Green RP-HPLC Method Development and Validation for Simultaneous Determination of Cefepime Hydrochloride and Avibactam Sodium in Bulk Drug and Laboratory-Prepared Mixture

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ABSTRACT

A simple, rapid, accurate, precise and environmentally friendly RP-HPLC Method Development and Validation. Chromatographic separation was accomplished on a C18 octadecylsilane column with methanol: phosphate buffer as the mobile phase at 0.9 mL/min and detection at 220 nm. The retention times for Avibactam Sodium and Cefepime Hydrochloride were 3.95 min and 9.22 min, respectively. We validated the developed method in compliance with ICH Q2 (R2) guidelines, the maximum concentration for avibactam sodium and cefepime hydrochloride showed excellent linear regressions by plotting calibration curves; correlation coefficient was 0.9990, 0.9991and range of concentrations were from5 to 25 µg/mL respectively. The accuracy studies observed the mean recoveries from Avibactam Sodium in a range of 98.20–99.91%. and 99.23–100.73% for Cefepime Hydrochloride. For precision studies, the %RSD values were found to be < 2%, indicating good reproducibility. The LOD and LOQ were determined to be 0.05 and 0.15 µg/mL for Avibactam Sodium, and 0.039 and 0.1197 µg/mL for Cefepime Hydrochloride respectively (results are displayed in Table3) Table3. The method showed appropriate robustness also during designed changes in chromatographic conditions. Greenness was determined with an Analytical Eco-scale score of 85 and MoGAPI score of 74. The proposed method can be effectively utilized for the routine quantitative assay of Cefepime Hydrochloride and Avibactam Sodium.

Keywords: Cefepime Hydrochloride, Avibactam Sodium, RP-HPLC, Method Validation, Green Analytical Chemistry.

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INTRODUCTION:

Multidrug-resistant (MDR) bacterial infections are now a leading cause of morbidity and mortality on a global scale, driven by the emergence of β-lactamase-producing Gram-negative pathogens. Emerging antimicrobial resistance in organisms such as Klebsiella pneumoniae, Escherichia coli and Pseudomonas aeruginosa has led to the limited effectiveness of traditional antimicrobial therapies, creating a need for inventive antibiotic combinations. [3]

Cefepime Hydrochloride is a fourth generation cephalosporin, a β-lactam class of antibiotic that demonstrates broad-spectrum bactericidal activity against both Gram-positive and Gram-negative microorganisms. [11, 12] It is effective in blocking the synthesis of bacterial cell wall by binding to penicillin-binding proteins and leading to bactericidal activity. Avibactam Sodium is a non-β-lactam β-lactamase inhibitor with a diazabicyclooctane nucleus capable of preventing β-lactams hydrolysis caused by resistant bacteria. The combination of cefepime hydrochloride and avibactam sodium is an effective treatment for complicated infections caused by multidrug-resistant Gram-negative organisms. [3, 13–15]

Several analytical methods have been reported for the determination of Cefepime Hydrochloride either alone or in combination with other drugs using RP-LC, RP-HPLC, TLC, and spectrophotometric techniques.[1,2,4–6] Similarly, analytical methods have been developed for Avibactam Sodium in combination with agents such as ceftazidime and aztreonam using RP-HPLC, RP-UPLC, and HPLC-MS/MS techniques.[7-10] Although these methods demonstrated satisfactory analytical performance, most of them were developed for different combinations and may not be directly applicable to the simultaneous estimation of Cefepime

Hydrochloride and Avibactam Sodium because of their distinct physicochemical characteristics.

A review of the available literature revealed limited information regarding the simultaneous estimation of Cefepime Hydrochloride and Avibactam Sodium by RP-HPLC. Therefore, the present study was undertaken to develop and validate a simple, rapid, accurate, precise, economical, and environmentally friendly RP-HPLC method for the simultaneous estimation of Cefepime Hydrochloride and Avibactam Sodium. The developed method was further evaluated using green assessment tools to establish its suitability for sustainable routine pharmaceutical analysis.

MATERIALS AND METHODS:

Materials:

Cefepime Hydrochloride obtained as a gift sample from Sumar Biotech, Gujarat and Avibactam Sodium was purchased from S.M Pharma and Chemicals, Mumbai. HPLC-grade Methanol and Milli-Q water were used throughout the study. Potassium Dihydrogen Phosphate (KH_2PO_4) and Sodium Hydroxide (NaOH) of AR grade were used.

Instrumentation:

Chromatographic analysis was performed using a Thermo Scientific Vanquish Series HPLC system equipped with a Vanquish Quaternary Pump and UV detector. Separation was achieved on a Hypersil ODS-C18 column (250 mm × 4.6 mm, 5 µm particle size).

Chromatographic conditions:

The mobile phase consisted of 0.02 M Potassium Dihydrogen Phosphate buffer (pH adjusted to 6.5) and Methanol in the ratio of 90:10 (% v/v). Isocratic elution was employed at a flow rate of 0.9 mL/min. The column temperature was maintained at 25°C. The injection volume was 20 µL, and detection was carried out at 220 nm using UV detection. The total run time for each chromatographic analysis was 15 minutes. The mobile phase was also used as the diluent.

Selection of Green Solvents

The mobile phase components were selected based on their environmental acceptability and analytical suitability. Methanol was chosen as the organic modifier due to its favorable environmental profile according to the SANOFI Solvent Selection Guide and CHEM21 Solvent Selection Guide. Phosphate buffer was employed as the aqueous component owing to its low toxicity and compatibility with RP-HPLC analysis. Furthermore, the optimized mobile phase contained only 10% methanol, thereby minimizing organic solvent consumption and reducing the environmental impact of the developed method.

Preparation of 0.02 M Potassium Dihydrogen Phosphate Buffer (pH 6.5)

A 0.02 M potassium dihydrogen phosphate buffer was prepared by dissolving 2.72 g of potassium dihydrogen phosphate (KH_2PO_4) in HPLC-grade distilled water. The pH of the solution was adjusted to 6.5 using 1 N sodium hydroxide solution. The resulting solution was transferred to a 1000 mL volumetric flask and the volume was made up to the mark with HPLC-grade distilled water.

Preparation of Mobile Phase:

A mixture of 100 mL methanol and 900 mL potassium dihydrogen phosphate buffer (pH 6.5) was prepared and thoroughly mixed.

Diluent: The mobile phase itself was used as the diluent for the preparation of standard and sample solutions.

Preparation of Standard Stock Solutions

Accurately weighed 10 mg each of Cefepime hydrochloride and Avibactam sodium were separately transferred into two 100 mL volumetric flasks. The drugs were dissolved and diluted to volume with methanol: phosphate buffer (10:90, v/v) to obtain standard stock solutions having a concentration of 100 µg/mL each.

Method optimization:

Method optimization was performed by systematically varying the mobile phase composition and with flow rate in order to achieve proper separation and satisfactory system suitability parameters.

Table No. 1: Method optimization Trials

Trial Run	Mobile Phase Composition (Phosphate Buffer: Methanol v/v)	Flow Rate (mL /min)
1	50:50	1
2	70:30	1
3	90:10	1
4	90:10	0.9

Method validation:

The RP-HPLC method was validated according to International Council for Harmonization (ICH) Q2 (R2) guideline on validation of analytical procedures.

Specificity:

We then injected the blank, placebo, standard and previously prepared samples into HPLC system to check whether there were some interferences at the retention times of Cefepime hydrochloride and avibactam sodium.

System Suitability:

Under optimized chromatographic conditions, system suitability was checked using six replicate injections of a mixed standard solution of Cefepime hydrochloride and Avibactam sodium. Data such as the retention time, theoretical plates, tailing factor and resolution was collected.

Linearity:

The linearity of the developed method was evaluated by preparing calibration standards in the concentration range of 5–25 µg/mL for Cefepime Hydrochloride and Avibactam Sodium.

Accuracy

Recovery studies were performed by spiking known quantities of Cefepime Hydrochloride and Avibactam Sodium standards at 80%, 100%, and 120% levels of the target concentration (10 µg/mL). The solutions were analyzed, and the percentage recovery was calculated.

Precision

Precision was evaluated as repeatability and intermediate precision. Repeatability was determined by six replicate injections of a standard solution (25 µg/mL), while intermediate precision was assessed by analyzing the same solution on three consecutive days. The results were expressed as %RSD for Cefepime Hydrochloride and Avibactam Sodium.

Sensitivity:

The sensitivity of the developed RP-HPLC method was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ) for Cefepime Hydrochloride and Avibactam Sodium. The LOD and LOQ values were calculated based on the standard deviation of the response and the slope of the corresponding calibration curve.

Robustness

The robustness of the developed method was evaluated by making deliberate variations in chromatographic conditions, including flow rate (±0.1 mL/min) and detection wavelength (±2 nm). Standard solutions (25 µg/mL) of Cefepime Hydrochloride and Avibactam Sodium were analyzed, and the effects on system suitability parameters were assessed.

Assay

A laboratory-prepared synthetic mixture containing Cefepime Hydrochloride (10 mg), Avibactam Sodium (10 mg), and mannitol as an excipient was transferred into a 100 mL volumetric flask and dissolved in methanol: phosphate buffer (10:90, v/v). The volume was made up to the mark with the same diluent, and appropriate dilutions were prepared to obtain a final concentration of 10 µg/mL of each drug. The solution was filtered through a 0.45 µm membrane filter and analyzed using the developed RP-HPLC method.

Statistical Analysis

All experiments were performed in triplicate where applicable, and results were expressed as mean ± standard deviation. Statistical evaluation was performed using percentage relative standard deviation (%RSD) and regression analysis.

Green Assessment:

Green assessment of developed RP-HPLC method was carried out using Analytical Eco -Scale and MoGAPI tools.

RESULT AND DISCUSSION:

Selection of Detection Wavelength:

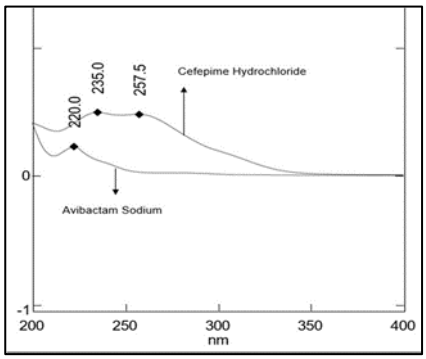


Figure No.1: Overlaid spectrum of Avibactam sodium and Cefepime hydrochloride

Considering the spectral behaviour of both drugs, 220 nm was selected as the common analytical wavelength. Avibactam sodium, being a weak chromophore, required detection at a lower wavelength to achieve adequate sensitivity, while Cefepime hydrochloride also showed sufficient absorbance at this wavelength. The selected wavelength provided adequate sensitivity for both analytes while maintaining acceptable baseline stability.

Selection of Stationary Phase:

Based on the nature of the analytes, a reversed-phase chromatographic system was selected. A C18 (octadecylsilane) column was used for the separation of Cefepime Hydrochloride and Avibactam Sodium, as it provided adequate retention and satisfactory peak separation under the optimized conditions.

Selection of Mobile Phase and flow rate:

The core goal of this study is to optimize chromatographic conditions by changing the mobile phase and flow rate so that satisfactory separation of Cefepime hydrochloride and Avibactam sodium could be achieved. At 1.0-mL/min flow rate, using phosphate buffer: methanol (50:50 and 70:30, v/v) mobile phase compositions as shown in Table-2; Avibactam sodium peak eluted before system peak with regard to retention time. Phosphate buffer: methanol (90:10, v/v) at the same flow rate pushed this peak beyond the system peak and did not give adequate resolution. A flow rate of 0.9 mL/min (phosphate buffer: methanol, 90:10 or v/v) resulted in narrow symmetrical well-resolved peaks for the two analytes. Accordingly, the optimized chromatographic condition was selected as phosphate buffer: methanol (90:10, v/v) 0.9 mL/min.

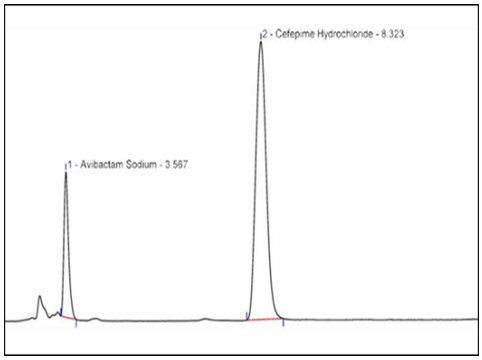


Figure No.2: Chromatogram at 1.0 mL/min

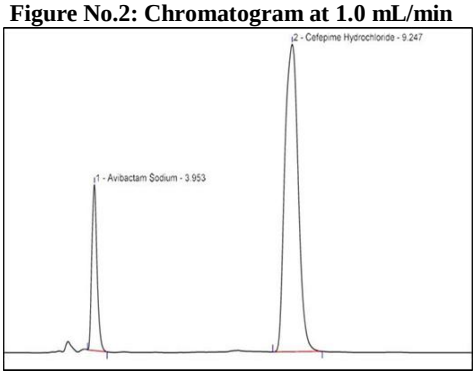


Figure No.3: Chromatogram at 0.9 mL/min

Method Development

Based on the optimization studies, the following chromatographic conditions were finalized

Table No.2: Optimized chromatographic conditions for method development

Parameter	Optimized Condition
Mobile phase	0.02M Potassium Dihydrogen Phosphate buffer (pH :6.5): Methanol (90:10v/v)
Mode of elution	Isocratic
Flow rate	0.9ml/min
Detection wavelength	220 nm
Injection volume	20 µL
Column temperature	25º C
Run time	15 min
Detection mode	UV detection

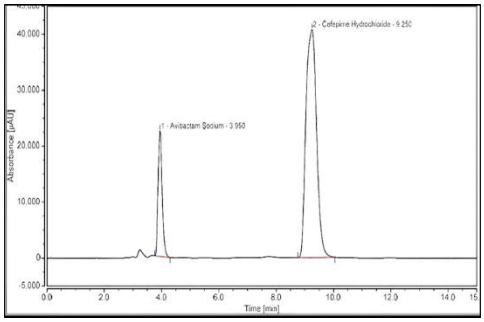


Figure No.4: Optimized RP-HPLC Chromatogram of Avibactam Sodium and Cefepime Hydrochloride

Method Validation:

Specificity:

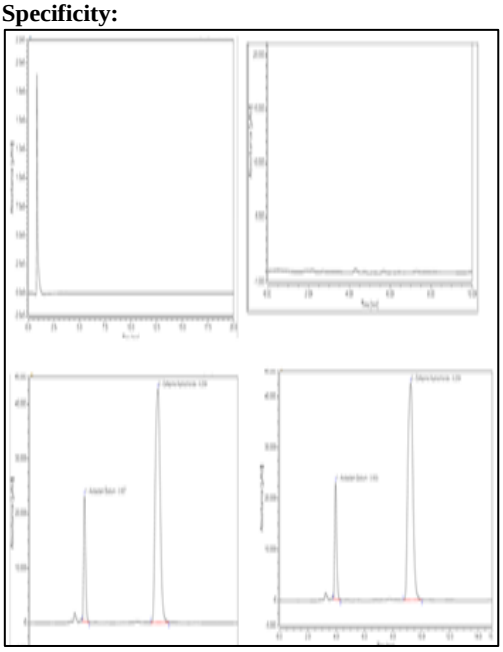


Figure No.5: Specificity chromatograms of blank, placebo, standard solution and sample solution

The specificity of developed RP-HPLC method was confirmed by injecting diluent, placebo, standard and sample solutions. A placebo consisting of Mannitol and other excipients was prepared and handled in the same way as that for the sample solution. As shown in Figure 4, there were no interfering peaks at the retention times of Avibactam Sodium (3.97 min), and Cefepime Hydrochloride (9.29 min) in the chromatograms of diluent and placebo sample. The standard and sample chromatograms demonstrate well-resolved peaks without interference which confirmed the specificity of the method.

System suitability:

Table No.3: System Suitability Parameters for Cefepime Hydrochloride and Avibactam Sodium

Parameter	Avibactam Sodium	Cefepime Hydrochloride
Retention Time (min)	3.950	9.220
Theoretical plates	5332	5521
Asymmetry Factor	1.44	1.19
Resolution	14.83	

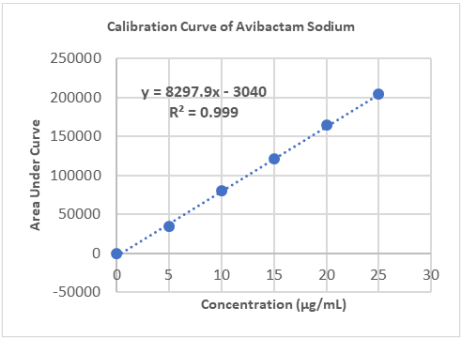
Theoretical plate counts greater than 5000 and asymmetry factors below 2 demonstrated satisfactory column efficiency and peak symmetry. Resolution of 14.83 indicated excellent separation between the analytes. The retention time, theoretical plates and resolution for drugs is as follows:

Linearity

Table No.4: Linearity Parameters for Cefepime

Avibactam Sodium (conc µg/mL)	Peak area	Cefepime Hydrochloride (conc µg/mL)	Peak area
5	34265.67	5	181378.7
10	80237	10	375510
15	120782.7	15	574160.7
20	164854.7	20	790255
25	203965.3	25	1007745
Regression equation	y=8389.7x -3040	Regression equation	y=40423x-15444
R^2	0.9990	R^2	0.9991

Hydrochloride and Avibactam Sodium



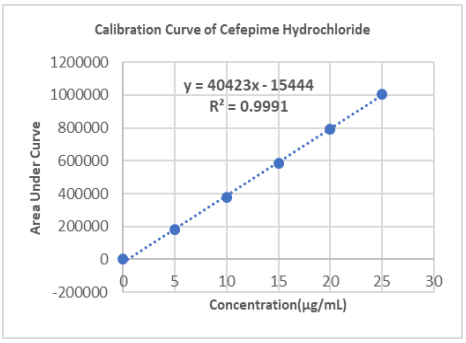


Figure No.6: Calibration curve of Avibactam Sodium and Cefepime hydrochloride

The calibration curves exhibited excellent linearity over the concentration range of 5–25 µg/mL with correlation coefficients of 0.9990 and 0.9991 for Avibactam Sodium and Cefepime Hydrochloride, respectively. These values demonstrate a strong proportional relationship between concentration and detector response, indicating suitability of the method for quantitative analysis. The linearity data and overlay chromatogram are presented in Table 8, Figure 5 respectively. Calibration curves are shown in Figure 6.

Precision:

Table 5. Intraday and Interday Precision Data of Cefepime Hydrochloride and Avibactam Sodium

Precision Parameter	Concentration (µg/mL)	Avibactam Sodium Mean AUC ± SD	% RSD	Cefepime Hydrochloride Mean AUC ± SD	% RSD
Repeatability	25	203028.7± 576.87	0.284	1004423± 3286.2	0.327
Intermediate	25	202826.4± 460.42	0.227	1011514.3 ±5764.3	0.57

Values are expressed as mean ± SD (n = 6) for repeatability and ± SD (n = 9) for intermediate precision. The %RSD values were below 2%, indicating excellent repeatability and intermediate precision of the developed method according to ICH acceptance criteria.

Accuracy

Table 6. Accuracy Study (% Recovery) of Avibactam Sodium and Cefepime Hydrochloride

Level	Initial Amount (µg/mL)	Amount Added (µg/mL)	Total Amount (µg/mL)	Avibactam Sodium Mean % Recovery ± SD	Cefepime Hydrochloride Mean % Recovery ± SD
8%	10	8	18	98.20± 0.16	99.06 ± 0.04
100 %	10	10	20	99.91 ± 0.04	99.23 ± 0.03
120 %	10	12	22	98.67 ± 0.11	100.73 ± 0.35

Values are expressed as mean ± SD (n = 3). Recovery values ranging from 98–102% confirmed the accuracy of the method and demonstrated the absence of matrix interference during quantification.

Sensitivity:

Table No.7: LOD and LOQ results

Parameter	Avibactam Sodium (µg/mL)	Cefepime Hydrochloride (µg/mL)
LOD	0.050	0.039
LOQ	0.150	0.1197

Low LOD and LOQ values indicated adequate sensitivity of the method for detecting and quantifying small concentrations of both analytes.

Robustness:

Table 8. Robustness Study of the Proposed RP-HPLC Method for Avibactam Sodium and Cefepime Hydrochloride

Parameter	Condition	Avibactam Sodium Mean AUC ± SD	% RSD	Cefepime Hydrochloride Mean AUC ± SD	% RSD
Flow rate (mL/min)	0.8	1228380 ±379.91	0.309	537791.3 ±1106.55	0.206
	0.9	223229.3 ±250.23	0.112	1053492.0 ±1285.95	0.122
	1.0	97099.3 ±1090.9	1.123	426150.7 ±2559.86	0.601
Detection wavelength (nm)	218	108030.3 ±471.12	0.436	475691.7 ± 1168.49	0.246
	220	222001.0 ±702.53	0.316	1050773.0± 3063.53	0.292
	222	107627.7 ±109.55	0.102	488851.3 ± 61.98	0.013

Values are expressed as mean ± SD (n = 3). Deliberate variations in flow rate and detection wavelength did not significantly affect analytical performance, as evidenced by %RSD values below 2%, confirming robustness of the method.

Assay:

Table 9. Assay Results of Laboratory Mixture Containing Avibactam Sodium and Cefepime Hydrochloride

Drug	Theoretical Concentration (µg/mL)	Mean Amount Found (µg/mL) ± SD	Mean % Assay ± SD	%RSD
Avibactam Sodium	10	9.99 ± 0.01	99.91 ±0.04	0.04
Cefepime Hydrochloride	10	9.84 ± 0.01	99.23 ±0.03	0.03

Values are expressed as mean ± SD (n = 3). Assay values close to 100% confirmed the suitability of the developed method for accurate quantification of Cefepime Hydrochloride and Avibactam Sodium in laboratory-prepared mixtures. **Green Assessment:**

1. Analytical Eco-scale Evaluation:

Table 10. Analytical Eco-Scale Evaluation of the Proposed RP-HPLC Method

Parameter	Penalty Points (PP)
Reagent consumption	2
Reagent hazard	3
Reagent penalty	6
Instrument energy	1
Occupational hazard	0
Waste generation	5
Waste treatment	3
Total waste penalty points	**8**
Total penalty points	**15**
Analytical Eco-scale score (100 − PP)	**85**

2: Modified Green Analytical Procedure Index (MoGAPI):

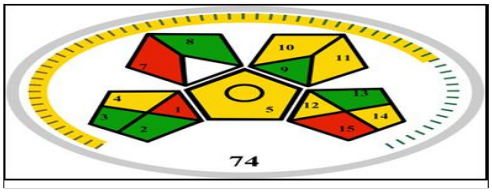


Figure No.6 MoGAPI assessment

Table No.11: MoGAPI assessment parameters

MoGAPI Parameter	Colour Zone	MoGAPI Parameter	Colour Zone
Sample collection	Red	Solvent consumption	Green
Sample preservation	Green	Solvent health hazards	Yellow
Sample transportation	Green	Solvent safety hazards	Yellow
Storage conditions	Yellow	Energy consumption	Yellow
Sample preparation	Yellow	Occupational hazards	Green
Scale of extraction	N/A	Waste generation	Yellow
Solvent usage	Red	Waste treatment	Red
Derivatization	Green	**Overall MoGAPI Score**	**74**

The Analytical Eco-scale score of 85 classifies the method as an excellent green analytical procedure. Similarly, the MoGAPI score of 74 indicates good environmental performance. The high greenness rating can be attributed to the low consumption of organic solvent (10% methanol) and reduced waste generation.

CONCLUSION

A novel, fast, simple accurate precise and green RP-HPLC method was developed & validated for the simultaneous estimation of Cefepime Hydrochloride and Avibactam Sodium in laboratory mixture. This approach adhered to ICH rules and hence showed an optimal linearity, accuracy, precision, robustness and sensitivity. Analytical Eco-scale and MoGAPI evaluated greenness scores of 85 and 74, respectively, confirming environmental acceptability of the method. Thus, the established method can be usefully applied for daily quantitative analysis of Cefepime Hydrochloride and Avibactam Sodium.

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