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Drug absorption studies of prodrug esters using the Caco-2 model: evaluation of ester hydrolysis and transepithelial transport

P. Augustijns *, P. Annaert, P. Heylen, G. Van den Mooter, R. Kinget

Laboratorium voor Farmacotechnologie en Biofarmacie, Katholieke Universiteit Leuven, O + N, Gasthuisberg, B-3000 Leuven, Belgium

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Abstract

The design of lipophilic ester prodrugs is a widely used approach to obtain enhanced oral delivery of poorly membrane permeable compounds. The present study was conducted in order to assess the influence of intestinal metabolism on transepithelial flux of prednisolone prodrugs using the Caco-2 system. In addition, distribution of esterase activity along the GI tract was evaluated using homogenates of scraped intestinal mucosa from various parts of small intestine and colon of rat and pig. Prednisolone acetate (lipophilic prodrug) and prednisolone hemisuccinate (hydrophilic prodrug) were selected as model compounds for transport studies. In transport studies using prednisolone acetate (100 μM), almost complete ester hydrolysis and an increased transepithelial flux of prednisolone were observed. Virtually no transport nor metabolism was observed when the hemisuccinate ester was used, illustrating its poor ability to cross membranes. Incubation studies with purified carboxylesterase showed that prednisolone acetate was rapidly degraded ($t_{1/2} = 2.94$ min), while prednisolone hemisuccinate degradation was very low. Studies on site dependency of esterase activity using *p*-nitrophenyl acetate as a substrate showed an important interspecies difference, rat intestine possessing much higher activity than pig intestine, and a gradual decrease of esterase activity along the GI tract for the two species tested. Esterase activity in Caco-2 monolayers was twice as high as observed in colon of rat and pig, but much lower than activities measured in the small intestine. It can be concluded that the rat may not be a suitable choice for oral bioavailability studies of ester prodrugs; it may also be advantageous to target ester prodrugs of hydrophilic compounds to the colon, thus preventing significant accumulation of the parent compound inside the mucosal cells.

Keywords: Caco-2; Ester prodrugs; Esterase activity; Intestinal absorption

* Corresponding author. Tel.: + 32 16 345828; fax: + 32 16 345996; e-mail: Patrick.augustijns@med.kuleuven.ac.be

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1. Introduction

In oral drug delivery programs, ester prodrugs are commonly used to enhance membrane permeation and transepithelial transport of hydrophilic drugs by increasing the lipophilicity of the parent compound, resulting in enhanced transmembrane transport by passive diffusion (Balant et al., 1990; Taylor, 1996). Acyloxyalkyl ester derivatives have, for instance, been introduced to increase oral bioavailability of phosphate or phosphonate compounds (Friis and Bundgaard, 1996). After absorption, the parent compound is expected to be released in the plasma through either enzymatic or chemical cleavage of the prodrug. This interesting approach can however be jeopardized if the prodrug is poorly resistant to esterase activity: rapid intracellular conversion of the prodrug by esterases inside the mucosal cells will result in the entrapment of the hydrophilic parent compound in the intestinal mucosa. Previous studies with the Caco-2 model conducted in our laboratory have shown that esterase mediated degradation of bis(pivaloyloxymethyl)-PMEA, a bis-ester prodrug of the antiviral compound phosphonylmethoxyethyladenine (PMEA), inside the cells was responsible for very high intracellular entrapment of negatively charged metabolites of bis (POM)-PMEA, thus diminishing the efficiency of the prodrug used (Annaert et al., 1997).

The objective of this report was to study the influence of metabolism on the transepithelial transport of ester prodrugs of a neutral compound in the Caco-2 system. Caco-2 monolayers are generally accepted as an in vitro model for drug transport studies as these cells have been shown to express most of the enzymatic, functional and morphological characteristics of the intestinal mucosa (Hidalgo et al., 1989; Audus et al., 1990; Hilgers et al., 1990; Gan et al., 1994). The Caco-2 system has already been used in several studies to characterise the biomembrane permeation of ester prodrugs (Hovgaard et al., 1995).

In this study, prednisolone acetate (lipophilic prodrug) and hemisuccinate (hydrophilic prodrug) were chosen as model prodrugs of a neutral compound, prednisolone. In addition to transport studies, esterase mediated degradation in homogenates of Caco-2 cells was compared to degradation in tissue homogenates from various sites in the GI tract of rat and pig using *p*-nitrophenyl acetate as a model substrate. These species were chosen as rats are commonly being used in drug bioavailability studies, while pigs are considered as the most suitable animal model for oral drug delivery since it resembles the human situation with respect to anatomy and physiology (Kararli, 1995; Gardner et al., 1996).

2. Materials and methods

2.1. Reagents

Hanks' balanced salt solution, Dulbecco's Modified Eagle Medium (DMEM) containing glutaMAX™, *N*-[2-hydroxyethyl]piperazine-*N*-ethanesulfonic acid (HEPES), 0.05% trypsin and 0.02% EDTA in PBS, non-essential amino acids (NEAA), penicillin-streptomycin (10000 IU/ml—10000 μg/ml) and fetal bovine serum were from Gibco BRL (N.V. Life Technologies, Paisley, UK). MeOH and CH_3CN were from BDH, Poole, UK. NaH_2PO_4, acetic acid, and sodium fluorescein were purchased from UCB, Leuven, Belgium; *p*-nitrophenyl acetate, Folin and Ciocalteu's phenol reagent (2 N), sodium potassium tartrate, D-glucose, bovine serum albumin, phloridzin, Leu-*p*-nitroanilide, Gly-Pro-*p*-NA, bestatin, diprotin A and carboxyl esterase (porcine liver) were from Sigma, Bornem, Belgium. Copper(II) sulphate pentahydrate (Baker, Deventer, Holland), sodium bicarbonate and n-octanol (Acros, Geel, Belgium) and sodium hydroxide (BDH, Poole, UK) were used as received. Prednisolone and prednisolone hemisuccinate were purchased from Akzo (Pharma division). Prednisolone acetate, phenylalanine and lysine were obtained from Fluka, Bornem, Belgium.

2.2. Caco-2 cell culture

Caco-2 cells were kindly provided by Dr Y.-S. Schneider (UCL, Belgium). Cells were grown in 75 cm^2 culture flasks at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were

passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1:7. The Caco-2 cells were maintained in high glucose (4.5 g/l) DMEM containing glutaMAX™, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 1% NEAA and 10% fetal bovine serum and grown in tissue culture flasks (75 cm^2, Nunc, Roskilde, Denmark). Cells were negative for *Mycoplasma* infection.

2.3. Growth of cells on membrane inserts

For transport experiments, Caco-2 cells were plated at a density of 40000 cells/cm^2 on Anopore membrane inserts (0.2 μm pore diameter, 25 mm diameter; Nunc, Roskilde, Denmark). Confluence was reached within 3–4 days after seeding and the monolayers were used for the experiments between days 18–24 post-seeding. Cell passages between 114 and 127 were used in the experiments. Apical (AP) and basolateral (BL) chamber volumes were maintained at 2 ml. Transepithelial electrical resistance (TEER) was measured using an EVOM Voltohmmeter (WPI, Aston, UK). Only monolayers having TEER values above 250 $\Omega \cdot cm^2$ were used in studies. Sodium fluorescein was used as a hydrophilic marker for cell monolayer integrity. Typical sodium fluorescein flux values across Caco-2 monolayers after the transport experiments with test compound were below 0.5% h^{-1}.

2.4. Caco-2 cell culture model validation (data not shown)

The Caco-2 system in our laboratory was validated by assessing the transport of propranolol (passive transcellular transport), phenylalanine (active carrier mediated transport; inhibited by lysine), glucose (active transport, inhibited by phloridzin), sodium fluorescein (passive paracellular transport). The presence of apical aminopeptidase and dipeptidylpeptidase activity was confirmed by incubation of the monolayers with specific substrates (Leu-*p*-nitroanilide and Gly-Pro-*p*-nitroanilide) and inhibitors (bestatin and diprotin A) for those enzymes.

2.5. Transepithelial transport studies

For the determination of the transepithelial flux of prednisolone and its prodrug esters across Caco-2 monolayers, the polarized monolayers were preincubated with transport medium (TM; Hanks' balanced salt solution, supplemented with 10 mM HEPES and 25 mM glucose) for 30 min, after which TEER values were measured to check monolayer integrity. The medium was then replaced by transport medium with test compound at the donor side (2 ml). Samples were taken from the apical (10 μl) and basolateral (100 μl) side, and the volume withdrawn was replaced with fresh transport medium, which was corrected for in further calculations. All flux experiments were conducted in triplicate. The transport was expressed as concentrations appearing at the basolateral side.

2.6. Degradation of p-nitrophenyl acetate by homogenates of Caco-2 monolayers, pig intestine, and rat intestine

Freshly scraped Caco-2 monolayers grown in a 75 cm^2 flask were homogenized in 5 ml TM using a cell disrupter (Branson Sonifier B15) for 10 s at 4°C. Duodenum, jejunum, ileum and colon ascendens of pig and rat were rapidly excised (within 30 min after sacrificing the animals). Segments of each part ($\pm$ 30 cm and 5 cm for pig and rat, respectively) were cut along the longitudinal axis and washed with ice-cold HBSS to remove intestinal contents. The intestinal mucosa of the various segments was removed by scraping with a glass microscope slide. The scrapings were homogenized at 4°C in 5 ml of cold TM using a cell disrupter (Branson Sonifier B15) for 10 s. After centrifugation of the crude Caco-2 and intestinal tissue homogenates at 14000 $\times$ g for 5 min, the supernatants were harvested and kept at 4°C. Protein content of all preparations (Caco-2 homogenate and duodenum, jejunum, ileum and colon from pig and rat) were determined according to the method of Lowry (Lowry et al., 1951) using bovine serum albumin as a standard. Prior to use in enzyme assays, protein content of all preparations was adjusted to 0.044 mg/50 μl.

Table 1
Physicochemical properties of prednisolone, prednisolone hemisuccinate and prednisolone acetate

	MW	Solubility[a] (M)	pK_a	Apparent octanol-TM partition coefficient
Prednisolone	360.4	6.8×10^{-4}	–	4.0
Prednisolone hemisuccinate	460.5	0.92	4.0	1.2
Prednisolone acetate	402.5	*	–	31

[a] Fleisher et al. (1986).
*Practically insoluble.

Initial velocities for degradation of 100 μM *p*-nitrophenyl acetate by esterases in Caco-2 homogenate and homogenates from various segments of pig and rat intestinal mucosa were determined by adding 50 μl enzyme preparation to 1 ml of prewarmed substrate solution. The absorbance of the mixture was followed at 405 nm to monitor the release of *p*-nitrophenol using a diode array spectrophotometer (HP 8452A). Initial velocities (nmol/s) of *p*-nitrophenol formation were calculated from the slope of the first linear part of the absorbance versus time curve. Preliminary studies had shown linearity for the degradation of 100 μM *p*-nitrophenyl acetate between 15 and 50 s for all enzyme preparations. All results are expressed as nmol/s/mg protein.

2.7. Degradation of prednisolone acetate and hemisuccinate in the presence of purified carboxyl esterase

Solutions containing 10 μM of prednisolone ester were incubated at 37°C with 0.1 IU/ml (or 10 IU/μmol) carboxyl esterase (from porcine liver). Samples (0.2 ml) were taken at predetermined time points and added to 0.2 ml methanol and vortexed to immediately arrest enzymatic activity. The samples were then centrifuged for 5 min at $14000 \times g$. The supernatant was injected into the HPLC for determination of the ester prodrug as well as free prednisolone.

2.8. HPLC-analysis of prednisolone and its esters

Prednisolone and its acetate and hemisuccinate esters were analyzed using a high-performance liquid chromatographic system equipped with a Model 6000A Pump, a Model U6K manual injector and a Model 480 Lambda-Max UV detector at 246 nm (Waters, Milford). UV-signals were monitored and the obtained peaks integrated using a Merck Hitachi D-2500 integrator. The column used was a reversed phase Merck Lichrospher 60 RP–select B (5 μM). The flow rate was 1 ml/min and the volume injected amounted to 50 μl. Methods were developed to separate prednisolone and each of its prodrug esters. For the determination of prednisolone and its acetate ester, the mobile phase consisted of 50% methanol and 50% water (v/v). For the separation of prednisolone and its hemisuccinate ester 0.875 ml of glacial acetic acid was added to 250 ml of a mixture of methanol and water (50:50) to obtain a mobile phase of pH 3.2. Retention times for prednisolone, prednisolone hemisuccinate and prednisolone acetate were 5.3, 7.9, and 9.8 min, respectively. Concentrations of prednisolone and its esters were calculated using calibration curves made up by standards of known concentration. All calibration curves showed sufficient linearity ($R^2 > 0.998$) for concentrations ranging from 0.62 to 10 μM. All samples were diluted to a final concentration within the range of the calibration graph. Intraday and interday variability, expressed as relative standard deviations were lower than 5% for all concentrations.

2.9. Octanol-TM partition coefficient

Prednisolone and its esters were dissolved in transport medium (pH 7.4) to give a final concentration of 100 μM. Three milliliters of the aqueous solution and 3 ml (prednisolone hemisuccinate) or 0.3 ml (prednisolone and prednisolone

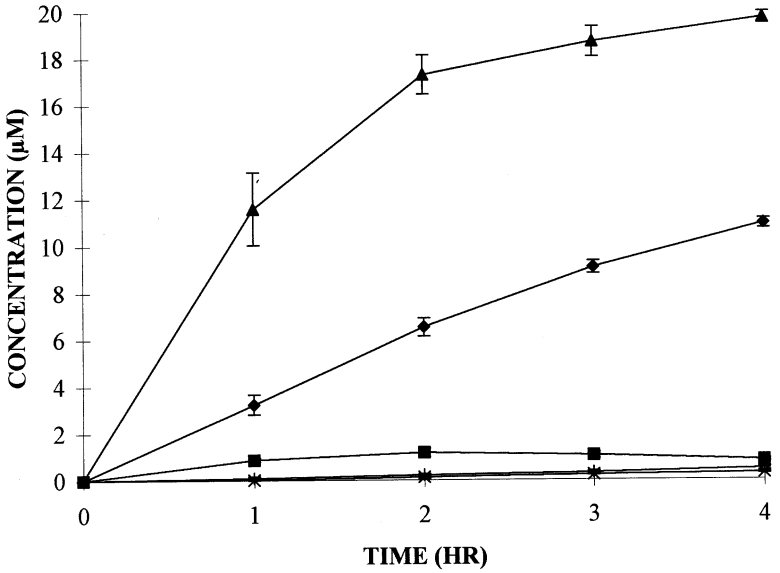

Fig. 1. Concentration time profiles of transepithelial transport of prednisolone and its acetate and hemisuccinate esters and of prednisolone generated during transport of these esters after addition of 100 μM solutions of prednisolone, prednisolone acetate and prednisolone hemisuccinate to the apical side of Caco-2 monolayers (◆, prednisolone; ▲, prednisolone from acetate ester; ×, prednisolone from hemisuccinate ester; ■, prednisolone acetate; +, prednisolone hemisuccinate). Error bars indicate the S.D. ($n = 3$).

acetate) of n-octanol were shaken for 1 h at 37°C. After separation of the two phases by centrifugation, the drug concentration was determined in the aqueous layer, and the concentration in the octanol phase was calculated from the initial and final concentration in the aqueous phase (Table 1). Transport medium and octanol were mutually saturated before the experiment.

3. Results and discussion

The design of ester prodrugs with increased lipophilicity is one approach to enhance transport across membranes and to increase the oral bioavailability of charged compounds. One of the prerequisites of an efficient prodrug ester is that the drug is eventually released, be it enzymatically or chemically. For a charged parent drug, however, it may be possible that, if resistance to esterase mediated cleavage of the prodrug moiety is too low, metabolism in the intestinal cells by intracellular esterases impedes the desired oral drug absorption enhancement due to the generation of the charged parent compound inside the cells, resulting in intracellular accumulation and diminished enhancement of transcellular flux, as it has recently been reported for bis(POM)-PMEA (Annaert et al., 1997).

It was the objective of this report to study the influence of esterase mediated metabolism on transcellular flux of ester prodrugs of a neutral drug, and to study site dependency of esterase activity along the gastrointestinal tract.

In a first set of experiments, the influence of metabolism on transepithelial flux was studied using the Caco-2 system as an in vitro simulation technique of the intestinal mucosa. Prednisolone was chosen as a model compound of a neutral drug as several ester prodrugs are commercially available, including the prodrugs used, prednisolone acetate and prednisolone hemisuccinate. Transport across Caco-2 monolayers was studied at a concentration of 100 μM, a concentration at which prednisolone and its two esters were all soluble, so that concentration dependent effects

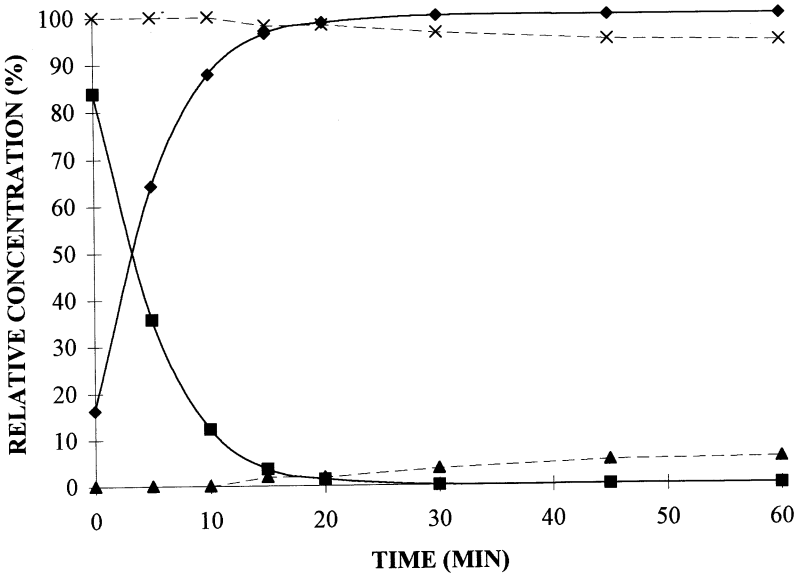

Fig. 2. Concentration time profile for degradation of 10 μM prednisolone acetate (■) and prednisolone hemisuccinate (×) in TM (pH 7.4) in the presence of 0.1 IU/ml purified carboxylesterase (from hog liver). The formation of prednisolone (◆ and ▲ for the formation form acetate and hemisuccinate esters, respectively) is also shown.

could be excluded. At this concentration, the test compounds also did not affect the integrity of the Caco-2 monolayers, as observed by stable TEER values during the experiment (data not shown). The results of this transport study are illustrated in Fig. 1. Transport of prednisolone across the monolayers appeared to be linear with time, and amounted to 2.5% h^{-1}; from mass balance data, it could be concluded that no intracellular entrapment of prednisolone occurred. The use of prednisolone acetate significantly enhanced transport of prednisolone, while only small amounts of intact prednisolone acetate could be observed at the basolateral side, suggesting extensive ester hydrolysis during transport. Deviation from linearity could be observed for the appearance of prednisolone at the basolateral side after adding its acetate ester to the apical side of the monolayers, which could be attributed to the absence of sink conditions when prednisolone acetate was used (appearance of prednisolone $>10\%$, so back-diffusion may have occurred from basolateral to apical side); experiments performed by replacing the inserts into wells containing fresh transport medium resulted in a linear appearance of prednisolone at the basolateral side after apical addition of prednisolone acetate (data not shown). After adding prednisolone hemisuccinate to the apical side of the monolayers, no significant metabolism nor transport could be observed. The role of esterase mediated degradation was assessed by incubation of both prodrugs with purified carboxyl esterase; ester hydrolysis is illustrated in Fig. 2 by a decline of the prodrug esters and the appearance of prednisolone. Prednisolone acetate appeared to be a better substrate for esterase than prednisolone hemisuccinate, which is in agreement with data of Krisch (1971) who reported that negatively charged compounds are poor substrates for carboxyl-esterase. The disappearance of the compounds due to metabolism or transport after addition to the apical side of the monolayers is given in Table 2, and illustrates that disappearance occurs in accordance with the apparent octanol-TM partition coefficient (Table 1) and the sensitivity to esterase mediated degradation. The results of this set of experiments illustrate that almost complete ester hydrolysis may occur before the ester prodrug reaches the basolateral side of the monolayers. The fact that pred-

Table 2
Concentrations of prednisolone and its esters remaining at the apical (donor) side of Caco-2 monolayers as a function of time after adding a solution of 100 μM

Time (h)	Prednisolone	Prednisolone acetate	Prednisolone hemisuccinate
0	99.8 ± 0.0	102 ± 0.0	100 ± 0.0
1	95.5 ± 1.6	34.1 ± 2.5	106 ± 6.8
2	94.0 ± 3.1	13.2 ± 2.3	102 ± 4.6
3	90.6 ± 0.4	4.48 ± 1.1	103 ± 0.4
4	82.6 ± 6.9	1.38 ± 0.5	103 ± 0.1

Values are average concentrations (μM) $\pm$S.D. of at least three determinations.

nisolone easily crosses the monolayers and is not trapped inside the cells explains the enhanced epithelial transport by using prednisolone acetate (prednisolone acetate is more lipophilic as prednisolone itself); however, in case of a charged parent compound, intracellular degradation would result in intracellular accumulation, and decreased transepithelial flux, as it has been reported for bis(POM)-PMEA (Annaert et al., 1997); limited enhancement of absorption of ester prodrugs due to high esterase activity in the intestinal mucosa has also been reported for acyclovir and L-Dopa (Narawane et al., 1993).

In a second set of experiments, the site specific esterase activity along the gastro-intestinal tract of rat and pig was studied in comparison with the Caco-2 system. *p*-Nitrophenyl acetate was used as a model substrate at a concentration of 100 μM. Under these conditions, the initial rates were linear with time. The results of this study are illustrated in Fig. 3 and clearly show that the enzymatic activity in homogenates from the small intestine of both species is much higher than that of the colon. This is in agreement with results of Narawane et al. (1993) who reported a 12-fold lower esterase activity in rabbit colon as compared to small intestine. Also an extensive interspecies difference was observed, the enzymatic activity in homogenates from rats being much higher as compared to the pig. Enzymatic activity of the Caco-2 system is lower than the enzymatic activity in the small intestine, and about two-fold higher than the activity measured in the colon of rat or pig. Additional kinetic studies in which the substrate concentration was varied between 10 and 1000 μM resulted in an estimated K_m and V_{max} of 483 μM and 5.8 nmol/s/mg protein for the esterase activity in homogenates from the Caco-2 system. These parameters were obtained by fitting the initial velocity vs. concentration data according to the Michaelis–Menten equation.

Based on the results of this study, it is concluded that the use of lipophilic ester prodrugs of neutral molecules is slightly advantageous to the use of the parent compound as long as solubility is not severely compromised. The use of hydrophilic ester prodrugs which would generate an increased concentration gradient due to increased solubility, did not seem to be advantageous as poor membrane permeation was observed.

Supposing that the enzymatic activity of pig intestine is closely related to that in humans, it could also be suggested that the rat is a less suitable animal model to test the oral absorption of ester prodrugs of charged drugs in the small intestine.

Due to the high variation in esterase activity, and the relatively low enzymatic activity in the colon of both species tested, it can also be suggested that colon-specific drug delivery will be advantageous for prodrug esters of charged compounds with moderate resistance to esterase activity. Supposing a satisfactory dissolution profile in the colon and limited luminal degradation, delivery in the colon combined with low enzymatic degradation in the colon cells will prevent intracellular accumulation in the intestinal lining, eventually leading to increased oral absorption by using lipophilic prodrugs.

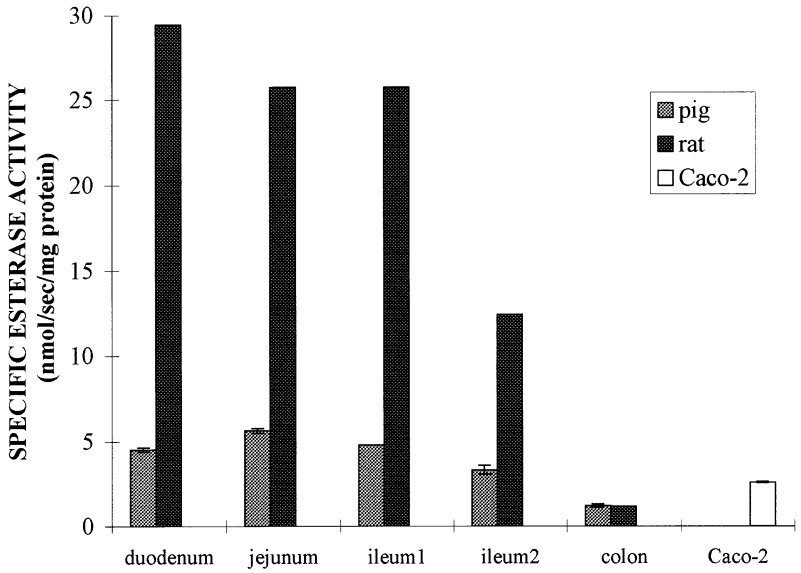

Fig. 3. Specific esterase activities (nmol/s/mg prot) in homogenates prepared from Caco-2 monolayers or freshly scraped intestinal mucosa of various segments of the small intestine and colon of rat and pig. Ileum1 and ileum2 refer to the proximal and distal parts of the ileum, respectively.

Although the pig model was considered representative for studying oral absorption in humans, one must bear in mind that studies assessing the enzymatic activity in homogenates from the human intestine should be conducted before results obtained in the Caco-2 system can be extrapolated to humans. It should however be mentioned that the ultimate utility of the Caco-2 system is not to enable a quantitative extrapolation to absorption in humans; it is primarily oriented to obtain information on the mechanism of absorption or absorption enhancement of drugs and to perform discrimination studies between (pro) drug candidates for evaluation of intestinal absorption.

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