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International Journal of Pharmaceutics 317 (2006) 155–160
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Investigation of factors responsible for low oral bioavailability of cefpodoxime proxetil

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Received 12 January 2006; received in revised form 4 March 2006; accepted 7 March 2006
Available online 12 March 2006

Abstract

Learning about the behavior of a drug in biological environment enables application of better formulation strategies to improve bioavailability of the same. Cefpodoxime proxetil (CP) is a prodrug, which is orally administered cephalosporin with only 50% absolute bioavailability. Despite previous studies, reasons responsible for low bioavailability of CP remain poorly understood. The present study tries to ascertain reasons for the low oral bioavailability of CP. The in vitro, in situ and ex vivo studies showed interesting results, where metabolism of CP into cefpodoxime acid (CA) inside the intestinal epithelial cell and preferential efflux of CA into lumen was identified as primary reason for low oral bioavailability of CP. Presence of specific carriers or transportation mechanism on the apical side membrane of enterocyte, than basal side of the same was observed.
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Keywords: Cefpodoxime proxetil; Bioavailability; Reasons; Factors; Oral; Closed loop method; Everted sac; Tissue uptake; Permeability

1. Introduction

It is important to understand the behavior of a drug molecule during its pre- and post-administration phases. A drug after oral administration has to successfully overcome a complex gastro intestinal tract (GIT) environment, consisting of varying pH, and digestive enzymes, before it gets absorbed into systemic circulation. Various approaches like salt formation, pro-drugs, solid-state modification are traditionally applied to improve absorption and therapeutic performance. Prodrugs are designed to improve solubility, permeability or stability aspect of the molecule, and consist of a very weak bond in their structure like ester linkage in case of ester prodrugs. This weak bond helps in easy reversal to active parent moiety in the intestinal epithelial cell or blood, but at the same time makes them susceptible to the actions of digestive enzymes and pH value, leading to pre-absorption degradation of pro-drug. This defeats the purpose for which the prodrug was designed. However, in such instances judicious application of formulation technology can be applied for achieving better results.

Cefpodoxime proxetil (CP) is a prodrug, third generation cephem type broad-spectrum antibiotic administered orally. CP is a non-crystalline, slightly basic compound and possesses an asymmetric carbon atom in the ester group and is supplied as a racemic mixture of R-isomer and S-isomer (Fig. 1) (Fuzimoto et al., 1987; Nakao et al., 1987; Miyauchi et al., 1989; Hamamura et al., 1995a). CP is known to exhibit a pH dependent solubility behavior, with highest solubility in acidic pH conditions, and the solubility falls as the pH increases (Hamamura et al., 1995a). CP is absorbed from the intestinal tract after oral administration and hydrolyzed to its parent moiety cefpodoxime acid (CA) by non-specific esterases in the intestinal wall/plasma (Kobayashi et al., 1988; Komai et al., 1988; Crauste-Manciet et al., 1997). The drug is absorbed throughout the GIT, and shows relatively more bioavailability in fed conditions than fasted conditions (Hughes et al., 1989). The absolute bioavailability of CP administered as tablet relative to cefpodoxime sodium intravenous infusion is about 50% (Borin, 1991).

Although CP is designed to improve the permeability and thus bioavailability of the parent molecule CA, it still has only 50% oral bioavailability, when administered orally. The rea-

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doi:10.1016/j.ijpharm.2006.03.004

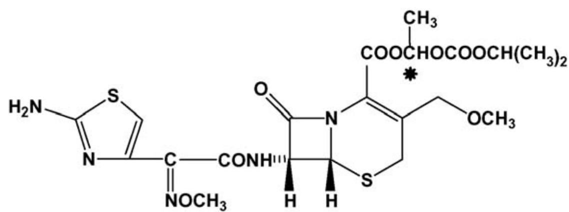

Fig. 1. Structure of cefpodoxime proxetil (*, asymmetric carbon).

sons for low oral bioavailability of CP remain poorly investigated. Reported studies have pointed possible reasons of low bioavailability as—the low solubility, typical gelation behavior of CP particularly in acidic environments (Hamamura et al., 1995a,b,c; Crauste-Manciet et al., 1997), and pre-absorption luminal metabolism into CA by the action of digestive enzymes (Crauste-Manciet et al., 1997). Concrete evidence for low bioavailability of CP still remains elusive and there is a need to understand the mechanistic aspects, to enable application of rational formulation strategies. This understanding may be applied for improving the delivery of other cephalosporin/non-cephalosporin prodrugs.

2. Materials and methods

2.1. Materials

The reference standards of CP and CA were obtained from Ranbaxy Research Laboratories Ltd., Gurgaon, India. Hydrochloric acid buffer for pH 1.2, sodium acetate buffer for pH 4.5, and phosphate buffers for pH 5.4 and 6.8 were used for various purposes. All salts and acids used in preparation of buffers are of analytical grade and those materials used in preparation of mobile phase and HPLC analyses are of chromatography grade.

2.2. Analytical methods

In-house developed and validated HPLC analytical methods were utilized for detecting quantities of CP and CA from the biological samples. The detection of CP and CA was performed by two separate HPLC methods based on reversed phase columns. A HPLC system (Shimadzu Corporation, Japan) equipped with a UV–vis spectrophotometric detector, and data acquisition software (CLASS-VP, version 6.14 SP1) was utilized for the purpose. Both HPLC methods employed acetonitrile:ammonium acetate buffer (pH 5.0) as mobile phase (at 36:64 and 10:90 for CP and CA, respectively), pumped at a flow rate of 1 ml/min, and analysis carried at a temperature of 30 °C and a detection of 235 nm. The method employed for quantifying CP had a calibration range of 5–150 μg/ml with a LOQ of 900 ng/ml, an accuracy of 98.45–101.63% and intra- and inter-day precision values of % RSD of 0.95–4.29. Similarly, the analytical method employed for quantification of CA had a LOQ of 50 ng/ml and operated in concentration range of 2.5–80 μg/ml with an accuracy of 93.68–107.09% and intra- and inter-day precision values with a % RSD in the range of 0.76–4.70.

2.3. Animal studies

All animal studies were done according to the guidelines of the Institutional Animal Ethics Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER), Punjab, India. Male Sprague-Dawley (SD) rats in the weight range of 250–275 g were used in various experiments. Rats were housed under standard laboratory conditions and fasted over night with water allowed ad libitum before conducting any experiment.

2.4. In vitro studies

2.4.1. Incubation studies

The effect of varying pH conditions and secretions of GIT on drug was evaluated by incubating CP respectively in buffers of various pH, without and with enzyme fractions. The stability of the drug was assessed in buffers of pH values—1.2, 4.5, 5.4, and 6.8 for 24 h at 37 °C, as they represent the local environments of stomach, duodenum, jejunum and ileum, respectively. Enzyme fraction was obtained by homogenization of mucosal scrapings of isolated small intestine (jejunal portion) of rats ($n = 2$), followed by preparation of microsomal fraction (Gibson and Skett, 1996), and the same was used to evaluate the effect of enzymes on CP. The reaction was initiated by adding equal assayed (Lowery et al., 1951; Peterson, 1979) quantities of protein (0.05 g) to the buffers of pH 1.2, 4.5, 5.4, and 6.8 containing drug solution (100 μmol) pre-equilibrated at 37 °C in a shaking water bath ($n = 3$). After 1 h, the enzymatic action was stopped by adding 100 μl of 10% trichloroacetic acid solution, followed by centrifugation at 16,000 × g for 15 min and analysis of supernatant by HPLC.

2.5. In situ studies

2.5.1. Studying the fate of CP in lumen

The actual fate of the drug after oral ingestion can only be obtained from in situ absorption studies, as they involve actual process of absorption of drug with the presence of blood circulation, mucus layer, and GI secretions. For the purpose, intestinal ‘closed loop’ technique (Levine and Perikan, 1961; Fiese and Perrin, 1968; Hori et al., 1988; Mariappan and Singh, 2003) was utilized. Closed loops (unwashed) were carefully made in the jejunal part of the lumen or stomach of the anesthetized rat, avoiding any damage to the tissue and the blood circulation ($n = 6$). About 1 ml of the drug solution prepared in various buffers of pH 1.2, 4.5, 5.4, and 6.8 was injected in to the loop (jejunum or stomach), and allowed to stay for 1 h. The contents of the loop were then extracted completely and analyzed for the remaining drug and metabolites, by HPLC.

2.6. Ex vivo studies

2.6.1. Determination of permeability coefficients for CP and CA

The formation of CA in the pre-absorption stage has been reported to lower oral bioavailability (Crauste-Manciet et al.,

1997). A more realistic assessment of this factor can be made by determining comparative permeability coefficients of CP and CA. The above aspects were evaluated using rat intestinal 'everted sac' method (Parsons and Paterson, 1960; Wiseman, 1961) for determination of permeability coefficients of both CP and CA.

Jejunal portion of the intestine was excised and everted immediately after sacrificing the rat. The everted intestine, as a sac of approximately 5 cm length, was taken with one end mounted on to a fine glass tube to remove samples from the inside (basal side), and other end secured with thread. A weight of 1 g was hanged in to the solution to keep the tissue in stretched position. After addition of drug to the outer side (apical side), samples were removed at 20 min interval for 2 h ($n = 3$ rats, with three sacs from each rat). The collected samples were immediately processed and analyzed by HPLC. The experiment was performed in Kreb's Ringer Buffer (KRB) solution at two different pH values (7.4 and 5.4). For CA the permeability coefficient was also calculated with out everting the sac that is from basal to apical side and compared with above results, to ascertain existence of efflux mechanism. Similarly, the samples were withdrawn periodically from the outer vessel (apical side) in which drug was added and analyzed.

2.6.2. Intestinal tissue uptake studies

The tissue-drug uptake studies can generate comparative data on absorption of CP and CA. Fresh everted jejunal rings of approximately 1 cm length were immediately prepared after sacrificing the animal and incubated in drug solution at 37 °C in a shaking water bath ($n = 3$, and jejunal portions isolated from two rats). At specified intervals of time (0.5, 1, 1.5, 2, 3, 4, 5, 7.5, 10, and 15 min), the rings were withdrawn and rinsed thoroughly in ice-cold buffer, dried on tissue paper, and immediately homogenized with 0.5% Triton + 2% trichloroacetic acid solution after weighing (wet tissue weight). The homogenate was centrifuged and analyzed for drug content by HPLC.

3. Results and discussion

3.1. In vitro studies

3.1.1. pH-stability

A strong influence of pH on the stability of CP was observed. The higher the pH of the buffer, lesser is the stability of CP, and buffer with pH 1.2 offered highest protection. There was a sudden increase in degradation of CP at pH 6.8. CP was stable for up to 12 h in all buffers except pH 6.8. Although at pH 5.4, the stability was less compared to those of pH 1.2 and 4.5, but the percentage of drug was maintained at 80% till 24 h. At pH 6.8, about 55% of its content was degraded within 8 h and complete degradation occurred in 24 h (Fig. 2). The major degradation product was found to be CA as analyzed by HPLC.

3.1.2. Enzymatic metabolism

Orally administered drug through GIT encounters not only varied pH environments but also secretions containing various enzymes. These enzymes consist of esterases, hydrolyases,

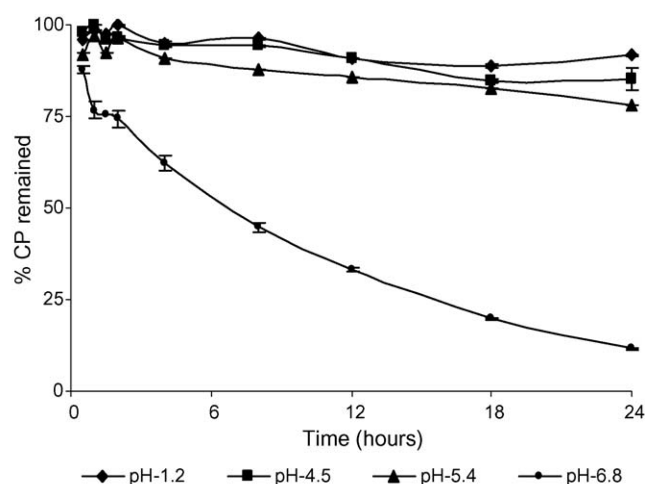

Fig. 2. Stability of CP at various pH values.

which can have a significantly influence weak ester linkage present in ester prodrugs like CP, and can convert them to their parent form. When CP was incubated with enzyme fraction, isolated from small intestinal scrapings, considerable amount of CP was metabolized into CA. The results are represented in Fig. 3. Here also the lower pH values clearly showed less or no degradation of CP, possibly because of the inactivation of enzymes. In buffers of pH 5.4 and 6.8, about 50% of the drug is metabolized in 1 h of incubation. The pH 5.4 is appearing as the critical point, where both chemical and enzymatic degradations become significant.

From the preceding studies, it can be inferred that, the drug is more soluble and stable in acidic pH (of stomach and duodenum) and prone to more degradation as it moves further to lower parts of GIT (i.e. jejunum, ileum and colon).

3.2. In situ studies

Although in vitro incubation studies provided an insight of effect of pH and enterocytic enzymes on CP, the in situ studies

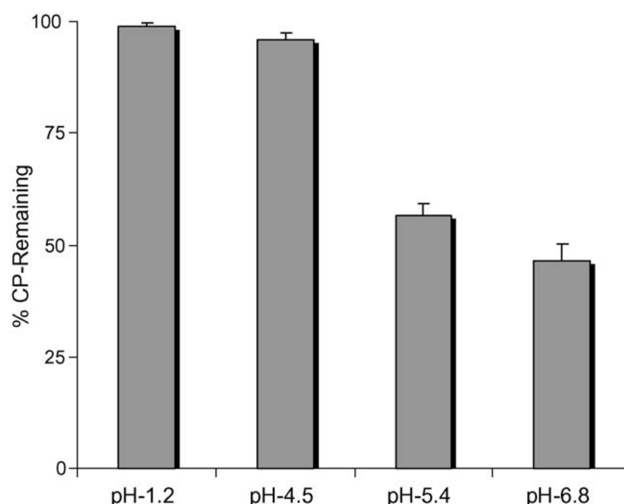

Fig. 3. The stability of CP in presence of enzymes at various pH values.

Table 1
Degradation of CP in jejunal and stomach closed loops

S. No.	pH of the drug solution injected	% CP remaining	% CA formed
1	4.5[a]	18.76 (5.40)	16.57 (3.72)
2	5.4[a]	18.05 (5.23)	16.77 (3.29)
3	6.8[a]	17.25 (4.67)	14.58 (2.89)
4	1.2[b]	76.52 (3.63)	1.25 (0.61)

Values in parenthesis indicate SD ($n = 6$). Note: the values of % CP remaining and % CA formed do not sum up to 100. Remaining quantity of drug is present inside the tissues.
[a] Jejunum.
[b] Stomach.

can provide a more realistic picture of fate of the drug in the GIT, as they take into account physiological conditions such as blood circulation. Apart from composite environment, once the drug enters the lumen, it is affected by biological factors such as gastric motility and blood circulation and these factors cannot be studied during an in vitro experiment performance.

The amount of CA formed and the amount of remaining CP in the intestinal loop after 1 h of incubation in the intestinal loop are tabulated in Table 1. The results show that CA is formed in higher amounts in jejunal loops at all pH conditions, compared to stomach. The pH of the buffer containing drug does not have any influence on the amount of CA formed in jejunal loops, as equivalent amount of CA is formed in all cases. In allegiance with the in vitro results, the closed loop studies performed in stomach region at pH 1.2, showed least formation of CA from CP. But the amount of CP remaining in stomach loops is high when compared to amount of drug extracted from loops made in jejunal region. This can be attributed to comparatively lesser absorption area in stomach, in comparison to jejunum, where larger area is available due to the presence of villi. Also, the disappeared drug does not necessarily mean absorption into the systemic circulation, as the drug may be concentrated in the epithelial cells or intestinal tissue. Thus, it can be inferred that CP has better stability towards metabolic conversion into CA in gastric contents as compared to jejunal contents.

3.3. *Ex vivo studies*

3.3.1. *Determination of permeability coefficients for CP and CA*

The poor oral bioavailability of CP is regarded as because of formation of CA in lumen which is absorbed lesser than CP. Lower bioavailability of CA can be because of (i) poor permeability as compared to CP, (ii) absorption by a mechanism other than passive such as carrier mediated, and/or (iii) presence of an efflux mechanism. The results obtained from in vitro and in situ studies confirmed susceptibility of CP to enzymatic degradation leading to formation of CA in the luminal contents. CP is devoid of carboxylic acid functional group, which is present in CA and hence shall be more lipophilic and more permeable than CA (Fuzimoto et al., 1987; Nakao et al., 1987; Miyauchi et al., 1989). Permeability coefficients were calculated using flux value obtained from the slope of cumulative concentration versus time plot.

Table 2
Apparent permeability coefficients of CP and CA as determined by everted sac method

pH of the medium	P_{eff} ($\times 10^{-5}$ cm/s)	
	CP	CA
7.4	1.54 (0.26)	1.13 (0.10)
5.4	1.10 (0.19)	1.18 (0.12)
Non-everted at 7.4	–	1.11 (0.34)

Values in parenthesis indicate SD ($n = 3$ rats, with three sacs from each rat).

The permeability coefficient, P_{eff}, obtained was similar for both CP and CA (Table 2). The results are against expectations as CP had been designed to improve permeability and absorption and is more lipophilic than CA. Hence, it appears that the P_{eff} values obtained were influenced by some other factors. The role of efflux mechanism in the overall process was also ruled out as CA yielded similar P_{eff} value when flux was measured without everting the intestine. The underlying factors responsible for these typical results might be at play in the epithelial cells or at interfaces, or in the buffer solution leading to poor permeability of CP. Additional experiments on CP in intestinal tissues were performed to understand this phenomenon.

3.3.2. *Stability of CP in presence of jejunal segment*

The metabolic conversion of CP to CA when exposed to enterocytes of the everted intestine (apical side) was measured. Samples were collected periodically from the outer vessel (apical side) and analyzed. CP undergoes rapid transformation to CA on exposure to enterocytes (Fig. 4). About 90% of the CP present was converted to CA within 15 min, and within 30 min of incubation, the conversion was nearly complete. This explains similar P_{eff} values for both CP and CA, as the minimum sampling point in the everted sac experiment was 20 min, by which time majority of the CP would have converted into CA. The reason for rapid conversion of CP to CA exposed to epithelial cells

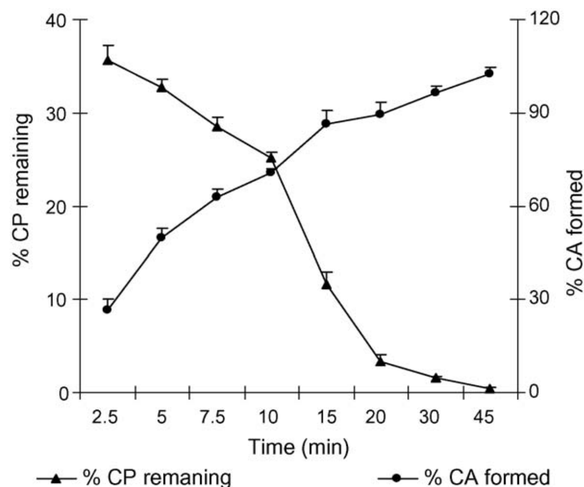

Fig. 4. Amount of CP degraded and amount of CA formed as a function of time in the presence of everted jejunal segment.

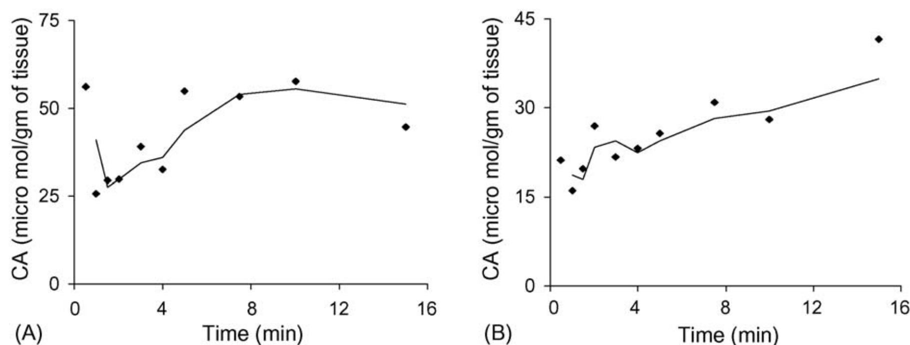

Fig. 5. Tissue uptake behavior of CP (A) and CA (B) (in plot (A), the concentration represented is sum of CP and CA found inside the tissue expressed in equivalents of CA).

can be (i) leaching of enzymes from the enterocytes into the buffer solution or (ii) post-absorption metabolism of CP to CA in the enterocyte and efflux into the buffer solution. However, the former is less probable, as the jejunal sac is first equilibrated for about 30 min in KRB solution, the leachables are then discarded and replaced with fresh buffer solution containing drug. Additional experiments were necessitated to identify and establish the exact reason responsible for rapid conversion of CP into CA.

3.3.3. Accumulation of CA in enterocytes

The propensity of the CA molecules formed inside the enterocyte by enzymatic action, to move towards apical or basal sides was evaluated. An everted sac experiment was set-up, with drug added to the buffer present in outer vessel (apical side). After 10 min of incubation, both apical and basal sides of the sac were thoroughly rinsed off the drug and the sac was mounted in fresh buffer solution. The amount of drug released on both sides of the segment was analyzed by HPLC.

Incubation of the jejunal sac for 10 min in the drug solution ensures absorption of CP into the enterocytes, which will be present inside the cell either in form of CP or CA. Mounting of the sac in fresh buffer after thorough washing provides equal sink conditions to the drug trapped inside the enterocyte to move off to any of the apical or basal sides. After 10 min of mounting in fresh buffer, the results showed nearly nine times more amount of CA being moved towards apical side (15.6 ± 3.34 μmol, $n = 3$) compared to that of basal side (1.86 ± 0.5 μmol, $n = 3$). The results obtained are interesting as CA present inside the enterocyte has a greater affinity to move towards apical side rather than basal side, which is against the expected movement during in vivo absorption. This phenomenon might be a contributing factor in the reduced absorption of CP. The reasons for such typical movement of CA towards apical side may be sudden change occurring in the physicochemical properties of the molecule such as pK_a and $\log P$-values, due to conversion of CP into CA, inside the epithelial cell. The accumulation of large amounts of CA inside the cell may be creating unfavorable state, resulting in its movement to apical side. It may also be possible that the CA molecule does not possess enough lipophilicity to cross the cell membrane and enter blood circulation. These assumptions were further evaluated with intestinal tissue-drug uptake study.

3.3.4. Intestinal tissue uptake studies

The rapid conversion of CP into CA is confirmed when exposed to enterocytes of the everted intestine. In such cases the determination of permeability of CP in a relatively smaller time and comparing it with that of CA would be difficult and unreliable. Alternatively tissue uptake rate was compared for CP and CA for a period of 15 min. The results provide interesting insights into the absorption of both CP and CA (Fig. 5). The jejunal rings showed presence of high amounts of CP inside the enterocyte in the initial stages as early as less than 1 min. But the concentrations inside the epithelial cells depleted fast and recurrence of CA concentration was observed with time. When CP was studied for uptake, its traces were observed in tissue for 5 min only. The point illuminated from earlier and present experiments, is that the CP permeates quickly, but is rapidly converted into CA, effluxed towards apical side rather than basal side leading to lowered absorption. With time, the accumulated CA is absorbed but slowly and to a lesser extent. When similar study was performed with CA, it exhibited a constant intake rate into the tissue giving an upward movement of concentration versus time profile.

4. Conclusions

The results of the in vitro, in situ and ex vivo studies clearly showed extensive metabolism of the prodrug into parent form. The conversion process occurs at two stages, one in the lumen before absorption, and the second inside the epithelial cell after permeation into it. Considering the degree of conversion of CP into CA in ex vivo studies, the latter is considered as a major contribution in the low bioavailability of CP. The prodrug is highly permeable, but is rapidly converted into CA and effluxed by an unknown mechanism, leading to a lowered bioavailability. This distinctive behavior of CA movement through the apical side of the cell membrane is either because of simple passive diffusion through apical membrane (as basolateral membrane is more lipid in nature and difficult to cross) as CA exists as zwitter ionic at physiological pH (Deslandes et al., 1996) or because of a specific transport mechanism present on apical membrane, similar to the one observed for cephradine on basolateral membrane of Caco-2 monolayers (Inui et al., 1992).

The bioavailability of CP can be improved by reducing or bypassing the metabolism of CP in the epithelial cell. As dissolution is a pre requisite for passive absorption of drugs, post-dissolution conversion of CP to CA prior to absorption into the systemic circulation will remain a hurdle in all types of conventional oral dosage forms. Hence, a formulation needs to be prepared for CP, which can bypass the passage of drug through epithelial cell and provide sufficient protection to the drug from the enterocyte enzymes. Formulations enabling lymphatic absorption, such as nano-particles, nano/micro emulsions can be prepared to improve bioavailability.

Acknowledgement

Mr. Vasu Kumar Kakumanu wishes to thank Ranbaxy Research Laboratories, Gurgaon, India for providing the research fellowship.

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