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# Liquid Chromatographic Method Development for the Estimation of Cefpodoxime Proxetil and Clavulanic Acid in Combined Dosage Form.

Dimal A Shah*, Piyush A Chaudhary, Usmangani K Chhalotiya, Sunil L Baldania and Kashyap K Bhatt

Indukaka Ipcowala College of Pharmacy, Beyond GIDC, P.B. No. 53, Vitthal Udyognagar- 388 121, Gujarat, India.

Research Article

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*For Correspondence

Indukaka Ipcowala College of Pharmacy, Beyond GIDC, P.B. No. 53, Vitthal Udyognagar- 388 121, Gujarat, India.
Mobile: +91 9824372379

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## ABSTRACT

Liquid chromatographic method was developed for simultaneous quantitative determination of cefpodoxime proxetil and clavulanic acid in their combined dosage form. The separation was achieved by $C_{18}$ (250 mm × 4.6 mm id, 5µm) column using methanol: water [60:40 (v/v)] as mobile phase at a flow rate of 1.5 mL $min^{-1}$ and detection at 225 nm. The retention time for CEF (S epimer) & CEF (R epimer) and CLV were found to be 7 & 8.2 and 1.93 min, respectively. The method was found to be linear in the range of 50-250 µg/ml for CEF and 30-150 µg/ml for CLV. The recovery was in the range of 98.14-99.94 % for CEF and 98.60-99.30 % for CLV. The developed method was statistically validated and found to be simple, precise, reproducible and accurate. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form.

## INTRODUCTION

Cefpodoxime proxetil (CEF) is an orally administered, extended spectrum, semi-synthetic antibiotic of the cephalosporin class. It is chemically 1-(Isopropoxy carbonyloxy) ethyl (6R, 7R)-7-[2-(2-amino-4-thiazolyl)-(Z)-2-(methoxyimino) acetamido]-3-methoxymethyl-3-cephem-4-carboxylate (Fig. 1).

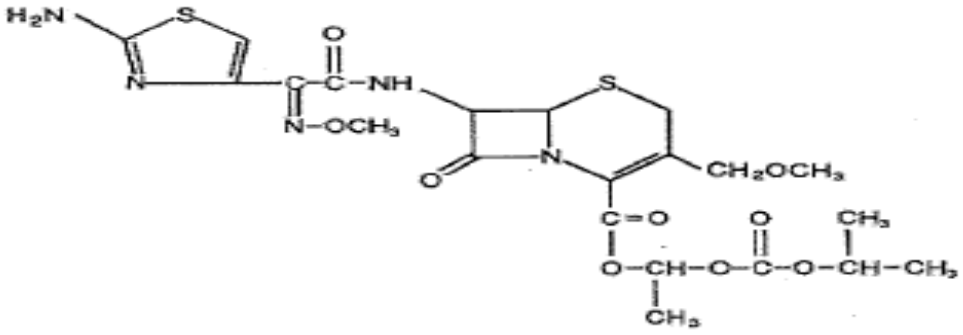

Figure 1: Structure of Cefpodoxime Proxetil

CEF is the prodrug of the bactericidal antibiotic cefpodoxime. The antibacterial action of cefpodoxime is through inhibition of bacterial cell wall synthesis probably by acylation of membrane bound trans peptidase enzymes; this prevents cross linkage of peptidoglycan chains, which is necessary for bacterial cell wall strength [1,2,3].

Clavulanic acid (CLV) is of semi synthetic origin and belongs to beta lactam class. It is chemically (2R,3Z,5R)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo [3.2.0] heptane-2-carboxylic acid (Fig. 2). It belongs to beta lactamase inhibitor class on the basis of mechanism of action. CLV competitively and irreversibly inhibits a wide variety of beta-lactamases, commonly found in microorganisms resistant to penicillins and cephalosporins.

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Binding and irreversibly inhibiting the beta-lactamase results in a restoration of the antimicrobial activity of beta-lactam antibiotics against lactamase-secreting-resistant bacteria. By inactivating beta-lactamase (the bacterial resistance protein), the accompanying penicillin/cephalosporin drugs may be made more potent as well [3,4].

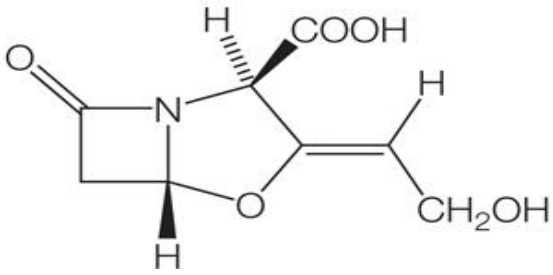


**Figure 2: Structure of Clavulanic acid**

CEF is ineffective in the presence of beta lactamase producing microorganisms. CLV which is a beta lactamase inhibitor protects cefpodoxime from hydrolysis by beta lactamases thus enhances the spectrum of cefpodoxime.

Literature survey revealed that various spectrophotometric [5,6], liquid chromatographic [LC] [7, 8] and HPTLC methods [9,10,11] have been reported for the estimation of CEF in biological fluids and in pharmaceutical dosage form. Liquid chromatographic methods [12,13,14] have been reported for the estimation of CLV in combination with other drugs.

Methods like spectrophotometry [15] and LC [15,16] have been reported for the estimation of combination of CEF and CLV in pharmaceutical dosage forms.

As CEF contains R and S epimers but none of the method has reported estimation of CEF with R and S epimers so, present study involved development of chromatographic methods for estimation of CEF- R ,CEF -S epimer and CLV.

## EXPERIMENTAL

### Reagents and materials

Analytically pure CEF was obtained as gift samples from Torrent Pharmaceutical Ltd., Ahmedabad, India and CLV was procured from Asia Develop Industrial Co. Ltd., China. HPLC grade methanol and water were obtained from SRL Ltd., Mumbai, India. Marketed tablet formulation A (OPOX CV, Hetero Labs. Ltd., India) containing labeled amount of cefpodoxime proxetil equivalent to cefpodoxime 200 mg and potassium clavulanate diluted equivalent to 125 mg of clavulanic acid was procured from local market.

### Instrumentation

The liquid chromatographic system consist of PerkinElmer series 200 LC (Shelton, USA) equipped with a series 200 UV detector, series 200 quaternary gradient pump and manual injector rheodyne valve with 20 µL fixed loop. The analytes were monitored at 225 nm. Chromatographic analysis was performed on Sunfire C18 column having 250 mm× 4.6 mm i.d. and 5 µm particle size. All the drugs and chemicals were weighed on Shimadzu electronic balance (AX 200, Shimadzu Corp., Japan). Mobile phase was degassed by ultrasonic vibrations prior to use. All determinations were performed at ambient temperature.

### Chromatographic conditions

A SunfireC-18 (250×4.6 mm i.d) chromatographic column equilibrated with mobile phase methanol: water (60:40,v/v) was used. Mobile phase flow rate was maintained at 1.5 mL $min^{-1}$ and effluents were monitored at 225 nm. The sample was injected using a 20 µL fixed loop and the total run time was 10 min.

### Preparation of standard stock solution

CEF (25 mg) and CLV (25 mg) were accurately weighed and transferred to two separate 25 ml volumetric flask and dissolved in few ml of methanol. Volumes were made up to the mark with mobile phase to yield a solution containing 1000 µg/ml of CEF and 1000 µg/ml of CLV.

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## Calibration curve for CEF and CLV

Appropriate aliquot of CEF stock solution was taken in different 10 ml volumetric flask. To the same flask different aliquots of stock solution of CLV was added. Volume was made up to the mark with mobile phase to obtain final concentration of 50, 100, 150, 200, 250 μg/ml of CEF and 30, 60, 90, 120, 150 μg/ml of CLV respectively.

## Validation

The method was validated for accuracy, precision, linearity, detection limit, quantitation limit and robustness.

*Linearity*

Linearity of the method was evaluated by constructing calibration curves at five concentration levels over a range of 50-250 μg/ml for CEF and 30-150 μg/ml for CLV. The calibration curves were developed by plotting peak area versus concentration (n = 5). The regression equations were constructed for both the drugs.

*Accuracy*

The accuracy of the method was determined by calculating recoveries of CEF and CLV by method of standard additions. Known amount of CEF (50%, 100%, and 150%) and CLV (50%, 100%, and 150%) were added to a pre quantified sample solution, and the amount of CEF and CLV were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

*Precision*

The intra-day and inter-day precision studies were carried out by estimating the corresponding responses 3 times on the same day and on 3 different days for three different concentrations of CEF (50, 150, 250 μg/ml) and CLV (30, 90, 150 μg/ml), and the results are reported in terms of relative standard deviation. The instrumental precision studies were carried out by estimating response of CEF (150 μg/ml) and CLV (90 μg/ml) for six times and results are reported in terms of relative standard deviation.

*LOD and LOQ*

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines.

$$LOD = 3.3 \times \sigma / S; \quad LOQ = 10 \times \sigma / S;$$

Where $\sigma$ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

*Specificity*

The specificity of the method was ascertained by analyzing CEF and CLV in presence of excipients like cellulose microcrystalline, magnesium stearate, sorbitol, talc, titanium dioxide,polyethylene glycols were used for tablet formulations. The peak of CEF and CLV were confirmed by comparing retention time of sample with those of standards.

*Robustness*

Small changes in the flow rate and the ratio of mobile phase were carried out and effects on the results were examined. Robustness of the method was determined in triplicate at a concentration level of 150 μg /ml of CEF and 90 μg/ml of CLV respectively. The mean and % RSD of peak areas were calculated.

*Solution stability*

Stability of sample solutions were studied at 25 ± 2°C for 24 Hrs.

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p-ISSN:2347-2340

### Analysis of marketed formulation

Twenty tablets were weighed accurately and finely powdered. Tablet powder equivalent to200 mg CEF (96mg of CLV) was taken in 100 ml volumetric flask Methanol (50 ml) was added to the above flask and the flask was sonicated for 15 minutes. The solution was filtered using 0.45 μm whatman filter paper in another 100 ml volumetric flask and volume was made up to the mark with the methanol.

Appropriate volume of the aliquot was transferred to a 10 ml volumetric flask and the volume was made up to the mark with the mobile phase to obtain a solution containing 100 μg/ml of CEF (48 μg/ml of CLV).The solution was injected using HPLC loop system and analyzed for CEF and CLV content using the proposed method.

## RESULTS AND DISCUSSION

### Optimization of the mobile phase

The objective of the method development was to resolve chromatographic peaks for active drug ingredients.

Various mixtures containing methanol, water and acetonitrile were tried as mobile phases in the initial stage of method development. Mixture of methanol: water (80:20, v/v), methanol: water (70:30, v/v), methanol: water (50: 50, v/v) and methanol: acetonitrile: water (80:20:20, v/v/v) were tried as mobile phase but satisfactory resolution of drug and peaks were not achieved.

The mobile phase methanol: water (60:40, v/v) was found to be satisfactory and gave three symmetric and well-resolved peaks for CEF and CLV. (Fig. 3) The retention time for CEF (S epimer) & CEF (R epimer) and CLV were 7 & 8.2 min and 1.93 min, respectively. The resolution between CEF (R & S epimer) and CEF & CLV was found to be 2.93 and 11, which indicates good separation of both the compounds. The asymmetric factors for CEF (R epimer) & CEF (S epimer) and CLV were 1.33& 0.88 and 1.02; respectively. The mobile phase flow rate was maintained at 1.5 ml $min^{-1}$.

Overlay UV spectra of both the drugs showed that CEF and CLV absorbed appreciably at 225 nm, so detection was carried out at 225 nm.

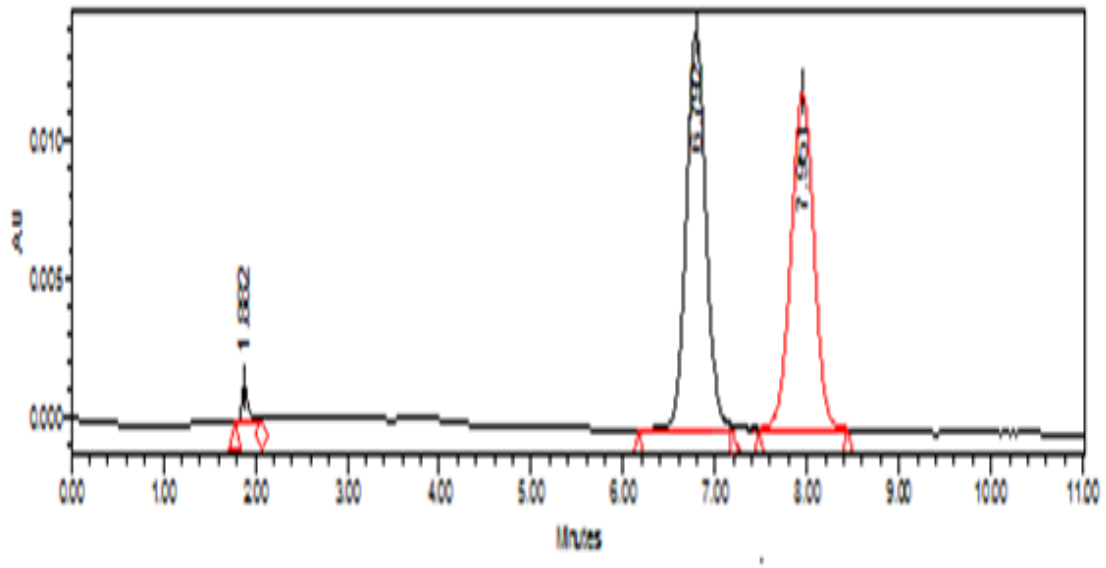


**Figure 3: Chromatogram of CEF and CLV (100 μg/ml and 60 μg/ml respectively) using mobile phase methanol: water (60:40, v/v) by RP-HPLC method.**

### Validation

*Linearity*

The calibration curve for CEF was found to be linear in the range of 50-250 μg/ml with a correlation coefficient of 0.998. The calibration curve for CLV was found to be linear in the range of 30-150 μg/ml with a correlation coefficient of 0.997. The calibration curve for CEF was plotted by making summation of peak area for S epimer & R epimer. The regression analysis of calibration curves are reported in table 1.

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Table 1: Regression analysis of calibration curve

| Parameter | CEF | CLV |
|---|---|---|
| Linearity | 50-250 µg/ml | 30-150µg/ml |
| Correlation coefficient (r) | 0.998 | 0.997 |
| Slope of Regression | 22157 | 197.71 |
| Standard deviation of slope | 267.17 | 3.13 |
| Intercept of Regression | 35914 | 130.44 |

*Precision*

Instrument precision was determined by performing injection repeatability test and the % RSD values for CEF and CLV were found to be 0.54 and 0.86 respectively. The intra-day and inter-day precision studies were carried out and the results are reported in table 2.

Table 2: Summary of Validation Parameters of HPLC

| Parameters | CEF | CLV |
|---|---|---|
| Range | 50-250(µg/ml) | 30-150 (µg/ml) |
| Retention time (min) | 7 & 8.2 | 1.93 |
| Tailing factor | 1.33&0.88 | 1.02 |
| Resolution | 2.93& 11 | |
| Theoretical Plates | 2280.55 | 1947.06 |
| Detection limit (µg/ml) | 0.54 | 7.27 |
| Quantitationlimit (µg/ml) | 1.6 | 24 |
| Accuracy(%) | 98.14 – 99.94 | 98.60-99.30 |
| Precision (%RSD) | | |
| Intra-day (n=3) | 0.57-1.17 | 0.75-1.05 |
| Inter-day (n=3) | 0.72-1.57 | 1.34-1.45 |
| Instrument precision (%RSD) | 0.54 | 0.86 |
| Specificity | Specific | Specific |

*Accuracy*

The accuracy of the method was determined by calculating recoveries of CEF and CLV by method of standard addition. The recoveries found to be 98.14 – 99.94 % and 98.60–99.30 % for CEF and CLV respectively. The high values indicate that the method is accurate (Table 2).

*Limit of detection and limit of quantification*

The detection limits for CEF and CLV were 0.54 µg/ml and 7.27 µg/ml, respectively, while quantitation limits were 1.63 µg/ml and 24 µg/ml respectively. The above data shows that a microgram quantity of both the drugs can be accurately and precisely determined.

*Specificity*

The specificity study was carried out to check the interference from the excipients used like microcrystalline cellulose, talc, magnesium stearate in the formulations by preparing synthetic mixture containing both the drugs and excipients. The chromatogram showed peaks for both the drugs without any interfering peak and the recoveries of both the drugs were above 98%.

*Robustness*

Robustness of the method was studied by changing the flow rate of the mobile phase from 1.5 mL $min^{-1}$ to 1.4 mL $min^{-1}$ and 1.6 mL $min^{-1}$. Using 1.4 mL $min^{-1}$ flow rate, retention time for CEF (R & S epimers) and CLV were observed to be 7.2 & 8.53 min and 2.01 respectively and with 1.6mL $min^{-1}$ flow rate, retention time for CEF (R & S epimers) and CLV were found to be 6.91 & 8.14 and 1.86 min respectively without affecting resolution of the drug. When a mobile phase composition was changed to methanol: water (70:30, v/v) by increasing percentage of water the retention time of CEF (R & S epimers) and CLV were observed to be 5.01 & 5.42 and 2.78 min respectively.When a mobile phase composition was changed methanol: water (50:50, v/v) by decreasing percentage of water the retention time of CEF (R & S epimers)and CLV were observed to be 7.6 & 8.4 min and 2.78 min respectively The assay result of both the drug was found to be more than 98% (Table 3).

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p-ISSN:2347-2340

Table 3: Robustness results of CEF and CLV in given formulations

| Parameter | Method condition | $R_t$ | | % RSD of peak area | |
|---|---|---|---|---|---|
| | | CEF | CLV | CEF | CLV |
| Flow rate | 1.4 ml/min | 7.2 & 8.53 | 2.01 | 0.48 | 1.18 |
| | 1.6 ml/min | 6.91 & 7.94 | 1.86 | 0.38 | 0.86 |
| Mobile phase ratio Methanol : Water | 70: 30 | 5.01 & 5.42 | 2.08 | 1.26 | 1.03 |
| | 50: 50 | 7.6 & 8.4 | 2.78 | 1.49 | 1.44 |

*Solution stability*

Stability of standard and sample solution of CEF and CLV were evaluated at room temperature for 24 hr. Both the drugs were found to be stable with a recovery of more than 98%.

### Analysis of marketed formulation

Marketed formulation was analyzed using proposed method which gave percentage recovery for CEF and CLV were more than 98% respectively for OPOX CV TAB. (Table 4).

Table 4: Analysis of marketed formulation

| Formulation | Labeled Amount of drug (mg) | | Amount of drug found (mg) | | % of drug found ± RSD (n = 3) | |
|---|---|---|---|---|---|---|
| | cefpodoxime | CLV | cefpodoxime | CLV | cefpodoxime | CLV |
| OPOX CV* | 200 | 125 | 199.45 | 124.13 | 99.58 ± 0.57 | 98.96 ± 0.34 |

*Each film coated tablet contains cefpodoxime proxetil equivalent to cefpodoxime 200 mg and potassium clavulanate diluted equivalent to 125 mg of clavulanic acid.

## CONCLUSION

Liquid chromatographic method has been developed for estimation of CEF and CLV in combined dosage form. RP-HPLC method was developed using mobile phase methanol: water (60:40, v/v) and stationary phase $C_{18}$. The method was found to be linear in the range of 50-250 µg/ml for CEF and 30-150 µg/ml for CLV. The recovery was in the range of 98.14-99.94 % for CEF and 98.60-99.30 % for CLV. The method was found to be accurate, precise, selective, specific, repeatable and reproducible. Limit of quantification for CEF and CLV was found to be 1.6 µg ml and 24 µg/ml respectively. Compared to reported HPLC [15-16] methods, the developed HPLC method is sensitive and contains simple mobile phase without buffer. In developed method, peak for CEF (S & R epimer) is well resolved and used for estimation of drug. The developed method was successfully applied for the estimation of both the drug from combined pharmaceutical dosage form.

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