The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
20(4): 496-505, 2025 www.thebioscan.com

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE ESTIMATION OF ANTIMICROBIAL DRUGS IN COMBINED DOSAGE FORM

Shefali Arora*, Kuldeep Ganju, Ankit Diwan, Rinshi Agrawal

Sagar Institute of Pharmacy and Technology (SIPTec), Bhopal (M.P.), India

Email Id: shefaliarora54@gmail.com

DOI: 10.63001/tbs.2025.v20.i04.pp496-505

KEYWORDS:
Enmetazobactam; Cefepime; RP-HPLC; Method Validation; Linearity; Precision; Recovery; LOD; LOQ; Combined Dosage Form.

Received on:

09-09-2025

Accepted on:

16-10-2025

Published on:

21-11-2025

ABSTRACT

A simple, precise, accurate, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Enmetazobactam (EZB) and Cefepime (CFP) in combined dosage form. Chromatographic separation was achieved on a C18 column (250 mm × 4.6 mm, 5 µm) using a mobile phase of methanol and acetonitrile (50:50 v/v) under isocratic conditions at a flow rate of 1.0 mL/min with detection at 254 nm. The retention times for EZB and CFP were found to be 3.75 min and 4.92 min, respectively. The method exhibited excellent linearity in the concentration range of 5–25 µg/mL for EZB and 10–50 µg/mL for CFP, with correlation coefficients (r^2) of 0.9991 and 0.9990, respectively. The percentage recoveries ranged from 98.19% to 98.73%, confirming the accuracy of the method. Precision and robustness studies showed %RSD values less than 2%, indicating high method reproducibility. The LOD and LOQ values were 0.35 µg/mL and 0.85 µg/mL for EZB, and 0.50 µg/mL and 1.50 µg/mL for CFP, respectively. The developed method was successfully applied to the assay of marketed formulations, with assay values of 99.77% for EZB and 99.31% for CFP. Hence, the proposed RP-HPLC method is rapid, reliable, and suitable for routine quality control analysis of Enmetazobactam and Cefepime in pharmaceutical dosage forms.

Introduction

High-Performance Liquid Chromatography (HPLC) is one of the most powerful analytical techniques widely used for the qualitative and quantitative estimation of pharmaceutical drugs in bulk and formulated dosage forms. The development and validation of an HPLC method for antimicrobial agents in combined dosage forms are essential to ensure drug quality, efficacy, and safety throughout production and storage. The increasing prevalence of microbial resistance has led to the formulation of fixed-dose combinations (FDCs) containing two or more antimicrobial agents to achieve synergistic effects, broadened spectrum of activity, and reduced resistance development (Patel et al., 2021).

The analysis of such combination products poses significant challenges due to differences in chemical structure, polarity, and UV absorption characteristics of the active pharmaceutical ingredients (APIs). Therefore, a robust and validated HPLC method is crucial to achieve accurate, reproducible, and specific quantification of each component in the presence of excipients or degradation products (Bala et al., 2020). Method development typically involves optimization of chromatographic parameters such as mobile phase composition, pH, flow rate, detection wavelength, and column selection to achieve well-resolved peaks and acceptable system suitability parameters (Dong, 2019).

Method validation, as recommended by the International Council for Harmonisation (ICH) guidelines (ICH Q2(R1)), ensures that the analytical procedure is suitable for its intended purpose. The critical validation parameters include specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), robustness, and system suitability (ICH, 2005). A well-validated method supports routine quality control, stability testing, and bioanalytical studies, thereby maintaining therapeutic efficacy and patient safety (Rao et al., 2022).

In recent years, several researchers have reported HPLC methods for simultaneous estimation of antimicrobial combinations such as amoxicillin–clavulanic acid, ciprofloxacin–tinidazole, levofloxacin–ornidazole, and azithromycin–ceftriaxone, among others, highlighting the growing need for precise and economical analytical techniques (Singh et al., 2023; Charde et al., 2020). Hence, the present study aims to develop and validate a simple, rapid, accurate, and stability-indicating HPLC method for the simultaneous estimation of antimicrobial drugs in combined dosage form as per ICH guidelines.

Material and Methods

Material

Enmetazobactam (EZB) and Cefepime (CFP) were obtained as gift samples from a reputed pharmaceutical manufacturer. Methanol and acetonitrile (HPLC grade) were procured from Merck India Pvt. Ltd. All other reagents and solvents used were of analytical grade. HPLC-grade water was prepared using a Milli-Q purification system. The marketed combined dosage formulation containing Enmetazobactam and Cefepime was purchased from a local pharmacy and used for assay analysis. All experiments were performed using a Waters HPLC system equipped with a UV detector

and a C18 column (250 mm × 4.6 mm, 5 µm).

Methods

Selection of mobile phase

Initially to estimate EZB and CFP 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 Acetonitrile: Methanol in the ratio of 50:50v/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 Diluent

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect retention and resolution of analyte. After various trials methanol was used as diluents.

Table 1: Separation Variable

Variable	Condition
Instrument	HPLC System (Waters)
Column	
Dimension	250 mm × 4.60 mm
Particle Size	5 µm
Bonded Phase	Octadecylsilane (C18)
Mobile Phase	
Methanol	50 parts
Acetonitrile	50 parts
Diluent	Methanol
Flow Rate	1.0 mL/min
Run Time	10 min
Temperature	Ambient (25 ± 2 °C)
Sample Size (Injection Volume)	20 µL
Detection Wavelength	254 nm
Detector Type	UV-Visible Detector

Retention Time	
Enmetazobactam	3.745 ± 0.3 min
Cefepime	4.885 ± 0.3 min
Resolution (R_s)	≥ 2 between Enmetazobactam and Cefepime
Theoretical Plates (N)	≥ 2000
Tailing Factor	≤ 2.0
pH of Mobile Phase	Not applicable (organic solvent system)
Mode of Elution	Isocratic
Detection Mode	Absorbance

Preparation of Stock Solution:

Accurately weighed 10 mg API of EZB 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 EZB and CFP respectively (Stock-B).

Preparation of Different Solution

0.5ml, 1ml, 1.5ml, 2.0ml and 2.5ml 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 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml, for EZB. In same manner 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml and 50µg/ml of CFP also prepared.

Linearity and Calibration Graph

To establish the linearity of analytical method, a series of dilution ranging from 5-25µg/ml for EZB and 10-50µg/ml for CFP 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 EZB 5µg/ml for EZB 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 EZB) and (5 to 25µg/ ml for (CFP) and areas for each concentration were recorded three times and mean area was calculated. The regression equation and correlation coefficient of curve are given and the standard calibration curve of the drug is shown in figure 6.5 & 6.6. 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

The stock solution was prepared. The precision are established in three differences:

Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range 5, 10, 15, 20 and 25µg/ml for EZB and 10, 20, 30, 40 and 50µg/ml for CFP indicates the precision under the same operating condition over short interval time.

Intermediate Precision

Day To Day 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 EZB 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, Acetonitrile: Methanol (50:50 % v/v) to (45:55 % 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 formulation

Accurately weighed quantity of powder equivalent to 10 mg of EZB 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 5µg/mL EZB and 20µg/mL CFP respectively. The amounts of EZB and CFP in tablets 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 developed reverse-phase HPLC method for the simultaneous estimation of Enmetazobactam (EZB) and Cefepime (CFP) in combined dosage form was found to be simple, precise, accurate, and reproducible.

The chromatographic conditions (Table 1) were optimized using a C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase of methanol : acetonitrile (50 : 50 v/v) under isocratic elution at a flow rate of 1.0 mL/min and detection wavelength of 254 nm. Both drugs were well resolved with retention times of 3.75 min for EZB and 4.92 min for CFP, confirming the efficiency of the separation system. The resolution (≥ 2) and tailing factors (≤ 2) were within acceptable limits, ensuring good peak symmetry and column performance.

The linearity study (Table 2) demonstrated an excellent correlation between concentration and peak area in the range of 5–25 µg/mL for EZB and 10–50 µg/mL for CFP, with correlation coefficients (r^2) of 0.9991 and 0.9990, respectively. This indicates the method's strong linear response within the selected range.

System suitability parameters (Table 3) such as theoretical plates, tailing factors, and retention times were within the prescribed limits of ICH Q2(R2) guidelines, confirming the system's reliability and reproducibility. The %RSD values for all parameters were below 2%, establishing the precision of the chromatographic system.

The recovery studies at 80%, 100%, and 120% levels (Table 4) revealed mean

recoveries between 98.19%–98.73%, confirming the accuracy of the method and the absence of interference from excipients in the formulation matrix.

Precision and robustness studies (Table 5) showed %RSD values well below 2%, demonstrating excellent method precision. Robustness results under small deliberate changes in chromatographic conditions (such as flow rate and mobile phase composition) remained consistent, indicating the method's reliability under varied conditions.

The LOD and LOQ values (Table 6) for EZB (0.35 µg/mL and 0.85 µg/mL) and CFP (0.50 µg/mL and 1.50 µg/mL) confirm the high sensitivity of the developed method, making it suitable for trace analysis.

The assay of marketed formulation (Table 7) revealed drug contents of 99.77% for EZB and 99.31% for CFP, which are within the acceptable range of 98–102%, demonstrating that the method is suitable for routine quality control analysis. The developed HPLC method fulfills all the validation parameters as per ICH Q2(R2) guidelines, confirming that it is accurate, precise, linear, specific, robust, and sensitive for the simultaneous determination of Enmetazobactam and Cefepime in combined dosage forms.

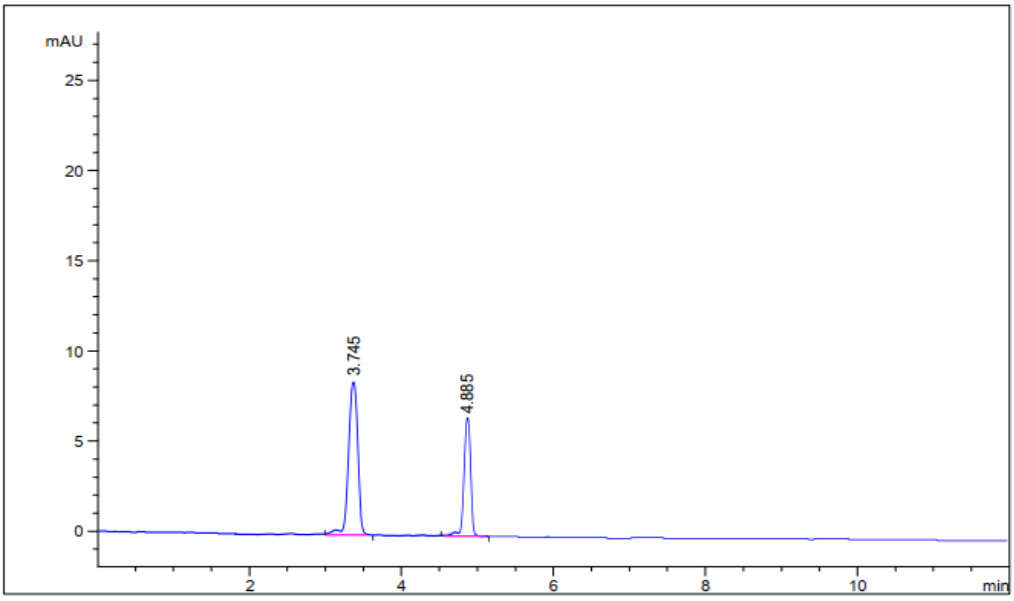

Figure 6.7: Chromatogram of Both the drug

Table 2: Results of linearity of EZB and CFP

Drug	Concentration (µg/mL)	Mean Area Under Curve (AUC)	Linearity Range (µg/mL)	Correlation Coefficient (r^2)	Slope (m)	Intercept (c)
EZB	5	583.811	5–25	0.9991	100.91	40.961
	10	1069.056				
	15	1560.657				
	20	2041.053				
	25	2559.201				
CFP	10	976.682	10–50	0.9990	97.85	45.612
	20	1828.964				
	30	2651.206				
	40	3573.158				
	50	4313.051				

Table 2: Results of system suitability parameters of EZB and CFP

Drug	Parameter	Mean	SD	%RSD
EZB	Retention Time (RT, min)	3.75	0.00	0.00
	Area Under Curve (AUC)	583.81	8.25	1.41
	No. of Theoretical Plates	2566.83	11.87	0.46
	Tailing Factor	1.20	0.03	2.50
CFP	Retention Time (RT, min)	4.92	0.04	0.81
	Area Under Curve (AUC)	976.68	5.25	0.54
	No. of Theoretical Plates	2958.00	15.76	0.53
	Tailing Factor	1.19	0.02	1.68

Table 3: Summary of Recovery Studies of EZB and CFP

Drug	Recovery Level (%)	Mean (%)	SD	%RSD
EZB	80	98.44	1.034	1.051
	100	98.63	0.839	0.850
	120	98.19	1.375	1.400

CFP	80	98.25	0.515	0.524
	100	98.50	0.726	0.738
	120	98.73	0.639	0.647

Table 4: Summary of Precision and Robustness Studies of EZB and CFP

Parameter	Drug	Mean (%)	SD	%RSD
Repeatability	EZB	98.84	0.073	0.074
	CFP	99.01	0.127	0.128
Day-to-Day Variation	EZB	98.42	0.120	0.122
	CFP	99.03	0.117	0.118
Analyst-to-Analyst Variation	EZB	99.52	0.047	0.047
	CFP	98.91	0.128	0.129
Robustness	EZB	98.57	0.106	0.108
	CFP	99.09	0.101	0.102

Table 5: LOD and LOQ of EZB and CFP

Name	LOD (µg/ml)	LOQ (µg/ml)
EZB	0.35	**0.85**
CFP	0.50	1.50

Table 6: Result of assay of tablet formulation

	EZB *	CFP*
Label Claim (mg)	**500mg**	**2000mg**
% Found (mg)	498.85	1986.36
% Assay	99.77	99.318
% RSD	0.228	0.192

*Average of three determination

Conclusion

The developed HPLC method was found to be simple, precise, accurate, and reproducible for the simultaneous estimation of Enmetazobactam (EZB) and Cefepime (CFP) in combined dosage forms. The method exhibited excellent linearity, recovery, precision, and robustness within

the specified concentration ranges. The low %RSD values confirmed the reliability and repeatability of the method, while the LOD and LOQ values indicated its high sensitivity. Thus, the validated method can be successfully applied for routine quality control analysis of Enmetazobactam and Cefepime in pharmaceutical formulations.

References

1. Patel, D. M., Shah, J. V., & Patel, R. K. (2021). Development and validation of RP-HPLC method for simultaneous estimation of amoxicillin and clavulanic acid in pharmaceutical dosage form. *Journal of Applied Pharmaceutical Science*, 11(2), 75–82.
2. Bala, S., Sharma, N., & Singh, R. (2020). Analytical method development and validation for pharmaceutical analysis: A review. *Journal of Analytical & Pharmaceutical Research*, 9(5), 142–150.
3. Dong, M. W. (2019). *Modern HPLC for Practicing Scientists.* 2nd Ed., John Wiley & Sons.
4. ICH Q2(R1). (2005). *Validation of Analytical Procedures: Text and Methodology*. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use.
5. Rao, R. N., Reddy, Y. N., & Nagaraju, D. (2022). Analytical method development and validation for simultaneous estimation of antibiotics in combined dosage form by RP-HPLC. *Asian Journal of Pharmaceutical Analysis*, 12(3), 181–189.
6. Singh, S., Gupta, A., & Kaur, G. (2023). RP-HPLC method development and validation for simultaneous estimation of ciprofloxacin and tinidazole in combined dosage form. *International Journal of Pharmacy and Pharmaceutical Sciences*, 15(1), 50–56.
7. Charde, S. Y., Welankiwar, A. S., & Chandewar, A. V. (2020). Simultaneous estimation of levofloxacin and ornidazole in tablet dosage form by RP-HPLC. *Indian Journal of Pharmaceutical Sciences*, 82(4), 623–630.