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Research paper

Investigation on physicochemical and biological differences of cefpodoxime proxetil enantiomers

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Abstract

Cefpodoxime proxetil (CP) is a prodrug of cefpodoxime acid (CA), and is supplied as racemic mixture of *R*- and *S*-enantiomers. CP has only 50% absolute bioavailability, and the reasons responsible for low bioavailability remain poorly understood. The present work ascertains physicochemical and biological properties of individual isomers of CP and explores their capacity to optimize delivery of CP. Both isomers showed similar pH stability behavior, but *R*-isomer was more susceptible to enzymatic metabolism compared to *S*-isomer, when incubated with enzymes collected from various segments of GIT. Based on the in vitro and in vivo results, use of *S*-isomer for development of a dosage form such as gastro-retentive dosage form can improve oral bioavailability of CP.

Keywords: Cefpodoxime proxetil; Enantiomers; Differences; Enzymes; Segments; Regional metabolism; GIT; Physicochemical; Biological

1. Introduction

Cefpodoxime proxetil (CP) is a prodrug of cefpodoxime acid (CA), and a third generation cephem type broad spectrum antibiotic administered orally. CP is a non-crystalline, slightly basic compound and is absorbed from the gastro-intestinal tract after oral administration and hydrolyzed to its parent moiety cefpodoxime acid (CA) by non-specific esterases in the intestinal wall/plasma [1–3]. The drug possesses an asymmetric carbon atom in the ester group (Fig. 1) and is supplied as a racemic mixture of *R*-isomer and *S*-isomer [4–7]. These *R*- and *S*-isomers of CP are equivalent in biological action and therapeutic efficacy, but differ in few physico-chemical properties [8]. Hamamura et al. reported that the two isomers exhibit different solubility values and *R*-isomer shows pH-dependent solubility and exhibits a typical micro-crystallization or gelation phenomenon in the acidic pH values [5].

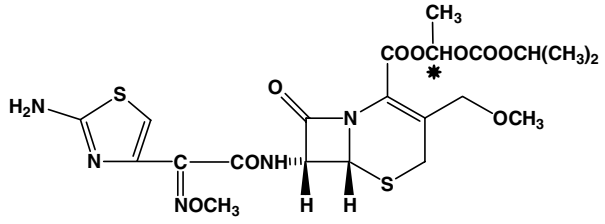

Fig. 1. Structure of Cefpodoxime proxetil (* – asymmetric carbon).

Although CP is designed to improve the permeability and thus bioavailability of the parent molecule CA, it still has only 50% oral bioavailability. The reasons for poor bioavailability of CP remain poorly investigated. Reported studies have pointed possible reasons of low bioavailability as, the low solubility and a typical gelation behavior of CP particularly in acidic environments [5,9,10], and pre-absorption luminal metabolism into CA by the action of digestive enzymes [1]. The detailed study of the properties and behavior of isomers can provide

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opportunity of applying rational formulation strategies, to improving absorption and bioavailability of CP. The objective of the present exercise is to study physicochemical and biological differences of enantiomers of CP and to suggest possibility of developing formulation containing pure isomers, with a view to improve the oral bioavailability of CP.

2. Materials and methods

2.1. Materials

The reference standards of CP and CA were obtained from Ranbaxy Research Laboratories Ltd, Gurgaon, India. The *R*- and *S*-isomers of CP were prepared in-house by preparative chromatography of CP. The isomers were separated on a reversed-phase preparative column chromatograph, and racemic CP was eluted slowly at a flow rate of 15–20 ml/h with a mobile phase consisting of water and acetonitrile in 32:68 composition. Fractions were collected periodically and analyzed by HPLC for their content. Fractions containing similar isomer in major proportion were mixed, concentrated in rotary evaporator and freeze dried. The purity of each isomer used was about 90%, as analyzed by HPLC. All reagents used were of analytical grade and materials used for preparation of mobile phase and HPLC analyses were of chromatography grade.

2.2. Analytical methods

In-house developed and validated HPLC analytical methods were utilized for detecting quantities of CP and CA in 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. The HPLC method for CP employed acetonitrile: ammonium acetate buffer (pH 5.0) pumped at a flow rate of 1 ml/min in 36:64 composition, and analysis was carried at a temperature of 30 °C and a detection of 235 nm. The HPLC method for determination of CA from plasma employed acetonitrile:phosphate buffer (pH 3.0) in 10:90, flow rate of 1 ml/min, and detection at 269 nm.

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 250–275 g were used in various experiments. The rats were housed under standard laboratory conditions and fasted overnight with water allowed ad libitum before conducting any experiment.

2.4. Differential scanning calorimetry

The separated and purified enantiomers of CP were analyzed under 80 ml/min of dry nitrogen purge with a heating rate of 5 °C/min, using Mettler Toledo DSC 821e (Greifensee, Switzerland) operating with Star software version Solaris 5.1, equipped with automated cooling accessory. The DSC instrument was calibrated for temperature and heat flow with indium. Samples of about 8–12 mg were taken in standard aluminum pans, sealed with a pin-hole and then thermograms were obtained in the range of 25–200 °C.

2.5. Powder X-ray diffractometry (PXRD)

A Philips PW1729 powder X-ray diffractometer (Philips, Holland) was used to analyze CP and its enantiomers. The radiation used was generated by a copper Kα source fitted with Ni filter at 0.154 nm wavelength at 20 mA and 35 kV. Samples were scanned over a range of 2θ values from 5° to 40° at a scan rate of 1°/min.

2.6. Optical rotation studies

The optical rotation and specific rotation of 0.2% methanolic solutions of each enantiomer were measured using AUTOPOL™ IV, an automatic polarimeter (RUDOLPH Research, Flanders, NJ, USA), at ambient temperature.

2.7. In vitro studies

2.7.1. pH-stability

The stability of enantiomers in various buffers was assessed. Enantiomers were incubated in buffers of pH values – 1.2, 4.5, 5.4, and 6.8 for 24 h at 37 °C. These pH values represent the local environments of stomach, duodenum, jejunum and ileum, respectively. At specified time points, samples were withdrawn and immediately analyzed by HPLC.

2.7.2. Enzyme incubation studies

2.7.2.1. Intestinal homogenate preparation. Enzyme fractions were prepared individually from four regions of the upper GIT of rats namely – stomach, duodenum, jejunum and ileum. Immediately after sacrificing the animals ($n = 3$), their abdominal portion was exposed and stomach, duodenum, jejunum and ileum sections of GIT were isolated, perfused with ice-cold saline and maintained at 0 °C. The intestinal segments were cut along the axis and the apical portion of individual region was scrapped with the help of a glass slide. The tissue scrapings were homogenized in Potter–Elvejhem tube with Teflon pestle at 0 °C in 0.25 M sucrose solution, filtered through nylon cloth and centrifuged at 4000*g* for 10 min at 4 °C preliminarily to separate macrosized tissue debris. The precipitate is discarded and supernatant was further

centrifuged at 12,500*g* for 15 min. at 4 °C [11]. The clear supernatant was collected, assayed and stored at 4 °C. These homogenates obtained from specific segments of GIT containing enzymes were normalized to the protein content after the protein estimation using bovine serum albumin (fraction V) (BSA) as standard by Lowery protein estimation method [12,13]. The method of estimation was based on formation of copper complex with the protein in highly alkaline conditions and the measurements were performed spectrophotometrically at a λ_{max} of 660 nm. The formation of colored complex and the intensity of color depend on the amount of protein present. All reagents are prepared fresh and the calibration curves were prepared with standard curve BSA in the range of 0.2–1.0 mg/ml each time during the measurement of enzyme protein component isolated from various segments of GIT. In test samples, equivalent volume of sample was added instead of BSA.

2.7.2.2. Metabolism studies. The susceptibility of each enantiomer towards enzymatic metabolism was investigated by incubating each isomer in 0.05 M phosphate buffer saline (pH 7.4) at 37 °C. Metabolism studies were performed at equal protein levels in all cases, and the reaction was initiated by adding 200 μl of drug solution to pre equilibrated normalized enzyme solution (100 μmol of isomer and 0.05 mg of protein in total) obtained from various segments of GIT ($n = 3$). The reaction was stopped by adding 100 μl of 10% trichloroacetic acid solution. The mixture was mixed well, and centrifuged at 16,000*g* for 10 min. The clear supernatant was separated and analyzed immediately by HPLC. Preliminarily esterase activity of the biological media was determined spectrophotometrically at 37 °C by following the initial formation (0–60 s) of *p*-nitrophenol from a 100 mM *p*-nitrophenyl acetate solution. Concentration profiles for each isomer were plotted and conversion rate constants were calculated from the results of HPLC analyses.

2.8. In vivo studies

Each isomer was administered to six rats by oral gavage needle, at 10 mg/kg (equivalent to CA) of aqueous suspension. Blood samples (about 0.4 ml) were collected in heparinized micro-centrifuge tubes at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 h after dosing. The blood was centrifuged at 6000*g* for 5 min and the plasma was separated and stored at −20 °C until analysis. The collected samples were treated according to a validated procedure and drug content was estimated. Data acquisition and processing was done on Shimadzu Class-VP software. The data were further processed and the various pharmacokinetic parameters are calculated using PC-NONLIN software. The computations and non-linear fit were performed with PC-NONLIN using Neldar-Mead algorithm without input of initial parameters, and fitted to model with first order input and fist order output with no lag time.

3. Results and discussion

3.1. Characterization

The results of DSC and PXRD studies showed absence of crystalline nature of both the enantiomers. The DSC thermograms did not show any melting endotherms, and PXRD spectra were devoid of any sharp peaks representative of crystalline phases in the material. Although both enantiomers were regarded as amorphous, their thermograms or PXRD spectra were not overlapping. This may be due to differences in the history of sample treatment during their preparation. Each isomer provided reverse rotation in the polarimeter (−0.107 for *S*-isomer and +0.257 for *R*-isomer) when 0.2% methanolic solutions were measured, confirming the *R*- and *S*-isomeric nature, which are supplied as a racemic mixture.

3.2. In vitro studies

3.2.1. pH-stability

Influence of pH on the stability of enantiomers of CP was observed. The pH values higher than 5.4 lead to lowering of the stability of CP. In mediums up to pH 5.4, the stability of CP was not affected for 12 h. The degradation kinetics did not differ for two isomers. A rapid increase in degradation of both isomers at pH 6.8 was observed. Both isomers were able to maintain more than 90% of the drug up to 12 h in all buffers except pH 6.8. At 24 h, the stability was less at pH 5.4 compared to those of pH 1.2 and 4.5, but the percentage of drug was maintained at 80%. At pH 6.8, about 55% of each isomer 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.

3.2.2. Enzymatic metabolism

Orally administered drug through GIT encounters not only varying pH environments but also secretions containing various enzymes like esterases, and hydrolyases, which

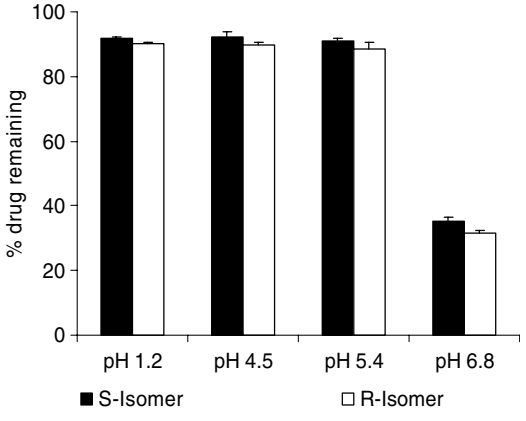

Fig. 2. Percentage of individual isomers remaining after incubation for 12 h in buffers of various pH.

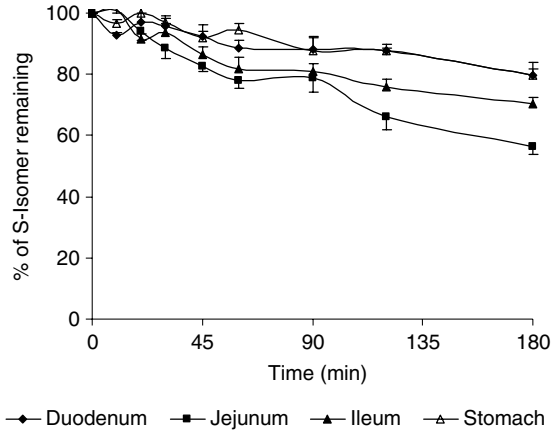

Fig. 3. Percentage of remaining isomer *S* after incubation with the enzyme fractions isolated from various regions of GIT ($n = 3$).

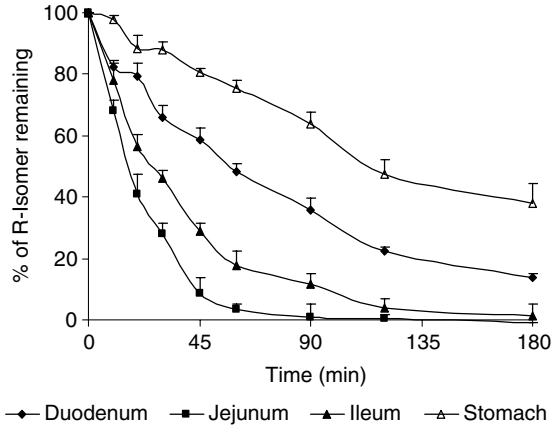

Fig. 4. Percentage of remaining isomer *R* after incubation with the enzyme fractions isolated from various regions of GIT ($n = 3$).

can significantly influence weak ester linkage of ester prodrugs like CP. Metabolism profiles of enantiomers of CP, after incubation with site specific enzyme fractions for 180 min, are presented in Figs. 3 and 4. Unlike pH stability behavior, the susceptibility towards enzymatic metabolism was different for both enantiomers, with *S*-isomer being more stable towards enzymatic metabolism. Another interesting phenomenon observed was that the enzyme fractions collected from the lower portions of GIT (jejunum and ileum) caused higher metabolism of CP to CA, as compared to those obtained from upper portions of GIT (stomach and duodenum). The rate of conversion was in the order of jejunum > ileum > duodenum > stomach. Both enantiomers of the drug were relatively stable in presence of enzyme fractions collected from stomach, when nearly 50% of *R*-isomer was metabolized within 30 min when incubated with jejunal or ileum homogenate. The results clearly indicate extensive metabolism of *R*-isomer in jejunum compared to *S*-isomer. From the results of incubation studies, it can be inferred that, the both isomers are more stable in regions of acidic pH (of stomach and duodenum) and prone to more degradation as it moves further to lower parts of GIT (i.e., jejunum, ileum).

3.3. In vivo studies

The results of pharmacokinetic study of enantiomers of CP performed in rats are presented in Fig. 5 and Table 1. The figure shows slow absorption of *R*-isomer compared to *S*-isomer. The C_{max} value obtained for the *R*-isomer was 2032 ± 924 ng/ml which is significantly lesser than that of *S*-isomer, 3652 ± 1235 ng/ml. This can be explained because of low solubility and high susceptibility of *R*-isomer to enzyme metabolism, renders delayed and incomplete absorption of *R*-isomer. The elimination rate constant (K_{10}) and absorption rate constant (K_{01}) values obtained for both isomers were similar to each other, but those obtained for *R*-isomer were slightly less than those of *S*-isomer. The value of volume of distribution (V) obtained was higher for *R*-isomer compared to *S*-isomer. The results showed apparent differences in the AUC values of enantiomers, but T_{max} and AUC values for both the isomers are statistically not different (at $p = 0.33$). Considering the higher resistance of *S*-isomer to enzymatic metabolism and minimal degradation in gastric regions, the benefits of a gastro-retentive (GR) system containing *S*-isomer can result into significant bioavailability advantage.

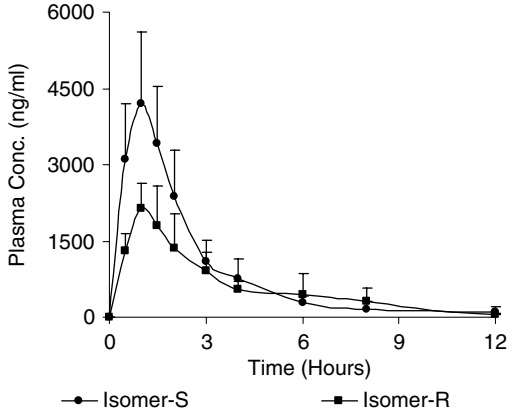

Fig. 5. Plasma concentration vs. time profiles observed when purified isomer *R* and isomer *S* are administered.

Table 1
Pharmacokinetic parameters obtained after oral administration of enantiomers of CP (values presented are means ± SD, and $n = 6$)

Parameter	*S*-isomer	*R*-isomer
C_{max} (ng/ml)	4094 ± 853	2032 ± 924
T_{max} (h)	0.91 ± 0.17	0.93 ± 0.35
AUC ((ng/ml) h)	9484 ± 1979	7981 ± 2453
Absorption rate constant, K_{01} (h^{-1})	1.22 ± 0.15	1.19 ± 0.44
Elimination rate constant, K_{10} (h^{-1})	0.99 ± 0.40	0.80 ± 0.29
V	386.62 ± 336.88	546.02 ± 307.60

4. Conclusions

The results of incubation studies at different pH values and with site specific enzyme fractions clearly showed extensive metabolism of the prodrug into parent form, with enzymatic metabolism contributing the most. Both isomers showed equivalent degradation profiles at higher pH values, but when incubated with enzyme fractions, the *R*-isomer is more susceptible towards hydrolysis than *S*-isomer. Hence, the two isomers differ in their solubility [5] and pre- and post-absorption degradation profiles. Considering the results of incubation and in vivo studies, the *S*-isomer can be judiciously utilized for improving oral bioavailability of CP. The higher stability of enantiomers in enzyme fractions collected from upper GIT section (stomach and duodenum) indicates potential of GR system in preventing pre-absorption conversion of CP to CA. Overall development of GR system using *S*-isomer can provide maximum benefit.

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