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Development and validation of isomer specific RP-HPLC method for quantification of cefpodoxime proxetil

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Abstract

The present work explains the development and validation of a simple and reliable isomer specific liquid chromatographic method for the quantitative determination of cefpodoxime proxetil (CP) in rat in situ intestinal perfusate samples. Chromatography was carried out by reversed-phase technique on a C-18 column with a mobile phase composed of 20 mM ammonium acetate buffer (pH 5.0) and acetonitrile in the ratio of 62:38 pumped at a flow-rate of 1 ml/min. The detection was carried out at 235 nm and a column temperature of 30 °C. The method was evaluated for the various validation parameters, such as linearity, accuracy, precision, LOD, LOQ, specificity, selectivity, and sample stability. The results of intra- and inter-day validation ($n = 3$) showed the method to be efficient and the same was applied in an in situ permeability study conducted for CP in rats.

Keywords: Racemic cefpodoxime proxetil; R, S-enantiomers; Separation of isomers; Reversed-phase; HPLC; Liquid chromatography

1. Introduction

Cefpodoxime proxetil (CP) is an oral third generation cephalosporin antibiotic, active against many gram positive and negative micro-organisms, and is widely used in treatment of variety of infections like skin, respiratory, urinary tract, and systemic [1]. CP is an ester prodrug of cefpodoxime acid (CA), where a proxetil radical is attached to CA (Fig. 1). In the biological system, CP undergoes ester hydrolysis and is converted into CA to exhibit its antibiotic activity [2,3]. CP has an asymmetric carbon at position 4 and is supplied as a racemic mixture of two enantiomers R and S forms. Few methods are reported to quantify CA [4–7], but analytical methods describing detection of CP are scarce only as a small part in few publications [8–11]. In the present study, a sensitive reversed-phase high performance liquid chromatographic (RP-HPLC) method was developed and validated, which can analyze both R and S isomers of CP in the samples obtained from rat in situ absorption studies. Although the R- and S-isomers of CP are reported to exhibit similar biological activity, they differ in their physicochemical and biological properties [10–14]. Thus, it would be beneficial to have an analytical method which can specifically quantify individual isomers of CP. In most of the cases, separation and quantification of isomers is performed on chiral columns, but the present study uses a simple method on a reversed-phase column. The separation of these enantiomers of CP on RP-HPLC was based on significant differences in physicochemical properties of individual enantiomers.

2. Experimental

2.1. Chemicals and reagents

The reference standards of racemic CP and R-, S-isomers of CP were obtained from Ranbaxy Research Laboratories Ltd., Gurgaon, India. All solvents and other chemicals were of HPLC grade. All samples and solvents were filtered through Millipore™ (nylon) 0.45 μm filters before use in HPLC.

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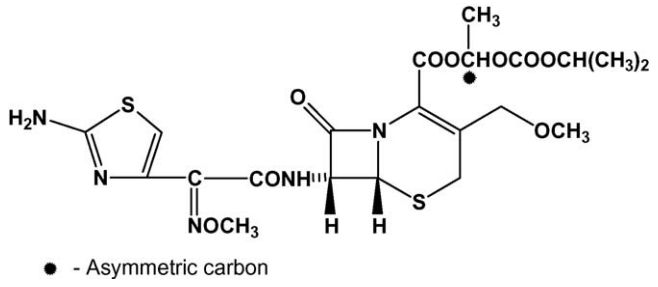

Fig. 1. Structure of cefpodoxime proxetil and it asymmetric carbon.

2.2. Apparatus and HPLC conditions

A HPLC system (Shimadzu Corporation, Japan) comprising of a solvent pump (LC-10AT VP), on line-degasser (DGU-14A), an auto injector (SIL-10AD VP) with temperature control, CTO-10AS VP column oven and UV–vis spectrophotometric detector (SPD-10AVP) was utilized for the purpose. Data acquisition, analysis, and reporting were done using Shimadzu CLASS-VP (Version 6.14 SP1) software. The chromatography was performed using LiChrocart® 250-4, LiChroSpher® 100 RP 18 (250 mm × 4.6 mm, 5 μm) and a guard column of LiChrocart® 4-4, LiChroSpher® RP 18 end capped (Merck, Germany). Mobile phase was pumped in a low pressure gradient mode at a flow-rate of 1 ml/min and column temperature of 30 °C. The analytical wavelength used for detection was 235 nm and samples of 40 μl were automatically injected using auto sampler.

2.3. Mobile phase

The chromatographic separation of enantiomers of CP was attempted using a mobile phase consisting of 20 mM ammonium acetate buffer (specified amount of ammonium acetate was dissolved in HPLC grade water and pH was adjusted to 5.0 ± 0.05 with glacial acetic acid) and acetonitrile (MeCN). The buffer and acetonitrile were filtered through 0.45 μm nylon filter and de-aerated in an ultrasonic bath before use.

2.4. Sample preparation

2.4.1. Preparation of standard solutions

Primary stock solution of CP was prepared in methanol to obtain a concentration of about 2 mg/ml. Consequently, the primary stock solution was diluted to prepare a secondary stock solution of 500 μg/ml concentration. Using the secondary stock solution, further dilutions were made to achieve various concentrations in the range of 5–150 μg/ml.

2.4.2. Sample preparation for analysis from the in situ samples

The samples obtained from rat in situ studies contain mucus, protein residues, and other exudates of intestine. These samples require purification, before injecting into the HPLC column to eliminate errors occurring from protein/intestinal tissue interference. A 10% trifluroacetic acid solution was added to all the samples to precipitate the protein material, and then centrifuged at 8000 × *g* for 10 min. The supernatant was then separated and analyzed by HPLC.

2.5. Validation

The proposed HPLC method was validated for various parameters, viz. linearity, range, specificity, sensitivity, precision, limit of detection (LOD), limit of quantification (LOQ), and system suitability. Calibration curves with seven concentrations, each in triplicate, were constructed in the concentration range of 5–150 μg/ml. Percentage recovery (accuracy) and precision were determined with six replicates of quality control (QC) samples. QC samples were prepared in blank in situ intestinal perfusate samples, at three different concentrations of 20, 50, and 100 μg/ml, following the same procedure as reported for calibration standards, using different primary stock. Calibration curves were prepared twice on the same day to assess intra-day variation, and inter-day variability was checked by constructing calibration curves on three consequent days. The results are expressed as % R.S.D. of concentration.

2.6. In situ absorption 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 (250–275 g) were housed under standard laboratory conditions and fasted over night with water allowed ad libitum. After inducing the anesthesia to the rats, the drug solution was injected into the ligated jejunal loop and the remaining drug was extracted after 1 h. The perfusate samples were extracted according to the extraction procedure explained earlier and injected into HPLC to estimate the drug content.

3. Results and discussion

Separation of enantiomers on a reversed-phase column is surprising, as generally enantiomers cannot be separated, without using a chiral column or chiral selector. Enantiomers of CP have considerable differences in their physicochemical properties, such as crystallization behavior and solubility [10,12–14]. The S-isomer exhibits 15.7 mg/ml of solubility against 1.7 mg/ml by R-isomer, at a pH of 0.7, and the difference between solubility of isomers continues even though the solubility falls as the pH increases. Similarly at pH 1.2, the solubility values are 2.0 and 8.5 mg/ml and at pH 5.0, the pH of mobile phase used for elution of isomers, the solubilities of each enantiomer were found to be 0.41 and 0.54 mg/ml for R- and S-isomers, respectively. But, at pH 6.8 and above, the solubility of each enantiomer decreases to 0.3 mg/ml and becomes equal. Such a difference in solubility behavior explains separation of R- and S-isomers on an achiral column and the more soluble S-isomer is eluted first during analysis. This is in line with previous reports where in a reverse phase C18 and C8 achiral columns without the use of any chiral column or chiral selector or chi-

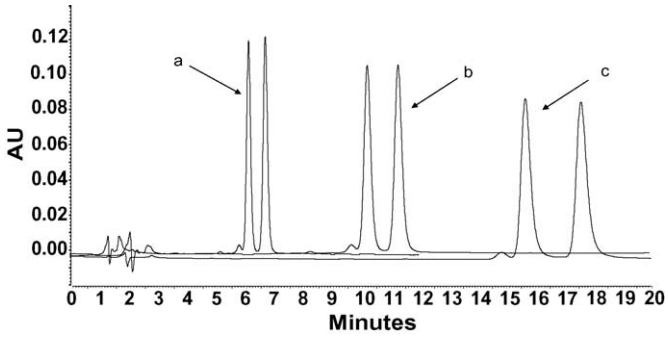

Fig. 2. Effect of concentration of organic solvent in the mobile phase on the separation of the S- and R-isomers of CP: (a) 45%, (b) 40%, (c) 35%.

ral derivitization process, have been utilized for analysis of CP [8–11].

3.1. Effects of elution conditions on the separation of isomers

The mobile phase consisted of mixture of ammonium acetate–glacial acetic acid (pH adjusting agent)–MeCN. The final composition optimized was in the ratio of buffer: MeCN: 62:38 (v/v). The amount of MeCN, which is the organic component present in the mobile phase, had a strong effect on the separation of isomers of CP. A decrease in percentage of MeCN from 45 to 35% had a noticeable effect on the separation of isomers. Although the retention times of individual peaks were increased, the baseline separation of each isomer was enhanced (Fig. 2). Although the run times increased with the decrease in MeCN concentration, it was acceptable because of accompanying enhanced resolution of individual peaks. The effect of column temperature on the elution of S- and R-isomers was evaluated (Table 1). Small changes in column temperature did not affect the elution, but higher increments of 5 °C caused shortening of the elution times. Higher values of column temperature not only reduced elution times, but also affected the resolution of the isomeric separation. Hence, to achieve a proper balance between the resolution, column temperature and also considering the stability of CP at higher temperatures [12], 30 °C was fixed as the column elution temperature. The flow-rate has an influence similar to that of amount of MeCN or temperature. Increase in the flow-rate, reduced the retention times and the resolution between the isomers (Table 2). The effect of flow-rate on elution pattern was evaluated between 0.75 and 1.5 ml/min

Table 1
Effect of column temperature on separation of CP isomers (mean ± S.D. for $n = 2$)

Temperature (°C)	Resolution factor Rs	Capacity factor K_1	Capacity factor K'_2	Separation factor $\alpha = K'_2/K'_1$
15	3.27 ± 0.15	5.93 ± 0.17	6.52 ± 0.14	1.10
20	2.95 ± 0.10	5.11 ± 0.13	5.36 ± 0.17	1.05
25	2.74 ± 0.03	4.67 ± 0.06	5.44 ± 0.10	1.16
30	2.85 ± 0.02	5.23 ± 0.09	5.97 ± 0.07	1.14
35	2.65 ± 0.03	4.76 ± 0.10	5.10 ± 0.09	1.07
40	2.23 ± 0.07	4.28 ± 0.21	4.77 ± 0.18	1.11

Table 2
Effect of flow-rate on separation of CP isomers (mean ± S.D. for $n = 2$)

Flow-rate (ml/min)	Resolution factor Rs	Capacity factor K'_1	Capacity factor K'_2	Separation factor $\alpha = K'_2/K'_1$
0.75	3.21 ± 0.09	6.47 ± 0.22	7.42 ± 0.18	1.15
1.00	3.08 ± 0.07	5.46 ± 0.16	6.24 ± 0.12	1.14
1.25	0.82 ± 0.06	4.30 ± 0.19	4.87 ± 0.09	1.13
1.50	1.96 ± 0.18	2.51 ± 0.11	2.80 ± 0.15	1.12

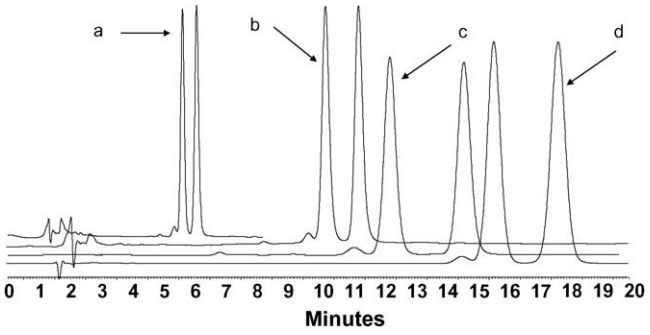

Fig. 3. Effect of flow-rate on the separation of the S- and R-isomers of CP: (a) 1.50 ml/min, (b) 1.25 ml/min, (c) 1.00 ml/min, (d) 0.75 ml/min.

(Fig. 3). Lower flow-rate values (less than 0.5 ml/min) resulted in too longer runtimes (up to 45–60 min), and higher flow-rates were not attempted because of their potential to damage the column. Finally, a flow-rate of 1 ml/min was fixed as optimum for elution of CP isomers.

3.2. Chromatography and specificity

Specificity of a method can be defined as absence of any interference at retention times of peaks of interest, and is evaluated by observing the chromatograms of blank rat intestinal perfusate samples and perfusate samples spiked with isomeric forms of drug. The elution peaks of individual isomers of CP are presented in representative chromatograms of blank and perfusate samples as shown in Fig. 4. Interfering peaks of any endogenous biological matter or buffer are not observed near the retention times of isomers of CP. The retention times of CP isomers are 12.5 and 14.5 min, respectively. The chromatographic run time was increased up to 20 min for better separation of isomers of

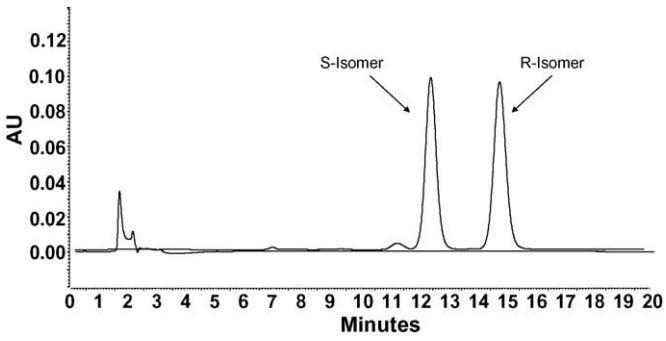

Fig. 4. Representative overlaid chromatograms of blank perfusate sample and perfusate sample spiked with CP, showing separation of individual isomers, S- and R-isomers, respectively.

CP. The run time was sufficient for routine, as well as, for crucial sample analysis.

3.3. Range and linearity

Sum of peak areas of both isomers were measured and served for quantification of CP. Calibration curves with seven concentrations were constructed for CP over the concentration range of 5–150 μg/ml. This concentration range was selected on the basis of anticipated drug concentrations in the in situ absorption studies. Peak areas of the drug versus concentration were plotted and found to be linear within the concentration range. Standard curves were constructed on three consecutive days and the evaluation parameters like regression, slope, intercept, and correlation coefficient, were calculated. The mean ± S.D. values of slope and intercept are 72477 ± 1749 and 19543 ± 1052 ($n = 4$), respectively, with a regression coefficient of 0.999. The standard curve had a reliable reproducibility over the standard concentrations of drug across the calibration range.

3.4. Sensitivity and selectivity

The sensitivity of a method is represented by the values of limit of detection (LOD) and limit of quantification (LOQ). The lowest concentration of the drug detectable by the proposed method is termed as LOD, while LOQ is the minimum quantifiable concentration of the drug by the suggested method. A number of methods have been practiced in the literature to calculate LOD and LOQ. The signal-to-noise ratio of 3:1 and 10:1 were taken as LOD and LOQ, respectively, which was calculated using Class VP software. The values of LOD and LOQ are further computed by dilution method, by analyzing diluted solution from the secondary stock solution till the peak area is obtained. The developed method was sensitive enough to detect 250 ng/ml. LOD and LOQ of spiked perfusate samples based on signal-to-noise ratio, where % R.S.D. is less than 20%, were found to be 250 and 900 ng/ml, respectively. The selectivity of the method was evaluated by spiking various drugs, which were either degradation components of CP or general permeability markers that are used for validating permeability models. The compounds examined were cefpodoxime acid, its Δ_2-isomer, phenol red, and many formulation excipients like polymers (PVP, HPMC), surfactants (poloxamer, sodium lauryl sulphate). Their effect on stability as well as elution of CP was studied.

3.5. Accuracy and precision

Accuracy and precision of the method represents the repeatability and robustness of the analytical method, and was determined by analyzing spiked or QC samples. The QC samples were analyzed at three different concentrations within the calibration range and each concentration for six times ($n = 6$). QC samples were prepared in blank in situ samples and are prepared from stock solutions different from the one used for preparing calibration curves. The percent recovery of method was found to be 100 ± 5% and with acceptable R.S.D. limits indicating that method is accurate and precise (Table 3). Intra- and inter-day constructions of calibration curves showed the intermediate precision of the method. It is expressed as percent R.S.D. for a statistically significant number of samples. The % R.S.D. values in the regression lines prepared on the same day or different days were within the limits (Table 3).

Table 3
Inter- and intra-day precision of CP isomers

Precision	Concentration (μg/ml)	Mean ± S.D. (μg/ml)	% R.S.D. (S.D. × 100)/mean	Recovery (%)
Inter day ($n = 3$)	19	18.71 ± 0.80	4.29	98.45
	47	47.77 ± 0.84	1.76	101.63
	95	95.70 ± 0.20	2.11	100.74
Intra-day ($n = 3$)	19	18.53 ± 1.18	6.37	97.52
	47	46.39 ± 1.43	3.10	98.71
	95	97.27 ± 0.92	0.95	102.39

3.6. Stability

The stability of the CP present in the perfusate samples, was evaluated in HPLC auto sampler tray (set at 4 °C), at ambient temperature and in refrigerated conditions. The drug spiked into blank perfusate samples were stored at these conditions, and analyzed at specified time points. The drug was stable for up to 24 h in HPLC auto sampler tray with more than 96.56 ± 3.97% assay value after 24 h. The drug present in the perfusate samples stored in freezer also maintained more than 97% of amounts at 24 h. The stability of CP towards hydrolysis is comparatively less in samples stored on ambient temperature/bench top, compared to the others, where 96.43 ± 4.11 and 88.19 ± 6.36% of amounts of CP were determined at 12 and 24 h, respectively.

3.7. Application of method to absorption studies in rats

Closed loop method, an in situ method was utilized to determine the permeability of CP in rats [15–18]. The validation exercise of the proposed method demonstrated its application in the analysis of CP present in the intestinal perfusate samples. The remaining drug was extracted from the jejunal loop and analyzed by HPLC after suitable treatment. The developed method could be easily applied to measure the concentrations of CP at very low levels. As the proposed method could easily quantify individual R- and S-isomers of CP and isomer specific conversion of CP into CA measurements were performed accurately. The results were presented in Table 4.

Table 4
Absorption of CP in jejunal closed loops ($n = 6$)

S. No.	pH of the drug solution injected	% CP remaining after 1 h
1	4.5	22.30 ± 9.60
2	5.4	18.05 ± 5.23
3	6.8	16.37 ± 5.21

4. Conclusions

Permeability or in situ absorption studies often require quantification of drug at very low concentrations. Also, for a sensitive drug such as CP, which undergoes metabolism in biological system and also exists as racemic mixture with isomers having different physicochemical properties, need a stable and robust analytical method for quantification. A rapid and reliable RP-HPLC method for determination of both isomers of CP from in situ rat intestinal permeability studies has been developed and validated. All the parameters were in the acceptable limits for the bioanalytical methods. The developed method is highly specific, accurate, and precise and involves minimal sample preparation with a short run time making it suitable for routine permeability studies. The proposed method is capable of quantifying both the isomers of CP, enabling to identify the effect of biological enzymes on individual isomers. The method was successfully employed to determine the permeability and stability of CP during in situ absorption studies.

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