $\pm$ 1.21	1.8	66
Glutathione	PEPT	D	32.5	4.98 $\pm$ 2.39		
Glutathione		E	33	0.84 $\pm$ 0.007		
Cimetidine	Pgp OCT	F	2.0	0.88 $\pm$ 0.25 ( $n=8$ ) <sup>**</sup>	0.30 $\pm$ 0.16	80
Cimetidine + verapamil		F	2.0	2.04 $\pm$ 0.5 ( $n=6$ ) <sup>**</sup>		
Nadolol	Pgp	B	6.0	0.51 $\pm$ 0.27 ( $n=9$ ) <sup>**</sup>		30
Nadolol + verapamil		B	6.0	1.65 $\pm$ 0.92 ( $n=9$ ) <sup>**</sup>		

FA: fraction absorbed in humans; AAT: substrate for amino acid transporters; ENT: substrate for the purine nucleobase transporter; OCT: substrate for the organic cation transporters; PEPT: substrate for peptide transporters; Pgp: substrate for P-glycoprotein. \*Statistical significance  $p < 0.001$ ; \*\*statistical significance  $p < 0.01$ .

<sup>a</sup> The active transport mechanism was taken from Matsson et al. (2005), Steffansen et al. (2004), and Varma et al. (2005).

<sup>b</sup> See Table 2 for details.

<sup>c</sup> Data are mean  $\pm$  S.D. of three separate determinations, each in triplicate.

<sup>d</sup>  $P_{eff}$  values were taken from Winiwarter et al. (1998), Takamatsu et al. (2001), and Sun et al. (2002).

<sup>e</sup> Fa values were taken from Steffansen et al. (2004), Zhao et al. (2001), and Trapani et al. (2004).

was selected. For these drugs both the absorbed fraction after oral administration in humans (FA) and the active transport mechanism were deduced from literature sources (Table 4), (Matsson et al., 2005; Steffansen et al., 2004; Varma et al., 2005). In particular, acyclovir is substrate for the purine nucleobase transporter (ENT) proteins, baclofen and L-dopa are substrates for amino acid transporters (AAT), benzylpenicillin, enalapril maleate, and glutathione are substrates for peptide transporters (PEPT). Furthermore, it should be also taken into account that some of these drugs are, at least in part, also passively transported (Matsson et al., 2005). In these experiments, two substrates for the P-glycoprotein (Pgp) mediated efflux transporter namely, Cimetidine and Nadolol, were also included. Cimetidine is also reported to be substrate for the organic cation transporters (OCT) (Matsson et al., 2005). The results obtained by using the selected drugs and following the previously reported experimental protocol are reported in Table 4. By comparing the  $P_{app}$  values observed for actively transported drugs with those previously reported for drugs absorbed by passive diffusion mechanism, it is apparent that the range of  $P_{app}$  values associated with the first group is wider than the second one ( $0.44 - 70.08 \times 10^{-6}$  cm/s and  $0.33 - 30.43 \times 10^{-6}$  cm/s, respectively).

In Table 4, the effective human intestinal permeability ( $P_{eff}$ ) values for some of the investigated drugs are also shown. Unfortunately, in fact, the available  $P_{eff}$  database is limited and strongly biased toward completely absorbed compounds (Matsson et al., 2005). Comparing the  $P_{app}$  values observed for actively transported drugs with the corresponding  $P_{eff}$  values it seems that the human intestine is at least 10–100-fold more permeable than the frog intestine.

In the previous study it was concluded that drugs completely absorbed in humans had  $P_{app}$  values greater than  $1 \times 10^{-6}$  cm/s, while drugs absorbed <90% had  $P_{app}$  values lower than  $1 \times 10^{-6}$  cm/s (Trapani et al., 2004). To use this permeability classification model to carrier transported drugs it should be taken into consideration that this type of transport is concentration-dependent. On the other hand, it is reasonable to expect that many active transporters may be saturated at therapeutic doses used for drugs actively transported. Therefore, in this study we tried to predict FA for the examined compounds (exhibiting significant active transport mechanisms in humans) by using the frog intestine and high concentrations of drugs (in the millimolar range), at which probably saturation of the carriers occurs. The choice of high concentrations allows also to circumvent problems connected with the use of radiolabeled materials which is only seldom available in the early stages of drug development.

### 3.2. Purine nucleobase transport system

As for the acyclovir, it is reported that this drug is substrate for the purine nucleobase transporter (ENT) proteins and enters human erythrocytes mainly by the same transporter system of adenine, guanine, and hypoxanthine (Matsson et al., 2005; Mahony et al., 1988). To explore whether this system is present also in frog intestine, the transport of acyclovir was assessed both in the absence and in presence of adenine (25 mM). As shown in Fig. 1, the permeation across the frog intestine was inhibited of about 66% in the presence of adenine supporting the conclusion that a similar purine



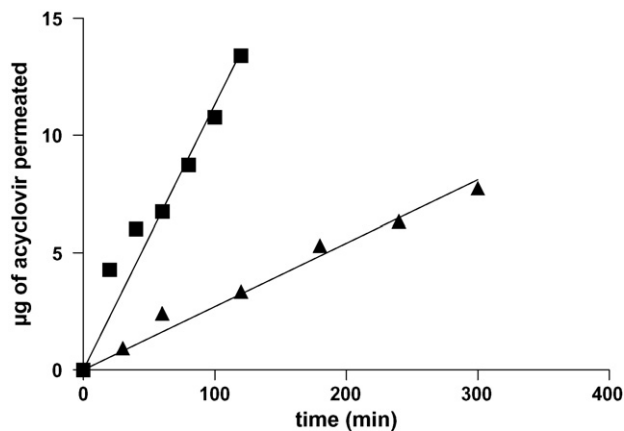


Fig. 1. Acyclovir transport in the presence (▲) and absence (■) of adenine.

nucleobase transport system should be also present in frog intestine.

Consistent with the presence of a carrier-mediated transport in frog intestine, the  $P_{app}$  value of acyclovir significantly increased at lower concentrations of the drug in the donor compartment as shown in Table 4.

### 3.3. Amino acid and peptide transporters

On the basis of the  $P_{app}$  values observed and the permeability classification model previously proposed (Trapani et al., 2004), baclofen ( $3.38 \pm 0.54$ ), benzylpenicillin ( $70.08 \pm 21.36$ ), L-dopa ( $33.07 \pm 3.79$ ), and enalapril maleate ( $2.56 \pm 1.21$ ) should exhibit high permeability indicative of an absorbed fraction in humans >90%. However, this is not the case of benzylpenicillin and enalapril maleate for which the reported FA is 15–30% and 65–70%, respectively. Benzylpenicillin, enalapril maleate and glutathione are, as mentioned above, substrates for peptide transporters (PEPT). On the other hand, Saitoh et al. (1997) reported that the restricted intestinal absorption of some  $\beta$ -lactam antibiotics is in part due to an energy-demanding efflux system, which is distinct from P-glycoprotein (Pgp)-mediated transport system. To gain insight into the involvement of such a type of efflux system, transport experiments with benzylpenicillin were carried out using a Glucose enriched frog Ringer with or without the known Pgp inhibitor verapamil (Sandström et al., 1998). As can be seen from Table 4, under these new conditions, the  $P_{app}$  values of benzylpenicillin observed (i.e.,  $0.78 \pm 0.28$  and  $1.19 \pm 0.11$ ) are more consistent with the reported FA of 15–30%. Therefore, these data indicated that (i) using the usual frog Ringer, the energy-dependent efflux system does not limit the absorption of benzylpenicillin; (ii) the intestinal absorption of this  $\beta$ -lactam antibiotic is restricted by an efflux system different from Pgp.

The finding of enalapril maleate raises concerns about the adequacy of the classification previously made where the cut-off  $P_{app}$  value of  $1 \times 10^{-6}$  cm/s seems very restrictive and the definition of a borderline region is more appropriate (Trapani et al., 2004). Taking into account that for carrier transported drug the range of  $P_{app}$  values is wider, we arbitrarily could set a drug in the borderline region when its  $P_{app}$  value ranges between

$1 \times 10^{-6}$  cm/s and  $3 \times 10^{-6}$  cm/s. Thus, enalapril maleate could be considered a borderline drug and prediction between *in vitro* frog intestinal sac system and FA become more uncertain.

To assess if glutathione permeability in frog intestine is a proton-dependent transport, the effect of the pH change (from pH 5.9 to pH 7.4) was studied. Results (Table 4) showed that there was a marked decrease in  $P_{app}$  value carrying out the experiment at higher pH, confirming so that there is a proton-dependent transport process involved in frog intestine.

Baclofen and L-dopa are substrates for the complex system of the AAT including both  $\text{Na}^+$ -dependent and  $\text{Na}^+$ -independent transport systems [the  $\text{Na}^+$ -stimulated (“pump”) ATPase activity] (Steffansen et al., 2004). This pump ATPase was observed also in frog intestinal epithelium (Csáky and Gallucci, 1977). To gain more information in this regard, the transport of L-dopa was assessed using a frog Ringer with or without  $\text{Na}^+$ . As shown in Table 4, the  $P_{app}$  value decreased significantly when the experiment was carried out in  $\text{Na}^+$ -free frog Ringer. Moreover, taking into account that the “pump ATPase” is specifically inhibited by Ouabain (Choi and Akera, 1977), the transport of baclofen and L-dopa was studied in the presence of 0.2 mM solutions of this digitalis steroid. Under these conditions, we found that the transport of both baclofen and L-dopa was markedly depressed (Table 4) indicating that  $\text{Na}^+$  is involved in the first step, namely at the luminal side, of the baclofen and L-dopa transport. Therefore transport of these drugs in frog intestine is a secondary active transport, i.e., there is no direct coupling with ATP energy, but the electrochemical potential difference due to pumping ions of the cell is utilized.

### 3.4. P-glycoprotein mediated efflux transporters

To evaluate the contribution of Pgp-mediated efflux system in determining the frog intestinal absorption of drugs, the transport of known Pgp substrates as Cimetidine and Nadolol was studied in the absence and presence of a specific Pgp inhibitor as verapamil (1 mM) (Sandström et al., 1998). Cimetidine is also reported to be substrate for the organic cation transporter 1 (Matsson et al., 2005). The mean permeability of Cimetidine and Nadolol in the absence of the inhibitor was found to be  $0.88 \pm 0.25 \times 10^{-6}$  cm/s and  $0.51 \pm 0.27 \times 10^{-6}$  cm/s, respectively. These values are consistent with the reported FA of 80% and 30%, respectively. In contrast, the mean permeability of the same drugs in the presence of the inhibitor significantly increased (i.e.,  $2.04 \pm 0.5 \times 10^{-6}$  cm/s and  $1.65 \pm 0.92 \times 10^{-6}$  cm/s, respectively). These results should be indicative of the functional activity of Pgp mediated efflux systems which can limit the absorption of some drugs in frog intestine.

In Fig. 2, the inhibition of the transport of benzylpenicillin, baclofen, and cimetidine in the presence of glucose, ouabain and Verapamil, respectively, is shown.

In Fig. 3, the  $\log P_{app}$  values observed for actively transported drugs were plotted versus the percentage of absorption in humans. As can be seen, an approximately sigmoidal relationship with a correlation coefficient ( $R^2$ ) of 0.883 was obtained. Although this correlation seems considerably strong, given the

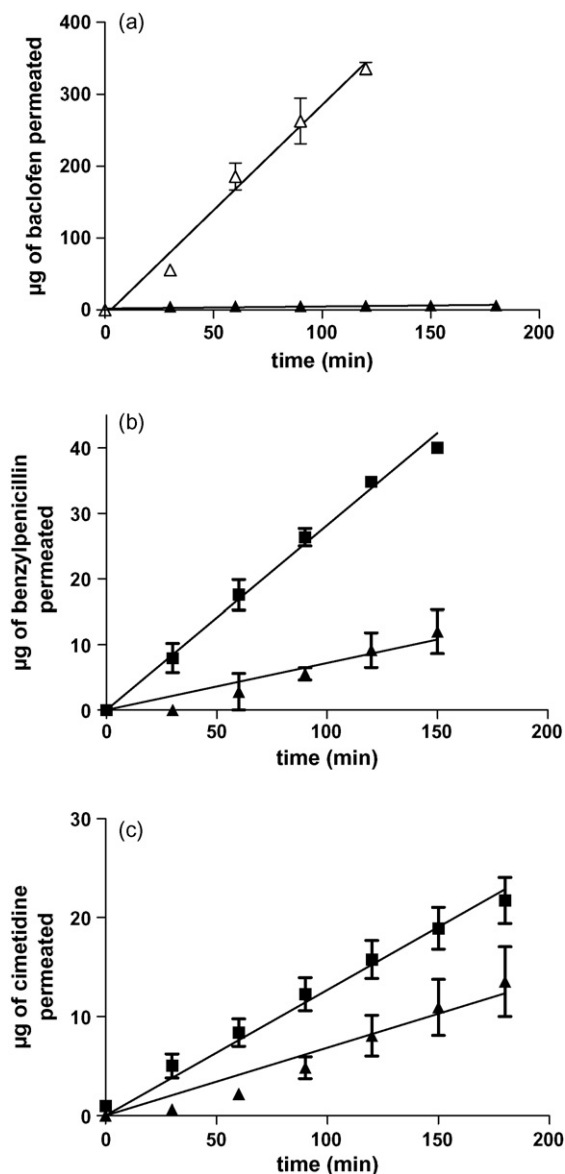


Fig. 2. (a) Permeability of baclofen without ( $\Delta$ ) and with ( $\blacktriangle$ ) ouabain  $2 \times 10^{-4}$  M; (b) permeability of benzylpenicillin without ( $\blacksquare$ ) and with ( $\blacktriangle$ ) glucose  $6 \times 10^{-3}$  M; (c) permeability of cimetidine without ( $\blacksquare$ ) and with ( $\blacktriangle$ ) verapamil  $1 \times 10^{-3}$  M.

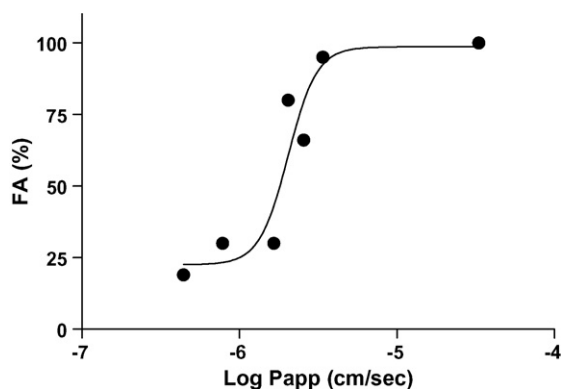


Fig. 3. Sigmoidal relationship between the fraction of drug absorbed in humans (Fa) and  $\log P_{app}$  values for the carrier-transported drugs examined in the frog intestinal sac.

limited data set points ( $n = 7$ ), further study is necessary to draw definitive conclusions in this regard.

#### 4. Conclusions

In this work, evidence for the expression of specific transporter systems in frog intestine was obtained on the basis of the effect of selected inhibitors. Although further studies are necessary to fully demonstrate that the compounds examined in this study (exhibiting significant active transport mechanisms in humans) are also carrier transported in frog intestine, results indicate that pharmaceutically relevant drug transporters should be also expressed in this last intestine. Moreover, it was also shown that prediction of the FA for the actively transported drugs in humans examined in this study may be successfully performed by using the *in vitro* frog intestinal sac system and high concentrations of drugs (in the millimolar range), at which probably saturation of the carriers occurs. In these cases, however, a borderline region characterized by  $P_{app}$  value between  $1 \times 10^{-6}$  cm/s and  $3 \times 10^{-6}$  cm/s should be considered. Therefore, drugs completely absorbed in humans are characterized by  $P_{app}$  values greater than  $3 \times 10^{-6}$  cm/s,  $P_{app}$  values lower than  $1 \times 10^{-6}$  cm/s characterize drugs absorbed  $<90\%$ , while for drugs actively absorbed with  $P_{app}$  value between  $1 \times 10^{-6}$  cm/s and  $3 \times 10^{-6}$  cm/s (borderline region) prediction of FA become more uncertain by the *in vitro* frog intestinal sac system. Moreover, it should also be taken into account that, when the method is applied to carrier transported drugs, a limitation arises from the seasonal variation in the expression of transporter systems of the frog intestinal epithelium and consequently a certain intra-laboratory variation could occur (Csàky and Gallucci, 1977). In this regard, it is advisable to carry out the transport experiments when the animals are not forced in hibernation and the active intestinal transport ability is restored. Finally, for the sake of completion and in addition to the previously mentioned distinct advantages of this new method for assessing intestinal permeability (Trapani et al., 2004) one should add some remarks. The presence of an apical mucus layer in the frog intestine is an advantage as it may influence the absorption of compounds. Moreover, the small size and the relative ease of maintaining large stocks of animals enable cost-effective screening of numerous compounds. It may represent a valuable alternative to the low speed and high cost of conventional animal models (typically rodents) for the assessment of intestinal permeability.

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