

Simultaneous Determination of Cefepime and Tazobactam in Injectables by Ultra-High Performance Liquid Chromatography Method

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ABSTRACT

Aim: Stability indicating ultra-high performance liquid chromatography (UHPLC) method was developed and validated for the determination of cefepime (CFPM) and tazobactam (TZB) in injectable dosage form. **Materials and Methods:** Separation was performed in a Dionex Ultimate 3000 UHPLC system equipped with chromeleon software using Acclaim 120 C₁₈ (250 × 4.6 mm, 5 μm particle size) column with mobile phase (pH 6.0) containing methanol and sodium acetate buffer in the ratio of 11:89 v/v with a flow rate of 1.8 mL/min and detection wavelength of 220 nm. Stress studies were performed using HCl, NaOH, H₂O₂, and ultraviolet radiation. **Results:** The method was found to be linear in the concentration range of 50-350 μg/ml (R² = 0.998) and 6.25-43.75 μg/ml (R² = 0.998) with the regression equation $y = 11068x + 115231$ and $y = 8317.1x - 9869.7$ for CFPM and TZB, respectively. The percentage of relative standard deviation (%RSD) of 0.63 and 1.39 for intra-day and 0.64 and 0.54 for inter-day precision, respectively for CFPM and TZB suggest the precision of the method as all these values are <2%. It was found from the stress studies that both the drugs are very susceptible to alkaline condition. The method has shown good, consistent recoveries for CFPM (100.6-102.0%) with mean RSD of 0.73% and TZB (98.4-100.6%) with mean RSD of 1.14%, which indicates the method is sufficiently accurate. **Conclusion:** The method was found to be accurate, precise, specific, robust, linear, and stability indicating for the determination of CFPM and TZB in injectable dosage form.

Keywords: Cefepime, injectables, simultaneous determination, tazobactam, ultra high performance liquid chromatography

INTRODUCTION

Cefepime (CFPM) is a broad spectrum fourth generation, semi synthetic cephalosporin effective against both Gram-negative as well as Gram-positive organisms.¹ It is chemically ([6R,7R,Z]-7-[2-(2-aminothiazol-4-yl)-2-(methoxyimino) acetamido]-3-[1-methyl pyrrolidinium-1yl] methyl)-8-oxo-5-thia-1-aza-bicyclo[4.2.0] oct-2-ene-2 carboxylate, as given in Figure 1, very much similar to the third-generation Cephalosporins structurally, except that it

has a N-methylpyrrolidinium at the 3-position, rendering it a zwitterion.^{1,2} The quantitative determinations of CFPM have been proposed by several methods, including the second-derivative spectrophotometry,³ micellar capillary electrokinetic chromatography,⁴ polarographic technique,⁵ liquid chromatography-ultraviolet (LC-UV) methods⁶⁻⁸ and LC-mass spectrometry.⁹ Tazobactam (TZB) is chemically (2S,3S,5R)-3-methyl-7-oxo-3-(1H-1,2,3-triazol-1-ylmethyl)-4-thia-1 azabicyclo [3.2.0] heptane-2-carboxylic acid 4,4-dioxide¹⁰ (Figure 2). It belongs to a class of penicillanic acid sulfones which acts by inhibiting bacterial β-lactamases. Infection caused by β-lactamase producing bacterial strains has recently become a major problem in hospitals. Several β-lactamase inhibitors have been developed against the target enzyme.¹¹ When they are combined with some penicillins or cephalosporins, the mixed ingredients have

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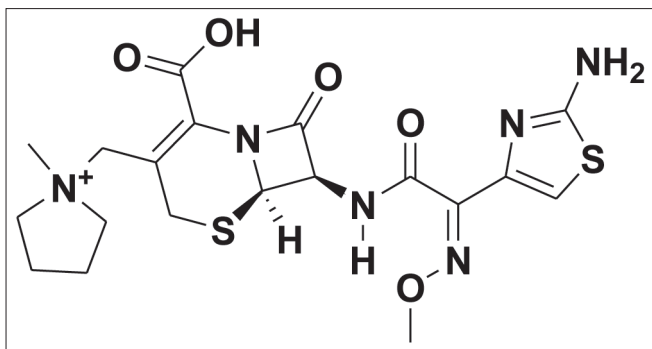


Figure 1. Chemical structure of cefepime.

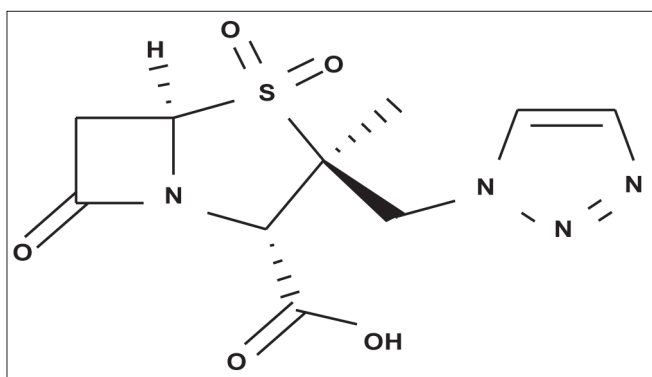


Figure 2. Chemical structure of tazobactam.

been shown to be effective against various β -lactamase producing bacteria both *in vitro* and *in vivo*.¹²⁻¹⁵ Various methods are available for the analysis of TZB in biological samples¹⁶⁻¹⁸ or in pharmaceutical formulations¹⁹⁻²¹ in combination with different drugs by high performance liquid chromatography (HPLC). An exhaustive review of the various analytical methods available for these drugs had been carried out by the authors.²² Until date, there is only one spectrophotometric method²³ and one chromatographic method²⁴ available for the simultaneous analysis of CFPM and TZB. However, these methods are suffering from various drawbacks such as use of very high buffer concentration, very low UV detection wavelength of 210 nm, very narrow linearity range of 4-24 $\mu\text{g}/\text{ml}$ for CFPM and 0.5-3 $\mu\text{g}/\text{ml}$ for TZB, very long run time of 12 min and mobile phase pH which is outside the region where the drug is most stable. Both these drugs CFPM and TZB contains a four member β -lactam ring, which is inherently strained and prone to hydrolysis and photolysis, limiting its stability.²⁵ CFPM is particularly labile, and its stability is highly pH dependent.²⁶ The most suitable pH region for the maximum stability of CFPM is from 4 to 6.²⁷ Thus, in order to overcome the existent limitations and the problem of unavailability of a simple method of analysis, the authors have two main objectives for the present work. The first objective is to develop

and validate a vastly improved HPLC assay method for simultaneous determination of CFPM and TZB in pure drugs and injectable dosage forms. The second objective is to develop the method using a pH value at which maximum stability of the drugs is reported.

MATERIALS AND METHODS

Reagents and chemicals

Qualified standards of CFPM and TZB were obtained as gift samples from Alkem Laboratories Limited, Sikkim, India. Methanol (HPLC grade), sodium acetate and acetic acid (AR grade) were obtained from S.D. Fine Chemicals Limited., Mumbai, India. HPLC grade water was obtained from Millipore direct Q3 (India). Commercially available sterile powder for injection vial (Actamase, Astra Zeneca) was procured from the local market.

Chromatography instruments and conditions

The chromatograph consisted of a Dionex Ultimate 3000 Ultra-HPLC (UHPLC) system with quaternary pump, auto injector, vacuum degasser, Ultimate 3000 diode array detector with Dionex Chromeleon software for data evaluation. The separation was accomplished using an Acclaim 120 C₁₈ (250 \times 4.6 mm i.d, 5 μm particle size) column and a mobile phase consisting of methanol and sodium acetate buffer (pH 6, adjusted with dilute acetic acid) in the ratio of 11:89 v/v in the isocratic mode with a flow rate of 1.8 ml/min. The column eluents were monitored at 220 nm with the overall run time of 7 min and the injection volume of 20 μl . All the solutions were filtered and degassed before use.

Preparation of standard solution

CFPM and TZB stock solutions were prepared by dissolving 100 mg and 12.5 mg of these drugs in the mobile phase to obtain the concentration of 2000 $\mu\text{g}/\text{ml}$ and 250 $\mu\text{g}/\text{ml}$, respectively. These were further diluted by mobile phase 10 times to obtain the concentration of 200 $\mu\text{g}/\text{ml}$ and 25 $\mu\text{g}/\text{ml}$ of CFPM and TZB, respectively.

Preparation of sample solution

An amount of injectable sample containing 100 mg CFPM and 12.5 mg TZB were transferred to a 50 ml volumetric flask, dissolved with 30 ml of mobile phase and sonicated for 10 min and finally made up the volume with the mobile phase. From this solution, 5 ml was transferred in a 50 ml volumetric flask and the volume was made up with the mobile

phase. This solution was filtered through a 0.45 μ membrane filter before use. The assay was performed 3 times individually weighing the respective injectable powder.

System suitability

The various system suitability parameters such as tailing factor, theoretical plates, resolution, and percentage of relative standard deviation (%RSD) of the area of five replicate injections were evaluated to verify that the analytical system is working properly and can give accurate and precise results.

Analytical method validation

It was performed as per International Conference on Harmonisation guidelines²⁸ and other available literatures.²⁹⁻³³

Specificity

It is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. The sample was subjected to various stress conditions such as acidic (0.1M HCl for 30 min) and basic (0.1M NaOH for 2 min) hydrolysis, oxidative (5% H₂O₂ for 30 min) degradation and photolytic degradation (UV light for 24 h). The chromatographic interference due to the presence of degraded products was studied. The blank chromatogram was also compared with the standard chromatograms to check the interference due to blank. Finally, the degradation in the various conditions were calculated with respect to the fresh sample.

Linearity

The linearity of the method was established by constructing calibration curves over a concentration range of 50-350 μ g/ml for CFPM and 6.25-43.75 μ g/ml for TZB. After injecting each solution into the UHPLC system the peak area of the chromatogram obtained was noted. The peak area against the corresponding analyze concentration was plotted and the slope, intercept and correlation coefficient were determined using linear regression analysis.

Precision

Precision of the method was evaluated in terms of intra- and inter-day precision. Intra-day precision was reported as %RSD on six separate weights of the sample at 100% test concentration against a qualified reference standard. Inter-day precision was also carried out similarly but in two different days and the %RSD was calculated.

Accuracy

Accuracy of the method was demonstrated by standard addition method at three levels. Known amounts of CFPM standard (50, 100, and 150 μ g/ml) and TZB standard (6.25, 12.50, and 18.75 μ g/ml) were added to the already analyzed sample solutions, and the analysis was carried out as per the method. At each level of addition, the procedure was repeated for three times.

Robustness

The robustness of the method was determined by introducing small deliberate variations in method parameters such as flow rate (1.7 and 1.9 ml/min), percentage of methanol in the mobile phase (10% and 12%) and pH (5.8 and 6.2). Only one parameter was changed at a time, and for all the changes the sample was analyzed in triplicate.

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were determined by the standard deviation of the response and the slope. For LOD and LOQ determination a formula of $3.3 \sigma/S$ and $10 \sigma/S$ was used respectively, where ' σ ' is the standard deviation of the y-intercept and 'S' is the slope of the calibration curve.

Solution stability

The stability of CFPM and TZB in the mobile phase was evaluated at room temperature by keeping the diluted test sample in a tightly closed volumetric flask and analyzing at 1 h interval against a freshly prepared standard solution. The %RSD of the peak areas obtained for the test samples were determined in different time intervals.

RESULTS AND DISCUSSION

Method development

The chromatographic conditions were optimized in such a manner to achieve good peak shapes and high resolution for CFPM and TZB. Internal standard was not used because it was not necessary as no extraction or separation step was involved.³⁴ Different mobile phase combinations containing methanol and sodium acetate buffer were tried. The pH of the buffer was adjusted to 6.0 by acetic acid so that it is at least 1.5 pH unit apart from the pKa of drugs for the pH ruggedness of the method. Moreover, highest stability of CFPM is also in the pH range of 4-6. Cephalosporins are highly degradable drugs due to their β -lactam ring thus for longer solution stability it is necessary to develop analytical methods at the pH at which the drug is most stable. The methanol content was also optimized for better resolution, selectivity and acceptable retention factor. Finally, a mobile phase containing methanol and

sodium acetate buffer (pH 6.0, adjusted with acetic acid) in the ratio of 11:89 v/v was selected as optimum for the estimation of CFPM and TZB with the retention time of 4.9 and 3.9 min, respectively.

Method validation

System suitability

A representative chromatogram for system suitability test is shown Figure 3 which displays a tailing factor of 1.2 for CFPM and 1.29 for TZB (<1.5 for both the peaks) with a resolution of 4.9 (more than 2). The %RSD of five replicate injections were 0.1 and 0.2 (<2%) with theoretical plates of 8,400 and 10,500 (more than 2,000) respectively for CFPM and TZB.

Specificity

The presence of degraded product peaks did not interfere with neither CFPM nor TZB peaks which establishes specificity and stability indicating the nature of the method. The blank chromatogram was also compared with the sample chromatogram, and it was evident that there is no interference from the blank as no peak appears in the retention time of either CFPM or TZB. The representative chromatograms of blank along with various stress conditions samples are presented (Figures 4-8). The percentage of degradation was also determined in the various stressed conditions and the data are presented in Table 1. The highest degradation was observed in alkali hydrolysis.

Linearity

The linearity plot was constructed by using the data for the peak areas obtained with their corresponding

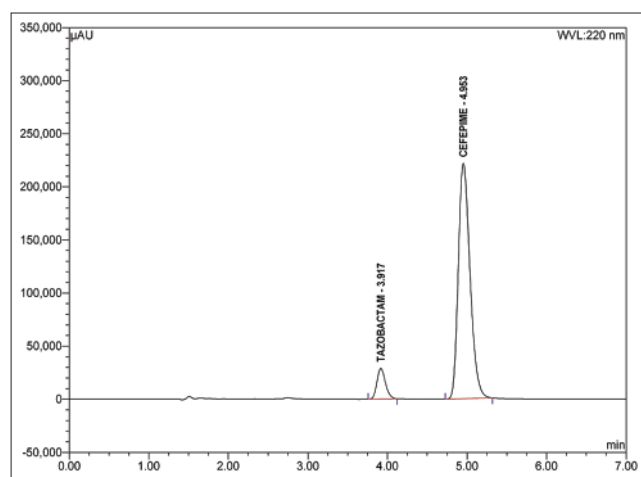


Figure 3. Typical UHPLC chromatogram of cefepime and tazobactam Rt at 4.95 ± 0.013 min and 3.92 ± 0.012 min.

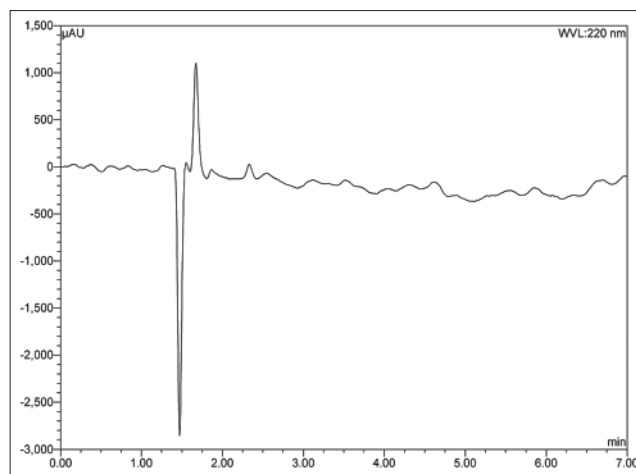


Figure 4. Blank chromatogram.

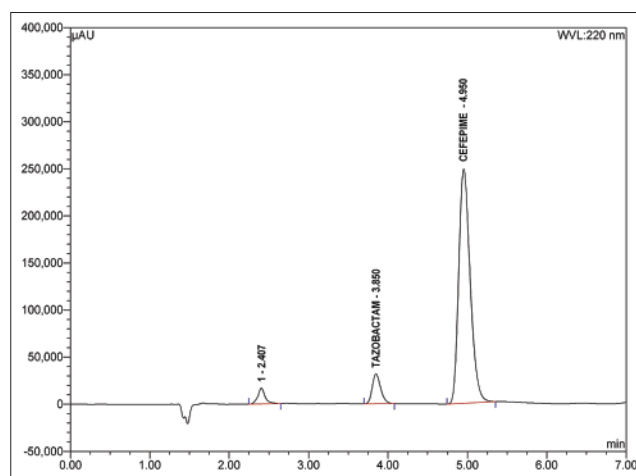


Figure 5. Cefepime and tazobactam degraded in 0.1 M HCl for 30 min at room temperature; degradation product peak R_t 2.047 min.

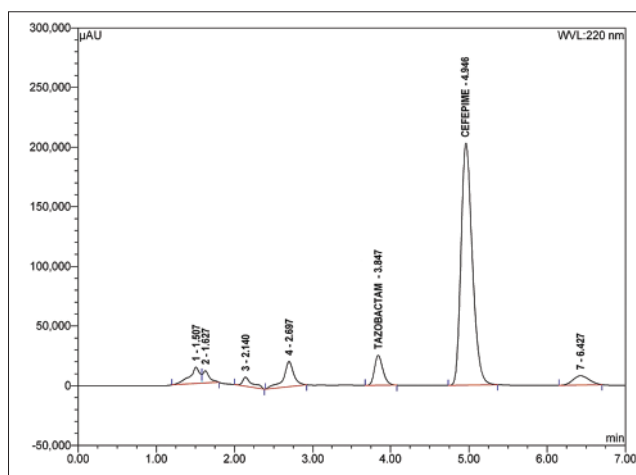


Figure 6. Cefepime and tazobactam degraded in 0.1 M NaOH for 2 min at room temperature; degradation product peaks R_t 1.507, 1.627, 2.140, 2.697, and 6.427 min.

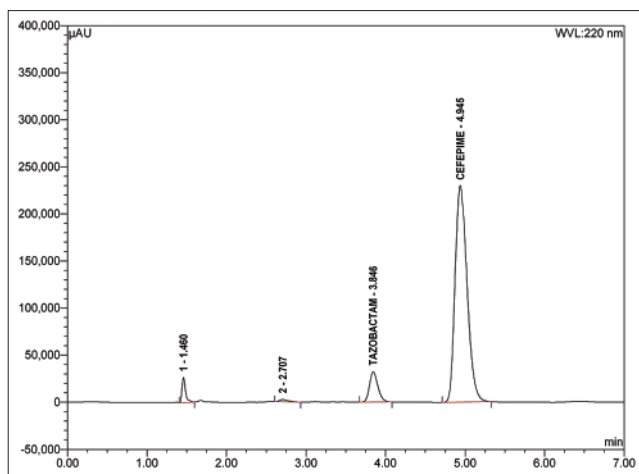


Figure 7. Cefepime and tazobactam degraded in H₂O₂ (5% v/v) for 30 min at room temperature; degradation product peaks R_t 1.450 and 2.707 min.

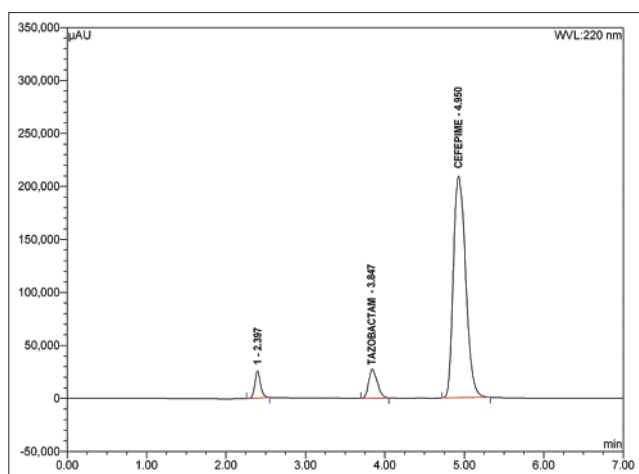


Figure 8. Cefepime and tazobactam degraded in UV light exposed for 24 h; degradation product peak R_t 2.397 min.

concentrations. The linearity plot was constructed within the concentration range that was selected on the basis of the anticipated drugs concentration during the assay of the dosage form. The method obeyed linearity in the concentration range of 50-350 µg/ml for CFPM and 6.25-43.75 µg/ml for T'ZB. The correlation coefficient value obtained was 0.998 for both the drugs with the regression equations of $y = 11068x + 115231$ and $y = 8317.1x + 9869.7$ for CFPM and T'ZB, respectively (Table 2).

Precision

The data obtained from the precision experiments are presented in Table 3. The method was found to be highly precise as the values of %RSD for intra-day precision were 0.78 and 1.58 and for inter-day precision were 0.70 and 0.59 for CFPM and T'ZB, respectively.

Table 1 Degradation study result

Stress parameters	Sample treatment	Cefepime		Tazobactam	
		Assay (%)	Degradation (%)	Assay (%)	Degradation (%)
Reference	Fresh solution	100.97	0	101.20	0
Acid hydrolysis	0.1 M HCl for 30 min	99.74	1.22	100.50	0.69
Base hydrolysis	0.1 M NaOH for 2 min	81.63	19.16	81.01	19.95
Oxidative degradation	5% H ₂ O ₂ for 30 min	93.15	7.09	94.49	6.64
Light degradation	UV light for 24 h	93.81	7.75	95.52	5.62

UV: Ultraviolet

Table 2 Regression data and sensitivity of the method

Drug name	Regression equation*	Correlation coefficient (R ²)	LOD (µg/ml)	LOQ (µg/ml)
Cefepime	$y = 11068x + 115231$	0.998	0.46	1.40
Tazobactam	$y = 8317.1x + 9869.7$	0.998	0.28	0.86

*Average of three readings. LOD: Limit of detection, LOQ: Limit of quantitation

Table 3 Precision study result of the method

Drug name	Drug concentration (µg/ml)	Intra-day precision (n=6)		Inter-day precision (n=6)	
		Mean peak area±SD	RSD	Mean peak area±SD	RSD
Cefepime	200	2539883±19887	0.78	2477744±17235	0.70
Tazobactam	25	235262±3728	1.58	229305±1351	0.59

RSD: Relative standard deviation

Accuracy

The method has shown good consistent recoveries for CFPM (100.6-102.0%) with mean RSD of 0.73% and T'ZB (98.4-100.6%) with mean RSD of 1.14%. Thus, it can be concluded that the recovery of the active substances from its matrix was correct, and the proposed method is sufficiently accurate. The data for the recovery study has been presented in Table 4.

Robustness

The small deliberate variations of method parameters did not affect the performance of the method significantly. The results obtained in all the changed conditions were in accordance with the original condition. The RSD of the percentage purity obtained in all the changed conditions were <2% which proves that the proposed method is sufficiently robust.

Solution stability

The RSD of the peak areas of CFPM and T'ZB in the sample were estimated. The RSD values of the peak areas were <1% for T'ZB for 7 h only, but CFPM was stable

even after this point of time. Hence, the sample prepared should be used within this stability period only.

LOD and LOQ

LOD was determined to be 0.46 µg/ml and 0.28 µg/ml while LOQ was determined to be 1.40 µg/ml and 0.86 µg/ml for CFPM and TZB, respectively.

Analysis of commercial formulations

The developed method was applied in the assay of CFPM and TZB in injectable formulation (Table 5). All the results obtained were tested by Student 't' test, and it did not show any significant difference with the label claimed of the drugs. The results obtained were also evaluated at 95% confidence interval to check that the determined amounts were within the limit. Almost all results were in the confidence interval for both drugs suggesting the analysis in injectable formulation were sufficiently accurate.

CONCLUSION

There is no official method available for the simultaneous determination of CFPM and TZB. The proposed method is an attempt to develop a simultaneous UHPLC procedure for the analysis of CFPM and TZB in the injectable dosage form. The results of the validation study exhibited correct estimation of drugs with acceptable linearity, accuracy and precision. The specificity study results suggested that there was no interference from any components of formulation or degradation products. The sample is stable for 7 h at the room temperature in the diluents used which overcomes the problem of fresh sample preparation every time. Thus, the method can be used for the routine quality control and stability sample analysis of CFPM and TZB.

Table 4 Accuracy study result of the method

Drug name	Level of addition (%)	Amount expected (µg/ml)	Mean recovery ±SD (n=3)	RSD (n=3)	Recovery ±SD (n=9)	RSD (n=9)
Cefepime	50	50	102.04±0.18	0.17	101.37±0.74	0.73
	100	100	100.62±0.62	0.62		
	150	150	101.47±0.47	0.46		
Tazobactam	50	6.25	98.43±0.40	0.40	99.29±1.14	1.14
	100	12.5	98.80±0.32	0.32		
	150	18.75	100.62±0.89	0.88		

RSD: Relative standard deviation

Table 5 Assay result for cefepime and tazobactam injectable formulation

Drug name	Amount declared (mg)	Mean recovery (%) ±SD*	95% confidence interval	RSD
Cefepime	1000	100.40±0.73	98.58-102.21	0.73
Tazobactam	125	99.12±0.52	97.83-100.41	0.52

*Average of three readings, RSD: Relative standard deviation

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