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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CEFTOLOZANE AND TAZOBACTAM IN INJECTION

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ABSTRACT

A simple, selective, linear, precise and accurate reverse-phase high-performance liquid chromatography technique was urbanized and validated for the concurrent determination of Ceftolozane and Tazobactam in powder for injection. The chromatographic separation was achieved on Altima C18 4.6×150mm, 5.0 µm column by means of a mobile phase consisting a mixture of Methanol: Water in a proportion of 65:35v/v at a flow rate of 1ml/min at room temperature and detection was carried out at 285nm. The clear chromatography peaks were identified with retention times of 2.09min for Ceftolozane and 6.07 min for Tazobactam. The proposed technique was validated according to ICH guidelines with respect to specificity, linearity, accuracy, precision, LOD, LOQ and robustness. The linearity was observed in the concentration range of 10-40µg/ml for Ceftolozane and 5-25µg /ml for Tazobactam. A linear regression coefficient for both drugs was 0.999. The percentage recovery of Ceftolozane and Tazobactam was 100.9% and 99.6%. The %RSD for repeatability and intermediate precision was less than 2%. LOD was 0.8µg/ml and 0.9µg/ml and LOQ was 2.5µg/ml and 2.9µg/ml for Ceftolozane and Tazobactam respectively. The results of validation parameters were met ICH requirements. Hence, the projected method can be used for the estimation of Ceftolozane and Tazobactam in powder for injection during regular and quality-control analysis.

KEYWORDS: Ceftolozane, Tazobactam, Simultaneous estimation, RP-HPLC, Injection.

INTRODUCTION

Ceftolozane (CZ) is a semisynthetic fifth-generation cephalosporin antibiotic effective against Gram-negative and positive bacterial infections such as *Pseudomonas aeruginosa* and *Escherichia coli*. It consisting a β- lactum ring fused with thiadiazole, and is chemically (6R,7R)-3-[[3-amino-4- (2-aminoethylcarbamoylamino)-2-methylpyrazol-1-ium-1-yl]methyl]-7- [[(2Z)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(2-carboxypropan-2-yloxyimino) acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate, Figure 1 with the molecular formula of $C_{23}H_{30}N_{12}O_8S_2$ and molecular weight is 666.689. It is a white to off-white powder, soluble in water (0.0884mg/ml). Ceftolozane is penicillin-binding proteins (PBPs) inhibitor. The bactericidal action is due to inhibition of cell wall biogenisis, by binding to PBPs of situated on the internal membrane of the bacterial cell wall.[1] Tazobactam (TZ) is a pencillin antibiotic, and is a penicillanic acid sulfone derivative with antibacterial activity, chemically it is (2S,3S,5R)-3-methyl-4,4,7-trioxo-3-(1H-1,2,3-triazol-1-ylmethyl)-4λ^6-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid, Figure 2 with a molecular formula of $C_{10}H_{12}N_4O_5$ and molecular weight is 300.289g/mol. Tazobactam is a crystalline white powder that is readily soluble in water and moderately soluble in ethanol. Tazobactam is an inhibitor of beta-lactamase. It irreversibly attaches to effective site of beta-lactamase, thereby permanently inhibits enzymatic activity and increases the effectiveness of added beta-lactam antibiotics by protecting them from beta-lactamase catalysis. Tazobactum is also employed as an antimicrobic and anti-infective agent.[2,4] Because of increasing bacterial resistance to cephalosporins, sucessfull efforts has been attentive on association of antipseudomonal ceftolozane with tazobactam for antimicrobial activity against *Pseudomonas aeruginosa* and *enterobacteriaceae*.[5,6] Ceftolozane/tazobactam is a novel antibaterial/β-lactamase inhibitor approved by the FDA in January 2015 and is marketed under the brand name Zerbaxa™ by Cubist Pharmaceuticals U.S, from January 22, 2015, is at present a completely owned subsidiary of Merck & Co., Inc.[7] Ceftolozane/ tazobactam 1.5g(1g/0.5g) fixed dose combination as powder for injection has been used for treatment of problematical urinary tract Infections such as pyelonephritis caused by Escherichia coli, Klebsiella pneumoniae, and in conjuction with metronidazole for cubersome Intra-abdominal Infections produced by Enterobacteriaceae, Escherichia coli and bacterial pneumonia. Most recently, in June 2019, it is approved for the cure of hospital-acquired pneumonia, including ventilator-associated pneumonia.[8] Number of drugs are introducing into the market yearly. There is a time delay between the date of the preface of a drug into the market and the date of its inclusion in pharmacopeias. Hence, standards and analytical methods either for the individual or

combination of drugs might not be formal in the pharmacopeias. Some analytical procedures are not approachable in the piblications due to patent regulations. Therefore, it becomes essential to build up a newer analytical procedure for such drugs. Literature survey reveals many analytical methods have been published for simultaneous estimation of Ceftolozane and Tazobactum in bulk, pharmaceutical dosage forms and in biological samples. These include high performance liquid chromatography,[9-14] stability indicating,[15,16] and LC-MS/MS methods.[17-20] The purpose of our study is that HPLC has an increasing growth in the analysis for the ascertainment of API in various pharmaceutical formulations, which make it the most accepted and suitable technique for the determination. There are very few HPLC methods are described for the analysis of Ceftolozane and Tazobactum in powder for injection. In view of that, a need was felt to develop a suitable HPLC method for the analysis of CZ and TZ. A fruitful strive has been done to design and develop a new, simple, accurate, reproducible, and economical method for simultaneous estimation of Ceftolozane and Tazobactum in powder for injection by RP-HPLC. The projected method was validated in consonance to specificity, linearity, precision, accuracy, LOD, and LOQ, and robustness as requisite by the International Conference on Harmonization Q2 (R1) guidelines to support the suitability of the method.[21]

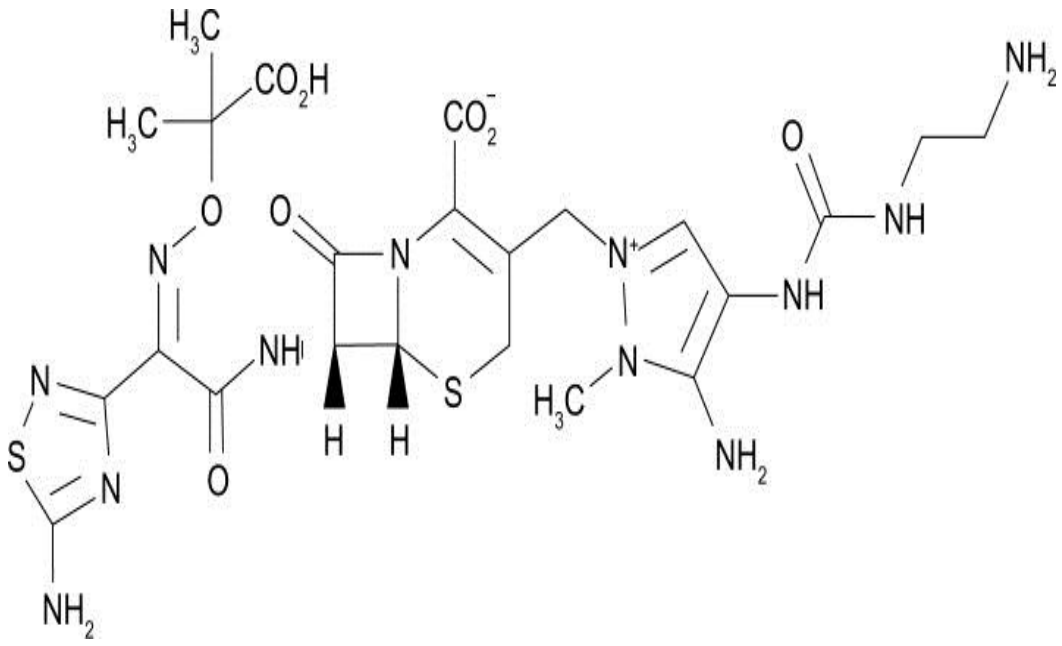

Figure 1: Structure of Ceftolozane.

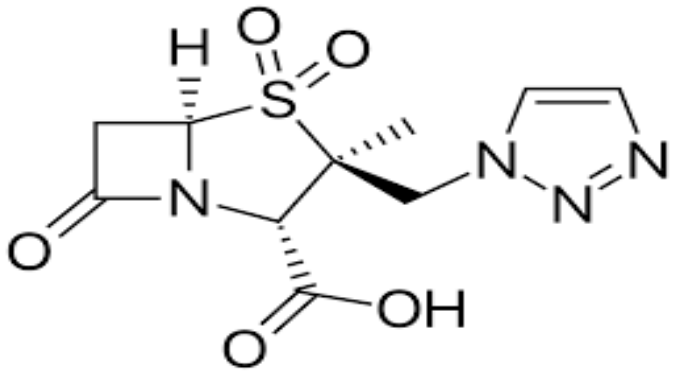

Figure 2: Structure of Tazobactum.

MATERIALS AND METHODS

Materials

Ceftolozane and Tazobactam standard drugs were obtained as contribution samples from Cubist pharmaceuticals, Hyderabad, India. Methanol and water of HPLC grade were procured from Lichrosolv, Merck (India) Ltd. Worli, Mumbai, India. The 0.45µ nylon filters were purchased from Millipore.1g of Ceftolozane equivalent to 1.147g of Ceftolozane sulphate and 0.5gm of Tazobactam equivalent to 0.537gm of tazobactam sodium powder for injection as Zerbaxa were purchased from pharmacy, Hyd.

Instruments

The HPLC system (Waters) auto sampler separation module 2695 consisted of high-pressure pump, PDA detector 996, and 10µL capacity injector loops. The system was well equipped with empower 2 software for monitoring and processing of data. Other types of equipment like Sartorius digital weighing balance, Lab India pH meter, and Lab man digital ultra sonicator were used for sample and standard preparations. The analytical column used was Altima C18 4.6×150mm packed with a particles size of 5.0µ.

METHODOLOGY

Experimental

The main objective of this experiment was to develop and validate a novel RP-HPLC method for simultaneous estimation of Ceftolozane and Tazobactum in powder for injection as we observed very few HPLC techniques are practicable from the literature study.

Selection of wavelength by UV-spectroscopy

From the UV-visible Spectro Photometric results, a detection wavelength of 285nm was selected. At this wavelength both the drugs exhibited maximum absorbance with superior peak intensity, excellent peak shape and height.

Selection of the method

Option of the technique be based on the nature of the sample such as Ionic or neutral molecule, molecular weight and solubility. The drugs used in the current study were polar; and hence RP- HPLC method was preferred for the original separation because of its accessibility and appropriateness.

Method Development

The starting point for method developments was following preface studies for the sample.

Preparation of solutions

Mobile phase

650ml of Methanol and 350ml of Water were mixed collectively and the solution was degassed in digital ultrasonic water bath for 10 minutes and then filtered through 0.45µ filter under vacuum.

Mobile phase was used as a diluent.

Standard solutions of Ceftolozane and Tazobactam

Each 10mg of Ceftolozane and Tazobactam working standards were accurately weighed and transferred into a 10ml of the clean dry volumetric flask respectively; about 7ml of diluent was added, sonicated for 30minutes and made up the volume to 10ml with diluent. From the above stock solutions, 30µg/ml of Ceftolozane and of 15µg/ml Tazobactam were prepared by diluting 0.3ml of Ceftolozane and 0.15ml of Tazobactam stock solutions to 10ml with the diluent.

Preparation of sample solutions

One vial powder was weighed and then the weight equivalent to 10mg of Ceftolozane and Tazobactam powder for injection was transferred into a 10ml of the clean, dry volumetric flask, dissolved completely in sufficient diluent, Sonicated for 30min, filtered the solution using 0.45-micron syringe filter, and then diluted to 10ml with diluent. From the filtered

solution 30µg/ml of Ceftolozane and 15µg/ml of Tazobactam were prepared by diluting 0.3ml of Ceftolozane and 0.15ml of Tazobactam to 10 ml with diluent.

Method development Experimental Trials

Various trials were accomplished through diverse chromatographic conditions during the progress of RP- HPLC.

Trial-1 Two peaks were observed. Tazobactum was eluted at 3.889mn and ceftolozane was eluted at 8.736mn. The peaks were recorded with improper baseline even though resolution was good.

Trial-2 Tazobactum was eluted at 3.681mn and ceftolozane was eluted at 4.052mn. Peaks shapes were good but, resolution of chromatogram was not acceptable. So, more trials were required for obtaining good peaks.

Trial-3 Tazobactum was eluted at 2.50mn and ceftolozane was eluted at 5.019mn. This trial showed less plate count. So, further trials were needed.

Trial-4 Ceftolozane was eluted at 2.362mn and tazobactum was eluted at 3.390mn. The peaks were separated with improper base line even the resolution and peaks shapes were good. So, further trials were essential.

Optimized chromatographic conditions

Optimization of mobile phase was considered from diverse parameters for example theoretical plate's count, resolution, and retention time. A mobile phase of Methanol and Water in the proportion of 65:35% v/v was found to be the most suitable for ideal separation of Ceftolozane and Tazobactam on Altima C18 4.6×150mm, 5µ column. At this ratio, mobile phase was forced with a flow rate of 1 ml/min. At this flow rate, the peaks were with good resolution. So, 1ml/min was remaining constant for entire analysis. The column was set at ambient temperature and had been equilibrated with mobile phase for 30minutes before the solutions were injected. 10µl of five replicates of both standard and sample solutions was introduced into the chromatographic system and the areas of Ceftolozane and Tazobactam peaks were measured. The detection of drugs was monitored at 285nm. The runtime was set at 15min. Under these optimized chromatographic conditions, the retention time for both the drugs was recorded from chromatogram and the % assay was also calculated using the following formula.

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg Wt}}{LC} \times 100$$

AS: Average peak area of standard preparation

AT: peak area of assay preparation,

WS: standard weight of ivacaftor/ lumacaftor in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

DS: dilution of standard preparation

P: purity of ivacaftor /lumacaftor

AV: average weight of tablets in mg

LC: labelled claim of ivacaftor/lumacaftor

Method validation

Validation of the analytical method confirm that the uniqueness of the method if they persuade the requirements of the method. After progress of method, it was intended to method validation according to ICH Q2 (R1) guidelines. Method validation includes different analytical presentation parameters such as specificity, linearity, accuracy, precision, LOD, LOQ and robustness.

RESULTS AND SCUSSION

Method development trials

Based on the observations of all trials performed in method development at various chromatographic conditions are noted in Table 1 and chromatograms are displayed from Figures 1-4, improved retention times, well resolved peaks and good peak intensity were observed at optimised chromatographic conditions are listed in Table 2. Hence, this was considered as the ultimate optimized method in the development process.

Method optimization

In simultaneous estimation of Ceftolozane and Tazobactam by RP-HPLC, the method was optimized through the evaluation of several solvent mixtures. A solvent system of Methanol: Water 65:35%v/v on Altima C18 4.6×150mm, 5µ column resulted in sharp, well-defined peaks with good resolution and low retention times were about 2.090min for Ceftolozane and 6.070min for Tazobactam at a flow rate of 1 ml/min. The optimized chromatogram is in Figure 7 and results are in Table 3. The % assay of drugs in the formulation was found to be

100.3% for Ceftolozane and 99.6% for Tazobactam respectively. Assay Results are revealed in Table 4.

Table No 1: HPLC Parameters for trials.

Parameter	Trial 1	Trial 2	Trial 3	Trial 4
Stationary phase(column)	zodiac C18 (4.6 ×150mm, 5µm)	Zodiac C18 (4.6×150mm) 5µ	Altima C18 (4.6×150mm 5µm)	Altima C18 (4.6×150mm 5µm)
solvent A	Methanol	Methanol	Methanol	Methanol
Solvent B	Water	Water	Water	Water
Elution mode	Isocartic	Isocartic	Isocartic	Isocartic
Mobile phase ratio	80:20	70:30	75:25	50:50
Flow rate	0.6 ml/min	0.7ml/min	08ml/min	0.8ml/min
Detector wave length	285 nm	285 nm	285 nm	285 nm
Detector used	PDA	PDA	PDA	PDA
Column oven temperature	ambient	ambient	ambient	ambient
Injection volume	10µl	10µl	10µl	10µl
Run time	10min	7min	10min	9min

Table No 2: HPLC Parameters for optimised method.

Parameters	Description
Stationary phase(column)	Altima C18 4.6×150mm, 5µ
solvent A	Methanol
Solvent B	Water
Elution mode	Isocartic
Mobile phase ratio	65:35
Flow rate	Iml/min
Detector wave length	285nm
Detector used	PDA
Column oven temperature	30
Injection volume	10µl
Run time	15min

Table No 3: Results for optimized parameters.

Name of the Drug	Retention time(min)	Peak Area	Resolution	Tailing factor	Theoritical plates
Ceftolozane	2.090	3468547	5.5	1.0	5565.5
Tazobactam	6.070	16289441		1.1	5355.2

Table No 4: Assay Results of Ceftolozane and Tazobactam.

parameters	Ceftolozane		Tazobactam	
	Standard	Sample	Standard	Sample
Average area	16266580	16276602.33	3438839	3468837
weight of powder	1.5gm			
Standard weight	10mg		10mg	
Sample weight	0.0102g		0.0279g	
Labeled amount	1.147g		0.537g	
Std.purity	99.7%		99.7%	
% Assay	100.3%		99.6%	

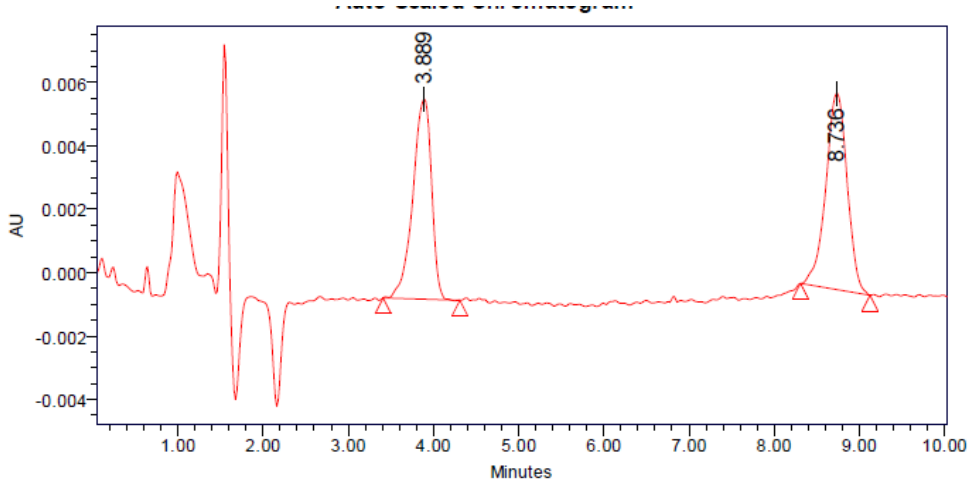

Figure 3: Chromatogram for Trial-1.

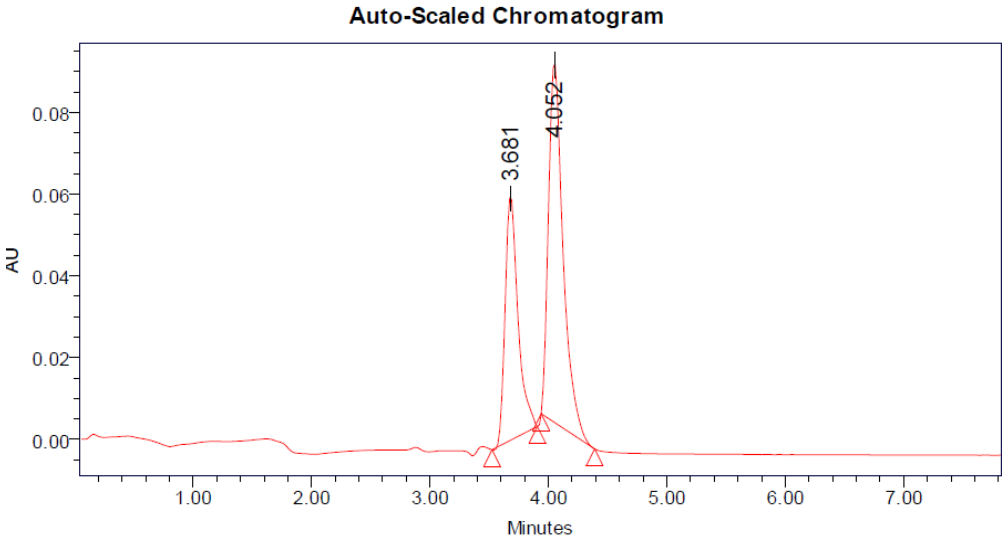

Figure 4: Chromatogram for trial 2.

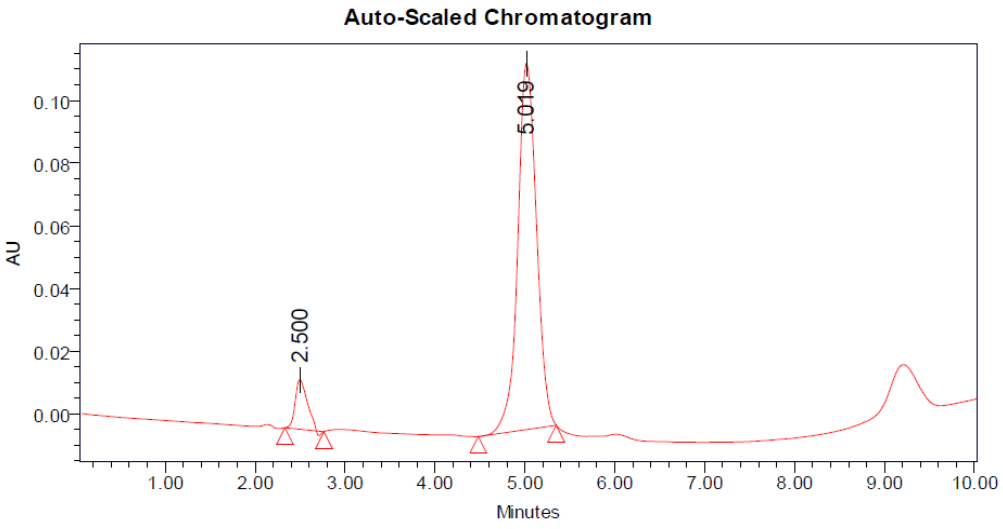

Figure 5: Chromatogram for trial 3.

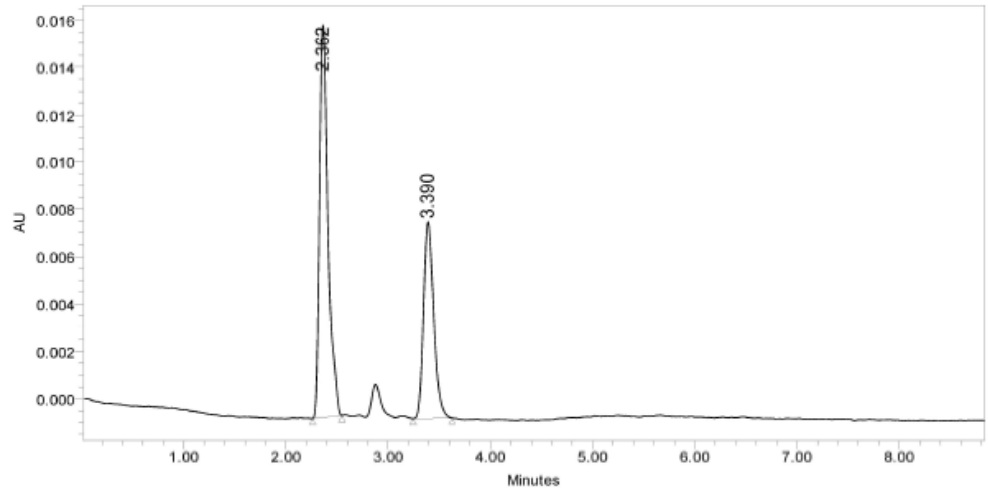

Figure 6: Chromatogram for trial 4.

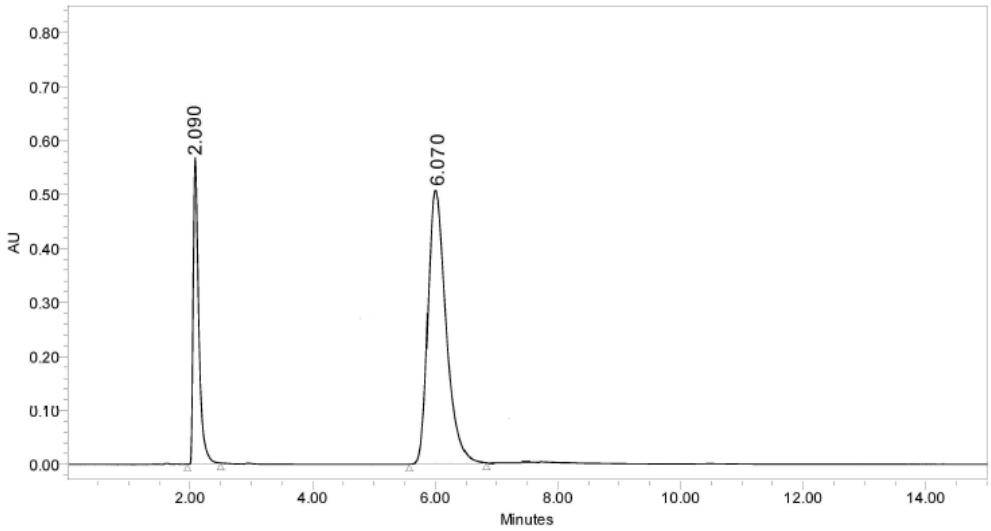

Figure 7: Optimized Chromatogram.

METHOD VALIDATION

System suitability

After equilibration of the column with mobile phase, five replicates of 10µl standard solutions were injected. The System suitability of the method was evaluated using parameters from the recorded chromatograms. The % RSD of replicating injections was less than 1%. The system suitability results are publicized in Table 5.

Table No 5: System suitability results for Ceftolozane and Tazobactam

	Ceftolozane				Tazobactam				
Injection No	**Rt (min)**	**Peak Area**	**Plate count**	**Tailing factor**	**Rt (min)**	**Peak Area**	**Plate count**	**Tailing factor**	**Resolution**
1	2.080	3569412	5568.0	1.0	6.056	3582264	5568.0	1.0	5.5
2	2.080	3465125	6359.2	1.1	6.056	3586491	5359.2	1.1	5.5
3	2.080	3598154	5565.5	1.0	6.056	3598154	5565.5	1.0	5.5
4	2.081	3586491	5355.2	1.1	6.057	3564125	5355.2	1.1	5.5
5	2.081	3582694	6348.0	1.0	6.057	3569412	5568.0	1.0	5.5
Mean		3560375				3580089			
SD		54225.61				13609.81			
%RSD		1.523031				0.380153			

Specificity

The specificity of the process was evaluated by injecting blank, sample, and standard preparations into the chromatographic system and chromatograms were compared. It was distinguished absolutely there was no interference due to excipients from the tablet dosage form and from solvent at the retention time of analytes peaks and furthermore peaks showed good resolution. The chromatogram of blank preparation is in Figure 6.

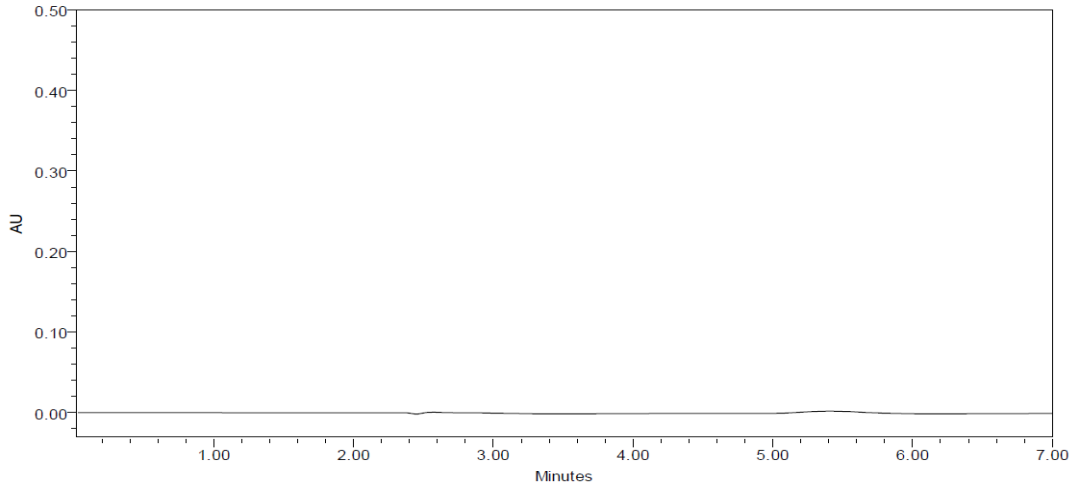

Figure 6: Chromatogram of blank preparation.

Linearity

The linearity of response (peak area) for Ceftolozane and Tazobactam was determined in a concentration range of 10-50µg/ml for Ceftolozane and 5-25µg/ml for Tazobactam. Each concentration level was injected in replicate into the HPLC system. The linearity was assessed by the value of the regression coefficient. The correlation coefficient for both drugs was 0.999 and good correlation was acquired among the peak area and concentration as in Figures 7 and 8 and the results are shown in Table 6.

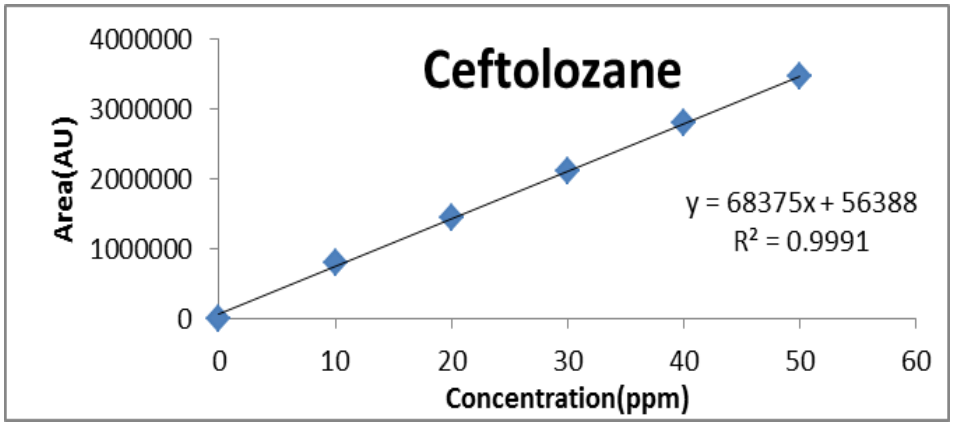

Figure 6: Calibration curve of Ceftolozone.

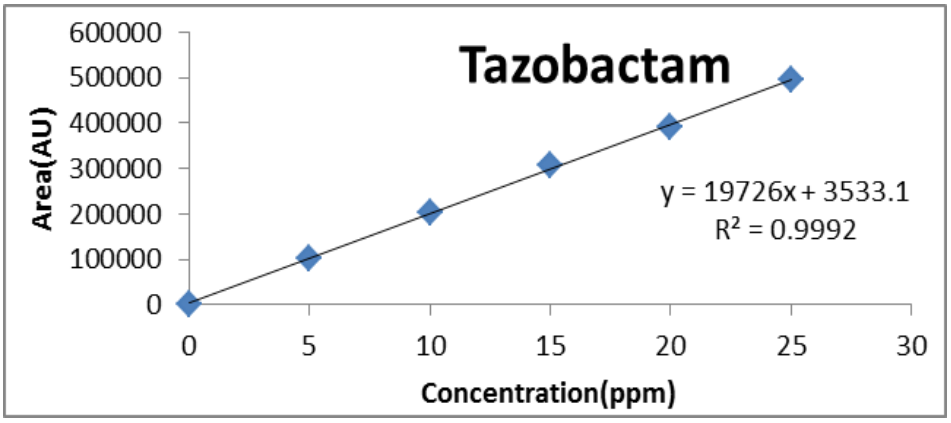

Figure 7: Calibration curve of Tazobactum.

Table No 6: Linearity results for Ceftolozone and Tazobactum.

	Ceftolozone		Tazobactum	
S. No	**Concentration (µg/ml)**	**Average Peak Area**	**Concentration (µg/ml)**	**Average Peak Area**
1	10	8040800	5	101025
2	20	1431841	10	204937
3	30	2108798	15	307270
4	40	2791392	20	392106
5	50	3458474	25	495281
Slope		68375		1972
Y-intercept		56388		3533
Correlation coefficient (R2)		0.999		0.999

Accuracy

The accuracy of the technique was resolute by recovery experiments and was performed by the standard addition method at three concentration levels of 50%, 100%, and 150%. Each strength was injected thrice into the chromatographic system and the percentage and mean % recoveries for both drugs were calculated and the results are given away in Table 7.

Table No 7: Recovery results of Ceftolozone and Tazobactum.

	Ceftolozone					Tazobactum				
Accuracy Level (%)	**Amount added (in µg)**	**Amount recovered (in µg)**	**% Recovery**	**Mean % recovery**	**%RSD**	**Amount added (in µg)**	**Amount recovered (in µg)**	**% Recovery**	**Mean % recovery**	**% RSD**
50	15	15	100	100.0	0.2	7.5	7.5	100	99.96	0.12
50	15	15.05	100.3			7.5	7.49	99.8		
50	15	14.98	99.8			7.5	7.51	100.1		
100	30	30	100	100.02	0.06	15	15	100	100.08	0.04
100	30	30.03	100.1			15	15.01	100.06		
100	30	29.98	99.96			15	15.02	100.1		
150	45	44.7	99.3	99.3	0.06	22.5	22.47	99.86	99.77	0.07
150	45	44.9	99.7			22.5	22.45	99.77		
150	45	44.6	99.1			22.5	22.43	99.68		

Precision

The precision of the method was evaluated by repeatability and intermediate precision studies. Intraday precision was assessed by six replicates of 100% accuracy and inter-day precision was evaluated by analyzing six injections of sample solution following the description of the analytical method by different analysts on dissimilar days using diverse HPLC and columns of the similar make but dissimilar lot number. The % RSD for the response factor of both drugs was found to be < 2% and results are revealed in Tables 8 and 9.

Table No 8: Repeatability Results of Ceftolozone and Tazobactum.

Injection No	**Ceftolozone**		**Tazobactum**	
	Rt (min)	**Peak Area**	**Rt (min)**	**Peak Area**
1	2.084	3569412	6.056	3582264
2	2.083	3465125	6.057	3586491
3	2.082	3598154	6.058	3598154
4	2.081	3586491	6.059	3564125
5	2.080	3582694	6.060	3569412
6	2.082	3569412	6.057	3580089
Mean	**2.082**	**3560375**	**6.057**	**13609.81**
SD	**0.001**	**54225.61**	**0.005**	**0.380153**
%RSD	**0.062**	**1.523031**	**0.08**	**3582264**

Table No 9: Intermediate precision Results of Ceftolozone and Tazobactum.

Injection No	Ceftolozone				Tazobactum			
	Rt (min)	Peak Area	Plate count	Tailing factor	Rt (min)	Peak Area	Plate count	Tailing factor
1	2.081	3481579	5568.0	1.0	6.061	15481579	5568.0	1.0
2	2.082	3458121	5359.2	1.1	6.062	15369852	5359.2	1.1
3	2.083	3426581	5565.5	1.0	6.063	15248454	5565.5	1.0
4	2.084	3465712	5355.2	1.1	6.064	15874692	5355.2	1.1
5	2.085	3451476	5568.0	1.0	6.064	15236547	5568.0	1.0
6	2.085	3452106	5359.2	1.1	6.064	15217547	5359.2	1.1
Mean	**2.083**	**3455929**			**6.063**	**15404779**		
SD	**0.004**	**18188.92**			**0.003**	**251289.4**		
%RSD	**0.19**	**0.5**			**0.04**	**1.6**		

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection and quantification was calculated from the signal to noise ratio. This ratio for LOD is 3:1 and LOQ is 10:1. The limit of detection and quantification was evaluated from the calibration curves by applying statistical calculations and results are shown in Table 10.

The limit of detection and quantification were expressed as:

$LOD = 3.3\ \sigma/S$

$LOQ = 10.0\ \sigma/S$ Where;

σ = Standard deviation of the response

S = Slope of the regression line

Table No 10: LOD and LOQ results for Ceftolozone and Tazobactum.

Name of the analyte	LOD µg/ml	LOQ µg/ml
Ceftolozone	0.8	2.5
Tazobactum	0.9	2.9

Robustness

Robustness of the method was demonstrated by analyzing the system suitability parameters under intentionally modified chromatographic conditions such as flow rate, and mobile phase ratio on the lower and higher side of the normal values. There was no considerable change in the retention time between the original method and modifications to the method. The results are illustrated in Table 11.

Table No 11: System suitability results for robustness study of Ceftolozone and Tazobactum.

Robust condition	Ceftolozone				Tazobactum			
	Rt (min)	Peak Area	Tailing	Plate count	Rt (min)	Peak Area	Tailing	Plate count
Normal	2.088	3425413	1.0	5568.2	6.068	2029854	1.1	5359.2
Flow rate 0.9 ml	3.111	3425282	1.2	5922.2	7.101	1738319	1.2	5999.1
Flow rate1.1 ml	1.880	3517879	1.2	5868.8	5.007	1638304	1.1	5989.2
Mobile phase 60:40v/v	3.101	3175485	1.2	5836.2	7.108	1973724	1.1	5387.2
Mobile phase 70:30v/v	1.881	3365431	1.1	5282.6	5.008	2102838	1.1	5938.1

CONCLUSION

In the present study, quantitative method of analysis of Ceftolozane and Tazobactum in injection was developed. The proposed solvent system gives good peak symmetry and resolution between the peaks and method is specific as no interference is observed from the solvent. ICH Q2 (R1) guidelines were used for validation of the meyhod. As of the experimental and validation results, the urbanized method for the concurrent estimation of Ceftolozane and Tazobactum in injection by RP-HPLC is simple, appreciably sensitive, precise, accurate, and high resolution. Also, the lesser solvent consumption and shorter retention time show the way to more acceptable, commercial and Eco- friendly chromatographic procedures. Hence, it can be suitably adopted for routine analysis of API content in the pharmaceutical formulations of Ceftolozane and Tazobactum in Educational institutions and Quality control laboratories.

CONFLICTS OF INTEREST

The authors have indicated that they have no competing interest regarding the content of this article.

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