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Intestinal uptake and toxicity evaluation of acetazolamide and its multicomponent complexes with hidroxypropyl-β-cyclodextrin in rats

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ABSTRACT

Large oral doses of ACZ lower the intraocular pressure (IOP), but usually lead to a multitude of systemic side effects, including gastrointestinal upset. The present study was undertaken to evaluate the effect of ACZ on the histological structure of rat duodenal mucosa and to assess a possible protective role of the complex formation of ACZ with HP-β-CD, either separately or in combination with a third compound, on the gut epithelial layer by histological and ultrastructural examinations of sections of rat duodenum exposed to ACZ or its formulations. In addition, the transport process of ACZ and its binary or ternary complexes across the duodenal mucosa by means of the single-pass intestinal perfusion (SPIP) method in rats was evaluated. Evidence was found that ACZ alters intestinal permeability and induces damage to the rat small intestine. In contrast, ACZ-induced intestinal injury may be abrogated by ACZ complexation. In addition, the complexation of ACZ with HP-β-CD, alone or in combination with a third compound, facilitated significant levels of ACZ uptake across the rat duodenal segment. Ternary complexes of ACZ with HP-β-CD in combination with TEA (triethanolamine) or calcium ions were found to provide an excellent approach that enabled an increased apparent permeability of ACZ across the duodenal epithelium, with a concomitant ability to preserve the integrity of the gut epithelium from ACZ-induced injury. These results could be useful for the design and development of novel ACZ formulations that can reduce GI toxicity, while still maintaining their essential therapeutic efficacies.

1. Introduction

Acetazolamide (ACZ) is an inhibitor of carbonic anhydrase (CA) and has been used for many years in the management of glaucoma. It is also prescribed in the treatment of various forms of epilepsy, to prevent or at least ameliorate the symptoms of acute high altitude sickness, and in the promotion of diuresis in cases of abnormal fluid retention (for example, in cardiac edema) (Chakravarty and Kannan, 1994; Nair et al., 1995; Kaur et al., 2002).

Oral administration of ACZ decreases the amount of aqueous humor produced by the ciliary epithelium. Although its potent ocular hypotensive effect is well known, its use is limited as despite large oral doses of ACZ being able to lower intraocular pressure (IOP), there are usually a multitude of systemic side effects due to the wide distribution of the CA enzyme. Among most-common side effects are diuresis and metabolic as well as respiratory acidosis (Goodfield et al., 1982; Gamm, 1984; Critchlow et al., 1984; Cowan et al., 1984). In spite of being generally well-tolerated, side effects from ACZ have also been noted to include gastrointestinal upset (Epstein and Grant, 1977).

To our knowledge, there is no published evidence which evaluates the effects of ACZ on the intestinal epithelium. It is known that following oral administration, the ACZ fraction dose absorbed (f_{abs}) and systemic bioavailability approaches 100%, reaching peak plasma concentrations approximately 1–3 h after oral administration (Ritschel et al., 1998; Granero et al., 2008a). However, the precise mechanisms that control the processes of its intestinal transport still remain to be elucidated. Despite the high oral bioavailability of ACZ, Lindenberg et al. (2004) tentatively classified ACZ as Class IV of the Biopharmaceutics Classificaiton Systems (BCS) (Amidon et al., 1995; Taub et al., 2002), indicative of low solubility and low permeability. Later, Wu and Benet (2005) classified ACZ as Class IV in their Biopharmaceutics Drug Disposition Classification System (BDDCS). Kasim et al. (2004) classified ACZ as BCS Class IV. However, their classification is based on solubility data in water, presumably at room temperature and

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calculated partition coefficients (log *P* values of −1.13 (C LogP®) and 0.14 (log *P*)). Also, a log *P* (*n*-octanol/water) value of −0.26 has been reported for ACZ (Remko and von der Lieth, 2004).

By virtue of the fact that ACZ has low solubility (0.72 mg/ml in water at 25 °C), the development of a new formulation of ACZ for oral administration is relevant in order to improve its physical and/or chemical properties.

High patient compliance makes the oral route the preferred mode of drug administration, which the use of oral drug delivery systems being able to improve the stability, pharmacokinetics and biodistribution of a wide variety of therapeutic agents, thereby, leading to more convenient drug administration or improved treatment efficacy with decreased side effects (Bernkop-Schnürch., 2013).

Macromolecules are generally used to alter the physicochemical properties or biopharmaceutical characteristics of an active compound, with molecular encapsulation being applied to many classes of drugs and the observed results have been promising, indicating an improvement in the physical, chemical, and biological properties of the included molecules. In particularly, drug encapsulation inside cyclodextrin (CD) cavities is an interesting alternative that has been applied to a variety of guest drugs, with these macromolecules, being good candidates for sophisticated drug delivery systems (Martin Del Valle, 2004; Brewster and Loftsson, 2007; Loftsson and Brewster, 2010; Jansook and Loftsson, 2009; Szejtli, 1998).

Cyclodextrins (CDs) are a family of cyclic oligosaccharides, obtained from starch by enzymatic degradation and composed of α-1,4-linked glucopyranose subunits. These macrocyclic carbohydrates possess apolar internal cavities which can form complexes with various guest molecules with the CD host being a dynamic rather than permanent process whereby, the guest molecule continuously associates and dissociates from the CD host (Kurkov and Loftsson, 2013). Among the wide variety of CDs available, hydroxypropyl-β-cyclodextrin (HP-β-CD) is a hydroxyl-alkylated-β-cyclodextrin derivative that combines relatively high water solubility with low toxicity and satisfactory inclusion ability (Irie and Uekama, 1997). In addition, ternary CD complexation using hydrophilic polymers, acidic or basic amino acids and polyhydroxy amines has emerged as another promising tool for the efficient solubilization of drugs having poor and pH dependent solubility. Thus, it is possible to increase the drug availability in aqueous CD formulations by including suitable auxiliary agents, thereby reducing the amount of the CD needed in the formulation (Loftsson et al., 2003; Asbahr et al., 2009; Hamai, 2009; Miller and Dahan, 2012).

In a previous study, we developed HP-β-CD with or without TEA (triethanolamine)-based formulations of ACZ, which increased the drug solubility (Granero et al., 2008b). Moreover; the freeze-dried products of multicomponent complexes of ACZ with HP-β-CD had excellent re-dissolution abilities and displayed better dissolution performances in comparison with the uncomplexed drug (Mora et al., 2013). Nevertheless, it is still uncertain whether chronic exposure to ACZ could have deleterious effects on the intestinal mucosa. Here, we have reported the first study regarding the impact of ACZ on the rat duodenal mucosa. Also, we investigated the effects of the HP-β-CD, alone or in combination with a suitable third component, on the intestinal permeability of ACZ, and whether these ACZ multicomponent complexes exerted any direct permeability-enhancing or protective effects on the rat intestinal epithelium. Therefore, the present study was undertaken to evaluate a possible membrane toxicity of ACZ on the gut epithelium by histological and ultrastructural examinations of sections of rat duodenum exposed to ACZ. In addition, the transport process of ACZ across the intestinal mucosa by means of the single-pass intestinal perfusion (SPIP) method was evaluated. Finally, the effects of HP-β-CD, alone or in combination with a third compound, on the interactions between ACZ and the intestinal epithelium of rat were evaluated in situ by means of the SPIP technique and by visualizing the histological and ultrastructural changes in sections of rat duodenum exposed to ACZ formulations.

2. Experimental

2.1. Materials

ACZ was obtained from Sigma® (99%, USA) and HP-β-CD (MW = 1325; degree of molar substitution, 7.0) was a gift from Ferroment S.A. (agent of Roquette in Argentina). TEA and Phenol red (PR) were obtained from Aldrich® (98%, USA), and verapamil (VER), propraponolol (PRO), atenolol (AT), Ibuprofen (IBU), chloramphenicol (CLOR), and metoprolol (MET) were provided by Parafarm® (Buenos Aires, Argentina). Allopurinol (ALLO) was obtained from Unifarma® (Buenos Aires, Argentina), with ketomine being purchased from Browener (Buenos Aires, Argentina), xilaxine from Köning (Buenos Aires, Argentina), and dehydrate calcium chloride from Cicarelli (Santa Fé, Argentina). All experiments were performed with analytical grade chemicals and solvents. A Millipore Milli-Q Water Purification System generated the water used in these studies.

2.2. Preparation of complexes in solid state

The preparation of the solid complex of ACZ:HP-β-CD at a 1:1 molar ratio and the multicomponent complex of ACZ:HP-β-CD:TEA at a 1:1:1 molar ratio was performed by the freeze-drying method. ACZ, HP-β-CD and/or TEA were accurately weighed and dissolved in distilled water. The whole solution was stirred using a magnetic stirrer for 24 h. After filtration, the solution was frozen overnight before being lyophilized over a period of 30 h using a freeze-drier (Lobaconco freeze dry system). The ACZ complex with TEA was prepared by dissolving equimolar amounts of ACZ and TEA in ethanol, and then the ethanol was removed in vacuum after treatment using ultrasound for 1 h.

2.3. Electron microscopy of rat epithelia

Rats were grouped into six categories of 3 rats each. Animals receiving only the vehicle solution (PBS) were designated as the control group and the test groups were perfused with the following ACZ formulations: ACZ free drug (966 μg/ml), 1:1 ACZ:HP-β-CD lyophilized complex (containing 966 μg/ml of ACZ), 1:1:1 ACZ:HP-β-CD:TEA lyophilized complex (966 μg/ml of ACZ), 1:1 ACZ:TEA complex (containing 966 μg/ml of ACZ), and 1:1 ACZ:HP-β-CD lyophilized complex (containing 966 μg/ml of ACZ) in combination with $CaCl_2$ (1468 μg/ml). At the end of these experiments, sections of gut loops were removed and opened longitudinally along the mesenteric border, before being fixed in Karnovsky's solution (4% formaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4) for 2 h at room temperature. Samples were washed three times with 0.1 M sodium cacodylate buffer pH 7.4 and treated with 1% osmium tetroxide for 2 h, and then stained in block with 1% uranyl acetate in 0.1 M acetate buffer, pH 5.2, for 20 min. After dehydration in a series of graded cold acetones, samples were embedded in Araldite. For light microscopy, 1-micron thick sections were cut serially and stained with a 1% toluidine blue and 1% borax solution. For ultrastructural studies, thin sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome, stained with uranyl acetate/lead citrate and examined using a Zeiss LEO 906E electron microscope.

2.4. Single-pass intestinal perfusion studies in rats

2.4.1. Solutions

The blank perfusion buffer solution was isotonic and consisted of 20.1 mM $Na_2HPO_4 \cdot 12H_2O$, 47.0 mM KH_2PO_4, 101.0 NaCl (PBS) with pH 6.5 ± 0.1 at 37 °C. Drug solutions used in the perfusion studies were prepared by dissolving each drug in PBS pH 6.5 and incubating these in a 37 °C water bath to maintain the temperature. The drug concentrations used in the perfusion studies were: ACZ, 99, 966, and 9800 μg/ml, with or without $CaCl_2$ (1468 μg/ml); 1:1 ACZ:HP-β-CD (containing 464 μg/ml of ACZ), 1:1 ACZ:HP-β-CD (containing 464 μg/ml of ACZ) with $CaCl_2$ (1468 μg/ml); 1:1:1 ACZ:HP-β-CD: TEA 8258 μg/ml (containing 966 μg/ml of ACZ), with or without $CaCl_2$ (1468 μg/ml); 1:1 ACZ:TEA 988 μg/ml (containing 591 μg/ml of ACZ), atenolol (AT) 403 μg/ml, with or without ACZ (966 μg/ml), propranolol (PRO) 159 μg/ml, with or without ACZ (966 μg/ml), verapamil (VER) 318 μg/ml, with or without ACZ (966 μg/ml), metoprolol (MET) 396 μg/ml, allopurinol (ALLO) 402 μg/ml, ibuprofen (IBU) 402 μg/ml, chloramphenicol (CLOR) 1016 μg/ml and phenol red (PR) 97 μg/ml, with or without ACZ (966 μg/ml).

2.4.2. Perfusion experiments

The experimental protocols were reviewed and approved by the Chemistry Faculty of the National University of Córdoba Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals". Male Wistar rats weighing 250–280 g were used for all studies ($n = 6$ for each group). Prior to each experiment, the rats were fasted overnight (12 h) with free access to water *ad libitum*.

The procedure for the single-pass *in situ* duodenal perfusions was performed as in previous reports (Dahan et al., 2009; Wu and Smith, 2013). Briefly, after fasting overnight, rats were anesthetized by an intraperitoneal injection of ketamine/xilazine (55/11 mg/kg) and affixed supine on a surface under suitable lighting to maintain a normal body temperature. The duodenum was exposed by a midline abdominal incision of approximately 5 cm, and two polyethylene cannula (outer diameter, 5 mm; inner diameter, 3 mm) were inserted through small slits at the proximal and distal ends (about 10 cm) and ligated by using silk suture. Care was taken to avoid disturbance of the circulatory system, and the exposed segment was kept moist with 37 °C normal saline solution. The proximal catheter was connected to an infusion pump (Samtronic ST100, Brasil), which was perfused with the test perfusion solution (0.2 ml/min). All perfusate solutions were incubated in a 37 °C water bath and were pumped through the intestinal segment. Saline was dropped onto the surgical area, which was then covered with gauze to avoid dehydration. The isolated segment was first rinsed with blank perfusion buffer PBS (phosphate buffer, pH 6.5) at a flow rate of 0.2 ml/min for 10 min which was followed by perfusion of phosphate buffer saline (pH 6.5) containing the test perfusion solution at a constant flow rate of 0.2 ml/min for 15 min in order to ensure steady state conditions. After reaching the steady state, samples were acquired from the distal part of the cannulated duodenum in pre-weighed Eppendorf tubes at 7 min intervals for an additional 75 min perfusion period. The equilibration time prior to sampling was sufficient to washout the intestinal segment and to reach initial steady state. At the end of the perfusion, the length of the segment was accurately measured and finally the animal was euthanized. At the end of the experiments, samples vials were weighed and stored at −20 °C until analysis, when they were thawed, deproteinized with H_3PO_4, centrifuged, filtered and the drug content in the supernatant injected into HPLC for quantification with a validated method.

The net water flux resulting from water absorption and efflux in the intestinal perfused segment was determined by the gravimetric method (Sutton and Rinaldi, 2001).

The effective permeability (P_{eff}) (cm/s) through the rat gut wall in the single-pass intestinal perfusion studies was determined assuming the "plug flow" model expressed by the following equations:

$$P_{eff} = \frac{-Q_{in} \ln\left(\frac{C_{out(corr)}}{C_{in}}\right)}{2\pi RL}$$

$$C_{out(corr)} = C_{out} x \frac{Q_{out}}{Q_{in}}$$

$$Q_{out} = \frac{M_{out}/D_{out}}{t}$$

$$V = \pi R^2 L$$

where Q_{in} (ml/min) is the flow rate of the perfusate inlet ($Q_{in} = 0.2$ ml/min), Q_{out} (ml/min) is the measured perfusate exit flow (ml/min), $C_{out(corr)}$ is the outlet concentration of perfusate which is corrected for volume change in segment using the gravimetric method, C_{in} denotes drug concentration measured in entering the perfusate, C_{out} is the concentration (μg/ml) of drug in the exiting solution, M_{out} (g) is the mass of collected perfusate per interval, D_{out} (g/ml) is the density of the collected perfusate per interval ($D_{out} = 1$), t is the interval time of sampling ($t = 7$ min). V is the volume of the perfused segment (ml), L is the length of the intestinal segment (cm), and R is the radius of the intestinal segment (0.18 cm).

2.4.3. HPLC analysis

The HPLC system consisted of an Agilent S1 100® chromatograph equipped with an Agilent Multiple Wavelength Ultraviolet–visible (UV–vis) Detector (MWD®) and a Chemstation software version A.10.02 (Agilent, Waldbronn, Germany). Chromatographic

Table 1
Chromatographic conditions for the drugs assayed.

Drug	Flow (ml/min)	Mobile phase	Injection volumen (μl)	λ (nm)	Temperature (°C)	t_R (min)
Acetazolamide	1.0	AcN/MeOH/water (3:2:95)	50	265	40	10.72
Atenolol[a]	1.0	AcN/MeOH/KH_2PO_4 (7.7:7.5:85)	50	230	25	5.51
Propranolol[a]	1.0	NH_4COOCH_3/AcN (30:70)	50	230	40	5.45
Metoprolol	1.0	AcN/KH_2PO_4 (25:75)	50	230	40	5.43
Verapamil[a]	1.0	NH_4COOCH_3/AcN (30:70)	50	230	40	7.31
Acyclovir	1.0	AcN/MeOH/water (3:2:95)	50	254	35	4.39
Allopurinol	1.0	AcN/MeOH/water (3:2:95)	50	254	40	4.56
Chloramphenicol	1.0	MeOH/water (65:35)	50	280	25	4.07
Ibuprophen	1.2	AcN/MeOH/KH_2PO_4 (7.7:7.5:85)	50	222	25	17.25
[a]Phenol red	1.0	AcN/KH_2PO_4 (20:80)	50	265	25	4.93

[a] The same chromatographic conditions were used for intestinal permeability markers assayed in combination with acetazolamide.

separations were performed on a Merck RP C18 250 mm × 4.6 mm i. d. filled with 5 μm particles, with a precolumn C18 Phenomenex®. HPLC experiments were carried out using isocratic conditions. The mobile phase was filtered through a 0.45 μm Millipore membrane and degassed prior to use, with the chromatographic conditions for the drugs described in Table 1. The calibration curves were prepared by spiking mixed working standard solution in blank intestinal perfusion buffer, and the HPLC methods were validated for various parameters: linearity ($R > 0.999$), precision (coefficient of variation <8%) and accuracy (relative error <11%). The specificity of the method was evaluated by analyzing the blank intestinal perfusion buffer collected from different rats to investigate the potential interferences at the LC peak region for the analytes.

2.4.4. Stability test

The drug stability was measured to ensure that the lower concentration from the intestinal perfusate was due to absorption. The drug was spiked into fresh blank perfusate at three concentrations obtained by passing the blank perfusion buffer through the duodenum segment *in situ* at a flow rate of 0.2 ml/min and immediately putting it into a water base at 37 ± 1 °C to mimic *in vivo* conditions. After 3 h, 1 ml of the incubated fluids was collected, deproteinized with H_3PO_4, centrifuged and filtered and the drug content in the supernatant was injected into HPLC for quantification.

2.5. Statistical analysis

A comparison of the 2 groups was made using an unpaired *t*-test (Mann–Whitney) with, a 2-tailed *P* value. Data obtained in the various groups were analyzed by analysis of variance (ANOVA). A *P* value of less than 0.05 was taken to indicate statistical significance. Data were expressed as means ± standard error of the mean.

3. Results

3.1. Histological findings

The effects of ACZ and its formulations on the rat duodenal mucosa were studied by means of light microscopy. The rat duodenal tissue sections perfused with the working blank perfusion buffer showed a normal pattern after 90 min (vehicle control) (Fig. 1A and B). However, the duodenum of rats treated with ACZ showed a remarkable morphological alteration in the mucosa compared with the control, which consisted of the thickening of the villi, together with edema, vascular dilatation and infiltration of mononuclear cells in the lamina propria (Fig. 1C and D). In contrast, light photomicrographs of representative sections of the rat duodenal epithelium treated with the binary systems: 1:1 ACZ:HP-β-CD (Fig. 1E and F) and 1:1 ACZ:TEA (Fig. 1G and H) and the ternary systems: 1:1:1 ACZ:HP-β-CD:TEA (Fig. 1I and J) and 1:1 ACZ:HP-β-CD:[calcium] (Fig. 1K and L) showed a similar appearance of the villi similar to that described for the control tissues, where the mucosa had numerous evaginations into the gut lumen (villi). Each villu had a core of lamina propia connective tissue containing capillaries and smooth muscles, with, the main absorptive cells of these villi, the enterocytes, being tall columnar cells with basal oval nuclei. Among these enterocytes, numerous mucus cells of a globet shape were distributed.

3.2. Ultrastructural features

TEM images of the control rat duodenal epithelium displayed a healthy ultrastructural morphology with an intact brush border at the mucosal surface (Fig. 2A and B). The enterocytes exhibited an integral plasma and nuclear membrane, with the nucleus revealing

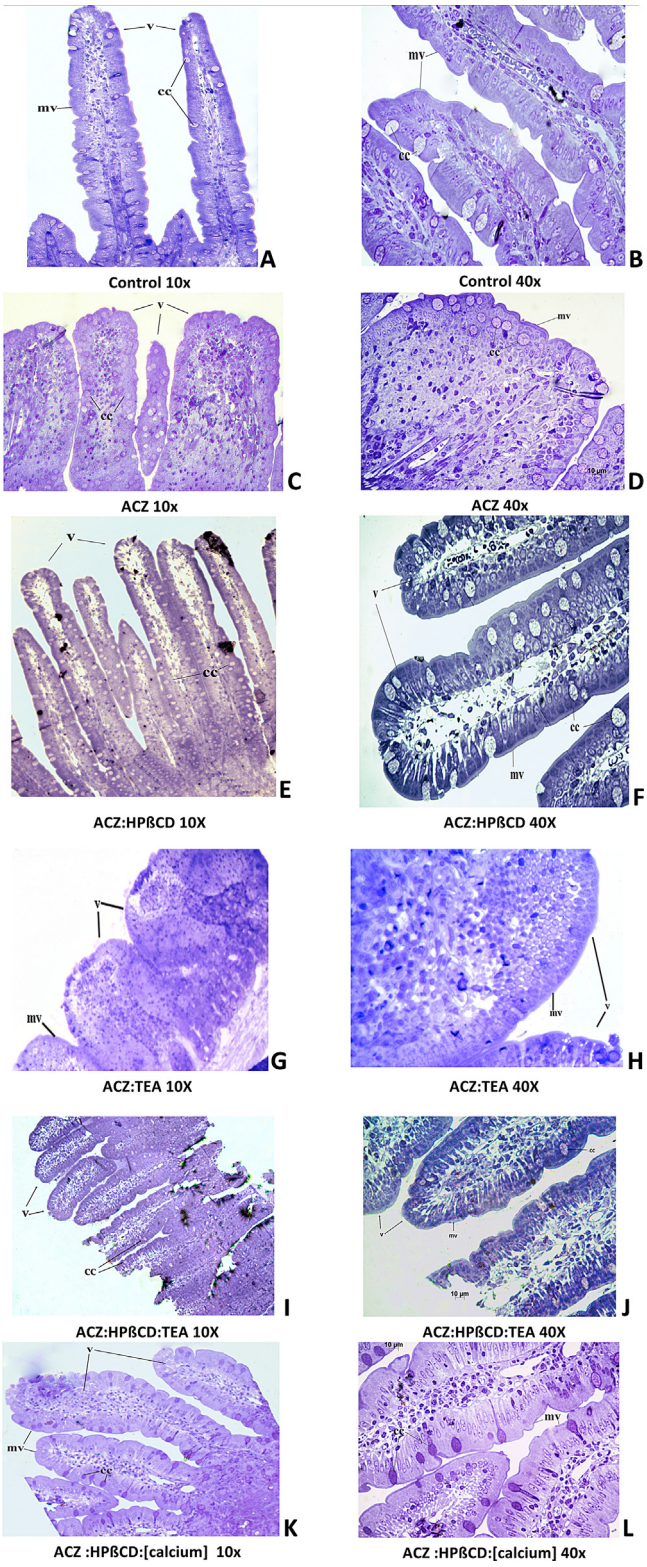

Fig. 1. Light micrographs of rat duodenal membrane following the in situ administration of various ACZ formulations. MOAR micrographs: A (10×) and B (40×) show rat duodenal segment of saline treated animals (control). C (10×) and D (40×) are of rat duodenal segment of ACZ treated animals. E (10×) and F (40×) show rat duodenal segment of ACZ:HP-β-CD treated animals. G (10×) and H (40×) show rat duodenal segment of ACZ:TEA treated animals. I (10×) and J (40×) show rat duodenal segment of ACZ:HP-β-CD:TEA treated animals. K (10x) and L (40×) show rat duodenal segment of ACZ:HP-β-CD:[calcium] treated animals. v: villi; mv: microvilli; cc: goblet cells.

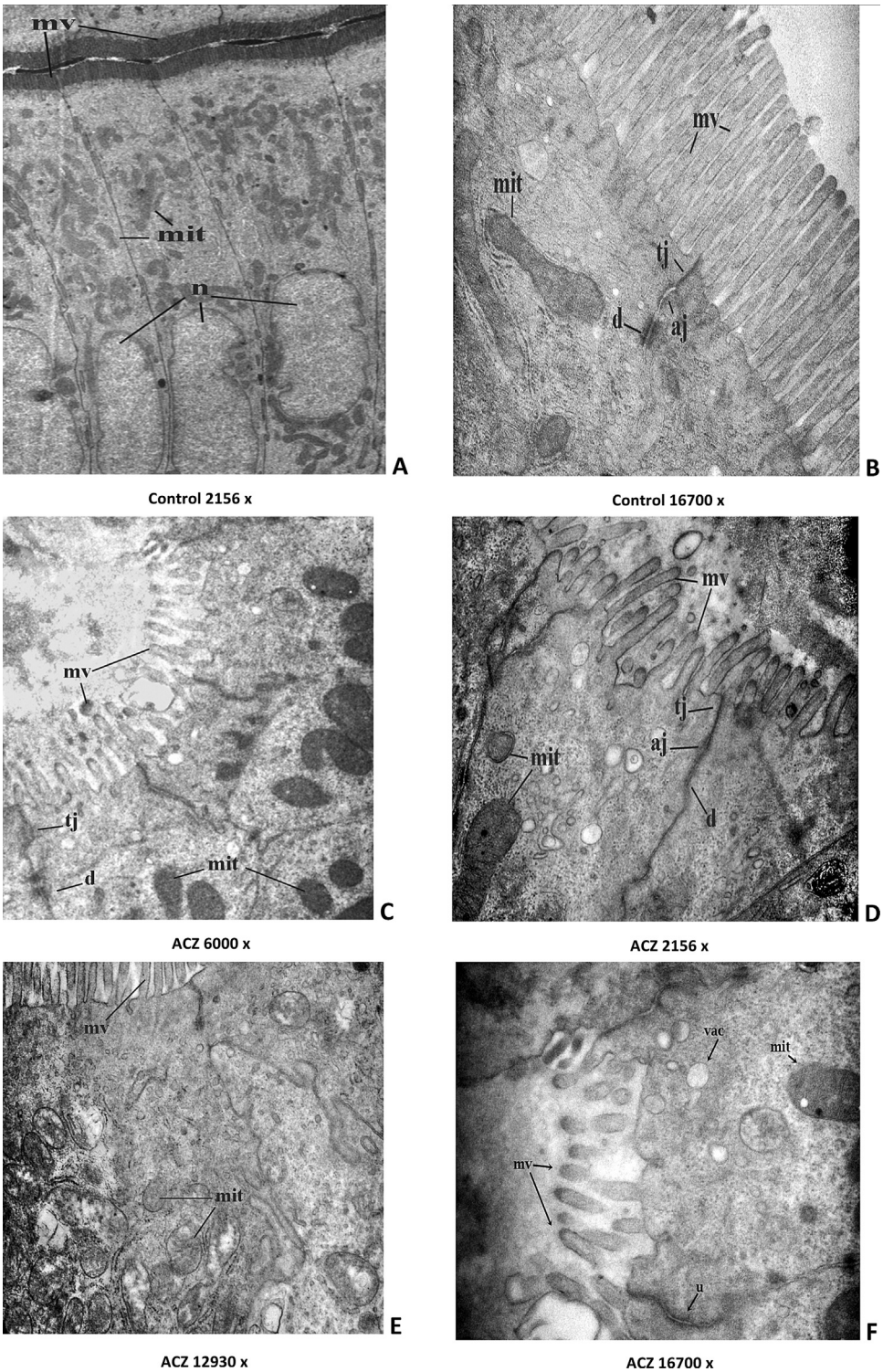

Fig. 2. Transmission electron (TEM) micrographs of rat duodenal segments perfused with: blank perfusion solution (A (2156×) and B (16,700×), (control)) and ACZ solution (C (6000×), D (2156×), E (12,930×), F (16,700×)). mv: microvilli; mit: mitochondria; tj: tight junctions; aj: adherens junctions; d: desmosomes, re: endoplasmic reticulum; n: nucleous; and vac: vacuole.

an extended area containing euchromatin. Furthermore, in the cytoplasm, numerous polymorphic mitochondria were observed and a well-developed endoplasmic reticulum with attached ribosomes, indicating an active enzyme synthesis. Junctional complexes including tight junctions (TJs), adherens junctions (AJs), and desmosomes (Ds) denoted good cell-to-cell adhesion along the lateral membrane.

The ultrastructural examination of the duodenal epithelium from ACZ treated rats revealed an alteration of the microvilli bordering on the apical surface (Fig. 2C–F). The perfectly smooth limiting membrane of these apical projections, seen characteristically in control preparations, was replaced by a series of round swellings over most of the microvillar surface. The microvilli were reduced in both in length and number and also showed irregular lengths and

arrangements. In some enterocytes, a vacuolization of the mitochondria and rough endoplasmic reticulum (RER) was seen. In addition, the exposure of the duodenal epithelium to ACZ resulted in a less structured morphology of the TJs and desmosomes. In contrast, the rat duodenal epithelium specimens treated with the binaries systems, 1:1 ACZ:HP-β-CD (Fig. 3A and B) and 1:1 ACZ:TEA (Fig. 3C and D), or the ternary complexes, 1:1:1 ACZ:HP-β-CD:TEA (Fig. 3E and F) and 1:1 ACZ:HP-β-CD:[calcium] (Fig. 3G and H), revealed that the integrity of the rat gut wall was preserved after exposure to these ACZ formulations. Enterocytes were united at their uppermost lateral membrane by a well-developed tight junction, with the supranuclear region of these enterocytes having several spherical mitochondria and numerous cisternae of rough endoplasmic reticulum, with attached ribosomes indicating an active enzyme synthesis and the luminal surface of the enterocytes possessing many closely packed parallel finger-like microvilli. The nucleus

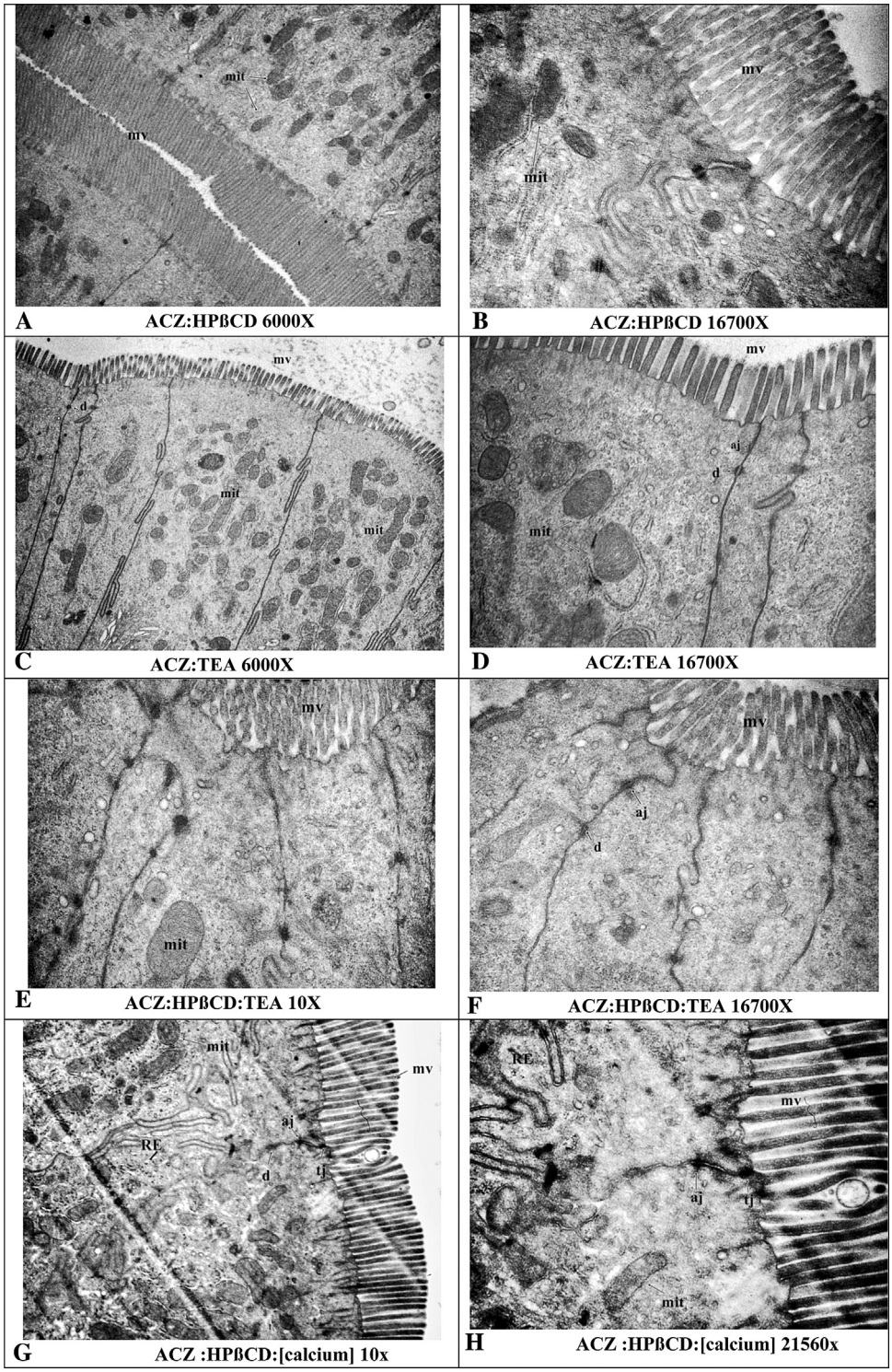

Fig. 3. Transmission electron (TEM) micrographs of rat duodenal segments perfused with: ACZ:HP-β-CD solution (A (6000×) and B (16,700×)); ACZ:TEA solution (C (6000×) and D (16,700×)); ACZ:HPßCD:TEA solution (E (10×) and F (16,700×)); ACZ:HP-β-CD:[calcium] solution (G (6000×) and H (16,700×)); mv: microvilli; mit: mitochondria; tj: tight junctions; and d: desmosomes.

Table 2
Intestinal permeability of intestinal marker substances from *in situ* single pass perfusion studies in rats ($n = 5$).

Treatment	$(P_{eff} \pm SD) \times 10^{-5}$ cm/s [without ACZ]	$(P_{eff} \pm SD) \times 10^{-5}$ cm/s [in combination with ACZ (966 μg/ml)]	AER[a]	f_a[b]
Verapamil (VER)	7.6 ± 5.3	8.97 ± 1.49	1.18	0.98
Propranolol (PRO)	7.7 ± 2.9	9.03 ± 3.26	1.17	0.99
Ibuprofen (IBU)	54.7 ± 12.7			0.99
Metoprolol (MET)	5.1 ± 0.8			0.95
Chloramphenicol (CLOR)	3.5 ± 1.4			0.70
Atenolol (AT)	4.4 ± 1.4	10.1 ± 0.6	2.33	0.50
Allopurinol (ALLO)	3.95 ± 0.46			0.52
Phenol Red (PR)		7.8 ± 2.3		

[a] AER: absorption enhancement ratio defined as ratio of P_{eff} of drug in the presence of ACZ to that of P_{eff} in the absence of ACZ.
[b] Data obtained from reference Hackelsberger et al., (1998).

showed a prominent nucleolus and an extended area containing euchromatin.

3.3. Stability test

Preliminary stability studies revealed no signs of compound degradation during perfusion experiments since all the analytes were found to be more than 95% stable in blank perfusion sample for 3 h at 37 °C (Table 4), and therefore, the disappearance of the drug from the perfusate was attributed to absorption.

3.4 In situ *intestinal perfusion experiments*

A set of compounds were selected to establish if a correlation existed between the P_{eff} values *in situ* and their respective f_a values *in vivo* in human, which could be used for prediction of f_a for the ACZ formulations. The permeability values (P_{eff}, cm/s) for the training set of compounds, which displayed a wide range of physicochemical properties and known human fraction absorbed values, were determined using the SPIP technique and are presented in Table 2.

The P_{eff} values obtained using the SPIP technique were well correlated with the f_a values, which were described by a sigmoidal correlation (R^2: 0.8, $P < 0.01$), according to the following equation:

$$a\left(1 - e^{-bx}\right), \quad (1)$$

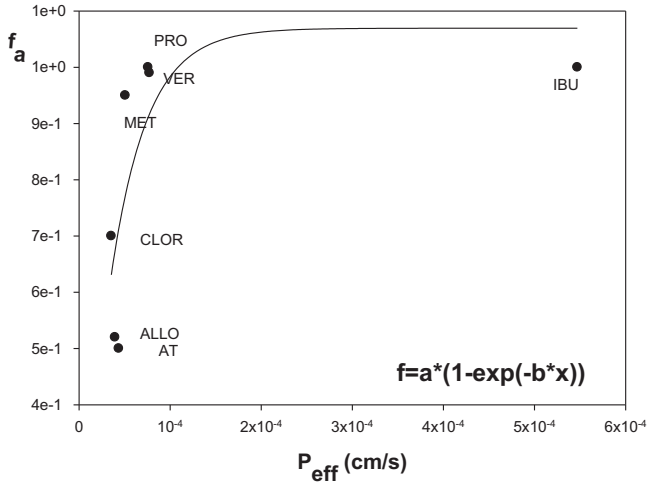

Fig. 4. Relationship between effective permeability coefficients (P_{eff}) in the in situ single-pass intestinal perfusion (SPIP) model in rats and their corresponding fraction of dose absorbed (f_a) in vivo in humans. F_a values of drugs cited from references were plotted against P_{eff} values (Table 2, second column without ACZ: $(P_{eff} \pm SD) \times 10^{-5}$ cm/s). A theoretical fitting line was obtained for seven model compounds using Eq. (1).

where a is 1.07 ± 0.14, b is $25.2 \times 10^3 \pm 8.3$ 10^3, and x is P_{eff}. As shown in Fig. 4, the relationship between the P_{eff} values and f_a in humans was well described by Eq. (1).

The P_{eff} cutoff value for highly permeability drugs was found to be 7.34×10^{-5} cm/s, which ensured >90% oral absorption. This correlation revealed that at $P_{eff} \leq 7.34 \times 10^{-5}$cm/s, dramatic changes in predicted oral f_a in humans were observed, whereas, at $P_{eff} \geq 7.34 \times 10^{-5}$ cm/s, the predicted oral f_a in human leveled off and is not any more significantly affected by changes in rat P_{eff}. This implies that drug substances with rat $P_{eff} \geq 7.34 \times 10^{-5}$ cm/s belong to the high-permeability class, according to the Biopharmaceutics Classification System (BCS) (Amidon et al., 1995; Taub et al., 2002). The determined mean P_{eff} values for different concentrations of ACZ and its formulations in PBS by the single pass intestinal perfusion technique in rats are listed in Table 3.

The *in vivo* permeability values of rat duodenum to ACZ were investigated using different concentrations of substance entering the single-pass perfused intestinal segments, and it was found that the effective intestinal permeability (P_{eff}) of ACZ exhibited a linear concentration-dependent increase. Nevertheless, in addition to the physical barrier, permeation of ACZ might be limited by the activity of *P*-glycoprotein (*P*-gp), which was highly expressed in the barrier and excretory tissues. Thus, the intestinal permeability coefficients of ACZ (P_{eff}) were evaluated in the absence or presence of the efflux pump inhibitor verapamil (VER), and it was found that the ACZ P_{eff} values were 27.9 ± 1.5 (10^{-5} cm/s) and 25.02 ± 2.32 (10^{-5} cm/s) ($P > 0.05$), respectively.

In order to evaluate whether or not ACZ affected the intestinal barrier function, an assessment of the integrity of the intestinal mucosa was performed by determining the ACZ effect on the transport of the paracellular marker compound atenolol (AT), a hydrophilic model compound which exhibits relatively low permeability across the intestinal epithelium, and therefore, any sudden increase in the AT permeability may be assumed to exclusively reflect leakage of the barrier. The P_{eff} of AT across the rat duodenal segment was approximately doubled by the addition of 99 μg/ml of ACZ to the perfusion solution (from $(4.4 \pm 1.4) \times 10^{-5}$ cm/s to $(7.89 \pm 0.6) \times 10^{-5}$ cm/s), increased 2.55-fold at a concentration of 990 μg/ml of ACZ (from $(4.4 \pm 1.4) \times 10^{-5}$ cm/s to $(11.10 \pm 1.2) \times 10^{-5}$ cm/s) and increased 3.12-fold at concentration of 9800 μg/ml of ACZ (from $(4.4 \pm 1.4) \times 10^{-5}$ cm/s to $(13.62 \pm 1.5) \times 10^{-5}$ cm/s), thereby indicating that ACZ demonstrated significant enhancement effects for this typical paracellular marker.

Next, we tested whether ACZ also facilitated transcellular transport in the rat duodenal segment by measuring the P_{eff} of propranolol (PRO) (a highly permeable tanscellular marker that is often used in rat intestinal perfusion studies) and also verapamil (VER) in the presence of ACZ (966 μg/ml). The P_{eff} values of these highly permeable markers, PRO and VER, in the presence of ACZ were increased by ~1.2-fold (Table 2). In addition, the permeation of the nonabsorbable marker phenol red (PR) through the rat duodenal segment, in combination with ACZ, was examined to

Table 3
P_{eff} values for ACZ formulations from *in situ* single pass perfusion studies in rats ($n = 5$).

Treatment	$(P_{eff} \pm SD) \times 10^{-5}$ cm/s	AER[a]
ACZ 99 μg/ml	11.4 ± 2.1	
ACZ 966 μg/ml	27.9 ± 1.5	
ACZ 9800 μg/ml	215.2 ± 5.6	
1:1 ACZ:TEA (equivalent to 591 μg/ml of ACZ)	17.1 ± 0.7	1.45
1:1 ACZ:HP-β-CD (equivalent to 464 μg/ml of ACZ)	12.2 ± 4.5	1.84
1:1:1 ACZ:HP-β-CD:[calcium] (equivalent to 464 μg/ml of ACZ)	20.5 ± 3.9	2.0
1:1 ACZ:HP-β-CD:TEA[calcium] (equivalent to 966 μg/ml of ACZ)	36.7 ± 1.5	1.82
1:1 ACZ:HP-β-CD:TEA (equivalent to 966 μg/ml of ACZ)	47.1 ± 10.2	2.11

[a] AER: absorption enhancement ratio defined as ratio of P_{eff} of ACZ complex to that of P_{eff} of the equivalent concentration of uncomplexed ACZ.

monitor intestinal integrity during perfusion, and it was found that the permeation of PR through the duodenal segment was significantly promoted by ACZ (Table 2).

The P_{eff} values of the enhancement ratio (R) were used to assess the ability of ACZ multicomponent systems to promote transport of ACZ across the duodenal segment. As shown in Table 3, all the ACZ formulations tested significantly enhanced the P_{eff} of ACZ across the rat duodenal segment, with R-values of 1.45, 1.84, 2.0, 2.11, and 1.82 being obtained for ACZ:TEA, ACZ:HP-β-CD, ACZ:HP-β-CD:[calcium], ACZ:HP-β-CD:TEA, and ACZ:HP-β-CD:TEA:[calcium], respectively.

4. Discussion

It has been clearly demonstrated that ACZ is not a substrate for *P*-glycoprotein (*P*-gp), the secretory efflux transporter, since in the presence of verapamil, a typical substrate for *P*-gp, the transporter of ACZ was not significantly altered and the uptake process was a nonsaturable one. In this regard, ACZ showed a linearly-increased permeability in a concentration-dependent manner, with the concentration dependent permeability kinetic confirming that the ACZ transport through the duodenal segment was passive. Increased intestinal permeability can occur by paracellular opening of tight junctions at the aqueous pore or by transcellular perturbation of the gut wall. In addition, ACZ showed appreciable permeability across the intestinal barrier.

Results of *in situ* permeation studies, summarized in Table 2, indicated that the intestinal permeability of selected model drugs lay in the range $3.54–54.70 \times 10^{-5}$ cm/s. Next, the sigmoidal relationship shown in Fig. 4 was used to determine the limiting P_{eff} value that divided the compounds into two classes: poorly or acceptably absorbed. A rather high cutoff value of the absorbed fraction dose (f_a) ≥ 90% was chosen for this classification, as recommended in the Biopharmaceutics Classification System (BCS) implemented by the FDA guide (FDA, 2000). As shown in Fig. 4, substances that had experimentally derived permeabilities of greater than approximately 7.34×10^{-5} cm/s could be described as "well-absorbed compounds". Data from the *in situ* rat permeability studies of the ACZ formulations were then extrapolated to predict the fraction dose absorbed in human (f_a) by fitting the data to Eq. (1), with the f_a of ACZ predicted based on its P_{eff} value, including the lower concentration of ACZ tested, being found to be ~1.0 in the present study.

Table 4
The mean (±SD) percentage of drug remaining after 3 h for rat intestinal perfusion fluid at 37 °C ($n = 3$).

Drug	% Of drug remaining
ACZ	97.5 (1.0)
ATE	96.4 (0.8)
PRO	101.8 (2.0)
VER	95.8 (1.6)
IBU	96.7 (1.3)
MET	98.3 (0.9)
CLOR	95.2 (2.1)
ALLO	96.7 (1.6)

Using a selection of hydrophilic and hydrophobic markers for the different transport routes across the isolated rat intestinal mucosa, the present data showed that the abilities of the highly permeable transcellular transported drugs propanolol (PRO) and verapamil (VER) to permeate across the rat duodenal segment were to some extent affected by ACZ. In addition, ACZ increased the permeability of the paracellular marker AT (Table 2).

Increased AT fluxes are widely accepted as being markers for paracellular transport, but this conclusion is only valid if there is confidence that the epithelial barrier has remained fully intact. However, ACZ induced extensive ultrastructural alteration on the rat duodenal epithelium. Moreover, the permeability of the duodenal segment to the nonabsorbable marker phenol red (PR) through the small intestine increased significantly in parallel with changes in the intestinal permeability of the transcellular (VER and PRO) and the paracelullar (AT) permeability markers (Table 2). In fact, the nonabsorbable marker phenol red can be used to assess ACZ-induced damage to the small intestine, since it may permeate the damaged intestine more easily due to the disordered barrier function of the intestinal epithelium.

Small intestinal permeability has been previously used to quantify mucosal alterations induced by acetylsalicylic acid and other nonsteroidal anti-inflammatory drugs (NSAIDs) (Davies, 1998), with these permeability changes having been detected by oral administration of probes such as, ^{51}Cr-EDTA, lactulose, cellobiose, and polyethylene glycol (Ford et al., 1995). Moreover, it has been demonstrated that measurement of the phenol red concentration excreted into urine after an oral or a rectal dose is a sensitive index of intestinal damage (Yáñez et al., 2006). Phenol red is a non-absorbable molecule almost completely ionized at pH values above 1, with its excretion probably reflecting both small intestinal and colonic permeability. An implicit advantage of this permeability test is that it reflects the functional integrity over a major area of the intestine.

Taken together, our results have demonstrated that ACZ induced remarkable membrane damage to the intestine over concentration ranges of 99–9800 μg/ml, thus enabling us to conclude that disruption of the intestinal mucosa caused the high transepithelial transport of ACZ across the rat duodenal segment. Although, the mechanisms behind this are still unclear, we hypothesize that ACZ might reduce the surface hydrophobicity on the duodenal mucosa, thereby increasing its epithelial permeability.

It has been shown in numerous studies that a decrease in mucosal hydrophobicity is associated with increased gut permeability (Qin et al., 2008; Lugea et al., 2000), with the

hydrophobicity of the intestinal segments increasing gradually from the duodenum to the end of the ileum and remaining at relatively high levels in the cecum, colon, and rectum (Qin et al., 2008). Furthermore, previous studies using the mucolytic agent *N*-acetyl cysteine (NAC) have provided proof-of-principle information that loss of the mucus layer is associated with both a loss of mucosal hydrophobicity and an increased intestinal permeability (Qin et al., 2008). Decreased hydrophobicity has also been documented for a variety of conditions including peptic ulcer disease (Goggin et al., 1991), Helicobacter pylori infection and gastritis (Hackelsberger et al., 1998; Goggin et al., 1992), as well as occurring along with damage to the stomach or colon caused by non steroidal anti-inflammatory drugs (Lugea et al., 1997), 2,4,6-trinitrobenzenesulphonic acid (Tatsumi and Lichtenberger, 1996), LPS (Dial et al., 2002), the nonionic surfact Brij 35 (Lugea et al., 2000) and dextran sodium sulphate (Lugea et al., 2000). In addition, pretreatment with phospholipids increases surface hydrophobicity, reduces colonic permeability and decreases susceptibility to chemical aggression (Dial et al., 2008).

In the present study, we found that ACZ complexes were able to markedly alleviate ACZ-induced mucosal injury, with this effect being clearly reflected in microscopic findings showing attenuation of tissue damage. Furthermore, all the complexes increased the transmural permeability to ACZ compared with the uncomplex drug (Table 3).

In this work, an inclusion complex between ACZ and HP-β-CD was formed *via* noncovalent interactions. Also, supramolecular systems composed of three molecular entities; two out of the three were the active drug ACZ and HP-β-CD, while the third component was a metal ion (Ca^{++}) or the basic surfactant triethanolamine (TEA) were evaluated. In the case of the ternary system with TEA, this surfactant is able to capture ACZ *via* a double effect: interaction with the lipophilic portion as a surfactant and ion pairing as a basic agent. In the case of the ternary assemblies gathering ACZ, HP-β-CD and Ca^{2+} ions, or assemblies gathering ACZ, HP-β-CD, TEA and Ca^{2+} ions, it is probably that appropriate moieties of the ACZ molecules jointly coordinated with Ca^{2+} to form HP-β-CD:ACZ: metal ions complexes in the presence of CD molecules. ACZ:TEA refers as the ion-pair complex formed between ACZ and the ionic surfactant TEA.

Based upon our laboratory experience, the complex formation of ACZ with HP-β-CD, in the presence or absence of a third compound, appeared to render the drug more lipophilic, which may facilitate its transit across the gastrointestinal mucosa, thus minimizing surface injury with no loss in functional bioavailbility and therapeutic efficacy. Therefore, ACZ complexes may act as drug delivery systems that facilitate the transit of ACZ across the duodenal mucosal gel layer, without disrupting its protective barrier. The defined mechanism of action of this kind of complex is based on the association of the CD with the therapeutic molecule, which renders the drug more hydrophobic. Consequently, large concentrations of the drug can cross the cell membrane *via* the passive transcellular route of uptake, and therefore, when the complex (drug–CD) is delivered orally, the CDs help the drug come into intimate contact with the intestinal wall. Loftsson et al. (2007) have suggested that hyrophillic CDs enhance flux through mucosa by increasing the concentration gradient over the unstirred water layer (UWL) thickness and/or by exerting a chaotropic effect on the UWL's structured water network, thereby increasing the availability of free drug molecules immediate to the membrane surface for absorption.

The greater efficiency of the ternary systems of ACZ with HP-β-CD, in the presence of TEA or calcium ions, in promoting the transepithelial delivery of ACZ in comparison with the ACZ:HP-β-CD binary complex, might be explained by the fast release of the drug directly on the underlying epithelium; thus, increasing the amount of ACZ available for absorption. Moreover, formation of ionic pairs in competition with the complex formation might shift the equilibrium in the direction of the complex dissociation, resulting in a decrease in the complex stability. Because the ionic pairing coexists with complex formation, contributions from both these processes allow drug molecules to traverse transcellularly across the gut epithelium. The ion pair ACZ:TEA gave a slightly lower value of permeability enhancement ratio in comparison with that obtained with the binary ACZ:HP-β-CD complex.

ACZ is highly polar ($\log P_{n\text{-octanol/water}} = -0.26$) (Remko and von der Lieth, 2004) and may exist with different charges depending on pH of the sample. Its $\log P_{n\text{-octanol/water}}$ into organic solvents is low which is related to the existence of highly hydrophilic groups in its structure. ACZ contains an acidic function (H–N group of the sulfonamide moiety) and it is classified as a weak acid ($pK_a = 7.4$ (Remko, 2010)). This type of chemical structure of ACZ causes that this compound is at physiological pH 7.4 partially ionized. By passive diffusion, ACZ penetrates poorly into biological membranes because of its highly polar character attributable in large part to the readily dissociable $-SO_2NH_2$ group.

Since ACZ has an acidic functional group, it is ionizable and forms an ion pair complex with the cationic surfactant TEA. Lipophilic counter ions, such as TEA, may provide Log *P* values in the range of 2–5 required to provide sufficient lipophilicity to achieve a higher membrane transport in comparison to the free drug in the pH range of the intestinal environment.

In conclusion, this study presents evidence that ACZ induces surface damage to the mucosa, probably by chemically interacting with the mucosal gel layer on the intestinal epithelium and disrupting the mucosal barrier properties. In contrast, ACZ-induced intestinal injury was abrogated by complex formation of ACZ with HP-ß-CD, with or without TEA or calcium ions. All the tested ACZ formulations were efficient in protecting the gut epithelium against the disrupting membrane integrity induced by ACZ and increasing the apparent permeability of ACZ across the duodenal epithelium. Moreover, ternary complexes of ACZ were found to be an excellent approach to enable an increase in the apparent permeability of ACZ across the duodenal epithelium, with a concomitant ability to preserve the integrity of the gut epithelium from ACZ-induced injury.

Our approach directly addresses the issue of gastrointestinal (GI) toxicity and mucosal lining integrity through the application of a specialized CD based formulation that facilitates the safe transit of ACZ across the mucus gel layer while maintaining the integrity of this barrier to luminal damaging agents. The data obtained could prove to be useful in the design and development of novel ACZ formulations and reduce GI toxicity while still maintaining their essential therapeutic efficacies.

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