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Assessment and modulation of acamprosate intestinal absorption: comparative studies using in situ, in vitro (CACO-2 cell monolayers) and in vivo models

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Abstract

The purpose of this study was to explore the intestinal absorption mechanism of acamprosate and to attempt to improve the bioavailability (BA) of the drug through modulation of its intestinal absorption using two enhancers (polysorbate 80 and sodium caprate) based on in situ, in vitro and in vivo models and comparing the results obtained. Intestinal transport of the drug, in the absence and in presence of polysorbate 80 (0.06, 0.28 and 9.6 mM) or sodium caprate (13 and 16 mM) was measured by using an in situ rat gut technique and Caco-2 cell monolayers. Additionally, the effect of sodium caprate on drug oral bioavailability, measured as urinary recovery, was quantified by performing in vivo experiments with the rat as animal model. Only sodium caprate was able to increase the absorption rate constant (k_a) of acamprosate in the mid-intestine of the rats from $0.29 \pm 0.07\,h^{-1}$ in the absence of the promoter to $0.51 \pm 0.19\,h^{-1}$ in the presence of C10 16 mM, along with the apparent permeability (P_{app}) obtained in Caco-2 cells (around two-fold). However, the drug bioavailability in rats (around 20%) did not improve in the presence of any of the concentrations tested (13, 16 and 50 mM). It is concluded that acamprosate absorption likely occurs via paracellular pathway and can be enhanced by sodium caprate in situ and in vitro but not in vivo—thus suggesting that although in situ and in vitro studies could be useful in early screening to select a potential promoter, in vivo studies in animal models are necessary to confirm the utility of the enhancer and to determine the influence of physiological variables.

Keywords: Enhancement; Acamprosate; Intestinal absorption; Paracellular; Bioavailability

1. Introduction

Numerous efforts are currently being made to ensure successful absorption after peroral drug administration, this being one of the greatest challenges in the field of drug delivery (Aungust, 1996; Langer, 1998; Ward et al., 2000). The poor permeation of drugs across the intestinal mucosa (usually due to their high hydrophilicity) is one of the common factors leading to failed absorption and thus to low drug bioavailability (BA). This seems to be the case of acamprosate ($(CH_3–CO–NH–CH_2–CH_2–CH_2–SO_3)_2Ca$, or calcium bis-acetylhomotaurinate), a relatively new psychotropic drug used for relapse prevention in alcoholism. Its oral bioavailability in humans is very low ($11 \pm 1\%$) when administered as enteric coated tablets, which implies the need for high doses (2 g/day) (Saivin et al., 1998). Surprisingly, until recently the absorption mechanism of acamprosate was completely unknown. In 1988, Chabenat et al. (1988) conducted a physicochemical study of acamprosate, showing it to be a highly hydrophilic compound almost completely dissociated in biological fluids—the acetylhomotaurinic acid molecule having a strongly charged functional group. These physicochemical properties of the drug, combined with the fact that acamprosate is not metabolized in either the intestine or liver (Saivin et al., 1998; Zornoza et al., 2002), leads us to postulate that the low

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permeability of the drug in the intestinal mucosa is the most probable reason for its reported low bioavailability. Hence, knowledge of its intestinal absorption mechanism would be crucial to maximize bioavailability. We started to address the problem in a previous paper involving a kinetic study of acamprosate intestinal absorption using an in vitro technique with intact rat jejunum. According to our results, the drug is predominantly transported in the rat mid-jejunum by passive diffusion. A lesser percentage of the absorption is mediated by the iminoacid carrier. Moreover, in this earlier paper we postulated the possible existence of secretion transport limiting its bioavailability (Más-Serrano et al., 2000). Nevertheless, we did not elucidate whether diffusion occurs via transcellular or paracellular routes—though others authors (Saivin et al., 1998) have postulated that acamprosate could cross the epithelial barrier via the paracellular route, albeit without providing any experimental evidence.

One of the strategies for enhancing the oral bioavailability of hydrophilic drugs involves the use of intestinal permeation enhancers (Aungust, 1996). A variety of exogenous compounds have been identified as promoters, including surfactants and medium chain fatty acids (MCFAs). Whereas non-ionic surfactants seem to enhance the transcellular pathway (Anderberg et al., 1992; Bermejo et al., 1991), MCFA increase hydrophilic drug permeability mainly through the paracellular route (Lindmark et al., 1998a,b). In fact, these latter compounds are included in the category of paracellular permeability enhancers (PPEs) (Ward et al., 2000). The use of such compounds could allow us not only to increase acamprosate absorption but also to explore the transport pathway of this drug. We selected polysorbate 80 (P80) as potential enhancer, since it reportedly increases the polarity of the absorbing intestinal membrane, rendering it more permeable to very hydrophilic substances (Bermejo et al., 1991), and is also able to reduce *P*-glycoprotein activity. Sodium caprate (C10), a MCFA capable of facilitating the transport of hydrophilic model compounds via the paracellular route (Anderberg et al., 1993; Sakai et al., 1997; Lindmark et al., 1998a,b), was also tested. The enhancement properties of P80 and C10 have been extensively documented by in vitro (mainly Caco-2 cells) and in situ animal studies (Bermejo et al., 1991; Anderberg et al., 1992, 1993; Sakai et al., 1997; Lindmark et al., 1998a). However, data are sparse regarding their promoting effect in vivo. The results obtained are moreover contradictory. While Chao et al. (1999) reported that sodium caprate can promote intestinal absorption of a small peptide in vitro and in vivo, Lennernäs et al. (2002) showed this fatty acid alone to have a limited effect on human permeability in vivo across the rectal epithelium when presented in solution. These latter authors also concluded that in vivo studies are needed to validate the results obtained in vitro or in situ, in order to avoid the risk of inaccurate predictions. Furthermore, it must be taken into account that in vivo studies are also required during formulation development, to determine the effective dose of the enhancer agent.

The present study explores the absorption mechanism of acamprosate through in situ, in vitro and in vivo experiments. We have also attempted to enhance acamprosate absorption using additives that can easily be formulated with the drug and therefore may potentially increase the bioavailability of acamprosate. On the other hand, the use of such compounds constitutes a valuable tool for evaluating the preferred route of acamprosate transport (i.e., transcellular or paracellular) (Knippt et al., 1997). In turn, the influence of the enhancers on drug oral bioavailability has been studied in order to confirm their usefulness in vivo.

2. Materials and methods

2.1. Chemicals and animals

^{14}C-Mannitol (MW 182; specific radioactivity 53.7 mCi/mmol) was obtained from New England Nuclear, Boston, MA (through Perkin-Elmer Life Sciences Inc.). The sodium salt of capric acid (C10) (99–100% purity), polysorbate 80, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, non-essential amino acids, glutamine and G-penicillin–streptomycin 10,000 U/ml were obtained from Sigma Chemical Co., St. Louis, MO. Hanks balanced salts solution (HBSS) and Hanks balanced salts solution without Ca^{+2}/Mg^{+2} (HBSS/CMF) were purchased from Biochrom AG, Berlin. ^{14}C-Acamprosate (MW 403.39; specific radioactivity 90.0 mCi/mmol) was a gift from Merck-Lipha Inc.

Male Wistar rats, bred and housed in the Animal Center of the Faculty of Pharmacy (University of Valencia, Spain), were used. The rats were housed under standard conditions of temperature (20 ± 2 °C), relative humidity (50%) and light and dark cycle (12 h/12 h). The animals were fasted prior to experiments, but having access to water ad libitum. They were randomly distributed to the experimental groups, each animal being used for one assay only. All procedures were conducted in strict adherence to EEC Council Directive 86/609 and Spanish law (RD 223/1988), and policies on the protection of animals. Experiments were approved by the Animal Care Committee of the Faculty of Pharmacy of the University of Valencia.

2.2. In situ experiments

2.2.1. Experiment 1: Topographic study of acamprosate absorption

All absorption tests were performed in male Wistar rats (n = 35) weighing 200–240 g, through an in situ rat gut technique (Bermejo et al., 1991). The animals were fasted for a period of 20 h, with free access to water. After anesthetizing the rats, the abdomen was opened and the small intestine was divided into three fractions of equal lengths: the proximal segment (0–33 cm from the pylorus), the mid-segment (the middle 33 cm of the small intestine) and the distal segment

(0–33 cm from the ileocecal junction). In each animal, one specific sector of the small intestine was randomly selected and cannulated. Posteriorly, 5 ml of an isotonic solution of 0.22 μM ^{14}C-acamprosate (0.1 μCi) buffered to pH 7.4 were perfused at 37 °C. The drug concentrations remaining in the perfused solution were measured every 5 min, for a total of 30 min. Water reabsorption was evaluated in each animal by measuring the remaining volume at 30 min, according to a previously reported procedure (Bermejo et al., 1991).

2.2.2. Experiment 2: Influence of polysorbate 80 on the intestinal absorption of acamprosate

To analyze the effect of the non-ionic surfactant polysorbate 80 on acamprosate absorption, we measured drug absorption in the absence (control group) and in the presence of a wide range of surfactant concentrations (below, equal to, and above the critical micellar concentration (CMC)). The CMC of P80 was previously determinated in our laboratory in the medium we have used to perform the experiments (Pla-Delfina et al., 1987). Five milliliters of an isotonic solution containing 0.22 μM ^{14}C-acamprosate (0.1 μCi) and polysorbate 80 (0.06, 0.24 (CMC) or 9.6 mM) buffered to pH 7.4 were perfused at 37 °C in the mid-segment. The in situ rat gut technique mentioned above was used to perform the experiments.

2.2.3. Experiment 3: Influence of sodium caprate on the intestinal absorption of acamprosate

The potential enhancing effect of C10 on drug absorption was tested using three experimental groups of animals. Acamprosate absorption was evaluated in the absence (control group) and in the presence of two concentrations of C10 (13 (CMC) and 16 mM). Five milliliters of an isotonic solution of 0.22 μM ^{14}C-acamprosate (0.1 μCi) and sodium caprate (0, 13 or 16 mM) buffered to pH 7.4 were perfused in the mid-segment. In three additional experimental groups, we analyzed the effect of previous exposure (40 min) to the promoter (16 mM) upon acamprosate intestinal absorption. In control group I, the mid-segment of the rat small intestine was pre-exposed for 40 min to a buffer (pH 7.4) solution. Posteriorly, an isotonic solution of 0.22 μM ^{14}C-acamprosate was perfused, and the absorption assay was performed as previously. In control group II, the mid-segment was pre-exposed identically as in control group I, though in the absorption experiment a solution containing 0.22 μM ^{14}C-acamprosate plus 16 mm C10 was perfused. In an additional group of animals, the mid-intestine was pre-exposed to an isotonic and buffered solution of 16 mM C10 for 40 min. After perfusing 0.22 μM ^{14}C-acamprosate plus 16 mM C10, the absorption test was carried out as in experiments 1 and 2.

2.2.4. Absorption rate measurements

Since acamprosate is absorbed mainly by passive diffusion (Más-Serrano et al., 2000), the first-order equation was fitted to the corrected remaining drug concentrations obtained at each time in experiments 1, 2 and 3, using a non-linear square regression program (Sigma plot 4.0):

$$A = A_0\, e^{-k_a t} \quad (1)$$

where A represents the remaining acamprosate concentration found in the luminal content at each sampling time, t, corrected for water reabsorption; A_0 is the calculated intercept at zero time; and k_a is the absorption rate constant of the drug.

2.3. In vitro transport experiments

2.3.1. Cell culture

Caco-2 cells originating from a human colorectal carcinoma (obtained from American Type Culture Collection (ATCC) (Rockville, MD) were maintained at 37 °C in an atmosphere of 5% CO_2 and in high-glucose DMEM supplemented. Cells were seeded in 23.1 mm i.d. Transwell® inserts (polycarbonate membrane, 3.0 μm pore size) in six-well plates at a density of 120,000 cells/cm^2. Confluent cell monolayers were studied 21 days after seeding. The transepithelial electrical resistance (TEER) of the filter-grown monolayers reached a value of at least 840 Ω cm^2 before use in the experiment. This cell line has been used in our laboratory to study the secretion process of several fluorquinolones demonstrating that *P*-glycoprotein is functional in our experimental conditions (Rodriguez-Ibañez et al., 2003).

2.3.2. Permeability studies in Caco-2 cells

Inserts were washed twice for 30 min with warm transport buffer, HBSS pH 7.4. All the experiments were performed in air at 37 °C and 95% relative humidity. In general, the basolateral chamber was bathed in 3 ml of HBSS, and 2 ml of the test solution, prepared in HBSS/CMF to avoid precipitation of the sodium caprate, was added to the apical chamber (t = 0). Several authors (Anderberg et al., 1993; Watson et al., 2001) have demonstrated that the absence of Ca^{2+}/Mg^{2+} in the apical chamber does not affect either monolayer integrity or mannitol flux if HBSS in the basolateral chamber always contains Ca^{2+}/Mg^{2+}; normal compound transport would thus be assured.

Acamprosate permeability was determined in the apical to basolateral (AP-BL) and basolateral to apical (BL-AP) direction. The concentration-dependent effect of the promoter was studied testing permeability values of ^{14}C-mannitol (8.2 μM), a well known paracellular pass marker, and ^{14}C-acamprosate (4.9 μM) in the absence and presence of two concentrations of sodium caprate (13 or 16 mM). In order to determine the possible time-dependent effect of the enhancer, monolayers (apical chamber) were previously incubated with sodium caprate 16 mM in HBSS/CMF for 40 min. The medium was then changed to HBSS/CMF containing 16 mM C10 and the analyte (^{14}C-acamprosate or ^{14}C-mannitol). In both cases and in all the experiments

performed, the total concentration of radiolabelled compound used was always 0.44 μCi/ml.

Samples from the basolateral chamber were collected 30, 60, 90, 120 and 150 min after addition of the test solution, and were then replaced with the same volume of fresh HBSS. Cumulative concentrations were calculated after correction for dilution caused by replacement with fresh HBSS. The apparent permeability coefficient (P_{app}, cm/s) was determined using the following equation:

$$P_{app} = \frac{dC}{dt}\frac{V_r}{AC_0} \quad (2)$$

where V_r is the volume of the receptor chamber (3 ml), A is the surface area of the membrane (4.2 cm^2), C_0 is the initial concentration in the donor chamber (μM), and (dC/dt) is the flux (J) (μM/s) determined by the linear slope of receptor drug concentration corrected for dilution versus time.

2.3.3. Measurement of TEER

After 1 h exposure of each monolayer to fresh HBSS, the TEER of the monolayers was measured (initial value at $t = 0$) using the MILLICELL electrical resistance system (Millipore Corp., Bedford, MA). At the end of each experiment ($t = 150$ min), the TEER of the monolayers was measured again. Results are presented as the percentage of the initial ($t = 0$) value in the same monolayer.

2.4. In vivo experiments

Acamprosate bioavailability after oral administration was studied in male Wistar rats (280–320 g). The animals were fasted overnight prior to drug administration. ^{14}C-Acamprosate (2.75 μM, 0.5 μCi) was dissolved in 2 ml of distilled water. The animals were randomly distributed into four experimental groups that received the ^{14}C-acamprosate solution orally with the addition of 0, 13, 16 or 50 mM C10 via gastric intubation. During the experiments, the animals were housed in metabolic cages in order to collect urine (0–24 h). We have previously used HPLC analysis to show that acamprosate is completely eliminated (98%) in the form of unchanged drug in the urine of animals (Zornoza et al., 2002). Moreover, we have proven that after oral administration of the drug, the total amount excreted in urine and in feces is very close to 100% (data not shown). Thus, urinary recovery following oral administration could be used as a measure of drug bioavailability. Twenty-four hours after drug administration, samples were diluted with 70 ml of distilled water.

2.5. Analytical method

Samples from in vitro and in situ experiments were analyzed for their ^{14}C-acamprosate or ^{14}C-mannitol content. The radioactivity content from urine samples was also measured.

In all cases, radioactivity was determined with a Microbeta Trilux® spectrometer through liquid scintillation counting (Ecoscint™ H; National Diagnostics, Atlanta, Georgia) of 150 μl (in vitro experiments) or 200 μl (in situ and in vivo experiments) of the sample. All samples were subjected to counting in triplicate. The coefficient of variation was always <2%. The concentration of the radiolabelled compound (acamprosate or mannitol) was calculated by reference to a standard calibration curve, obtained by analyzing known amounts in the liquid scintillation spectrometer.

2.6. Statistical methods

Statistical comparisons were performed by the one-way analysis of variance (ANOVA). When significant differences were found, the multiple comparison Tukey test was applied. Statistical significance was accepted for $P < 0.05$.

3. Results

3.1. In situ experiments

3.1.1. Experiment 1: Topographic study of acamprosate absorption

After fitting the first-order equation (Eq. (1)) to the corrected remaining acamprosate concentrations at each sampling time, the following first-order absorption constants, k_a, were obtained: proximal segment ($n = 12$) $k_a = 0.38 \pm 0.13\ h^{-1}$, mid-segment ($n = 13$) $k_a = 0.35 \pm 0.09\ h^{-1}$ and distal segment ($n = 12$) $k_a = 0.35 \pm 0.13\ h^{-1}$. The analysis did not reveal any statistically significant differences among the k_a-values tested ($P = 0.797$). Thus, k_a-values seem to be invariable along the rat small intestine. Based on these results, the existence of a preferential absorption zone in the small intestine can be discarded. It must be stressed that the k_a-values obtained are quiet low in comparison with those corresponding to well absorbed compounds such as flumequin (k_a-value $= 6.87 \pm 1.09\ h^{-1}$, bioavailability 94%) (Sanchez-Castaño et al., 2000) and antipyrine (k_a-value $= 2.75\ h^{-1}$, bioavailability near 100%) (Merino et al., 1989; Peris-Ribera et al., 1992). Probably this fact could explain the low oral bioavailability of acamprosate.

Since k_a-values did not differ along the small intestine, we have chosen the mid-intestine for the enhancement experiments, because we recorded less variability in this segment, with the need for fewer animals to obtain reliable values.

3.1.2. Experiment 2: Influence of polysorbate 80 on the intestinal absorption of acamprosate

Fig. 1 summarizes the k_a-values (h^{-1}) obtained after fitting Eq. (1) to the corrected remaining acamprosate concentrations measured in the absence and presence of the non-ionic surfactant. As can be seen, the k_a-values decreased significantly ($P < 0.001$, ANOVA) to 63% ($0.21 \pm 0.05\ h^{-1}$), 66% ($0.22 \pm 0.04\ h^{-1}$) and 42% ($0.14 \pm$

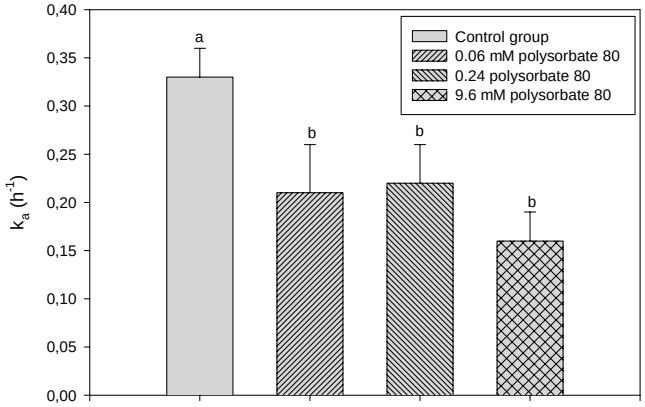

Fig. 1. Effect of polysorbate 80 on acamprosate intestinal absorption in situ in rat mid-small intestine. Bars represent the intestinal absorption rate constants of acamprosate, k_a, in the absence (control group) and in the presence of polysorbate 80 at concentrations of 0.06, 0.24 and 9.6 mM. The standard deviation of each mean value is also plotted ($n = 6$). Groups with different letters are statistically different at a 5% significance level (Tukey test).

$0.02\,h^{-1}$) of the control value ($0.33 \pm 0.03\,h^{-1}$) in the presence of 0.06, 0.24 and 9.6 mM of surfactant, respectively. Hence, acamprosate absorption in the rat small intestine not only does not increase in the presence of the surfactant but actually decreases.

3.1.3. Experiment 3: Influence of sodium caprate on the intestinal absorption of acamprosate

The k_a-values of acamprosate in the absence and in the presence of C10 are summarized in Fig. 2. As can be seen, k_a increased significantly ($P < 0.0001$) to 179% ($0.52 \pm 0.17\,h^{-1}$) and 176% ($0.51 \pm 0.19\,h^{-1}$) of the control value ($0.29 \pm 0.07\,h^{-1}$) in the presence of 13 and 16 mM of the promoter, respectively (Fig. 2A).

On the other hand, the effect of prior 40 min exposure of the rat intestine to C10 (16 mM) did not increase the k_a-value significantly with respect to the value obtained with C10 16 mM in the absence of pre-exposure [200% ($0.50 \pm 0.18\,h^{-1}$) and 191% ($0.45 \pm 0.13\,h^{-1}$) of control I value ($0.25 \pm 0.13\,h^{-1}$)] (Fig. 2B).

These in situ results suggest that sodium caprate could be an effective excipient for use in enhancing acamprosate absorption.

3.2. In vitro experiments

The AP-BL ($1.074 \times 10^{-6} \pm 0.158 \times 10^{-6}$ cm/s) and BL-AP ($1.098 \times 10^{-6} \pm 0.045 \times 10^{-6}$ cm/s) permeabilities of ^{14}C-acamprosate were determined. The BL-AP/AP-BL permeabilities ratio was 1.022 ± 0.156 (S.D. was calculated using the delta method). This ratio was close to unity, which may indicate that there is not any transporter implicated in the absorption process. The ratio also indicates that no drug secretion occurs under our experimental conditions.

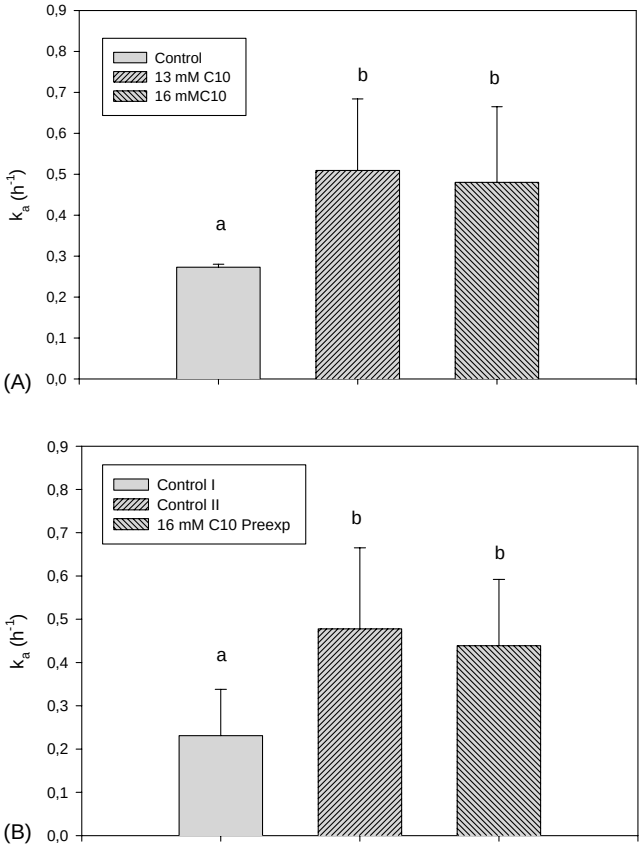

Fig. 2. (A) Enhancing effect of sodium caprate on acamprosate intestinal absorption in situ in rat mid-small intestine. Bars represent the intestinal absorption rate constants of acamprosate, k_a, in the absence (control) and in the presence of 10 and 13 mM sodium caprate. (B) The plot shows the k_a of acamprosate obtained in three additional groups. In control group I the intestine was pre-exposed for 40 min to a buffer solution followed by the perfusion of a solution of 0.22 μM ^{14}C-acamprosate. In control group II, the pre-exposition to a buffer solution was followed by the perfusion of 0.22 μM ^{14}C-acamprosate plus 16 mM C10. In the last group, the intestine was pre-exposed to a solution of 16 mM C10 for 40 min followed by the perfusion of 0.22 μM ^{14}C-acamprosate plus 16 mM C10. The standard deviation of each mean value is also plotted ($n = 10$). Groups with different letters are statistically different at a 5% significance level (Tukey test).

Table 1 summarizes the permeability coefficients of ^{14}C-acamprosate and ^{14}C-mannitol in the absence and in the presence of C10 under several experimental conditions. As is shown by the statistical analysis, the permeabilities of both compounds were enhanced as the concentration of the promoter or the duration of exposure was increased—exhibiting a clear concentration- and time-dependent effect of sodium caprate in the absorption of both compounds. In Fig. 3, the P_{app}-values of acamprosate and mannitol in the absence and presence of C10 at different exposure times were plotted.

The concentration- and time-dependent effects of C10 on TEER, an indicator for opening of the tight junction (Sakai et al., 1997) are shown in Figure 4. The TEER values in the control monolayer did not change in the course of

Table 1
Comparison of apparent permeability coefficients (P_{app}) of the transport of ^{14}C-mannitol and ^{14}C-acamprosate[a]

Enhancer	P_{app} (10^7 cm/s)	
	^{14}C-Mannitol	^{14}C-Acamprosate
Control	2.80 ± 0.85 b ($n = 4$)	10.33 ± 1.58 b ($n = 6$)
13 mM C10	4.95 ± 1.33 bc ($n = 4$)	12.26 ± 0.80 c ($n = 4$)
16 mM C10	7.27 ± 0.90 c ($n = 4$)	14.89 ± 0.79 d ($n = 4$)
16 mM C10 (40 min pre-exposition)	21.52 ± 3.28 d ($n = 5$)	24.11 ± 1.99 e ($n = 5$)

Groups with different letter (b–e) are statistically different at a 5% significance level (Tukey test).
[a] Calculated from the results of in vitro transport of mannitol and acamprosate across Caco-2 monolayers and represented as the mean ± S.D. (standard deviation).

the experiments (150 min) with acamprosate and mannitol. In contrast, the TEER values significantly decreased in a concentration-dependent manner when C10 was added together with acamprosate or mannitol—the maximum reduction being attained when the monolayers were previously pre-exposed to the fatty acid salt during 40 min (21.28 and 24.93% of the control value for the drug and mannitol, respectively).

In the same way as the in situ results, these experiments suggest that sodium caprate is a good candidate for enhancing acamprosate absorption.

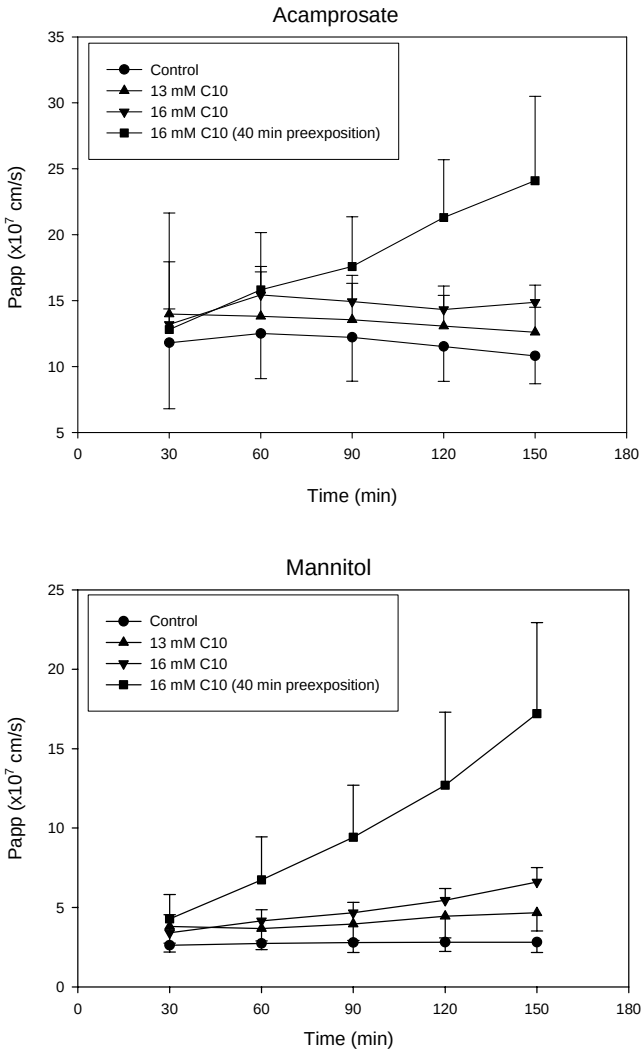

Fig. 3. Time-dependent effects of sodium caprate on the transport of acamprosate and mannitol across Caco-2 cell monolayers. The graph represents P_{app}-values determined during different time intervals after addition of C10 13 or 16 mM. The time-dependent effect of 40 min pre-exposure to C10 16 mM was also plotted. Mean values ± S.D. ($n = 4$–6).

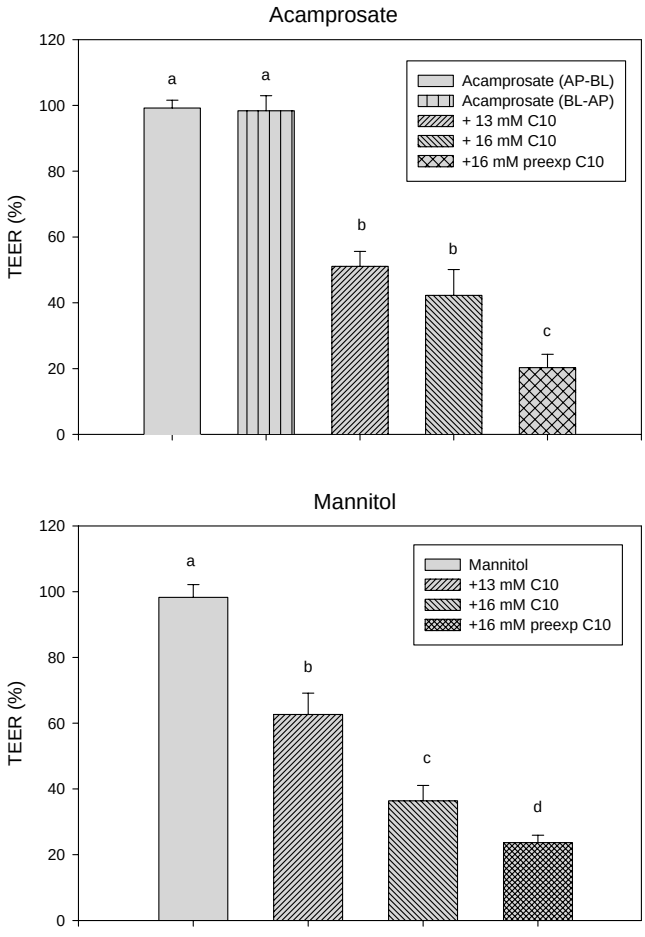

Fig. 4. Transepithelial electrical resistance (TEER) of the Caco-2 monolayers measured at the end of the different permeability studies (150 min) with acamprosate and mannitol. The TEER values are represented as the percentage of the intial value ($t = 0$ min) in the same monolayer. Groups with different letters are statistically different at a 5% significance level (Tukey test).

Table 2
Percentage of ^{14}C-acamprosate excreted in urine (U_0^{24}) in the absence and presence of C10 after oral administration in the rat[a]

C10 concentration (mM)	n	U_0^{24} (%) (mean ± S.D.[b])
0	12	19.87 ± 4.57
13	8	24.18 ± 8.81
16	8	23.82 ± 5.12
50	8	23.40 ± 6.71
Significance	–	n.s.

[a] n.s.: non-significant in the ANOVA test at a 5% significance level.
[b] S.D.: standard deviation.

3.3. In vivo experiments

Table 2 summarizes the percentage administered oral dose eliminated in the urine of the animals over the 0–24 h post-administration period in the four experimental groups (0, 13, 16 and 50 mM C10). Although a slight tendency to increase acamprosate recovery in urine is observed when administered with sodium caprate, the statistical analysis revealed no significant differences.

4. Discussion

4.1. In situ and in vitro experiments

For an in-depth exploration of the intestinal transport of acamprosate, we planned several experiments using an in situ rat gut technique. We first conducted a topographic study to assess drug absorption in the proximal (duodenum), middle (jejunum) and distal (ileum) segments. These experiments allowed us to detect or discard possible preferential absorption at some point along the intestine attributable to localization of the iminoacid carrier or simply to differences in permeability along the intestinal canal, as has been reported for different marker molecules (Artursson et al., 1993). As the results show, the first-order rate constant does not vary in the different intestinal regions tested, yielding a mean k_a-value of 0.35 h^{-1}, which is quite low. These data confirm that diffusion is the preferential mechanism of absorption and suggest that the low permeability is the main cause for the low bioavailability of acamprosate—discarding the possibility that preferential carrier localization could be responsible of this behavior. These conclusions, while partly redundant with respect to our previous studies (Más-Serrano et al., 2000), provided a valuable basis for designing the following permeation enhancement experiments with acamprosate. In fact, we selected the mid-segment of the rat small intestine to improve acamprosate absorption, because the absorption rate constant obtained in this region was less variable (see Section 3).

According to the above cited physicochemical characteristics of acamprosate, this is the typical type of compound that may benefit from permeation-enhancing excipients (Aungust, 2000). A critical issue to consider in evaluating the usefulness of any particular enhancer is the selection of its concentration, because some enhancers show scant separation between toxicity and efficacy (increased permeability) (Ward et al., 2000). In these experiments, we selected a concentration range based on the data found in the literature (Bermejo et al., 1991; Anderberg et al., 1992, 1993; Sakai et al., 1997; Lindmark et al., 1998a). Polysorbate 80, at the concentrations tested, was unable to increase acamprosate absorption. This lack of effect of P80 could lead us to discard the transcellular route as the preferential route for acamprosate absorption. Nevertheless, the experiment is not conclusive, since the effects of non-ionic surfactants on intestinal permeability are quite complex.

While P80 failed to enhance acamprosate absorption, effective absorption increases occurred with sodium caprate. This fatty acid has been extensively characterized as an absorption-enhancing excipient (Aungust, 2000), and there is extensive agreement that sodium caprate increases the intestinal absorption of hydrophilic drugs mainly through the paracellular pathway (Anderberg et al., 1993; Sakai et al., 1997; Lindmark et al., 1998b; Söderholm et al., 2002). Accordingly, we present the first experimental evidence that acamprosate absorption may occur preferentially via the paracellular route. The results reported here also indicate that under our conditions and in the concentration range studied, the promoting effect of C10 (between 1.75- and 2-fold) is not concentration-dependent. Regarding the time dependency of the promoting effect, we designed the experiment on the basis of the results reported by Anderberg et al. (1993). These authors showed that, in Caco-2 cells, at concentrations above 13 mM (particularly at 16 mM), sodium caprate exhibits a clearly time-dependent effect on the transport of mannitol (a typical paracellular probe) and penicillin V. Nevertheless, according to our results, 40 min of tissue pre-exposure to sodium caprate does not increase acamprosate absorption versus the respective control group (Fig. 2B). Curiously, on performing in vitro studies with Caco-2 cells we observed the time- and concentration-dependent effect, as will be commented below.

In the in vitro experiments, we obtained a permeability value (AP-BL) of 1.03 × 10^{-6} cm/s for acamprosate. Moreover, no significant asymmetry was observed for acamprosate in Caco-2 cells (AP-BL and BL-AP fluxes were not statistically different). This observation, together with the lack of effect of verapamil on acamprosate intestinal absorption (data not shown), may allow us to discard the possibility that the existence of secretion transport of the drug limits its oral bioavailability (Más-Serrano et al., 2000).

On the other hand, on comparing the permeabilities of acamprosate and mannitol, the permeability of the former is seen to be about three-fold greater than that of mannitol. Both compounds have a similar molecular weight (182 Da for mannitol and 181.65 Da for acetylhomotaurine); accordingly, their hydrodynamic radii, estimated according to Tavelin et al. (2003), are effectively comparable (around 3.6 Å)—these values in turn corresponding to the average

pore radius of the Caco-2 cells. Nonetheless, several authors (Artursson et al., 1993; Pauletti et al., 1997) have shown that characteristics other than molecular weight (e.g., shape, flexibility of the structure and charge) are also important for paracellular permeability. Mannitol is a neutral compound while acamprosate is negatively charged—a fact that could explain the observed differences in the P_{app}-values. The effect of charge on permeability is not clearly defined, the reported results being controversial (Pauletti et al., 1997). Watson et al. (2001) have reported similar findings with PEG 282 and mannitol. Although their hydrodynamic radii are similar, PEG 282 exhibits three- to four-fold greater permeability than mannitol across Caco-2 cells.

As pointed out above, our in vitro results show the effect of C10 to be concentration- and time-dependent, the maximum enhancement effect being obtained after pre-exposure experiments (P_{app}-value was 233% of the control value). The same behaviour has been observed with mannitol, though in this case the permeability increased to 760% of the control value. These differences are probably related to the different intrinsic permeabilities observed for both compounds and to the mechanism of action of sodium caprate.

The improvement of acamprosate permeability was accompanied by a decrease in the TEER values. According to Fig. 4, the TEER values show a significant decrease as the concentration of sodium caprate increases, yielding a maximum reduction after the pre-exposure experiments. These data correlate well with those obtained in the acamprosate permeability studies, demonstrating a greater permeability of the drug in the presence of C10, attributable to tight junction aperture. The results of the TEER measurements in the mannitol experiments exhibit a truly similar profile, even though the observed increments in mannitol permeability are greater than those obtained with acamprosate, as we have pointed out above. It should be taken into account that Artursson et al. (1993) have reported that permeability to marker molecules affords a more correct description than TEER of the selective barrier function in tighter epithelia such as Caco-2 cell monolayers. In addition, others investigations have shown that changes in TEER and mannitol permeability are not always correlated (Balda et al., 1996).

Overall, the results from the in situ and in vitro experiments support the idea that poor absorption may be the main cause of the low oral bioavailability of the drug. Moreover, these studies nicely illustrate that sodium caprate is able to significantly enhance acamprosate intestinal absorption in the concentration range tested. As a result, this compound would be a good candidate for use as an excipient in formulation to increase drug absorption. However, the performance of in vivo studies in experimental animals is required to validate the efficiency of this fatty acid.

4.2. In vivo experiments

We chose the rat as animal model for performing the in vivo studies because, as reported by He et al. (1998), this species is better than the dog for predicting the human bioavailability of paracellularly absorbed drugs. These authors have demonstrated a good correlation between the BA of some hydrophilic substances, which are predominantly excreted unchanged following intravenous administration, in humans and rats—the latter tending to underpredict BA in humans. Acamprosate is a hydrophilic drug excreted 95% as unchanged drug (on average) in urine after intravenous administration (Zornoza et al., 2002). No studies have been published to date in relation to acamprosate bioavailability after oral administration in rats. Hence, we first planned a series of experiments to address this aspect. Urine collection over 24 h after drug oral administration was used to estimate BA, since this drug is stable in plasma, is not metabolized in the biological medium, and the total amount excreted in urine and feces is close to 100% (80% in feces and 20% in urine; data not shown). Collectively, these data strongly support the idea that urine recovery following an oral dose yields a close measure of drug bioavailability and the extent of its intestinal absorption. As can be seen in Table 2, the estimated oral BA of acamprosate in rats is close to 20%, doubling that obtained in humans (11%) (Saivin et al., 1998).

Published studies (Chao et al., 1999) indicate that C10 concentrations greater than those used in our in situ and in vitro experiments are able to increase the in vivo bioavailability of an enteral peptide without causing detrimental alterations of the intestinal mucosa. Therefore, a greater concentration of the fatty acid (50 mM) was also assayed in the in vivo enhancement experiments. According to the results obtained, although the percentage drug excreted in urine is always high in the presence of sodium caprate, no significant differences between the values were found. Thus, the enhancing effect of C10 on acamprosate absorption observed in situ and in vitro has not been confirmed in vivo.

This lack of effect in vivo is in concordance with previous data presented by Lennernäs et al. (2002). There could be several explanations for this observation. In fact, these and other authors (Takahashi et al., 1994) have indicated that fatty acid permeability into the tissue upon which it acts is very important for obtaining a promoting effect for poorly permeable compounds. In addition, Raoof et al. (2002) have shown that sodium caprate presents a rapid onset and short duration of action, which could be one of the reasons why no significant efficacy of C10 was detected in our in vivo studies. Accordingly, an increase in enhancer residence time in the intestine and the prolongation of sodium caprate exposure to the intestinal membrane was associated with improved oral BA (Raoof et al., 2002) Furthermore, as can be seen in Fig. 3, the time-dependent effect of sodium caprate on the intestinal permeability of mannitol and acamprosate is clearly evident in the case of pre-exposure to C10 16mM. It seems to exist an initial phase (lasting less than 30 min), characterized by a rapid increase in permeability. This initial phase has been reported by other authors (Lindmark et al., 1998a). In our experiments, this phase could not be studied since our first sample was taken at 30 min. In the

pre-exposure group, a second phase is observed, characterized by a more prolonged and important increase in permeability. This time-dependent effect could account for the lack of effect of sodium caprate in vivo in our experimental conditions. Another crucial and related point is the concentration of the enhancer at the site of drug absorption. In contrast to the conditions of the in situ and in vitro experiments, when C10 is administered in vivo, calcium is present in the luminal content and can reduce fatty acid concentration by precipitation—thus probably reducing its efficacy. The same effect causes fatty acid dilution by intestinal fluids in vivo. In this context it could be worth testing greater C10 concentrations (100–200 mM). It is worth pointing out that Chao et al. (1999) used higher sodium caprate concentrations (100–167 mM). Likewise, the enhancement of intestinal absorption detected in vivo by Raoof et al. was observed using C10 concentrations greater than 50 mM (80, 160 and 300 mM). The interaction of sodium caprate with mucus might offer an additional explanation for this lack of effect of C10 observed in vivo, as occurs with other enhancers (Schipper et al., 1999; Ward et al., 2000). In fact, Schipper et al. (1999) have observed that although chitosans (potent absorption enhancers for poorly absorbed drugs) are effective in Caco-2 cells and are able to affect the disappearance of atenolol from the perfusion solutions (using an in situ rat perfusion model), they do not seem to influence the BA of the drug. It was suggested that the presence of the anionic mucus layer in the intestine prevented chitosan interaction with the epithelial surface, thus inhibiting the absorption-enhancing effects of these promoters. Our findings are effectively similar, since while sodium caprate was able to enhance acamprosate disappearance from the perfusion solution (in situ experiments), it failed to improve the BA of this drug.

In contrast to our findings, Chao et al. (1999) have observed an in vivo enhancing effect of C10 on intestinal absorption of a small peptide. Such discrepancy could be explained taking into account that in the in vivo experiments of the above authors the enhancer was directly delivered to the terminal ileum, whereas we administered the drug orally. In this sense, it has often been the case that the BA of a drug administered with an enhancer is far lower after oral dosing than after administration directly into the intestine (Aungust, 2000).

Taken as a whole, the lack of effect of C10 on acamprosate BA could be explained as the sum of all the above cited factors, which prevail in vivo and prevent us from obtaining measurable drug absorption enhancement.

4.2.1. Concluding remarks

Our results provide the first experimental evidence of acamprosate absorption via the paracellular pathway, and clearly show poor membrane permeability to be the main cause of its low oral bioavailability. The in situ and in vitro studies suggest that sodium caprate could be useful as an acamprosate absorption-enhancing excipient, even though this fatty acid has been unable to improve oral drug bioavailability.

The comparison of the results obtained in the in situ, in vitro and in vivo experiments indicates that although in situ and in vitro studies could be very useful in early screening to select the compound to be used for promoting drug absorption, in vivo studies are needed to confirm the usefulness of the potential enhancer and to determine the influence of both formulation and physiological variables.

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