ELSEVIER

International Journal of Pharmaceutics 149 (1997) 241–249

international journal of pharmaceutics

Cefpodoxime proxetil esterase activity in rabbit small intestine: a role in the partial cefpodoxime absorption

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Received 5 December 1996; accepted 13 January 1997

Abstract

The luminal and mucosal de-esterification of the prodrug ester cefpodoxime proxetil was studied in rabbit intestine in vitro. An enzymatic hydrolysis of the ester, releasing the active third-generation cephalosporin, was observed in both luminal washing and mucosal homogenate. The mucosal activity was mainly recovered in the soluble fraction, whereas the brush-border membranes were almost devoid of activity. Eserine and diisopropyl fluorophosphate were potent inhibitors of cefpodoxime proxetil hydrolysis in both luminal washing and mucosal homogenate, suggesting the participation of choline esterases in the hydrolysis of cefpodoxime proxetil. The luminal and mucosal activities were equally sensitive to $HgCl_2$ and acetylsalicylic acid inhibitions but slight differences were observed concerning the 50% inhibitory concentration (IC_{50}) of two drug esters, bacampicillin and enalapril. In vitro experiments run with rabbit jejunum mounted in Sweetana-Grass diffusion chambers showed that an extensive hydrolysis of cefpodoxime proxetil occurred in the mucosal compartment and that the accumulation of cefpodoxime in the serosal compartment was very slow. These observations support the hypothesis that the partial oral bioavailability of cefpodoxime proxetil results from a hydrolysis by luminal choline esterases. © 1997 Elsevier Science B.V.

Keywords: Absorption; Cefpodoxime–proxetil; Intestinal esterase; Metabolism

1. Introduction

Third-generation cephalosporins differ from previous oral cephalosporins in that they lack an α-amino group. First- and second-generation α-

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PII S0378-5173(97)04881-3

amino-cephalosporins are absorbed through the di- and tripeptides transport system and have a very high bioavailability per os (Nakashima et al., 1984; Okano et al., 1986). The lack of the α-amino group results in a dramatic decrease of the intestinal absorption of cephalosporins, except for cefixime which seems to share the same transporter as α-amino-cephalosporin (Inui et al., 1988; Kramer et al., 1993). Prodrug ester has been developed for the last decade in order to increase the oral bioavailability of poorly absorbed drug. Cefpodoxime proxetil is an orally absorbed broad spectrum third-generation cephalosporin ester. This prodrug ester is de-esterified in vivo to its active metabolite cefpodoxime. In humans, the absolute bioavailability of cefpodoxime proxetil administered as a 100-mg tablet is about 50% (Borin, 1991). Moreover, in the faeces, cefpodoxime proxetil accounted for less than 0.5% of the administered dose, most of it being recovered as free cefpodoxime. This suggests that degradation of the prodrug ester occurs in the intestinal lumen.

Non-specific esterases have been found in the intestinal epithelium of various species including human, rat and rabbit (Holmes and Master, 1967; Négrel et al., 1976; Shindo et al., 1978; Inoue et al., 1979a,b,c, 1980; Campbell et al., 1987). There have been very few reports concerning the role of these enzymes in the hydrolysis of prodrug antibiotic esters (Shindo et al., 1978; Campbell et al., 1987). The aim of the present work was to localize and characterize the activity responsible for cefpodoxime proxetil hydrolysis in the intestinal tract and to investigate the absorption of this third-generation cephalosporin in vitro.

2. Materials and methods

2.1. Materials

Cefpodoxime proxetil was kindly provided by Roussel Uclaf, France. Bacampicillin, enalapril, eserine and diisopropyl fluorophosphate (DFP) were purchased from Sigma (La Verpillière, France). All other reagents were of the highest grade available.

2.2. Methods

2.2.1. Intestinal washings and mucosal preparations

Male New Zealand rabbits weighing 2.5–3 kg were fasted overnight before being sacrificed by intravenous (i.v.) injection of 2 ml/kg sodium pentobarbital. After an abdominal mid-line incision, the small intestine was exposed and a 20-cm segment beginning 5 cm distal to the pylorus was ligatured at both ends. The segment was filled with 5 ml of washing buffer which was left inside for 10 min. The intestine was then evacuated, the washing was spun down at 3800 × *g* for 10 min to remove particular material, aliquoted and frozen at −22°C.

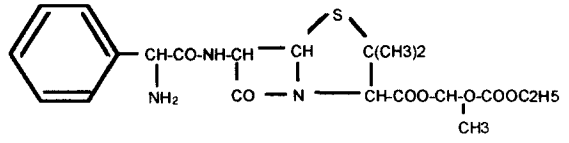
A 40-cm segment immediately proximal to the preceding one was quickly removed, washed with ice-cold saline, opened lengthwise and the mucosa was scraped off gently with a glass slide. Mucosal scrapings were suspended in 20 ml ice cold buffer per g mucosa (10 mM 2-morpholinoethanesulfonic acid (MES), 300 mM mannitol (pH 6); 10 mM 2-hydroxyethylpiperazine-2-ethanesulfonic acid (HEPES), 300 mM mannitol (pH 7); or 10 mM HEPES, 300 mM mannitol (pH 8)), and homogenized in a waring blender at the maximum speed for 1 min. The homogenate was filtered on cheesecloth and either frozen at −22°C or used to prepare cytosolic and brush-border membrane fractions. Brush-border membranes were purified from the mucosal homogenate by differential Ca^{++} precipitation and centrifugation as previously described (Mahé et al., 1989).

The enrichment factor of the preparation in membrane-specific enzymatic activity was 18 and 15 for aminopeptidase-*N* and alkaline phosphatase, respectively.

The soluble fraction of the mucosal homogenate was prepared by ultracentrifugation of the homogenate at 100 000 × *g* for 1 h. The supernatant was collected and represented the soluble mucosal fraction.

2.2.2. Assay of cefpodoxime proxetil esterase activity

The enzymatic preparation (5 μg protein) was preincubated at 37°C for 5 min in 200 μl of 10



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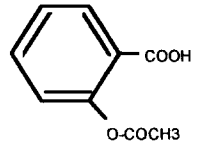

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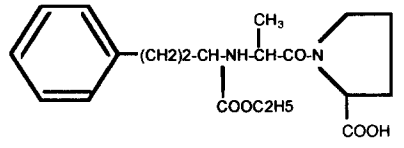

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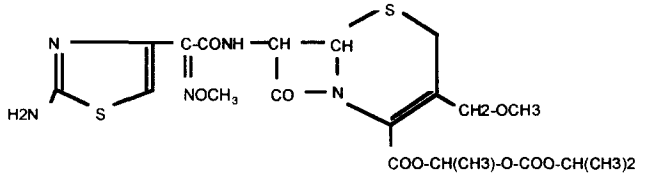

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Fig. 1. Organic structural formulae of cefpodoxime proxetil and drug esters used as competitors: (1) bacampicillin; (2) acetylsalicylic acid; (3) enalapril; (4) cefpodoxime proxetil.

mM HEPES buffer (pH 7) + 300 mM mannitol. When necessary, 10 mM HEPES buffer (pH 8) + 300 mM mannitol or 10 mM MES buffer (pH 6) + 300 mM mannitol was used in place of the former. The reaction was started by the addition of 50 μl cefpodoxime proxetil (final concentration 100 μM). After a 30-min incubation, the reaction was stopped by addition of trifluoroacetic acid (TFA) and the samples were centrifuged at 10 000 $\times$ g for 15 min. The amount of cefpodoxime released was measured by high-performance liquid chromatography (HPLC) analysis of the supernatant.

The effect of eserine, diisopropyl fluorophosphate (DFP), EDTA and dithiothreitol (DTT) on the hydrolysis of cefpodoxime proxetil was measured by preincubating the enzymatic preparation with the above mentioned compound for 15 min at 37°C.

The specificity of cefpodoxime proxetil esterase was studied by adding different drug esters (Fig. 1) simultaneously to the cefpodoxime proxetil at final concentrations in the range 30 μM–1 mM. Competitor selection was based on structural homology with cefpodoxime proxetil, i.e. the presence of a nitrogen distant from two carbons from the ester bond and of an α-methyl group in the alcohol substituent ($>N-CR_1R_2-CO-O-$

CHR_3-CH_3) for enalapril and bacampicillin, or known affinity of intestinal esterase for acetylsalicylic acid (Inoue et al., 1979b).

2.2.3. Transport experiment

The intestinal absorption of cefpodoxime proxetil was measured in vitro using rabbit jejunum mounted in Sweetana-Grass diffusion chambers (Grass and Sweetana, 1988). Male New Zealand White rabbits weighing 2.5–3.5 kg were killed by i.v. injection of sodium pentobarbital. Segments of the distal jejunum were removed, rinsed free of intestinal contents, and the serosal and external muscular layers stripped off with fine forceps. The tissue was opened along the mesenteric border and 4-cm-long segments mounted between two halves of Sweetana-Grass diffusion chambers (Precision Instruments design, Los Altos, CA). Care was taken to avoid Peyer's patches. Each side of the tissue (exposed area 2.51 cm^2) was bathed with 6 ml isotonic Ringer solution consisting of (in mM) 140 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 120 Cl^-, 25 HCO_3^-, 2.4 HPO_4^{2-} and 0.4 $H_2PO_4^-$ (pH 7.4). Oxygenation of the tissue was ensured by a gas lift of O_2/CO_2 (95:5) and the temperature was maintained at 37°C throughout the study with an aluminium block heater. The experiment was started by the addition of cefpodoxime proxetil (final concentration: 50 μM) to the mucosal compartment. Thereafter, samples of 500 μl were withdrawn every 15 min from the serosal compartment for 90 min and replaced by the same volume of warm Ringer solution. Aliquots of the mucosal compartment were also collected immediately after the addition of cefpodoxime proxetil and at the end of the transport experiment. The amounts of cefpodoxime and cefpodoxime proxetil recovered in the serosal compartment were measured by HPLC.

2.2.4. HPLC analysis of cefpodoxime

The enzymatic incubation media were diluted with a 2% trichloroacetic acid solution. The dilution was analyzed on an optimized system composed of a Supelcosil LC18 (250 × 4.6 mm, 5 μm particle size) column (Supelco, St Germain en Laye, France) and of a ternary mobile phase (acetate buffer 0.05 M (pH 3.8) + methanol + acetonitrile, 87:10:3, v/v/v). The flow rate was 1 ml/min.

The separation was performed at ambient temperature and monitored at 235 nm. Linearity and both between- and within-day reproducibility were assessed. Interassay coefficients of variation were within the range 8.3–3.5% for cefpodoxime concentrations between 0.2 and 2 μM. The limit of quantification was 0.05 μM.

3. Results

Using intestinal washing or crude mucosal homogenate, we were able to observe a significant hydrolysis of cefpodoxime proxetil (Fig. 2). The specific activity was higher in the luminal washing than in the homogenate and was slightly higher for neutral or basic pH. Moreover, the hydrolysis of cefpodoxime proxetil by mucosal homogenate was significantly activated by the temperature in the range 4–50°C whereas its spontaneous hydrolysis at pH 7 remained insignificant (Fig. 3).

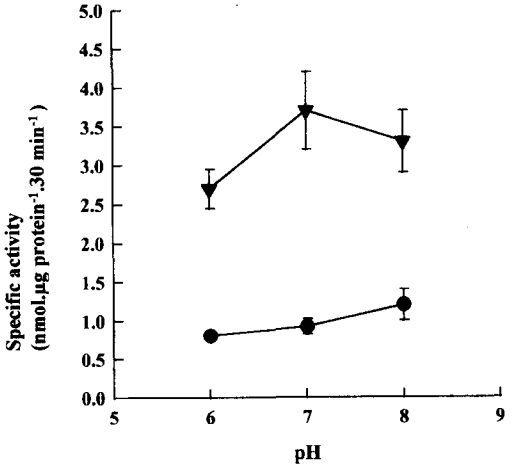

Fig. 2. Effect of incubation pH on the hydrolysis of 100 μM cefpodoxime proxetil in mucosal homogenate (●) and intestinal washing (▼). The incubation buffer consisted of 10 mM MES + 300 mM mannitol (pH 6), 10 mM HEPES + 300 mM mannitol (pH 7), or 10 mM HEPES + 300 mM mannitol (pH 8). The specific activity was calculated from the amount of free cefpodoxime released in the incubation medium, the amount of protein present in the reaction mixture being 4.6 μg for the mucosal homogenate and the intestinal washing.

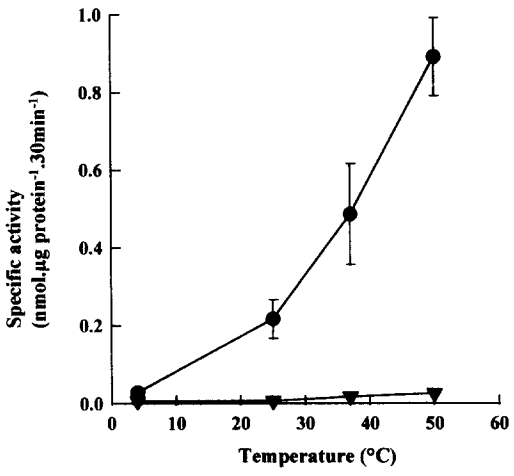

Fig. 3. Effect of temperature on the rate of hydrolysis of 100 μM cefpodoxime proxetil in crude mucosal homogenate (●) or in 10 mM HEPES + 300 mM mannitol (pH 7) alone (▼). The specific activity was calculated from the amount of free cefpodoxime released in the incubation medium, the amount of protein present in the reaction mixture being 4.6 μg.

The distribution of the cefpodoxime proxetil esterase activity was investigated by fractionation of the mucosal homogenate by Ca^{2+} selective precipitation or ultracentrifugation. The brush-border membrane fraction was almost devoid of cefpodoxime proxetil esterase activity (0.08 ± 0.08 nmol/μg protein per 30 min) whereas the specific activity measured in the soluble fraction prepared by ultracentrifugation was similar to that of the crude homogenate (1.08 ± 0.01 and 0.92 ± 0.01 nmol/μg protein per 30 min, respectively).

The characterization of the esterase activity was achieved using different inhibitors (Table 1). Both luminal and mucosal activity were inhibited by 100 μM DFP (-67 and -56%, respectively) and by 100 μM eserine (-74 and -86%, respectively). Moreover, a complete inhibition was achieved using 1 mM $HgCl_2$.

In contrast, the chelating agent EDTA was only moderately effective to prevent the cleavage of the ester bond (-27% for both luminal washing and mucosal homogenate) and no inhibition was observed with the thiol reacting agent DTT.

Competition experiments were run with drug esters using intestinal washing and mucosal homogenates (Fig. 4). The competition curves obtained with acetylsalicylic acid were similar for the washing and the homogenate (A), the effect being almost proportional to the concentration of the competitor. For bacampicillin and enalapril, however, the competition was more pronounced in the luminal washing than in the homogenate for low concentrations of the competitors (B and C). Moreover, at high concentration, bacampicillin efficiently reduced the hydrolysis of cefpodoxime proxetil in the intestinal washing, whereas its effect remained restrained. The 50% inhibitory concentration (IC_{50}) calculated from the competition curves are lower for enalapril than for bacampicillin or acetylsalicylic acid (Table 2). Apart for the later, the IC_{50} values are much lower in the intestinal washing than in the mucosal homogenate.

The absorption of cefpodoxime across rabbit jejunum was studied for 90 min after the addition of 50 μM cefpodoxime proxetil to the mucosal compartment of the Sweetana-Grass diffusion chamber. Cefpodoxime was not detected in the serosal compartment before 30 min. After 30 min, the amount of cefpodoxime recovered in the serosal compartment increased linearly, indicating that absorption occurred at a steady state (flux = 0.46 ± 0.10 pmol cefpodoxime/h per cm^2). The apparent permeability coefficient P_{app} for cefpo-

Table 1
Distribution of cefpodoxime proxetil esterase activity in intestinal mucosa and luminal washing, and effect of esterase inhibitors

Inhibitors	Specific activity (nmol/μg protein per 30 min)	
	Luminal washings	Crude mucosal homogenates
Control	3.70 ± 0.50	0.92 ± 0.01
DFP (100 μM)	1.20 ± 0.07*	0.40 ± 0.02*
Eserine (100 μM)	0.96 ± 0.04*	0.12 ± 0.04*
$HgCl_2$ (1 mM)	0.00 ± 0.00*	0.00 ± 0.00*
DTT (1 mM)	3.80 ± 0.15	0.73 ± 0.10
EDTA (1 mM)	2.70 ± 0.24*	0.68 ± 0.05*

Cefpodoxime esterase activity was measured from the release of free cefpodoxime in the incubation buffer (pH 7.0). The amount of protein in the reaction mixture was 5–7 μg and the final concentration of cefpodoxime proxetil was 100 μM. Mean $\pm$ S.D. of six observations.
* Significantly different ($p < 0.05$) from paired control.

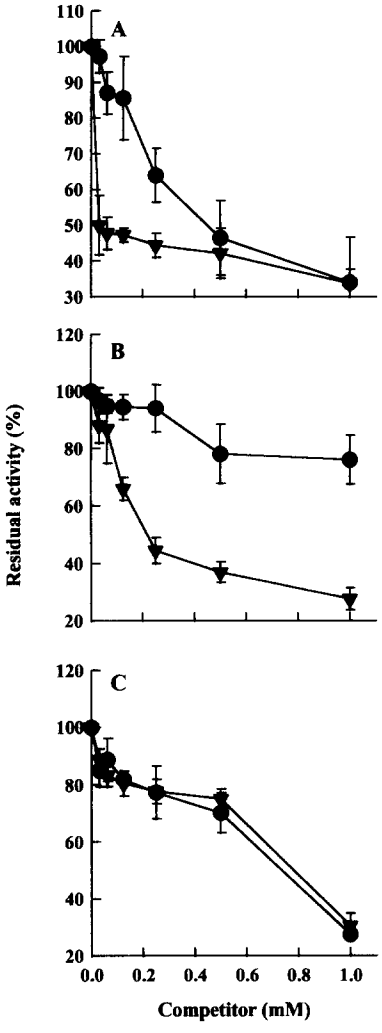

Fig. 4. Effect of drug ester competitors on the hydrolysis of cefpodoxime proxetil in crude mucosal homogenate (●) and intestinal washing (▼). Enalapril (A), bacampicilline (B) or acetylsalicylic acid (C) were added at concentrations ranging from 0.03 to 1 mM in the reaction mixture together with 100 μM cefpodoxime proxetil. The residual activity is the ratio between the activity measured in the presence of the competitor and the activity measured without any competitor. Results are mean ± S.D. of six observations.

doxime is 2.59×10^{-9} cm/s. Cefpodoxime proxetil was never recovered in the serosal compartment of the chamber. The HPLC analysis of the content of the mucosal compartment of the chamber at the end of the experiment revealed that all the cefpodoxime proxetil was converted to cefpodoxime after 90 min (Fig. 5).

Table 2
IC_{50} of different prodrug ester competitors for cefpodoxime proxetil hydrolysis

	IC_{50} (mM)	
	Crude mucosal homogenate	Luminal washing
Bacampicillin	>1	0.25
Acetylsalicylic acid	0.70	0.70
Enalapril	0.40	0.05

The different competitors were added in the incubation medium at concentrations ranging from 3×10^{-6} to 10^{-3} M and the concentration that reduces the rate of hydrolysis of cefpodoxime proxetil from 50% (IC_{50}) was determined graphically.

4. Discussion

The hydrolysis of cefpodoxime proxetil was investigated using mucosal homogenate and intestinal washing. Our results show that cefpodoxime proxetil esterase activity was present in luminal washing and in the mucosal homogenate. Both luminal and mucosal activities were sensitive to

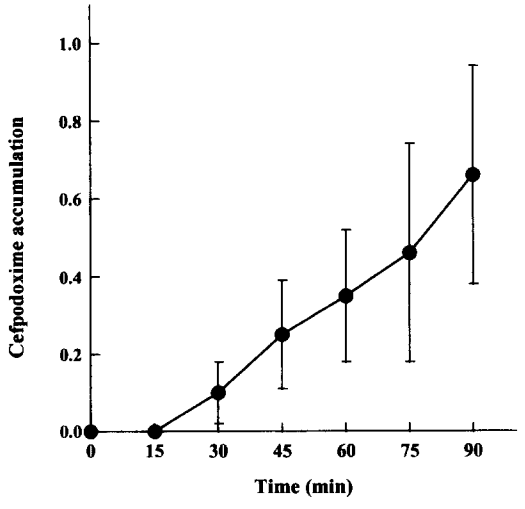

Fig. 5. Transepithelial transport of cefpodoxime proxetil across rabbit jejunum in vitro. The initial concentration of cefpodoxime proxetil in the mucosal compartment was 50 μM. The amount of cefpodoxime recovered in the serosal compartment was measured at 15-min intervals by HPLC and expressed as pmol/cm^2. Results are mean ± S.D. of eight observations.

$HgCl_2$ and may be classified as choline esterase. The in vitro transport experiment shows that the absorption of cefpodoxime is poor and that a de-esterification of the prodrug occurs in the mucosal compartment. Our observations provide some explanations for the partial oral bioavailability reported for cefpodoxime.

The prodrug ester cefpodoxime proxetil has been designed to enable the oral delivery of the third-generation cephalosporin cefpodoxime. The fate of the prodrug in the intestinal tract has not been studied yet. Using luminal washing and crude mucosal homogenate, we have observed a hydrolysis of the ester leading to the release of free cefpodoxime. This hydrolysis is clearly related to an enzymatic activity since we did not observe any spontaneous hydrolysis of the prodrug even for temperatures as high as 50°C, whereas its de-esterification by mucosal homogenate was clearly activated by increasing the temperature in the range 4–50°C.

The cefpodoxime proxetil esterase (CPE) activity was high in the luminal washing and could be inhibited by both DFP and eserine. Therefore, this activity can be classified as a B esterase according to Aldridge's classification (Aldridge, 1953) and a choline esterase according to Walker and Mackness (1983). An esterase hydrolyzing the prodrug ester cefuroxime axetil has been purified from rat intestinal washing (Campbell et al., 1987). This enzyme was classified as a carboxyl esterase since its activity was inhibited by DFP but remained unaffected by eserine. Therefore, it appears that cefpodoxime proxetil, although it exhibits some similarity with the prodrug ester cefuroxime axetil, is not hydrolyzed by the same esterase in the rabbit intestinal lumen.

A cefpodoxime proxetil esterase activity was also observed in crude mucosal homogenate, although it was lower than in the intestinal washing. This activity was almost completely recovered in the ultracentrifugation supernatant, thus indicating a soluble and probably mainly cytosolic enzyme. On the contrary, no enrichment was observed in a purified brush-border membrane fraction. The presence in intestinal epithelial cells of esterolytic enzymes hydrolyzing drug esters has been reported by different authors (Shindo et al., 1978; Inoue et al., 1979a,c). These enzymes are recovered in the endoplasmic reticulum and in the cytosol of the villus cells, whereas the brush-border membrane is almost devoid of any activity (Inoue et al., 1979a). The mucosal enzymes were classified as carboxyl esterases (Inoue et al., 1980). Our results indicate that the mucosal enzyme was inhibited by both DFP and eserine, thus belonging to the choline esterase group.

It appears that the luminal and mucosal cefpodoxime proxetil esterase activities share many characteristics. Both enzymes belong to the choline esterase group, are sensitive to $HgCl_2$ and are resistant to the thiol reacting agent DTT. Moreover, similar inhibition patterns are observed with increasing doses of acetylsalicylic acid. However, some discrepancies appear concerning the effect of drug ester competitors. The IC_{50} of bacampicillin is lower for the luminal washing than for the mucosal homogenate, the same result being observed with enalapril. This may indicate that the luminal cefpodoxime proxetil esterase has a broader specificity than the mucosal one. Alternatively, a higher IC_{50} with mucosal homogenate may be the consequence of a binding of the drug competitor to a mucosal protein, thus resulting in a decreased effective concentration of the drug. Enalapril may bind to the angiotensin converting enzyme and bacampicillin to the H^+-peptide cotransporter, both proteins being present in the mucosal homogenate. Therefore, it is not possible to rule out the possibility that the same esterase is responsible for the luminal and the mucosal hydrolysis of cefpodoxime proxetil. Indeed, the in vitro transport experiment provides some arguments for an epithelial origin of the luminal enzyme.

The cefpodoxime proxetil added in the mucosal compartment of the Sweetana-Grass diffusion chamber was completely hydrolyzed after a 90-min incubation, suggesting that an esterase was released from the tissue in the course of the experiment.

Whatever its origins, the presence in the intestinal lumen of an esterase hydrolyzing cefpodoxime proxetil is probably important regarding the bioavailability of this cephalosporin. In contrast with first-generation cephalosporins, the lack of

an α-amino group next to the amide bond in the cefpodoxime moiety may reduce its affinity for the intestinal brush-border peptide transporter that enables the absorption of most oral amino cephalosporins and β-lactam. The proxetil moiety is used to increase the lipophilicity of the molecule and its absorption by diffusion across biological membranes. The hydrolysis of the ester bond in the intestinal lumen may result in a decreased bioavailability of the drug and is likely to be responsible for the very low transepithelial absorption observed in our experiment. Indeed, it has been shown that the absolute bioavailability of cefpodoxime proxetil was about 50% and that free cefpodoxime was recovered in the faeces (Borin, 1991). The same kind of observations have been done with cefuroxime axetil, another prodrug ester of cephalosporin which undergoes a de-esterification in the intestinal lumen (Campbell et al., 1987). Whether it is possible to increase the oral bioavailability of cefpodoxime by using esterase inhibitors remains questionable. It has been shown that DFP, an esterase B inhibitor, promotes the absorption of pivampicillin, a hydrophobic ester of ampicillin, in vitro. Our observations show that less toxic compounds such as acetylsalicylic acid or enalapril also reduce the luminal de-esterification of cefpodoxime proxetil. However, these drugs may also affect the systemic conversion of the ester prodrug into the active cephalosporin and further investigations are needed to evaluate the role of drug association in promoting the absorption of ester prodrugs.

In conclusion, the above experiments demonstrate that a hydrolysis of cefpodoxime proxetil occurs in the intestinal lumen and that the choline esterase responsible for the cleavage of the ester bond may in part be of mucosal origin.

This hydrolysis probably affects the oral bioavailability of the drug and is responsible for the faecal elimination of cefpodoxime. Hence, reducing the luminal cleavage of the ester prodrug may promote the absorption of cefpodoxime and reduce its side effects on the intestinal microflora.

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