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HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ENMETAZOBACTAM AND CEFEPIME

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ABSTRACT

The present study aimed to develop and validate a simple, precise, accurate, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Enmetazobactam (EMB) and Cefepime (CFP) in bulk and pharmaceutical formulations. Chromatographic separation was achieved using a C18 Hypersil BDS column with an optimized mobile phase, resulting in sharp and well-resolved peaks for both analytes. The method exhibited excellent linearity within the concentration ranges of 5–25 µg/mL for EMB and 10–50 µg/mL for CFP, with correlation coefficients (r^2) of 0.9987 and 0.9990, respectively. Accuracy studies revealed mean recoveries close to 100%, confirming the method's reliability. Precision results showed %RSD values within acceptable limits for repeatability, intra-day, and inter-day analyses, while robustness tests indicated that minor variations in analytical conditions did not significantly affect the results. The method also demonstrated high sensitivity, with low LOD and LOQ values for both drugs. Assay of the marketed formulation showed drug content of 99.716% for EMB and 99.271% for CFP, indicating suitability for routine quality control. The validated method fulfills ICH Q2(R1) guidelines and is suitable for routine analysis, stability testing, and quality assessment of EMB and CFP in combined pharmaceutical dosage forms.

Introduction

Cefepime, a fourth-generation cephalosporin, is widely used for the treatment of severe infections caused by Gram-negative and selected Gram-positive bacteria. Its broad-spectrum bactericidal activity and stability against many β-lactamases make it a key therapeutic agent in hospital settings, particularly for complicated urinary tract infections, pneumonia, and sepsis (1,2). However, the global rise of extended-spectrum β-lactamase (ESBL)-producing Enterobacterales has significantly reduced the clinical efficacy of many β-lactam antibiotics, including cefepime. To address this resistance challenge, enmetazobactam, a novel sulfone-based β-lactamase inhibitor structurally related to tazobactam, has been developed to restore β-lactam activity by

inhibiting ESBL enzymes, specifically class A β-lactamases (3,4). The fixed-dose cefepime–enmetazobactam combination has demonstrated improved antibacterial efficacy in Phase III clinical trials and has gained regulatory interest for the management of multidrug-resistant infections (5,6).

With the increasing clinical adoption of this novel combination, the development of reliable analytical methods for its simultaneous estimation in bulk drug and pharmaceutical formulations is essential. High-performance liquid chromatography (HPLC) remains the most preferred analytical tool due to its high resolution, sensitivity, reproducibility, and ability to separate structurally related β-lactam compounds and their degradation products (7). Although analytical methods have been reported individually for cefepime or for cefepime combined with other β-lactamase inhibitors, comprehensive, stability-indicating RP-HPLC methods specifically designed for the cefepime–enmetazobactam combination are still limited (8,9). A robust and validated HPLC method is therefore necessary to ensure accurate quantification, quality control, and regulatory compliance for this emerging combination therapy.

Analytical method validation is a important step to ensure the reliability and consistency of results. Regulatory guidelines, particularly those outlined in the International Council for Harmonisation (ICH) Q2(R1), emphasize key validation parameters, including specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability (10). Given the susceptibility of β-lactam antibiotics to hydrolysis and thermal degradation, the development of a stability-indicating method capable of distinguishing active pharmaceutical ingredients from their degradation products is vital for both manufacturing and stability studies (11).

Therefore, the present study aims to develop and validate a simple, precise, accurate, and stability-indicating RP-HPLC method for the simultaneous estimation of enmetazobactam and cefepime in bulk and pharmaceutical formulations, in accordance with ICH guidelines. This method is expected to support routine quality control, stability assessments, and future formulation development involving this novel antibacterial combination.

Material and Methods

Material

The analytical standards of Enmetazobactam (EMB) and Cefepime (CFP) were obtained as gift samples from a pharmaceutical company. The marketed formulation containing 2 g of cefepime and 500 mg of enmetazobactam as a dry injection, was used as the sample for analysis. HPLC-grade methanol, water, and acetonitrile required for the chromatographic studies were procured from Merck Specialties Pvt. Ltd., Mumbai, and used throughout the experimental work.

Instruments

The HPLC analysis was performed using a Waters HPLC system equipped with a 515 pump and fitted with a C18 Hypersil BDS column (25 cm × 4.6 mm) supplied by Agilent Technologies. An Ultrasonic Fast Clean water bath and an Electroquip digital pH meter were used for sample preparation and pH adjustments. Weighing operations were carried out using a Shimadzu AUX-200 analytical balance. UV analysis was conducted using a Labindia 3000 Plus UV–Visible spectrophotometer with quartz cuvettes (Shimadzu Corporation, Kyoto, Japan), and the same Shimadzu analytical balance was used for accurate weighing during UV measurements.

Methods

Selection of Mobile Phase

Initially to estimate Enmetazobactam and Cefepime in fix dosage form number of mobile phase in different ratio were tried. Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was 10mM NaH_2PO_4: Methanol in the ratio of 10:90v/v. The mobile phase was filtered through 0.45µ filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min.

Selection of separation variable

Table 1: Separation Variable

Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5µ
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase	

10mM NaH_2PO_4	10
Methanol	90
Diluent	Methanol
Flow rate	1.0 ml/min
Temperature	Ambient
Sample Size	20 µl
Detection wavelength	250nm
Retention time	
Enmetazobactam	3.445 ± 0.3min.
Cefepime	6.152 ± 0.3 min.

Preparation of Stock Solution:

Accurately weighed 10 mg API of EMB and CFP was transferred into 10 ml volumetric flask separately and added 5ml of methanol as diluents, sonicated for 20 minutes and volume was made up to 10ml with methanol to get concentration of solution 1000µg/ml (Stock-A)

Preparation of Sub Stock Solution:

5 ml of solution was taken from stock-A of both the drug and transferred into 50ml volumetric flask separately and diluted up to 50 ml with diluent (methanol) to give concentration of 100µg/ml of EMB and CFP respectively (Stock-B).

Preparation of Different Solution

1ml, 2ml, 3ml, 4ml and 5ml of stock-B were taken separately in 10 ml volumetric flask and volume was made up to 10ml with (methanol). This gives the solutions of 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml and 50µg/ml, for CFP. In same manner 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml EMB of also prepared.

Linearity and Calibration Graph

To establish the linearity of analytical method, a series of dilution ranging from 5-25 µg/ml for CFP and 10-50µg/ml for EMB were prepared. All the solution were filtered through 0.45µm membrane filter and injected, chromatograms were recorded and it was repeat for five times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

System Suitability Parameters

Separation variables were set and mobile phase was allowed to saturate the column at 1.00 ml/min. After complete saturation of column, six replicates of working standard of EMB 1µg/ml for EMB and 10µg/ml CFP was injected separately. Peak report and

column performance report were recorded for all chromatogram.

Validation of developed Method

The method was validated for the parameters reported below.

Linearity

Linearity of analytical procedure is its ability (within a given range) to obtain test which are directly proportional to area of analyte in the sample. The calibration plot was contracted after analysis of five different concentrations (from 5 to 25 µg/ ml for EMB) and (5 to 25µg/ ml for (CFP) and areas for each concentration were recorded three times and mean area was calculated. The response ratio (response factor) was found by dividing the AUC with respective concentration.

Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present such as impurities, degradation products and matrix components.

Accuracy

Recovery studies were performed to calculate the accuracy of developed method to preanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed.

Precision

Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range 1, 2, 3, 4 and 5µg/ml for EMB and 5, 10, 15, 20 and 25µg/ml for CFP indicates the precision under the same operating condition over short interval time.

Intermediate Precision

Intermediate precision was also performed within laboratory variation on different days and different analyst in five replicate at five concentrations. Results of day to day intermediate precision for EMB and CFP reported in table.

Robustness

As per ICH norms, small but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was change from, 10mM NaH_2PO_4: Methanol (10:90 % v/v) to (15:85 % v/v).

Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

Analysis of both the drug in Injectable Sample

The dry injectable formulation were grinded to fine powder, an accurately weighed quantity of powder equivalent to 20 mg of CPF was transferred to 10 ml volumetric flask containing methanol. The solution was sonicated for 25 min and the final volume was made with mobile phase. The mixture was then filtered through a 0.45 µm filter. The stock solution was further diluted sufficiently with methanol to get sample solution of drug concentration of 20µg/mL CFP and 5µg/mL EMB respectively. The amounts of EMB and CFP in injectable s formulation were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with formulation.

Results and Discussion

The primary objective of this study was to develop and validate a simple, precise, and reliable RP-HPLC method for the simultaneous estimation of Enmetazobactam (EMB) and Cefepime (CFP) in bulk and pharmaceutical formulations. The chromatograms obtained for EMB (Figure 1), CFP (Figure 2), and their combined analysis (Figure 3) demonstrated sharp, symmetric, and well-resolved peaks, indicating excellent selectivity of the developed method toward both analytes. The absence of interfering peaks at the respective retention times confirmed the specificity of the method.

The linearity study revealed a strong correlation between peak area and concentration for both drugs across the selected ranges, with correlation coefficients ($\mathbf{r^2}$) of 0.9987 for EMB and 0.9990 for CFP (Table 2). These values exceed the minimum acceptance criteria (>0.999 is ideal; >0.995 acceptable), confirming excellent linearity and proportional response of the method. The slopes and intercepts further established the method's suitability for quantitative analysis.

Accuracy, evaluated through recovery studies at 80%, 100%, and 120%, showed mean recoveries close to 100% for both analytes, with low standard deviations (Table 3). This confirms that the method can accurately estimate the true amount of both drugs in the matrix without interference from excipients or solvents.

Precision results (%RSD) for repeatability, day-to-day, and analyst-to-analyst variability were all within acceptable limits (<2%), demonstrating that the method is highly precise and reproducible (Table 4). Robustness testing showed minor variations without significantly affecting results, indicating that the method can withstand

small deliberate changes in chromatographic conditions.

Sensitivity of the method was reflected in the low LOD and LOQ values 0.25 µg/ml (LOD) and 0.75 µg/ml (LOQ) for EMB, and 0.50 µg/ml (LOD) and 1.50 µg/ml (LOQ) for CFP (Table 5). These values confirm the method's suitability for detecting and quantifying very low concentrations, which is essential for stability and degradation studies.

The assay results for the marketed formulation showed drug content of 99.716% for EMB and 99.271% for CFP, with %RSD values well below 1% (Table 6), demonstrating the method's applicability for routine quality control. The results are in excellent agreement with the label claim, reflecting the accuracy and reliability of the developed method.

The combination of sharp peak resolution, excellent linearity, high accuracy, precision, robustness, and low detection limits confirms that the developed RP-HPLC method is a validated, stability-indicating, and reliable analytical tool for routine estimation of Enmetazobactam and Cefepime in bulk and pharmaceutical dosage forms. The method can be effectively used in quality control laboratories, formulation development, and stability testing.

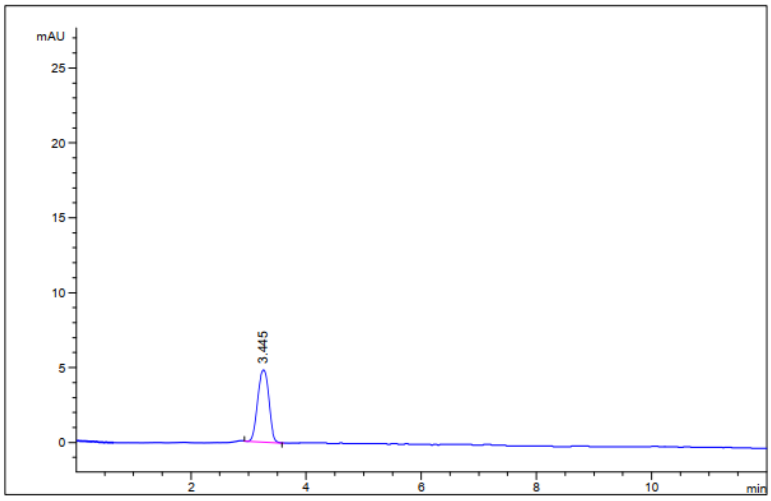

Figure 1: Chromatogram of EMB

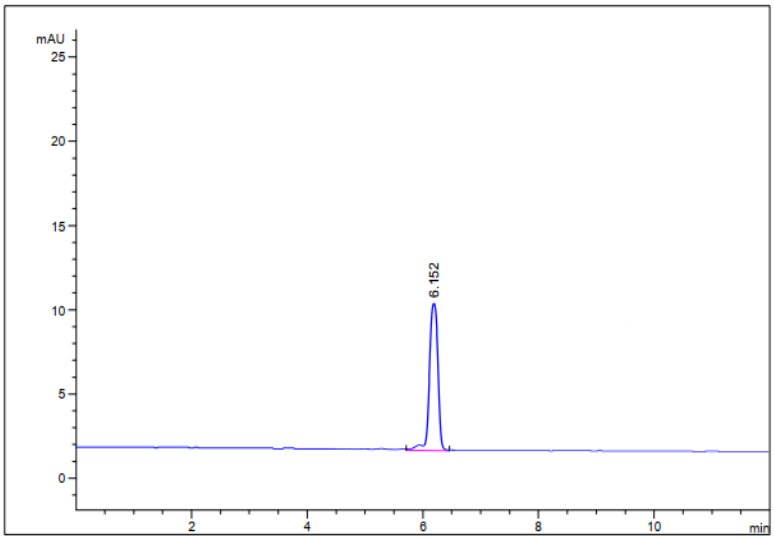

Figure 2: Chromatogram of CFP

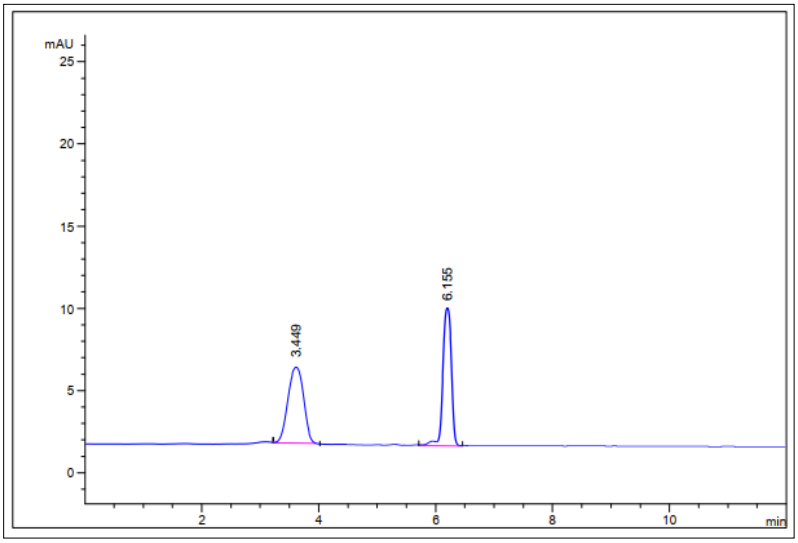

Figure 3: Chromatogram of Both the drug

Table 2: Results of Linearity of Enmetazobactam (EMB) and Cefepime (CFP)

S. No.	Parameter	EMB	CFP
1	Linearity	5-25µg/ml	10-50µg/ml
2	Correlation Coefficient (r^2)*	0.9987	0.9990
3	Slope (m)*	243.2	230.24
4	Intercept (c)*	50.297	16.355

Table 3: Results of Recovery Studies on Marketed Formulations

Recovery Level %	% Recovery (Mean±SD)*	
	EMB	CFP
80	98.51±0.798	99.16±0.155
100	98.72±1.002	98.90±1.132
120	98.94±0.559	98.58±0.613

Table 4: Results of validation (%R.S.D.)

PARAMETER		(Mean±SD)	
		EMB	CFP
Precision (% R.S.D.)*	Repeatability	98.709±0.098	99.020±0.119
	Day to Day	98.405±0.079	99.033±0.117
	Analyst to Analyst	99.201±0.083	98.341±0.201
	Robustness	96.051±0.687	99.093±0.101

*Average of five determination

Table 5: LOD and LOQ of EMB and CFP

Name	LOD (µg/ml)	LOQ (µg/ml)
EMB	0.25	0.75
CFP	0.50	1.50

Table 5: Result of assay of tablet formulation

	EMB*	CFP*
Label Claim (mg)	**500mg**	**2000mg**
% Found (mg)	498.58	1985.42
% Assay	99.716	99.271
% RSD	0.225	0.632

*Average of three determination

Conclusion

The present study successfully established a simple, accurate, precise, and robust RP-HPLC method for the simultaneous estimation of Enmetazobactam (EMB) and Cefepime (CFP) in bulk and pharmaceutical dosage forms. The chromatographic conditions resulted in well-resolved, sharp

peaks with no interference, confirming excellent specificity. Validation parameters fulfilled the ICH Q2(R1) requirements, with strong linearity, high accuracy, low %RSD in precision studies, and satisfactory robustness. The low LOD and LOQ values demonstrated the sensitivity of the method, while the assay results of the marketed formulation showed drug content within acceptable limits. Overall, the developed method is reliable and suitable for routine quality control, stability studies, and analytical evaluation of EMB and CFP in combined dosage forms.

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