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Transport of cephalosporins across artificial membranes and rabbit ileum

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Abstract

The aim of this study is to determine transepithelial transport of aminocephalosporins (cephalexin, cefaclor and cefadroxil) with in vitro models using artificial and biological membranes. The three antibiotics have higher partition coefficient (K_p) at pH 5 than at pH 6 and cephalexin passes through the lipid barrier faster than cefadroxil and cefaclor. With a cephalexin concentration of 1 mM, the diffusion rate constant (K_d) through the artificial intestinal barrier was 0.563 ± 0.001 and 0.426 ± 0.031 ($\times 10^{-3}$ cm/min) at pH 5 and 6, respectively. The transepithelial passage of the three aminocephalosporins through rabbit ileum tissues is measured in an Ussing chamber. The mean transepithelial mucosal to serosal fluxes (Φ_{m-s}) were 52.54 ± 6.45 nmol/h/cm^2 and 27.51 ± 2.43 nmol/h/cm^2 for cephalexin and cefadroxil, respectively. No effect on intensity short-circuit (I_{sc}) and conductance (G) of the tissue were observed with cephalexin. In Grass cell diffusion with pH ranged from 6 to 7.4, showed a maximal cephalexin transport at pH 6. The concentration dependence of cephalexin absorption and transport was examined over a concentration range of 0.1–5 mM. The cephalexin transport involves Michaelis–Menten kinetics. Moreover, D-glucose does not affect the cephalexin (CFX) flux across the ileal tissue. Copyright © 1996 Elsevier Science B.V.

Keywords: α-amino group; Artificial membranes; Epithelial transport; Passive diffusion; Michaelis–Menten kinetic; pH-dependence

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1. Introduction

Aminocephalosporins are amphoteric drugs which are orally absorbed in man. As these antibiotics have an important bioavailability (Pfeffer et al., 1977; Lode et al., 1980), their transport mechanism has been therefore extensively investigated (Kimura et al., 1985; Iseki et al., 1988; Yoshikawa et al., 1989; Dantzig and Bergin, 1990; Sugawara et al., 1991; Gochoco et al., 1994). Concerning oral β-lactams, different authors suggest an absorption obedient to Michaelis–Menten kinetics at lower doses and a kinetic order 1 at higher concentrations (Tsuji et al., 1987). As shown in Fig. 1, the drug possesses some structural features in common with dipeptides, most notably an α-amino group, a terminal carboxylic acid group, and the presence of a peptide bond. The intestinal absorption of natural di- and tripeptide has been demonstrated to be a pH-dependent process, mediated by a di-/tripeptides carrier located on the apical membrane of small intestinal absorptive cells. The pH gradient required for the absorption of these molecules is generated by the combined action of a brush-border membrane Na^+/H^+ exchanger and a basolateral N^+/K^+-ATP_{ase} membrane (Ganapathy and Leibach, 1985). The di-/tripeptide carrier is also believed to mediate the transport of a number of structurally related molecules including β-lactam and cephalosporin antibiotics (Nakashima et al., 1984; Kramer et al., 1990). Much of the current knowledge on peptide transporters has been obtained from studies of intestinal brush border membrane vesicles (BBMV) (Ganapathy and Leibach, 1985). Nevertheless studies of BBMV provide no information on the mechanism involved in peptide efflux across the basolateral membrane of enterocytes. Other authors used the Caco-2 and HT-29 cells (Dantzig and Bergin, 1988, 1990; Inui et al., 1992), which are models that have been characterized by a polarized intestinal epithelium (Hidalgo et al., 1989; Wilson et al., 1990) and shown to possess the di-/tripeptides carrier (Dantzig and Bergin, 1990). Many reports have been made in the literature about the carrier mediated transport of cephalosporins with brush border membrane vesicles and the human intestinal cell line. Few authors such as Gochoco et al. (1994) have studied the transport of cephalexin in single layers of Caco-2 cells to examine the relative contribution of paracellular and transcellular fluxes to transepithelial transport of this drug. On the other hand, in this field, no experiment has been described with in vitro models using artificial and biological membranes.

The purpose of this study has been to test different in vitro models to simulate the transepithelial transport of cephalexin through intestinal barriers. Passive diffusion studies are conducted using the Stricker absorption simulator and the partition coefficients within an artificial lipid barrier are determined, to compare the diffusion rate (K_d) and the partition coefficient (K_p) of cephalexin with cefadroxil and cefaclor at different values of pH. Rabbit ileum isolated in an Ussing chamber or a Grass cell allows the total transepithelial transport (transcellular, paracellular diffusion and carrier mediated transport) to be assessed. The monitoring of electrical parameters, as well as the influence of pH and concentration on cephalexin transport are investigated for biological membranes. Moreover, experiments are carried out to evaluate the relative importance of the paracellular transport. An important goal of in vitro transport models is to simulate the same conditions as in vivo and to try to predict in vivo transport of cephalexin.

2. Materials and methods

2.1. Tested drugs

Cephalexin (CFX) and cefaclor (CFC) were kindly provided by Elli Lilly. Cefadroxil (CDX) was purchased from Sigma Chemical (St. Quentin Fallavier, France).

These antibiotics were orally absorbed in man and belong to aminocephalosporins with α-amino group (Fig. 1).

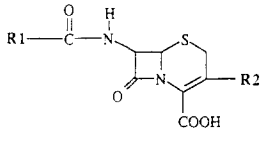
2.2. Determination of the apparent partition coefficient

2.2.1. Chemicals

Dodecanol-1 (lauric alcohol) and octanoic acid (caprylic acid) making up the lipidic phases were purchased from Prolabo (Paris, France). The aqueous phases used for the determination of the partition coefficient and diffusion rate constant were prepared and composed with a phosphate buffer containing Na_2HPO_4 and KH_2PO_4 at different rates for different pH values studied (5, 6, 7.4).

2.2.2. K_p determination

The cephalosporin partition coefficient between the lipid and aqueous phases was determined previously as described by Reese et al. (1964). It was determined at 37 ± 1°C in a stoppered pyrex tube with horizontal shaking at 180 rpm (Shaker Baudard) (France) containing 5 ml of the buffered solution (phosphate buffer) added with the antibiotic at 1 mM and 3 ml lipid phase, for 90 min. The assays were determined at pH 5 and 6. The lipid phase consisted of a mixture of dodecanol + octanoic acid (0.92/4, w/w). After shaking, the two phases were separated by centrifugation; the antibiotic concentration in the aqueous phase was determined through spectrophotometer measurements (Spectronic 1001).



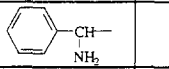
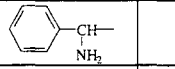
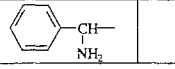
DRUGS	R1	R2
Cephalexin $pKa_1 = 2.56$ $pKa_2 = 6.88$		CH_3
Cefaclor $pKa_1 = 1.5$ $pKa_2 = 7.1$		Cl
Cefadroxil $pKa_1 = 1.4$ $pKa_2 = 7.5$		CH_3

Fig. 1. Chemical structures of cephalosporin antibiotics.

2.2.3. Calculations

The partition coefficient (K_p) was calculated from

$$K_p = 1.66 \times C'_0(C_0 - C'_0)$$

where C_0 is the drug concentration in the aqueous phase before shaking, C'_0 is the antibiotic concentration at 90 min. The volume of the aqueous phase was 5 ml and the volume of the lipid phase was 3 ml.

2.3. Intestinal absorption simulator

2.3.1. Material and apparatus

This model, described by Decroix and Chaumeil (1989) allows the diffusion behavior of organic substances to be studied under conditions simulating those in the intestinal tract. The essential part of this device is the artificial lipid barrier, which has a permeability towards 'passively' transported drugs similar to that of the natural intestinal membrane. Therefore, it allows an in vitro determination of the absorption. The Sartorius absorption simulator SM 16750, consists of two 130 ml compartments containing a buffered phase. The first simulated either of the intestinal phases (pH 5 or 6) and the second one, the plasmatic phase (pH 7.4). The composition of these aqueous buffers were the same as those used to determine the partition coefficient. These two compartments were divided by a lipid barrier and connected through a peristaltic pump. The lipid barrier comprises a membrane filter made of cellulose nitrate with a 1 μm pore size (Sartorius, France) and wetted with a mixture of dodecanol and caprylic acid in the same proportions as those used for partition coefficient determination.

2.3.2. Experimental procedure and calculations

The apparatus requires a period of about 30 min to reach the experimental temperature (39 ± 1°C) and phase equilibrium. After this period the drug was dissolved at 1 mM in the first container at pH 5 or 6 and the experimental procedure consisted essentially of monitoring the cephalosporin concentration changes with time in the two compartments. The plasmatic phase was bound to a spectrophotometric recorder which

provided a direct measurement of drug concentration increase in this compartment. The experiment duration was 2 h.

The diffusion rate constant (K_d) according to Stricker's experiments (Stricker, 1973) through the artificial gastro-intestinal barrier was calculated from the initial rate:

$$K_d = \frac{(C_{p2} - C_{p1})}{T_2 - T_1} \times \frac{V}{C_{i0}A} \quad [\text{cm/min}]$$

where C_{p1} is the substance concentration in the plasmatic phase at time T_1 (mM); C_{p2}, the substance concentration in the plasmatic phase at time T_2 (mM); C_{i0}, the initial concentration in the intestinal compartment (mM); V, the volume of the plasmatic phase (110 ml); A, the barrier area (80 cm^2); and T_x, the time (min).

2.4. Diffusion cell experiments and transport studies

2.4.1. Chemicals

The intestinal and plasmatic phases were reproduced using an isotonic Ringer solution (containing the following (in mM): 140 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 120 Cl^-, 25 HCO_3^-, 2.4 HPO_4^- and 0.4 $H_2PO_4^-$), at pH 7.4 with 95% O_2–5% CO_2.

Dulbecco's buffer solution (phosphate buffer saline (PBS)) was prepared from a 10 × solution (Gibco, Life Technology, SARL, France). After dilution, this buffer contained the following components, (in mmol/l): 137 NaCl, 3 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$.

The pH was adjusted to 6, 7, and 7.4 with concentrated NaOH or HCl.

D-glucose was purchased from Sigma Chemical (St. Quentin Fallavier, France).

2.4.2. Transport experiment

Male New Zealand white rabbits weighing 2.3–2.8 kg were fasted for 24 h and killed by an intravenous pentobarbital sodium injection (Sanofi, France). A portion of distal ileum ending approximately 10 cm proximal to the ileoappendiceal attachment was removed and prepared for study through rinsing free of intestinal contents, removal of the serosa, muscularis propria (Stripping), taking off Peyer's patches described previously (Donowitz et al., 1980a,b) and opening along the mesenteric border. Four to six pieces of tissue from each animal were studied simultaneously by being mounted as flat sheet between two half Ussing chambers (Ussing and Zerhan, 1951; Shultz and Zalusky, 1964a,b) or Grass diffusion cells (Precision Instrument Design, Los Altos, CA, USA) (Grass and Sweetana, 1988) with a surface area of 3.14 and 2.52 cm^2, respectively; attached to separate mucosal and serosal reservoirs.

The tissue was immersed on each side into 12 or 7 ml of a Ringer solution in an Ussing chamber and Grass diffusion cells, respectively, and oxygenated (O_2/CO_2 in 95/5, v/v). The bubbling was adjusted to ensure a permanent blending of the solution in the two compartments. All experiments were conducted at 37°C using a constant-temperature bath connection passing through a water jacket for both models. The electrical parameters were studied only for the Ussing chamber. The difference between both models, namely the Ussing and Grass diffusion cells, is that the electrical parameters can be monitored in the first and not in the second. Nevertheless, Grass model was chosen for most studies because this allows the use high series (Boisset and Desjeux, 1994).

In Ussing's chamber the spontaneous transmucosal electrical potential difference (PD) was continuously shorted by a short-circuit current (I_{sc}) delivered by an automatic voltage clamp system (WPI, New Haven, CT USA) as previously described (Tomé et al., 1987).

The delivered I_{sc} was corrected for fluid resistance and continuously monitored an a chart recorder. Every 30 s, tissue samples were automatically clamped at + 1 mV to calculate the electrical conductance (G) according to Ohm's law. In all experiments, 30 min were necessary for stabilization of electrical parameters before starting flux measurements. The studied drug was added at concentrations dictated by the experiment, into the mucosal compartment and 0.5 ml aliquots were collected from mucosal and serosal at tissues 30, 60, 90, 120 min for high performance liquid chromatography (HPLC) analysis. The extracted

volumes were replaced with buffer. Mucosal to serosal fluxes (Φ_{m-s}) were measured on adjacent pieces of tissue from the same animal. Among the different experiences, we studied the effect of cephalosporins on electrical parameters and the transport of the antibiotics at different concentrations, the drug transport with widening tight junction produced by D-glucose and influence of intestinal pH on drug absorption were monitored during the experiment.

2.4.3. Calculations

The mucosal to serosal and serosal to mucosal fluxes respectively, (Φ_{m-s}) and (Φ_{s-m}) were calculated from:

$$\Phi = \frac{Q_n - Q_{n-1}}{A \times (t_n - t_{n-1})} = \frac{dQ}{2.52 \times dt} \quad [\text{nmol/h/cm}^2]$$

dQ/dt is the transepithelial flux measured at each time for samples in the serosal or mucosal compartment, Q is the cumulative quantity (nmol) of drug and $A = 2.52$ cm^2 is the area of contact epithelium between the two parts (serosal and mucosal).

The rate of cephalexin absorption was measured at 30, 60, 90, and 120 min and the initial absorption rates were calculated through a linear fit to these points.

2.4.4. High-pressure liquid chromatography

Two standard curves were obtained for each studied pH by integration of the peak area after injection of various amounts of cephalexin (0.1–1 μg) or (1–10 μg) for serosal and mucosal aliquots respectively. Samples were filtered in 0.45 μm pore size filter (Millipore) and injected in a Shimadzu HPLC system equipped with a Kromasil C18 column, 5 μm (250 × 4.6 mm) (Touzart and Matignon) and an ultraviolet dosage at 254 nm. The column was maintained in the bath at 40°C, and the mobile phase, a mixture (13:87 v/v) of acetonitrile (Prolabo, France) and water containing 1% trifluoro-acetic acid (TFA) (Sigma Chemical, St. Louis, MO, USA) was used at a flow rate of 1.5 ml/min. Under these conditions cephalexin were eluted in 7–10 min.

Table 1
Partition coefficient at pH 5 and 6 for CFX, CFC and CDX

K_p	pH 5	pH 6
Cephalexin	0.067 ± 0.011	0.053 ± 0.010
Cefaclor	0.030 ± 0.007	0.018 ± 0.004
Cefadroxil	0.063 ± 0.013	0.043 ± 0.024

Values are means ± S.D. for six determinations.

2.5. Statistical analysis

The means ± S.E. of the results are reported with the number (n) of observations.

The difference observed between the means of each group are compared according to Student's t-test.

3. Results

3.1. Evaluation of intestinal diffusion with artificial barriers

3.1.1. Influence of pH on the partition coefficient and diffusion rate constant of cephalosporins

The first partition coefficient obtained with dodecanol + octanoic acid, gave higher values at pH 5 than at pH 6 (Table 1). At 1 mM and at pH 5 CFX and CDX show a better K_p than CFC. Both results, were significantly different ($^*p < 0.01$). These values were very small, and give information on the lipophilic character of the drug studied.

Experiments on Stricker's intestinal absorption simulator show that the three antibiotics have the same tendency for a better diffusion at pH 5 than at pH 6 and that CFX passes through the lipid barrier faster than CDX and CFC (Table 2).

Table 2
Rate constant diffusion at pH 5 and 6 for CFX, CFC and CDX (cm/min)

K_d ($\times 10^{-3}$ cm/min)	pH 5	pH 6
Cephalexin	0.563 ± 0.003	0.426 ± 0.025
Cefaclor	0.395 ± 0.003	0.018 ± 0.024
Cefadroxil	0.464 ± 0.006	0.318 ± 0.005

Values are means ± S.D. for nine determinations.

With a cephalexin concentration of 1 mM, the diffusion rate constant K_d through the artificial intestinal barrier was 0.563 ± 0.001 and 0.426 ± 0.031 ($\times 10^{-3}$ cm/min) at pH 5 and 6, respectively. For both pH values the uptake of the three antibiotics increased linearly with time. In absolute values these values were high compared with the low partition coefficient. At pH 7 the studies of diffusion rate constant for the different drugs was not possible, as the lower concentration can not be determined.

3.2. Evaluation of intestinal transport with biological membranes

3.2.1. Transepithelial passage of CFX, CDX and CFC in Ussing's chamber

To measure the transepithelial passage of the three aminocephalosporins through rabbit ileum tissues mounted in an Ussing chamber, some drug was added at the concentration of 1 mM on the mucosal side of the tissue and its fluxes were measured in the mucosal and serosal compartments. Fig. 2 shows an important increase in the CFX concentration in the serosal compartment until 120 min. Both molecules crossed the rabbit ileum intact except CFC, whereas an HPLC analysis showed a different peak at 60 min, indicating a degradation of products.

The mean transepithelial mucosal to serosal fluxes (Φ_{m-s}) were 52.54 ± 6.45 and 27.5 ± 2.43 nmol/h/cm^2 for CFX and CDX, respectively. Fig. 3 shows the evolution of cephalosporins on the mucosal side where a small decrease of CFX and CDX occurs, whereas a strong decrease in CFC concentration was observed as the experiment proceeded.

3.2.2. Electrical measurements in an Ussing chamber

The addition of CFX and CFC at 1 mM on the mucosal side of the ileum isolated in an Ussing chamber had no effect on short-circuit current (I_{sc}), but adding 1 mM of CDX on the mucosal part had an influence on the membrane rejected as an increase in I_{sc} (Fig. 4).

No modification of the conductance (G) of the tissue was observed, and it remained stable during the experiment (Table 3). This lack of effect was indicative of cellular integrity during the experiment.

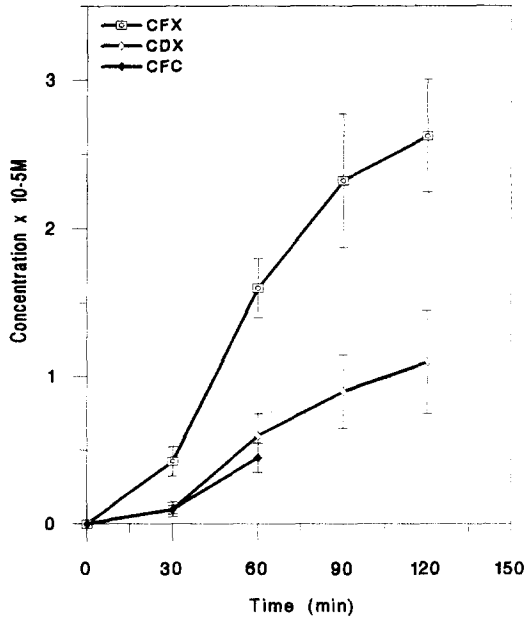


Fig. 2. Time-course of transepithelial transport of cephalexin, cefadroxil and cefaclor. At $t = 0$, 1 mM of drug on the mucosal side of the tissue was added, the concentrations were measured up to 120 min. Each point represents the mean of 12 determinations (three animals).

3.2.3. Transport of cephalexin on Grass cell diffusion

In order to compare the two biological membrane models, namely Ussing's chamber and Grass cell diffusion, we chose CFX to study its transport in Grass cell diffusion. This choice was based on the best flux obtained in the first model and on the good stability of this molecule. With the same concentration as used in the Ussing chamber (1 mM), CFX crossed the ileum isolated in the Grass diffusion cells with a flux of 51.9 ± 3.25 nmol/h/cm^2. This result was similar to that obtained with Ussing's chamber, so the transepithelial flux of CFX is not significant in the two models. The tissue viability cannot be determined with electrical parameters in the Grass cell, but studies of Grass and Sweetana (1988) who monitored intestinal tissue viability based on electrical

potential, concluded that the intestinal tissues were viable for a period of at least 180 min. The continuation of this study, was investigated on a Grass diffusion cell and all experiments were conducted until 120 min. Cephalexin has been chosen to determine transport mechanism of these aminocephalosporins, because its transepithelial passage was maximum in comparison with CDX and CFC.

3.2.4. *Effect of pH on cephalexin transport*

Fig. 5 shows the effect of pH on transepithelial flux of cephalexin (1 mM) on Grass cells at 37°C. The varying mucosal pH ranged from 6 to 7.4 (PBS) in comparison with Ringer solution (pH 7.4), and a maximum transport occurred when the pH was 6. The CFX transport in the mucosal–serosal direction is twice as large at pH 6 ($\Phi_{m-s} = 11.42 \pm 2.24$) as the fluxes obtained at pH 7–7.4 ($\Phi_{m-s} = 5.0 \pm 1.03$) in nmol/h/cm^2.

3.2.5. *Concentration dependence of cephalexin uptake on Grass cells*

The concentration dependence of cephalexin uptake and transport was examined over a concentration range of 0.1–5 mM. Data in Fig. 6 show that the uptake of cephalexin involve Michaelis–Menten kinetics, and the value for the apparent K_m of cephalexin accumulation from the apical side was 10.5 mM. The V_{max} value was 493 nmol/h/cm^2.

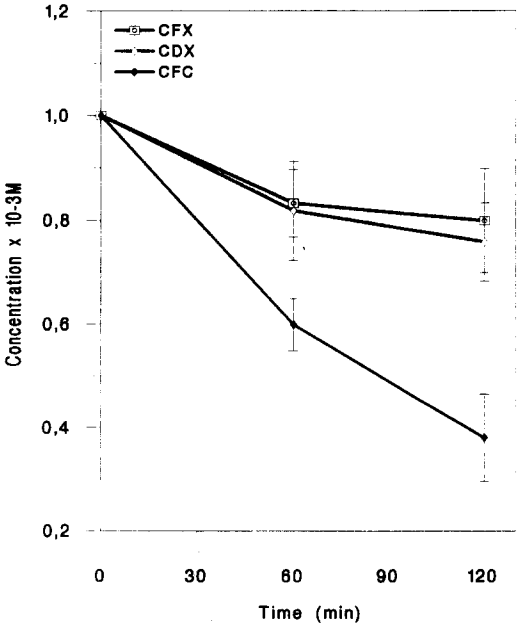


Fig. 3. Evolution of cephalosporins concentration in the mucosal compartment (12 determinations).

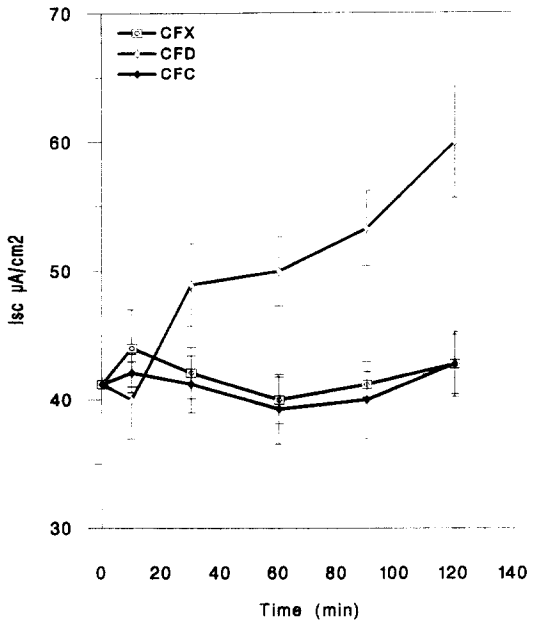


Fig. 4. The intensity short-circuit (I_{sc}), in the presence of the three cephalosporins, as function of time (nine experiments).

Table 3
Electrical parameters after addition of CFX, CFC and CDX to the mucosal side of rabbit ileum in an Ussing chamber

Electrical parameters	I_{sc} (µA/cm^2)	PD (mV)	G (mS)
Control	40.9 ± 1.21	−2.31 ± 0.06	17.7 ± 0.90
Cephalexin	41.8 ± 1.82	−2.4 ± 0.15	17.4 ± 1.32
p	NS	NS	NS
Control	51.80 ± 1.56	−2.45 ± 0.32	21.1 ± 2.51
Cefaclor	50.66 ± 1.40	−2.70 ± 0.26	18.7 ± 1.40
p	NS	NS	NS
Control	40.5 ± 1.86	−1.90 ± 0.12	21.32 ± 1.82
Cefadroxil	48.66 ± 2.36	−2.31 ± 0.05	21.06 ± 0.64
p	<0.001	<0.05	NS

Values are means ± S.E. for 24 tissues. I_{sc}, PD, and G values were measured between 45 and 120 min after addition of drugs.

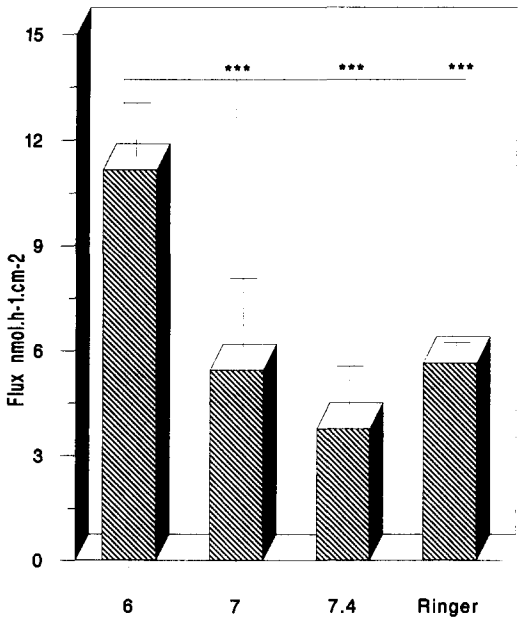


Fig. 5. pH-dependence of transepithelial transport of cephalexin (1 mM through isolated rabbit ileum. Each point represents the mean ± S.E. of 18 determinations (three rabbits). *** $p < 0.001$, significant difference from pH 6 flux using student's t-test.

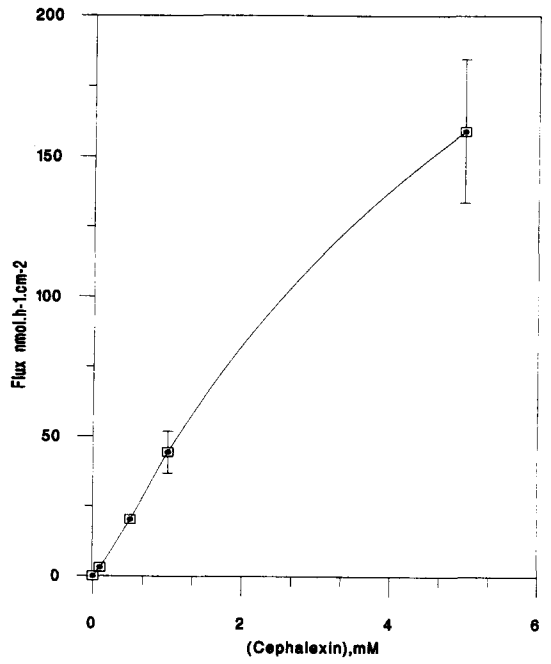


Fig. 6. Concentration dependence of the transepithelial transport of cephalexin. The flux is expressed as nmol/h/cm^2. Each point is the mean of 12 points determined from two independent experiments (two rabbits).

These pharmacokinetical parameters were obtained through a mathematical computer sitting process, extrapolating the saturable transport.

3.2.6. Effect of D-glucose on the cephalexin transport rate in the ileal mucosa

D-glucose does not affect the cephalexin flux across the epithelium isolated on a Grass diffusion cell. With 25 mM of D-glucose present in the transport medium on both sides of the tissue, the CFX transport was not affected and the mucosal to serosal flux remains unchanged (Table 4).

4. Discussion

These experimentations allowed to determine the transepithelial transport of cephalosporins with in vitro models using artificial and biological membranes.

Artificial models such as Stricker's and Reese's apparatuses simulate a diffusional passage through lipid barriers, dependent on lipophilic characteristics of molecules. Thus, the K_d and K_p values provide information on the relative importance of transcellular transport through the phospholipidic barrier of the enterocyte. The biological model used was Ussing's chamber and its variant, the Grass diffusion cell, where the three ways of transepithelial transport (transcellular, paracellular and carrier-mediated transport) were studied. Several factors originating from the

Table 4
Effect of D-glucose on cephalexin transport in Grass cell diffusion

Drug	Φ_{m-s} (nmol/h/cm^2)
Control CFX (1 mM)	38.75 ± 5.23
CFX + 25 mM D-glucose	35.90 ± 6.45
Control CFX (5 mM)	119.42 ± 25.75
CFX + 2 mM D-glucose	102.35 ± 20.4

Values are means ± S.E. for 12 determinations for each experiment.

biological system such as pH, will affect the bioavailability of drugs (Ungell, 1994).

For instance the secretion of hydrochloric acid in the stomach, H^+ or HCO_3^- from pancreas or glands in the intestinal wall, will change the pH profile both longitudinally in the lumen and from the lumen towards the epithelial surface.

This change in pH will affect the degree of ionization of the drug molecule if the drug has a pK_a-value in that specific region. This pH change can be a good predicting factor of absorption of different molecules. Indeed, for many drugs there is a correlation between the ability of the molecule to pass through the lipid membrane and the degree of absorption. It is the reason why the pH influence on transepithelial passage of cephalosporins has been studied.

4.1. pH-influence on cephalexin transport

The partition of cephalosporins within the lipid phase has been carried out with Reese's model. The partition coefficient (K_p) results are listed in Table 1. The obtained values seem very low, which show that the cephalosporins cannot pass through the lipidic barrier with only passive diffusion. Nevertheless, the K_p values were significantly higher ($p < 0.005$) at pH 5 than at pH 6 for cephalexin, cefaclor and cefadroxil. At pH 5, cephalexin and cefadroxil have twice the K_p-value of cefaclor. The better results pH 5 than at pH 6 can be explained by the ionization of there amphoteric cephalosporins having a higher percentage of zwitterion species at pH 5 than pH 6 (98.34 and 88.32%, respectively). The diffusion rate constant (K_d) determined with Stricker's model are shown in the Table 2. These values confirm a weak passive diffusion with a K_d higher at pH 5 than at pH 6. Therefore, the two experiments show that the maximum absorption occurs at pH 5, for which value the zwitterionic specy is maximum (98%). At the pH values studied, the cephalosporins have different ionized species. The zwitterionic is the most liposoluble one allowing hydrophilic and amphoteric drugs to pass through the intestine epithelium by passive diffusion. At pH 7.4, the partition coefficient and rate diffusion coefficient of the three drugs were undeterminable. That is explained by an increase in the anionic form and a decrease in the zwitterionic species (56%) proving the important role of the zwitterionic species in the lipid barrier passage.

Yasuhara et al. (1977) and De Young et al. (1978) tried to investigate the influence of pH on the intestinal absorption of cephalexin and cefradine in situ in the rat. They reported a maximum absorption in the pH range (5 and 6) when the zwitterionic concentration is maximum, and confirmed by Kimura et al. (1985) with aminopenicillins.

These findings were in accordance with the results obtained on Grass cells when the intestinal fluxes were maximum at pH 6 and not at pH 7.4 (pH of the ileal part) (Fig. 5), which confirms that the transport of aminocephalosporins was pH-dependent. At pH 7.4 no significant difference was observed between Ringer's buffer and PBS. At pH 6, the intestinal tissue was not altered, as opposed to pH 5. At the later pH value, the transepithelial electrical resistance (TEER) decrease is due to the aggressiveness of PBS, causing a perforation of the membrane. Therefore, the fluxes become undeterminable. In addition to the pH-partition hypothesis related to the chemical structure of the molecule, another energetic phenomenon can be involved in the transepithelial transport needing an acidic environment. Hobgen et al. (1959) explain the kinetics of weakly acid and basic transport through intestinal epithelia according to the 'pH-partition hypothesis' which suggest the pH in the surface of the brush membrane is more acidic than the one measured in the intestinal lumen when the medium was less buffered.

This pH-dependent and energetic transport was confirmed by different authors (Tsuji et al., 1987; Inui et al., 1988; Kramer et al., 1990). When they studied the cephalosporin transport, they showed that they rapidly dissipate the H^+ gradient across biological and artificial lipid membranes with ionophores such as FCCP or nigericin. In spite of this pH-dependence and the best transport at pH 6, we studied the cephalexin transport in the rabbit ileum isolated in an Ussing chamber or a Grass diffusion cell at pH 7.4 (Ringer buffer) to reproduce the physiological environment of the intestinal tract and in conditions such that the ileal part has a maximum viability.

4.2. Transport characteristics of cephalosporins on the biological membrane

Employing an Ussing chamber, the transport of cephalexin, cefaclor and cefadroxil was studied at 1 mM, and Fig. 2 shows an increase in the cephalexin and cefadroxil concentrations in the serosal compartment until 120 min (without reach saturation).

The mucosal to serosal fluxes (Φ_{m-s}) were 52.54 ± 6.45 and 27.5 ± 2.43 nmol/h/cm^2, respectively. For cefaclor, a great degradation was observed during experiments starting at 60 min, confirmed by a strong decrease in the concentration in the mucosal part (Fig. 3).

Nevertheless, CFX and CDX concentrations show a weak and stable disappearance on the mucosal side. With the results previously obtained on artificial membranes, a weak diffusional passage has been shown. Thus, with biological membranes, one can observe an important transport probably due to another mechanism such as carrier-mediated transport or perhaps an association of paracellular and transcellular transports. The current short-circuit was not modified when the CFX and CFC were added in the mucosal compartment, showing no toxicity of both drugs on the tissue (Fig. 4). The stability of I_{sc} during the experiment is also in agreement with an Na^+-independent transport system. The absence of any cephalexin effect on I_{sc} is also in agreement with a proton-dependent transport system similar to the peptide transport system. The intestinal absorption of natural di- and tripeptides has been demonstrated to be a pH-dependent process, mediated by a di-/tripeptide carrier located on the apical membrane of small intestinal absorptive cells (Dantzig and Bergin, 1990). The structural analogy between aminocephalosporins and peptides explains the similar proton-dependent transport system. Nevertheless, an I_{sc} increase is noticed after introduction of 1 mM of cefadroxil in the mucosal part. This effect is not clear and could be in relation with either an ion dependent (Na^+) transport or a specific cellular effect of this molecule. For the three drugs, the conductance (G) remains stable during the experiment (120 min), which is indicative of the cellular integrity of the tissue during the transport (Table 3).

The choice of Grass diffusion cell instead of an Ussing chamber to continue the cephalexin study transport was based on different parameters. The first and most important one was the absence of the difference between the results obtained from both models. More specifically, the mean flux (Φ_{m-s}) of 1 mM cephalexin was the same as the one obtained with the Grass diffusion cell, that is 51.9 ± 3.25, in comparison with 52.54 ± 6.45 nmol/h/cm^2 obtained on an Ussing chamber.

At 1 mM, cephalexin was transported through the ileum mucosa and the concentration increased during the experiment up to 120 min without any equilibrium being reached, points to a non saturable transport in this case because the concentration cephalexin added in the mucosal compartment was lower to saturate different sites of the carrier. Grass and Sweetana (1988) reported that no statistical differences in permeability measurements were found between the two systems. However, variability was much greater with the Ussing chamber apparatus. On the other hand, from the stand point of chemical composition, Ussing's chamber is generally made of two or three different materials, including the glass reservoir, acrylic cell and connecting tubing contrary to the Grass diffusion cell which was fabricated to have only one type of material (acrylic). This detail is very important because with the very lipophilic drugs such as progesterone, it is impossible to conduct a study using an Ussing chamber due to the large drug adsorption on the tubing. Moreover the relationship between the surface area of the tissue and the volumes of fluid in the bath medium is critical for the quantification of the low permeabilities, especially with relatively insoluble substances. Compared with the 10 ml volume of the Ussing chamber, the Grass system uses 7 ml reservoirs. In addition, the surface area in Ussing's chamber is twice that of the Grass cell, which makes it more difficult to mounted the tissue on half of the acrylic chamber. Finally, the temperature of Grass cells is easier to control, since a group of six cells is heated together and a greater surface of the device is actually heated when compared with the Ussing chamber system which controls the temperature cells one by one.

Taking the above parameters into accounts, the more important factor influencing this change in this system was the use of the Grass diffusion cell in high series, which makes it easier to assemble tissue and quicker to clean the cells. Nevertheless, this system does not allow the electrical parameters to be monitored, but the tissue viability can be studied by comparison with a transport paracellular marker such as mannitol or PEG 4000. In this case it is not possible to use such markers but the transport studies have been made until 120 min, in accordance with Grass and Sweetana (1988), who showed that the epithelium can be used up to 180 min at 37°C. At different concentrations between 1 and 5 mM, the transport in Grass chamber seems to take place according to non linear kinetics (kinetic order 0) (Fig. 6) and tends to confirm a Michaelis–Menten Kinetics. These results were obtained at lower concentrations, without saturation and are in accordance with the literature, where different authors reported mixed kinetics. This hypothesis was confirmed by the study of aminocephalosporins with α-amino groups such as cefadroxil perfused in situ in rat (Sanchez-Pico et al., 1989) and cephradine on brush border membrane vesicles (Okano et al., 1986). Nevertheless, Tsuji et al. (1987) show this phenomena for cefixime (cephalosporin without an α-amino group), where non saturable passive diffusion occurs at high concentrations and saturable Michaelis–Menten kinetics occur at lower concentrations. The higher concentrations were no tested to confirm the passive diffusion because solubility of cephalexin was not absolute beyond 5 mM. Nevertheless, it is obvious that the passive diffusion was demonstrated on the Stricker absorption simulator.

At low concentrations, saturable transport is certainly energy-dependent and these results are in accordance with the literature relating to the transport of di- and tripeptides generated by the combined action of Na^+/H^+ exchanger and Na^+/K^+-ATP_{ase}. Previous experiments described the transcellular transport of cephalosporins through the intestinal epithelia. Now, it is important to examine the relation aspect of paracellular and transcellular fluxes of cephalexin transport.

To confirm and obtain the paracellular diffusion of cephalexin through the ileum, we tried to expand the tight junctions with 25 mM of D-glucose on both sides of the tissue.

The results were not conclusive and there was no significant difference between the cephalexin fluxes with and without D-glucose (Table 4). Therefore, the passive diffusion of CFX through a tight junction route was not perfect and it was in agreement with amphoteric structure and the partition coefficient obtained.

In conclusion, many authors have described intestinal transport of cephalosporins with BBMV, cell cultures and experiments in vivo, but few experimentations described models with artificial and biological membranes. Because of difficulties raised by the viability of cell cultures and by in vivo experiments it is important to determine at first, characteristics of aminocephalosporins transport with simple models such as Stricker and Reese's models and rabbit ileum isolated. These models allow one to examine the relative contribution of paracellular and transcellular flux to transepithelial transport of cephalexin. Moreover, they can verify a transport proton dependent, energy dependent, sodium independent and obedient to Michaelis–Menten at low concentration and a small passive diffusion at these concentrations. These models complete in vivo experimentations in order to improve bioavailability of drugs.

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