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***ORIGINAL RESEARCH ARTICLE***

# Development and Validation of RP-HPLC Method For Simultaneous Estimation of Cefixime and Cloxacillin in Tablet Dosage Form

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## ABSTRACT

A rapid, sensitive and specific RP-HPLC method involving U.V detection was developed and validated for the estimation of Cefixime and Cloxacillin in tablet dosage form. The method was validated in terms of linearity, accuracy, precision, specificity, robustness, limit of detection and limit of quantitation. The mobile phase used acetonitrile: tetra-butyl ammonium hydroxide buffer in the ratio of 45:55 and pH adjusted to 4 with orthophosphoric acid. The detection of combined dosage form was carried out at 225 nm at constant flow rate of 1ml/min. Hydrochlorothiazide was used as internal standard. The retention time of Cefixime, Cloxacillin and hydrochlorothiazide were found 5.75 min, 11.90 min and 3.74 min respectively. Linearity was observed in 10-50 µg/ml for Cefixime ($r^2$ = 0.9994) and 25–125 µg/ml for Cloxacillin ($r^2$ = 0.9998). Detection limit for Cefixime and Cloxacillin is 0.05µg/ml and 0.18 µg/ml respectively and quantification limit for Cefixime and Cloxacillin is 0.15µg/ml and 0.11 µg/ml. The proposed method was successfully applied for the quantitative determination of Cefixime and Cloxacillin in tablet dosage form.

**Keywords:** Cefixime, Cloxacillin, Hydrochlorothiazide, RP-HPLC method.

## INTRODUCTION

Cefixime ($C_{16}H_{15}N_5O_7S_2$, $3H_2O$), chemically (6R, 7R)-7-[[(Z)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy) imino] acetyl] amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid trihydrate, is a cephalosporin antibiotic. [1-3] Cloxacillin ($C_{19}H_{17}ClN_3NaO_5S$, $H_2O$), chemically Sodium (6R)-6-[3-(2-chorophenyl)-5- methylisoxazol-4-carboxamido] penicillariate monohydrate, is a Beta- lactum antibiotic. [4] Previous literature survey revealed that, few HPLC methods were reported for the estimation of Cefixime and Cloxacillin individually [7-13]. But, there was no any method reported till date for both drugs in combination. Hence, the present study is to attempt and develop accurate, simple and sensitive method for simultaneous estimation of Cefexime and Cloxacillin in tablet dosage form.

## MATERIALS AND METHODS

***Instruments:*** HPLC system with intelligent HPLC pump (JASCO PU-2080 plus), Rheodyne injector with injection volume 20 µl, HiQ sil C-8 (4.6*250 mm, internal diameter 5 µm) column, U.V Spectrophotometer (Shimadzu-1700), pH meter (Metrohm) etc.

***Chemicals and reagents:***

Reference standard of Cefixime and Cloxacillin were obtained from Maxheal Pharmaceutical Pvt. Ltd., Nashik. Tablets of three different brands, T1 (Cefi–XL-200), T2 (Xcept-200) and T3 (Mahacef-200) having combination of Cefixime (200 mg) and Cloxacillin (500 mg) were used. HPLC grade acetonitrile from Merck Specialties Pvt. Ltd., Mumbai, Tetrabutylammonium hydroxide and orthophosphoric acid from S. d. Fine Chemicals, Mumbai, All other chemicals and reagents used were of AR grade.

***Preparation of mobile phase:***

Mobile phase were prepared by mixing of 450 ml of acetonitrile with 550 ml of tetrabutylammonium hydroxide buffer, whose pH

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was previously adjusted to pH 4 by addition of orthophosphoric acid. The mobile phase prepared was degassed by ultrasonication for 20 min, so as to avoid the disturbances caused by dissolved gases. The degassed mobile phase was filtered through 0.45µ filter to avoid the column clogging due to smaller particles.

***Preparation of standard stock solutions:***

25 mg of Cefixime and 25 mg of Cloxacillin weighed separately and dissolved in 10 ml of mobile phase and volume was made up to 25 ml so as to get the concentration 1 mg/ml. From this standard stock solution, appropriate dilution was made using mobile phase to get combined standard solution containing two drugs in the ratio of 1:2.5. Final concentration of solution was prepared as 100 µg/ml of Cefixime and 250 µg/ml of Cloxacillin. Hydrochlorothiazide was used as internal standard having 70 µg/ml concentrations in each solution. All solutions were shown to be stable during the period of study.

***Sample preparation for injection:***

20 tablets, each containing 200 mg Cefixime and 500 mg Cloxacillin were weighed and crushed to fine powder and quantity of powder equivalent to 25 mg Cefexime and 62.5 mg Cloxacillin weighed and transferred to 25 ml volumetric flask. Mobile phase was added to same flask and shaken for 20 min. The volume was made up to 25 ml with mobile phase and filtered. The concentration of filtrate obtained was 1000 µg/ml of Cefixime and 2500 µg/ml of Cloxacillin. From this solution appropriate dilutions were made so as to obtain 50 µg/ml of Cefixime and 125 µg/ml of Cloxacillin as final concentrations and injected into the system to get the chromatogram. Before making of the volume of dilution, internal standard, hydrochlorothiazide was added to get 70 µg/ml of concentration in final solutions and this solution was used for estimation.

***Chromatographic conditions:***

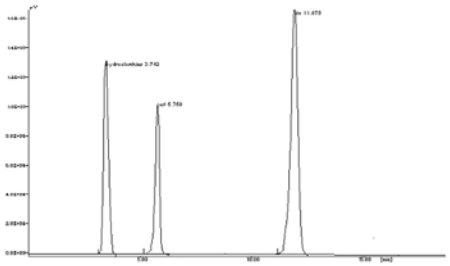
A mobile phase consisting of acetonitrile: 0.1M tetrabutylammonium hydroxide buffer whose pH was previously adjusted to 4 with orthophosphoric acid (45:55 v/v) were found ideal to resolve the peaks of Cefixime and Cloxacillin. The detection of combined dosage form was carried out at 225 nm with constant flow rate of 1 ml/min at ambient column temperature of HiQ Sil C8 column.

**METHOD VALIDATION PARAMETERS:**

The method was validated for linearity, accuracy, precision, specificity, robustness, limit of detection and limit of quantitation by the following procedure,

1. ***Linearity:*** Suitable dilutions using mobile phase were made from the standard stock solutions containing 1000 µg/ml of Cefixime and 1250 µg/ml of Cloxacillin to prepare range of standard solutions of five different concentrations of analyte for further experimental work. In each dilution hydrochlorothiazide of 70 µg/ml concentration was used as internal standard. Five replicates of each concentration were injected. Chromatograms were recorded. The peak area was plotted against concentration to get calibration curve. The plots of peak area Vs respective concentration of Cefixime and Cloxacillin were found to be linear in range of 10-50 µg/ml and 25-125 µg/ml with coefficient of correlation ($r^2$) 0.9994 and 0.9998 for Cefixime and Cloxacillin respectively as shown in ***Fig.1***.

**Fig 1: Typical chromatograms of a mixture of standard Cefixime and Cloxacillin.**



2. ***Accuracy:***To check the accuracy of proposed method, level of recovery carried out at 80, 100 and 120 % of concentration as per standard addition method. To perform recovery studies of the test concentration, a powder of pre-analyzed tablet sample containing 200 mg of Cefixime and 500 mg of Cloxacillin was weighed such that it should contain 50 mg of Cefixime and 125 mg of Cloxacillin, then transferred into 100 ml volumetric flask, add about 50 ml of mobile phase and sonicated for 20 min. with intermediate shaking and volume make up to the mark. 50 µg/ml of Cefixime and 125µg/ml Cloxacillin of pure drugs were used as standard concentrations. The recovery studies were carried out five times, at each level of recovery. The results of studies along with its evaluation as shown in **Table 1.**

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**Table-1: Statistical data of recovery study.**

| Tablet Formulation | Level of % recovery | (%) Mean* | | SD | | SEM | |
|---|---|---|---|---|---|---|---|
| | | Cefixime | Cloxacillin | Cefixime | Cloxacillin | Cefixime | Cloxacillin |
| T1 | 80 | 99.56 | 99.46 | 0.1929 | 0.5474 | 0.1114 | 0.3161 |
| | 100 | 99.61 | 99.82 | 0.1858 | 0.0611 | 0.1073 | 0.03528 |
| | 120 | 100.03 | 99.98 | 0.4065 | 0.1172 | 0.1200 | 0.0677 |
| T2 | 80 | 100.24 | 99.962 | 0.2564 | 0.9112 | 0.1425 | 0.5101 |
| | 100 | 99.95 | 100.34 | 0.6447 | 0.5684 | 0.3841 | 0.2545 |
| | 120 | 99.89 | 99.78 | 0.2356 | 0.7111 | 0.0989 | 0.3152 |

***Mean of five determination readings (n=5), T1 and T2 are two different brands of tablet formulations.***

3.***Precision*:** One set of three different concentrations of combined working standard solution of Cefixime and Cloxacillin were prepared. All the solutions were analyzed thrice, in order to record any intra-day variation in the result. The result obtained for intra-day variations are shown in the **Table 2.** For inter-day variation study, three different concentrations of the combined standards were analyzed for three days. The result obtained for inter-day variations are shown in the **Table 3**.

**Table-2: Intra day precision.**

| Parameters | Cefixime (µg/ml) | | | Cloxacillin (µg/ml) | | |
|---|---|---|---|---|---|---|
| | 10 | 20 | 30 | 25 | 50 | 75 |
| MPA* | 421347.2 | 790582.3 | 1184277.2 | 931465.1 | 1822685.0 | 2713908.2 |
| SD | 142.76 | 357.97 | 15.23 | 233.54 | 283.50 | 537.60 |
| % RSD | 0.03 | 0.05 | 0.01 | 0.03 | 0.02 | 0.03 |

**MPA-indicates mean peak area of three peaks, SD: Standard Deviation, RSD: Relative Standard Deviation*

**Table-3: Inter day precision.**

| Parameters | Cefixime (µg/ml) | | | Cloxacillin (µg/ml) | | |
|---|---|---|---|---|---|---|
| | 30 | 40 | 50 | 75 | 100 | 125 |
| MPA* | 1184773.5 | 1580952.1 | 1925604.4 | 2713472.8 | 3605497.9 | 449627.2 |
| SD | 127.07 | 335.24 | 451.87 | 394.74 | 360.35 | 367.2 |
| % RSD | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.03 |

**MPA-indicates mean peak area of three peaks, SD: Standard Deviation, RSD: Relative Standard Deviation*

4. **Specificity:**The specificity of the RP-HPLC method was determined by complete separation of Cefixime and Cloxacillin with parameters like retention time, resolution and tailing factor (T). The peaks obtained for Cefixime and Cloxacillin were sharp and have clear baseline separation. Here tailing factor for peaks of Cefixime and Cloxacillin was less than 1.5 and resolution was satisfactory. The average retention time ± standard deviation for Cefixime and Cloxacillin were found to be 5.70±0.0428 min and 11.91±0.098 min, respectively for five replicates. The peaks obtained for Cefixime and Cloxacillin were sharp and have clear baseline separation.
5. **Limit of Detection (LOD) and Limit of Quantitation (LOQ):** LOD is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. LOQ is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. The LOD and LOQ were separately determined based on the standard calibration curve. LOD = 3.3×D/S and LOQ = 10×D/S, where D is standard deviation of y-intercepts of regression line and S is the slope of the calibration curve. The validation parameters are given in ***Table- 4***.

**Table 4: Validation and system suitability parameters.**

| Parameters | Cefixime | Cloxacillin |
|---|---|---|
| Retention time (Min.) | 5.75±0.02 | 11.93±0.03 |
| Width | 0.0871±0.0014 | 0.0763±0.0018 |
| Area (µV.sec) | 423066.4±514 | 931235.67±656 |
| Plates | 4886.685±251 | 5511.62±325 |
| Linearity (µg/ml) | 10 – 50 | 25 – 125 |
| Resolution | - | 6.334±0.115 |
| Asymmetry | 1.013±0.011 | 1.291±0.01 |
| Regression equation | y = 38560x + 2028 | y = 35881x + 1907 |
| Correlation Coefficient | 0.9994 | 0.9998 |
| Percent recovery | 99.0 – 100.5 | 99.0 - 100.79 |
| Precision (%RSD) | < 2 | < 2 |
| LOD (µg/ml) | 0.04653 | 0.06074 |
| LOQ (µg/ml) | 0.1437 | 0.18407 |



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6. **Robustness:** This was done by small changing in the chromatographic conditions and found to be unaffected by small changing like ± 0.1 change in pH and ± 2% change in volume of organic solution of mobile phase.

**Statistical Treatment of Analytical Data Using ANOVA Test:**

The proposed method for simultaneous estimation of Cefixime and Cloxacillin was found to be simple, precise, accurate, specific and sensitive in analysis of three marketed formulations. To check the statistical significance in this method, one way one-way analysis of variance (ANOVA) test was applied. Statistical evaluation of Cefixime and Cloxacillin tablets and also with ANOVA test as given in **Table-5 & 6**. For cefixime, F = 0.3228 = (MS treatment/ MS residual). The table value of F = 1.173 and calculated F value = 0.3228. So Calculated value < Table value and for Cloxacillin, F = 0.1172= (MS treatment/ MS residual). The table value of F = 1.173 and calculated F value = 0.172. So Calculated value < Table value. Hence, it is concluded that there is no significant difference between all brands.

**Table-5: Statistical evaluation of Cefixime and Cloxacillin tablets.**

| Tablet formulations | No. of points | % Mean | SD | SEM | 95% confidence interval From | To |
|---|---|---|---|---|---|---|
| **Cefixime** | | | | | | |
| T1 | 5 | 100.26 | 0.1498 | 0.06698 | 99.84 | 100.21 |
| T2 | 5 | 100.02 | 0.1663 | 0.07439 | 99.82 | 100.23 |
| T3 | 5 | 99.96 | 0.1069 | 0.0468 | 99.83 | 100.10 |
| **Cloxacillin** | | | | | | |
| T1 | 5 | 99.98 | 0.945 | 0.0422 | 99.86 | 100.10 |
| T2 | 5 | 100.01 | 0.0756 | 0.0338 | 99.91 | 100.11 |
| T3 | 5 | 99.94 | 0.0469 | 0.0209 | 99.88 | 99.99 |

*SD: Standard Deviation, SEM: Standard Error Mean*

## RESULT AND DISCUSSION:

The proposed method describes a new RP-HPLC method for the determination of Cefixime and Cloxacillin in combined tablet dosage form (Zenflox-NT) employing JASCO PU-2080 plus HPLC system, UV–2075 plus Intelligent UV/VIS detector, HiQ sil C-8 (4.6×250mm) column and mobile phase comprising of acetonitrile:tetrabutylammonium hydroxide buffer (45:55 v/v) and its pH adjusted to 4 with orthophosphoric acid was found to be satisfactory and gave two symmetrical and well resolved peaks for Cefixime and Cloxacillin. The resolution between Cefixime and Cloxacillin was found to be 6.3, which indicate good separation for both the compounds. The retention time for Cefixime and Cloxacillin were 5.75±0.02 min and 11.93±0.03 min respectively. Flow rate kept at 1.0 ml/min and UV detection performed at 225 nm.

**Table-6: Statistical evaluation of Cefixime and Cloxacillin by ANOVA test.**

| Source of variation | Degree of freedom | Sum of squares | Mean square |
|---|---|---|---|
| **Cefixime** | | | |
| Treatments (between columns) | 2 | 0.01324 | 0.006620 |
| Residual (within columns) | 12 | 0.2461 | 0.02051 |
| Total | 14 | 0.2594 | --- |
| **Cloxacillin** | | | |
| Treatments (between columns) | 2 | 0.01317 | 0.006587 |
| Residual (within columns) | 12 | 0.06740 | 0.056171 |
| Total | 14 | 0.08057 | --- |

The method was validated as per ICH guidelines. Linearity for detector response was observed in 10 – 50 µg/ml for Cefixime and 25 – 125 µg/ml for Cloxacillin and found to be linear with $r^2$ = 0.9994 and 0.9998 for Cefexime and Cloxacillin, respectively. Percent recovery for both Cefixime and Cloxacillin was found in range and 99.05 – 100.79 % and 99.02 – 100.55 %, respectively indicating accuracy of the proposed method. The percent RSD for both the tablet analysis and recovery studies is less than 2% indicating high degree of precision. The detection LOD for Cefixime and Cloxacillin were 0.04653µg/ml and 0.06074µg/ml, respectively. LOQ for Cefixime and Cloxacillin were 0.1437µg/ml and 0.18407µg/ml. The LOD and LOQ showed that the method is sensitive for Cefixime and Cloxacillin. The results of robustness study also indicate that the method is robust and is unaffected by small variation in chromatographic conditions. It was observed that excipients present in formulation did not interfere with peaks of Cefixime and Cloxacillin. Statistical analysis of the method was done by using one way analysis of variance (ANOVA). Hence, results of our study

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suggest that, there is no significant difference between three different analyzed brands of Cefixime and Cloxacillin in combined tablet dosage form. The method was found to be simple, specific, accurate, precise and reproducible.

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