

Transepithelial transport of ambroxol hydrochloride across human intestinal Caco-2 cell monolayers

Věra Štětínová, Libuše Smetanová, Dagmar Kholová, Zbyněk Svoboda and Jaroslav Květina

Institute of Experimental Biopharmaceutics, Joint Research Centre of the Academy of Sciences of the Czech Republic and PRO.MED.CS Praha a.s., Heyrovského 1207, 500 03 Hradec Králové, Czech Republic

Abstract. This study aimed i) to characterize the transepithelial transport of the mucolytic agent ambroxol hydrochloride across the intestinal barrier, ii) to classify the ambroxol according to Biopharmaceutics Classification System (BCS) and iii) to predict ambroxol absorption in humans. Transport of ambroxol (100, 300 and 1000 $\mu\text{mol/l}$) was studied in a human colon carcinoma cell line Caco-2 in apical to basolateral and basolateral to apical direction, under iso-pH 7.4 and pH-gradient (6 vs. 7.4) conditions. The relative contribution of the paracellular route was estimated using Ca^{2+} -free transport medium. Ambroxol samples from receiver compartments were analysed by HPLC with UV detection (242 nm).

Results showed that ambroxol transport is linear with time, pH-dependent and direction-independent, displays non-saturable (first-order) kinetics. Thus, the transport seems to be transcellular mediated by passive diffusion. Estimated high solubility and high permeability ($P_{\text{app}} = 45 \times 10^{-6}$ cm/s) of ambroxol rank it among well absorbed compounds and class I of BCS. It can be expected that the oral dose fraction of ambroxol absorbed in human intestine is high.

Key words: Ambroxol — Caco-2 cells — Transport — Biopharmaceutics Classification System

Introduction

Ambroxol (2-amino-3,5-dibromo-*N*-[trans-4 hydroxycyclohexyl]benzylamine) is a metabolite of bromhexine used in the treatment of respiratory disorders with productive cough. The major pharmacodynamic actions of ambroxol are surfactant stimulation, mucokinetic and secretagogue activity (Püschmann and Engelhorn 1978; Disse 1987; Malerba and Ragnoli 2008). In addition to the mucolytic activity, ambroxol possesses antioxidant and antiinflammatory properties (Štětínová et al. 2004).

The aim of the present study was to investigate permeability and transepithelial transport mechanisms of the orally administered ambroxol hydrochloride across the human intestinal barrier. Although there are many factors that limit bioavailability of drug, the intestinal epithelium has proved in many cases to be a rate-limiting factor for absorption of orally administered drug molecules (Artursson and Bergström

2004). For this reason, the transepithelial flux of ambroxol was studied using the human colonic carcinoma cell line Caco-2, a standard model of human intestinal absorption as an *in vitro* tool for prediction of absorption in humans (Artursson et al. 2001). The objectives of this study were: i) to determine whether the ambroxol transport across the Caco-2 monolayer involved an active or a passive mechanism, ii) to evaluate the transcellular and paracellular pathway involves in ambroxol transport, iii) to determine effects of the pH on ambroxol transport, iv) to classify ambroxol according to Biopharmaceutics Classification System (BCS) (Amidon et al. 1995), and v) to predict ambroxol absorption in humans. BCS was devised to allow prediction of pharmacokinetic performance of drug products based on their permeability and solubility which are the fundamental parameters controlling rate and extent of drug absorption (Wu and Benet 2005).

Materials and Methods

Materials

Ambroxol hydrochloride was purchased from Erregierre S.p.A. (Italy). Phenol red, 4-(2-hydroxyethyl)piperazine-1-

Correspondence to: Věra Štětínová, Institute of Experimental Biopharmaceutics, Joint Research Centre of the Academy of Sciences of the Czech Republic and PRO.MED.CS Praha a.s., Heyrovského 1207, Hradec Králové 500 03, Czech Republic
E-mail: stetinova@uebf.cas.cz

ethanesulfonic acid (HEPES), 2-morpholinoethanesulfonic acid (MES), acetonitrile, methanol, dextromethorphan and scintillation liquid (Universal LSC cocktail) were supplied by Sigma-Aldrich (Praha, Czech Republic). Ammonium acetate and formic acid 98–100% were from Merck (Říčany, Czech Republic). Dulbecco's modified Eagle's medium (DMEM) with high glucose, Hanks' balanced salt solution (HBSS) with/without Ca^{2+} , Dulbecco's phosphate buffered salt solution (DPBS), trypsin-EDTA (1 : 250), antibiotic-antimycotic solution were obtained from PAA Laboratories (BioTech, Praha, Czech Republic). Fetal bovine serum was purchased from Gibco Invitrogen (KRD Molecular Technologies, Praha, Czech Republic) and ^{14}C -mannitol (100 $\mu\text{Ci/ml}$) from Moravěk Biochemicals and Radiochemicals (M.G.P. Zlín, Czech Republic).

Cell culture

The Caco-2 cell line was purchased from the European collection of cell culture, Sigma-Aldrich (Praha, Czech Republic) and was used between passages 67 and 77. The cells were routinely grown (Bourdet and Thakker 2006) in plastic tissue culture flasks (75 cm^2 growth area, TPP AG, Switzerland) in DMEM containing 25 mmol/l glucose and supplemented with 4 mmol/l L-glutamine, 10% fetal bovine serum, 1% non-essential amino acids, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 0.25 μg amphotericin. Cell line was cultured at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity, and passaged at about 90% confluence, using 0.05% trypsin (1 : 250) – 0.02% EDTA in calcium-free and magnesium-free DPBS. The medium was changed three times a week.

Incubation of Caco-2 cells in polycarbonate well inserts

For ambroxol transport experiments, the cells were seeded onto polycarbonate filter cell culture chamber inserts (dimensions 13 × 11 mm, area available for growth 0.5 cm^2 , pore diameter 0.4 μm) of tissue culture plates containing 24 wells (TPP AG, Switzerland) at the density of 2.5×10^5 cells/ cm^2 . Medium was changed three times a week for 21–25 days, and daily during the last 3–5 days (500 μl to the insert compartment – the apical side, and 650 μl to the well compartment – the basolateral side). Caco-2 cells were grown for at least 21 days to allow formation physiologically and morphologically (polarization, differentiation) well developed confluent cell monolayers prior to initiating drug transport studies (Hidalgo et al. 1989; Vachon and Beaulieu 1992; Walle and Walle 1998).

Monolayer integrity

Two or three days before start of the experiment the monolayer integrity of each insert was checked by 500 $\mu\text{mol/l}$

phenol red permeability (Fleet and Wood 1999; Garcia-Casal et al. 2000). After 1 h incubation of phenol red added to the apical side, 500 μl sample from each insert was taken from basolateral side and measured by spectrophotometer Spekol 11 at 558 nm.

The integrity of the monolayer during the transport studies was confirmed simultaneously by ^{14}C -mannitol (0.5 $\mu\text{Ci/ml}$) permeability (Baird and Prosser 1998) done at least in triplicate. 50 μl of apical or 500 μl of basolateral samples were mixed with 10 ml of the scintillation liquid and counted in the Beckman liquid scintillation analyser (LS 5000TD) for 1 min.

Transport studies

Transport experiments were performed with HBSS transport medium (Walle and Walle 1998; Ingels and Augustijns 2003) buffered with 25 mmol/l HEPES (pH 7.4) or 25 mmol/l MES (pH 6.0). The prepared Caco-2 cell inserts were rinsed twice with prewarm HBSS and equilibrated with HBSS at 37°C for 30 min before the transport experiments (Walle and Walle 1998).

The transport experiments from apical to basolateral (AP-BL) side were initiated by replacing the transport medium with the diluted ambroxol (100, 300 and 1000 $\mu\text{mol/l}$) in the transport medium (550 μl) on the apical side. The samples of 500 μl from the basolateral compartment was withdrawn at 30, 60, 90 and 120 min for high performance liquid chromatography (HPLC) analysis and the same volume of the prewarmed transport medium was added to the basolateral compartment. Between sampling, the cells were kept in an incubator at 37°C and 5% CO_2 .

Similarly, for basolateral to apical (BL-AP) transport, the drug was added to the basolateral compartment (650 μl) and the volume of 300 μl was withdrawn from the receiver side in the same time intervals.

All used solutions and transport media were sterile filtered just before the experiments. All experiments were carried out under sink conditions so that the concentrations of the drug in the receiver compartment would not exceed 10% of applied dose in the donor side.

To determine paracellular component of the ambroxol transport (300 $\mu\text{mol/l}$) the HBSS Ca^{2+} -free was used (for washes, 30 min preincubation and for the transport study).

Design of the experiments

- AP-BL transport – absorptive direction (iso-pH 7.4 = pH 7.4 in both sides),
- BL-AP transport – secretory direction (iso-pH 7.4),
- AP-BL transport (pH 6 apical/pH 7.4 basolateral),
- AP-BL transport (pH 7.4 in both sides) with the Ca^{2+} -free transport medium.

HPLC analysis of ambroxol

The quantification of ambroxol was performed by HPLC. The modified method of Su et al. (2007) was used. Chromatographic separation was performed on an analytical column Discovery®C18 (250 × 4.6 mm, 5 μm; Supelco, USA) with mobile phase consisting of 30 mmol/l ammonium acetate (0.4% formic acid)-acetonitrile (64 : 36, v/v) at a flow-rate of 1 ml/min, the total run 7 min for each sample. Samples were diluted with mobile phase. Dextromethorphan was used as an internal standard. The detection and quantification was performed by UV detection at 242 nm (ambroxol) and 280 nm (dextromethorphan). The peak area was measured and the peak area ratio of ambroxol to internal standard was calculated. The linear calibration curve was assembled within the concentration range of 0.25–100 μmol/l ($r > 0.999$). Samples were stored at –20°C until HPLC analysis.

Data analysis

Cumulative transport of ambroxol

Calculated cumulative amount of ambroxol (nmol) were plotted *versus* time.

Percent transport of ambroxol

Percent transport was calculated as the ratio of the cumulative concentration in the receiver chamber to the concentration in the donor chamber ×100. Percent transport was plotted *versus* time.

Calculation of the apparent permeability coefficient

The apparent permeability coefficient (P_{app} , cm/s) was calculated according to the equation (Artursson and Karlsson 1991):

$$P_{app} = (dQ/dt) \times (1/A \times C_0)$$

where dQ/dt is the permeability rate, the amount of drug appearing in the receiver compartment in function of time (nmol/s), C_0 is the initial concentration in the donor chamber (nmol/ml), and A is the surface area of the monolayer (cm²).

Calculation of the flux rate

The flux rate (nmol/min/cm²) of the ambroxol was determined from the slope of the plot of the cumulatively transported amount *versus* time using linear regression analysis and the flux rates were plotted *versus* ambroxol concentrations to investigate whether the flux rate is concentration-dependent/independent.

Evaluation of the bidirectional flux

Transport in both directions across monolayer enables to calculate an uptake or efflux ratio (Ungell and Karlsson 2004):

$$P_{app} \text{ uptake ratio} = P_{app} \text{ AP-BL} / P_{app} \text{ BL-AP}$$

$$P_{app} \text{ efflux ratio} = P_{app} \text{ BL-AP} / P_{app} \text{ AP-BL}$$

Calculation of recovery

Recovery (mass balance) was calculated using the following equation:

$$\text{recovery (\%)} = [(M_{r-120} + M_{d-120}) / M_{d-0}] \times 100$$

where M_{r-120} is the cumulative amount of drug transported to the receiver side at the end of the experiment, M_{d-120} is the amount of drug remaining on the donor side at the end of the experiment, M_{d-0} is the amount of drug on the donor side at the start of the experiment (Ungell and Karlsson 2004).

Statistical analysis

All values are represented as mean ± SD (standard deviation). Statistical differences were determined using Kruskal-Wallis one way analysis of variance (ANOVA) with Mann-Whitney U test for double comparison or a Student's *t*-test. The differences were considered significant when $p < 0.05$. All treatments were carried out at least in triplicate.

Results

Ambroxol solubility

Equilibrium solubility (Yazdanian et al. 2004) was 452 μg/ml (1085 μmol/l) in the transport medium HBSS (pH 7.4). Our results of dose-relative solubility (Yazdanian et al. 2004) determined according to FDA (Food and Drug Administration) guidance on BCS ranged ambroxol among highly soluble drugs (Polli et al. 2004) as the highest dose strength (60 mg) is soluble in 250 ml over the pH range 1–7.5.

The assessment of the monolayer integrity

The P_{app} value of phenol red after 1 h was $0.82 \pm 0.28 \times 10^{-6}$ cm/s and percent cumulative transport reached $0.23 \pm 0.08\%$. The phenol red recovery was $108 \pm 2.4\%$.

The P_{app} of mannitol after 2 h was $2.26 \pm 0.62 \times 10^{-6}$ cm/s and corresponded to the percent cumulative transport $1.63 \pm 0.44\%$. The ¹⁴C-mannitol recovery was $107 \pm 2.2\%$. There was no effect of ambroxol on the P_{app} of ¹⁴C-mannitol (Table 1).

Table 1. Permeability, percent transport and efflux ratio of ambroxol across Caco-2 monolayer. Transport was observed in the AP-BL and BL-AP directions under iso-pH 7.4 and pH-gradient (6 vs. 7.4) conditions.

Compound concentration	P_{app} (cm/s $\times 10^{-6}$)		Percent transport (%)		Efflux ratio
	AP-BL pH 7.4-7.4	BL-AP pH 7.4-7.4	AP-BL pH 7.4-7.4	BL-AP pH 7.4-7.4	
Phenol red	0.82 \pm 0.28		0.23 \pm 0.08		
Man	2.26 \pm 0.62		1.63 \pm 0.44		
Man+Amb (300 μ mol/l)	2.10 \pm 0.25				
Amb (100 μ mol/l)	37.75 \pm 1.15	34.30 \pm 0.48	27.18 \pm 0.67	19.02 \pm 0.22	0.90
Amb (300 μ mol/l)	43.10 \pm 3.39 ^{*1)}	43.52 \pm 2.35 ^{*1)}	30.92 \pm 2.65	24.10 \pm 1.24 ^{*1)+}	1.01
Amb (1000 μ mol/l)	45.90 \pm 0.95	43.27 \pm 5.17	33.05 \pm 0.56	23.97 \pm 2.34	0.94
Amb (300 μ mol/l)	AP-BL pH 6-7.4		AP-BL pH 6-7.4		
	5.08 \pm 0.75 [#]		3.66 \pm 0.38 [#]		

Effect of ambroxol concentration on P_{app} values and percent transport. * significant differences among ambroxol concentrations: ^{*1)} 100 vs. 300 μ mol/l, ^{*2)} 100 vs. 1000 μ mol/l (non significant), ^{*3)} 300 vs. 1000 μ mol/l (non significant). Ambroxol P_{app} values and percent transport AP-BL vs. BL-AP: + significant differences between AP-BL and BL-AP directions. Effect of pH conditions on ambroxol P_{app} values and percent transport: # significant difference in comparison with ambroxol (300 μ mol/l) at iso-pH condition. Man, mannitol; Amb, ambroxol. Efflux ratio = P_{app} BL-AP / P_{app} AP-BL. Values are represented as mean \pm SD, $p < 0.05$.

Transport studies

Cumulative transport of ambroxol

The cumulative transport of ambroxol was linear for up to 2 h over the concentration range examined and was pH-dependent (Fig. 1).

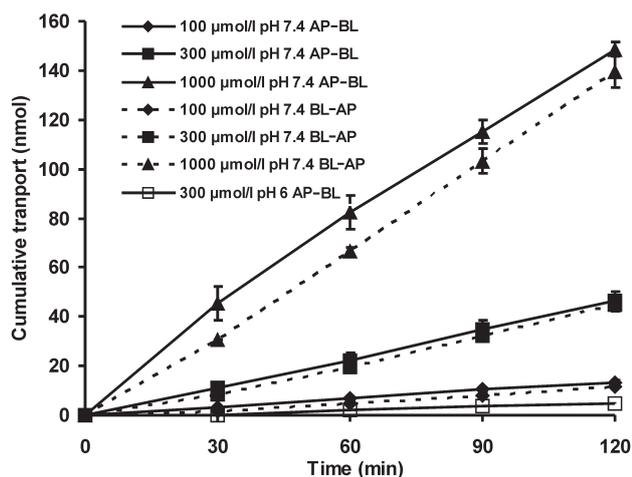


Figure 1. Time course of cumulative bidirectional transport: apical-basolateral (AP-BL; solid line) and basolateral-apical (BL-AP; dashed line) of ambroxol (100, 300 and 1000 μ mol/l) under iso-pH 7.4 and pH-gradient (6 vs. 7.4) conditions. Each point represents the mean \pm SD of cumulative transport across Caco-2 monolayers.

Percent transport of ambroxol

The transported cumulative amount of ambroxol (100, 300 and 1000 μ mol/l) expressed as a percentage of the amount added to the donor chamber ranged from 19 to 33% (Table 1). Percent transport in BL-AP direction was slightly lower than

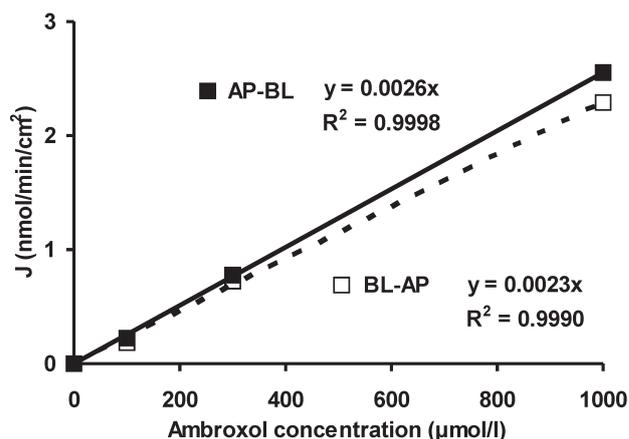


Figure 2. Comparison of AP-BL and BL-AP transport of ambroxol across Caco-2 monolayers. The flux rate J was determined in the time course (30–120 min) of AP-BL or BL-AP transport at various concentrations. The transport was characterized by linear regression analysis resulted in linear equation $y = kx$ and coefficient of determination R^2 .

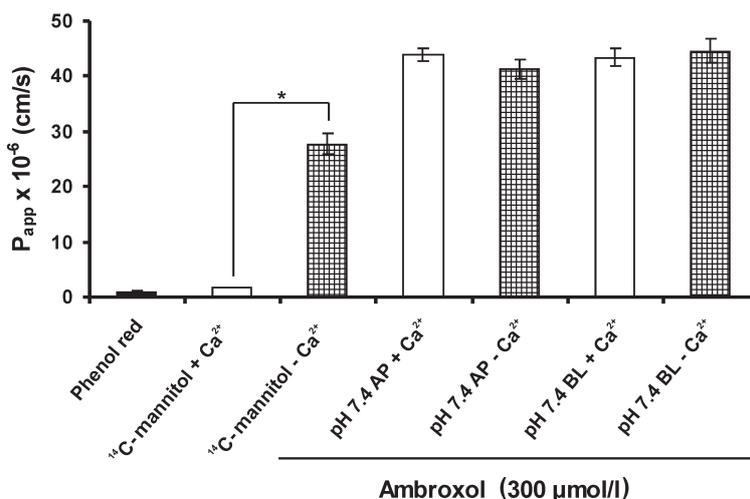


Figure 3. Ambroxol and ^{14}C -mannitol (a marker of paracellular transport) permeability AP-BL in absence or presence of Ca^{2+} in transport medium. * significantly different from values obtained in the presence of Ca^{2+} . Each column represents the mean \pm SD of permeability coefficient (P_{app}), $p < 0.05$.

AP-BL transport. Under the pH-gradient (pH 6 apical), the transported amount of ambroxol was significantly decreased and the percent transport reached only 3.7% (Table 1).

Calculation of P_{app}

P_{app} of ambroxol (Table 1) was independent of concentration over the entire concentration range (100–1000 $\mu\text{mol/l}$). The ambroxol P_{app} was $(43.1\text{--}45.9) \times 10^{-6}$ cm/s at the concentration of 300 and 1000 $\mu\text{mol/l}$ and slightly lower (37.7×10^{-6} cm/s) at 100 $\mu\text{mol/l}$. Furthermore, no differences in the P_{app} of ambroxol in both directions at doses of 100, 300, 1000 $\mu\text{mol/l}$ were observed and the calculation of the efflux ratio oscillated around 1. At pH 6 in the donor apical chamber, the P_{app} of ambroxol decreased approximately 8-times and was 5.1×10^{-6} cm/s (Table 1).

Calculation of the flux rate

The range of concentrations used (100–1000 $\mu\text{mol/l}$) was limited by the lack of ambroxol solubility in the transport medium (1085 $\mu\text{mol/l}$). AP-BL and BL-AP fluxes were similar (2.6 and 2.3 nmol/min/cm² at 1000 $\mu\text{mol/l}$). The fluxes were a simple linear function of ambroxol concentrations, indicating passive diffusion (Fig. 2).

Recovery

The ambroxol recovery defined as the amount recovered in the apical and basolateral compartment at the end of the experiment oscillated about 90–95% for both directions.

Evaluation of the paracellular transport

The transport of the paracellularly transported ^{14}C -mannitol was significantly enhanced 16-times by the use of HBSS Ca^{2+} -free transport medium (27.7×10^{-6} cm/s) in comparison with transport medium with Ca^{2+} . However, the P_{app} of ambroxol in both transport directions was not changed (Fig. 3) and oscillated about 43×10^{-6} cm/s.

Discussion

BCS devised by Amidon et al. (1995) categorizes drugs into four classes according to their solubility and permeability which are the fundamental parameters controlling rate and extent of drug absorption.

To predict the oral absorption of drugs in humans, the drug permeability determined in Caco-2 cell monolayers (one of the *in vitro* models for studying membrane permeability and oral absorption) is used (Van de Waterbeemd 2005). Despite the well documented activity and pharmacokinetics of ambroxol after oral administration, there are few published data available on the exact transport mechanisms. Thus, the purpose of this study was to characterize the transepithelial transport mechanisms of ambroxol across the Caco-2 cell monolayer system as a model of human intestinal absorption *in vitro*.

The transport parameters of ambroxol were monitored for 2 h (corresponding to time to reach ambroxol peak plasma concentration in humans) (Vergin et al. 1985) in the AP-BL (absorptive), as well as in the BL-AP (secretive) direction. No superiority in any transport direction was seen. The cumula-

tive amount transported with respect to time showed that the transport was linear with time in both directions, and was concentration- and pH-dependent. Furthermore, similar cumulative percent transport plotted *versus* time for all tested ambroxol concentrations (100, 300 and 1000 $\mu\text{mol/l}$) and for both directions signalized the passive, non-saturable process. Small differences observed (slightly lower percent transport from basolateral compartment than from apical compartment) may be a consequence of differences in the surface areas of the apical and basolateral side of the monolayer and volume differences of the donor compartments.

Our findings of similar P_{app} values and the efflux ratio (0.9–1) over the ambroxol concentration range (100–1000 $\mu\text{mol/l}$) and no observed difference between the flux rates in the two opposite directions also suggested a passive diffusion mechanism of the ambroxol transport across the Caco-2 monolayers.

As hydrochloride form of ambroxol was used, the possibility of paracellular transport was supposed. However, the molecular weight of ambroxol hydrochloride 414.6 is a limiting factor for this transport pathway. The molecular weight limit for the Caco-2 monolayer seems to be 300 g/mol (Ungell and Karlsson 2004). Also the high value of ambroxol P_{app} (45×10^{-6} cm/s) indicated transcellular transport. In general, compounds limited to paracellular transport are not efficiently absorbed across the Caco-2 monolayer due to the small available absorptive area and the restriction caused by tight junctions (Ungell and Karlsson 2004).

The assessment of the paracellular component of the transport in the Caco-2 cells can be studied with opening the tight junctions by using the Ca^{2+} -free transport medium (Delie and Rubas 1997; Ungell and Karlsson 2004; McMillan et al. 2005). When this Ca^{2+} -free medium was used, the P_{app} of ^{14}C -mannitol (positive control for the paracellular pathway) was significantly increased but the P_{app} of ambroxol was not enhanced. According to this fact it can be concluded that there is no evidence for the paracellular mechanism of ambroxol transport.

We also studied the effect of pH that is a very important factor affecting absorption of ionizable drugs in the gastrointestinal tract. pH demonstrates two main effects on drugs: 1. the effect on the degree of dissociation of weak electrolytes and thereby affection of the passive compound diffusion, and 2. the effect on the establishment of a proton gradient as a driving force for some transporters. The pH of the apical solution has a direct impact on the transport experiments using monolayers, since the solution is in direct contact with the membrane and should therefore mimic the microclimate pH. Our experimental schedule applying pH gradient (6.0 vs. 7.4) better reflect the gradient under physiological condition and also reflect the absorption across the jejunum (the main part of absorption of most drugs) (Ungell and Karlsson 2004). The passive diffusion of ambroxol as a weak base was

enormously decreased at pH 6. Obtained results are in a good agreement with the theoretical assumption of transport mechanisms in gastrointestinal tract for weak bases in acid conditions: lowering of the pH increases ionization of weak bases and decreases their permeability.

Taken together obtained results showed that the transport of ambroxol seems to be transcellular mediated by passive diffusion.

Determined high permeability of ambroxol and dose-relative high solubility enable to classify ambroxol as class I of BCS.

Regarding prediction of ambroxol fraction absorbed, transport rates of passively absorbed drugs in the Caco-2 cells have been shown to correlate with the fraction absorbed in humans (Artursson and Karlsson 1991; Lennernäs et al. 1996). The high P_{app} of ambroxol (45×10^{-6} cm/s) ranged ambroxol among well-absorbed compounds (Yee 1997). From the nomogram (Ungell and Karlsson 2004) showing the oral fraction of different drugs absorbed in humans plotted *versus* their P_{app} obtained in Caco-2 cell monolayers it can be approximated that the fraction of ambroxol absorbed in humans is high, reaching almost 100%. Predicted ambroxol absorbed fraction corresponds with the high absolute bioavailability 70–83% reached in human studies (lower bioavailability of ambroxol determined in human studies – in spite of its rapid and extensive absorption after oral administration – is probably caused by hepatic first-pass effect (Hammer et al. 1978; Vergin et al. 1985).

Conclusion

The results of our experiments in the Caco-2 monolayer model showed that the ambroxol transport is linear with time, pH-dependent and direction-independent, displays non-saturable (first-order) kinetics. Thus, the transport of ambroxol seems to follow the transcellular passive diffusion. The high solubility and high permeability classifying ambroxol as class I of BCS indicates a well-absorbed compound. It can be supposed that the oral dose fraction of ambroxol absorbed in humans is high.

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