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A VALIDATED STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF TOBRAMYCIN AND CEFTAZIDIME IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

The proposed study, a new stability- indicating RP-HPLC method has been developed for estimation of Tobramycin and Ceftazidime in bulk and pharmaceutical dosage form. The present method was a sensitive, precise, and accurate RP-HPLC method for the analysis of Tobramycin and Ceftazidime. To optimize the mobile phase, various combinations of buffer and organic solvents were used on Hypersil BDS C18 (250mm X 4.6 mm, 5µ) column. Then the mobile phase containing a mixture of Phosphate Buffer:Acetonitrile in the ratioof 55:45%v/v was selected at a flow rate of 1.0 ml/min for developing the method and the peaks with good shape and resolution were found resulting in short retention time, baseline stability and minimum noise. The retention times of Tobramycin and Ceftazidime were found to be 4.255 and 2.823 min respectively. Quantitative linearity was obeyed in the concentration range of 7.5-30 and 62.5-375 µg/mL of Tobramycin and Ceftazidime respectively. The limit of detection and limit of quantitation were found to be 0.020 µg/mL and 0.061µg/mL (TOBRA); 0.246 µg/ mL and 0.746 µg/mL (CEFTA) respectively, which indicates the sensitivity of the method. The high percentage recovery indicates that the proposed method is highly accurate. No interfering peaks were found in the chromatogram indicating that excipients used in injection formulations didn't interfere with the estimation of the drugs by the proposed HPLC method.

Keywords: Liquid chromatography, Tobramycin and Ceftazidime

INTRODUCTION

Tobramycin binds irreversibly to one of two aminoglycoside binding sites on the 30 S ribosomal subunit, inhibiting bacterial protein synthesis. Tobramycin may also destabilize bacterial memebrane by binding to 16 S 16 S r-RNA. An active transport mechanism for aminoglycoside uptake is necessary in the bacteria in order to attain a significant intracellular concentration of tobramycin..The chemical structure of Tobramycin was given in fig 1.

Ceftazidime is a semisynthetic, broad-spectrum, beta-lactam antibiotic for parenteral administration. Ceftazidime is bactericidal in action exerting its effect by inhibition of enzymes responsible for cell-wall synthesis, primarily penicillin binding protein 3 (PBP3). A wide range of gram-negative organisms is susceptible to ceftazidime in vitro, including strains resistant to gentamicin and other aminoglycosides. In addition, ceftazidime has been shown to be active against gram-positive organisms. It is highly stable to most clinically important beta-lactamases, plasmid or chromosomal, which are produced by both gram-negative and gram-positive organisms and, consequently, is active against many strains resistant to ampicillin and other cephalosporins. Ceftazidime has activity against the gram-negative organisms Pseudomonas and Enterobacteriaceae. Its activity against Pseudomonas is a distinguishing feature of ceftazidime among the cephalosporins.The chemical structure of Ceftazidime was given in fig 2.

The review of literature revealed that several analytical methods have been reported for Tobramycin, Ceftazidime [7-13] in spectrophotometry, HPLC, HPTLC, LC/MS individually and in

combination. To date, there have been no published reports about the stability indicating studies and simultaneous estimation of Tobramycin and Ceftazidime by HPLC in bulk drug and in pharmaceutical dosage forms. This present study reports for the first time stability indicating simultaneous estimation of Tobramycin and Ceftazidime by RP-HPLC in bulk drug and in pharmaceutical dosage form.

MATERIALS AND METHODS

Chemicals and Reagents: Tobramycin and Ceftazidime were obtained as gift sample from Spectrum Pharma Research laboratory in Hyderabad and marketed formulation was purchased from local market. Acetonitrile, Water, were obtained from Merck. Mumbai and Potassium dihydrogen ortho phosphate, Triethylamine, Ortho Phosphoric Acid obtained from RANKEM Mumbai. All solvents used in this work are HPLC grade.

Instrument and chromatographic condition: RP-HPLC waters 2695 separation module equipped with 2996 Photodiode Array Detector was employed in this method. The Empower 2 software was used for LC peak integration along with data acquisition and data processing. The column used for separation of analytes is Hypersil BDS C18, (250 x 4.6 mm, 5µ). Mobile phase consisting of Phosphate Buffer: Acetonitrile in the ration of 50:50 % v/v at a flow rate of 1.0 ml/min. It was filtered through 0.45µm nylon filter and sonicated for 5 min in ultrasonic bath. Samples were analysed at 260 nm at an injection volume of 10 µL.

Preparation of Phosphate Buffer pH 3.6: Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then PH adjusted to 3.6 with dil. Orthophosphoric acid solution.

Preparation of Solutions:

Tobramycin stock preparation (300µg/ml): Accurately weighed and transferred 3 mg of Tobramycin in to 10ml of clean dry volumetric flask, add 7ml of diluent (water : acetonitrile 50:50), then sonicated for 10min and make up the volume with diluent.

Ceftazidime stock preparation (2500µg/ml): Accurately weighed 25mg of Ceftazidime and transferred into 10ml of clean dry volumetric flask, add 7ml of diluent (water : acetonitrile 50:50), then sonicated for 10 min and make up the final volume with diluent.

Standard Preparations:

Tobramycin Standard Preparation *(30µg/ml):*From the above Tobramycin stock solution 1ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent.

Ceftazidime Standard Preparation *(250µg/ml):* From the above Ceftazidime stock solution 1ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent.

METHOD VALIDATION

The validation of the method was carried out as per ICH Guidelines **.** The parameters assessed were specificity, linearity, precision, accuracy, stability, LOD and LOQ.

Specificity: Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences including degradation products and related substances.

Accuracy: The accuracy was determined by calculating % recoveries of Tobramycin and Ceftazidime. It was carried out by adding known amounts of each analyte corresponding to three concentration levels (50, 100, and 150%) of the labelled claim to the excipients. At each level, six determinations were performed and the accuracy results were expressed as percent analyte recovered by the proposed method.

Precision: Precision of an analytical method is usually expressed as the standard deviation. The repeatability studies were carried out by estimating response of Tobramycin and Ceftazidime six times. The intra-day and inter-day precision studies (intermediate precision) were carried out by estimating the corresponding responses three times on the same day and on three different days for three same concentrations and the results are reported in terms of relative standard deviation.

Linearity: The purpose of the test for linearity is to demonstrate that the entire analytical system (including detector and data acquisition) exhibits a linear response and is directly proportional over the relevant concentration range for the target concentration of the analyte. The linear regression data for the calibration plot is indicative of a good linear relationship between peak area and concentration over a wide range. The correlation coefficient was indicative of high significance.

Robustness: Robustness of the method was investigated under a variety of conditions including changes of composition of buffer in the mobile phase, flow rate and temperature. This deliberate change in the method has no affect on the peak tailing, peak area and theoretical plates and finally the method was found to be robust.

Limit of Detection & Limit of Quantitation: The LOD can be defined as the smallest level of analyte that gives a measurable response and LOQ was determined as the lowest amount of analyte that was reproducibly quantified. These two parameters were calculated using the formula based on the standard deviation of the response and the slope. LOD and LOQ were calculated by using equations, LOD=3.3 × s/s and LOQ=10 × s/s, where s = standard deviation, S= slope of the calibration curve.

Assay of Tobramycin and Ceftazidime in injection: Assay of marketed product was carried out by using the developed method. Sample solutions were prepared and injected into RP-HPLC system. The sample solution was scanned at 260 nm. The % drug estimated was found to be 99.34 and 99.17% respectively as tobramycin and ceftazidime. The chromatogram showed two single peaks of Tobramycin and Ceftazidime was observed with retention times of 4.255 and 2.823 min (Figure 3)

Forced Degradation studies

Stress studies are performed according to ICH guidelines under conditions of hydrolysis (acidic and alkaline), photolysis, oxidation, and thermal studies.

Oxidation: To 1 ml of stock solution of Tobramycin and Ceftazidime, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain 30µg/ml & 250µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies: To 1 ml of s tock s solution Tobramycin and Ceftazidime, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c .The resultant solution was diluted to obtain 30µg/ml & 250µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies: To 1 ml of stock solution Tobramycin and Ceftazidime, 1ml of 2 N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 30µg/ml & 250µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies: The standard drug solution was placed in oven at 105^0c for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 30µg/ml & 250µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies: The photochemical stability of the drug was also studied by exposing the 30µg/ml & 250µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 30µg/ml & 250µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

RESULTS & DISCUSSIONS

Optimized Chromatographic conditions: To establish and validate an efficient method for analysis of these drugs in pharmaceutical formulations, preliminary tests were performed. Different chromatographic conditions were employed for the analysis of the Tobramycin and Ceftazidime in both bulk and pharmaceutical dosage form. Finally the analysis was performed by using Phosphate Buffer: Acetonitrile in the ration of 50:50 % v/v at a flow rate 1.0 ml/min. Samples were analysed at 260nm at an injection volume of 10 µL and separation was carried by using Hypersil BDS C18, (250 x 4.6 mm, 5µ) column. The proposed method was optimized to give a sharp peak with minimum tailing for Tobramycin and Ceftazidime. (Fig 4). The optimized conditions were given in table 1.

Forced degradation studies were performed to establish the stability indicating property and specificity of the proposed method. Degradation studies were carried out under conditions of hydrolysis, dry heat, oxidation, UV light and photolysis and the drug substances were degraded in all conditions. Acid and base hydrolysis was performed by exposing the drug substances with 2N HCl and 2N NaOH at 60 ^{o}C for 30min and it was showed degradation of Tobramycin and Ceftazidime with degraded products peak at retention time 2.637 min. Degradation studies under oxidative conditions were performed by heating the drug sample with 20% H_2O_2 at 60 ^{o}C and degraded product peaks were observed. Both Tobramycin and Ceftazidime are sensitive to acid and alkali and there was no

degradation occurs under UV light and thermal conditions. The results of forced degradation studies were given in table 2. Precision was evaluated by a known concentration of Tobramycin and Ceftazidime was injected six times and corresponding peaks were recorded and % RSD was calculated and found within the limits. The low % RSD value was indicated that the method was precise and reproducible and the results were shown in the table (Table 3). Accuracy of the method was proved by performing recovery studies on the commercial formulation at 50, 100 and 150% level. % Recoveries of Tobramycin and Ceftazidime ranges from 100.91 to 99.76% in simultaneous equation method and the results were shown in the (Table 4). Linearity was established by analyzing different concentrations of Tobramycin and Ceftazidime respectively. The calibration curve was plotted with the area obtained versus concentration of both Tobramycin and Ceftazidime (Fig 5&6). In the present study six concentrations were chosen ranging between 7.5-45 µg/mL of Tobramycin and 62.5-375µg/mL of Ceftazidime. The regression equation and correlation coefficient for Tobramycin and Ceftazidime was found to be y = 6480.x + 298.1. And R^2=0.9990 and y = 3222.x + 2111. And R^2=0.9990 respectively and results were given in table 5. Robustness of the method is the ability of the method to remain unaffected by small deliberate changes in parameters like flow rate, mobile phase composition and column temperature. To study the effect of flow rate of the mobile phase it was changed to 0.1 units from 1.0 mL to 0.9 mL and 1.1 mL. The effect of column temperature also checked by changing temperature to ± 5 ^{o}C. This deliberate change in the above parameters has no significant effect on chromatographic behaviour of the samples and results were given in table 6. LOD and LOQ of Tobramycin and Ceftazidime were evaluated based on relative standard deviation of the response and slope of the calibration curve. The detection limits were found to be 0.020µg/mL and 0.061µg/mL for Tobramycin and Ceftazidime respectively. The quantitation limits were found to be 0.246µg/mL and 0.746µg/mL for Tobramycin and Ceftazidime respectively. The results were given in the table 7.

CONCLUSION

A new stability- indicating RP-HPLC method has been developed for estimation of Tobramycin and Ceftazidime in bulk and pharmaceutical dosage form. The developed method was validated and it was found to be simple, sensitive, precise, robust and it can be used for the routine analysis of Tobramycin and Ceftazidime in both bulk and pharmaceutical dosage forms. The forced degradation studies were carried out in accordance with ICH guidelines and the results revealed suitability of the method to study stability of Tobramycin and Ceftazidime under various degradation conditions like acid, base, oxidative, thermal, UV and photolytic degradations. Finally it was concluded that the method is simple, sensitive and has the ability to separate the drug from degradation products and excipients found in the dosage form.

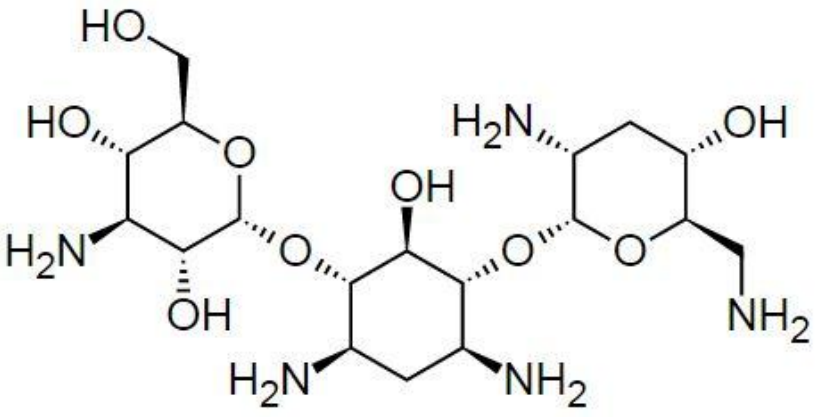

Fig 1: Chemical Structure of Tobramycin

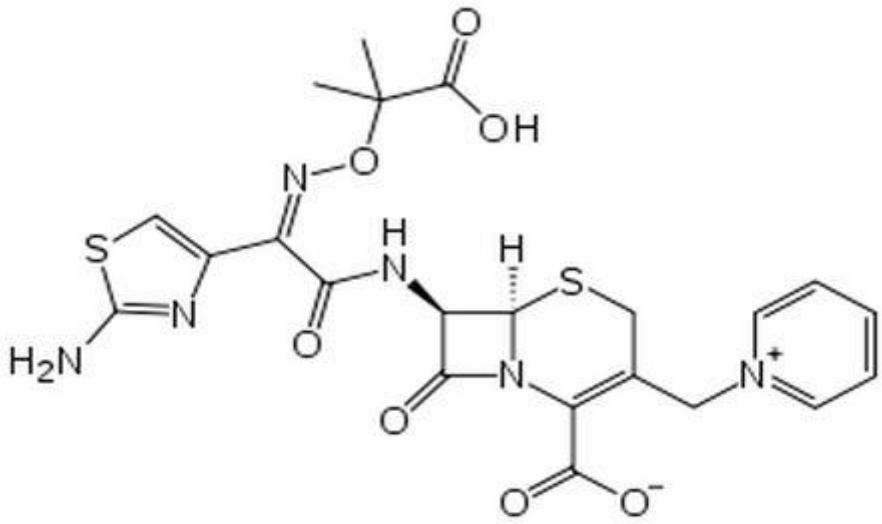

Fig 2: Chemical Structure of Ceftazidime

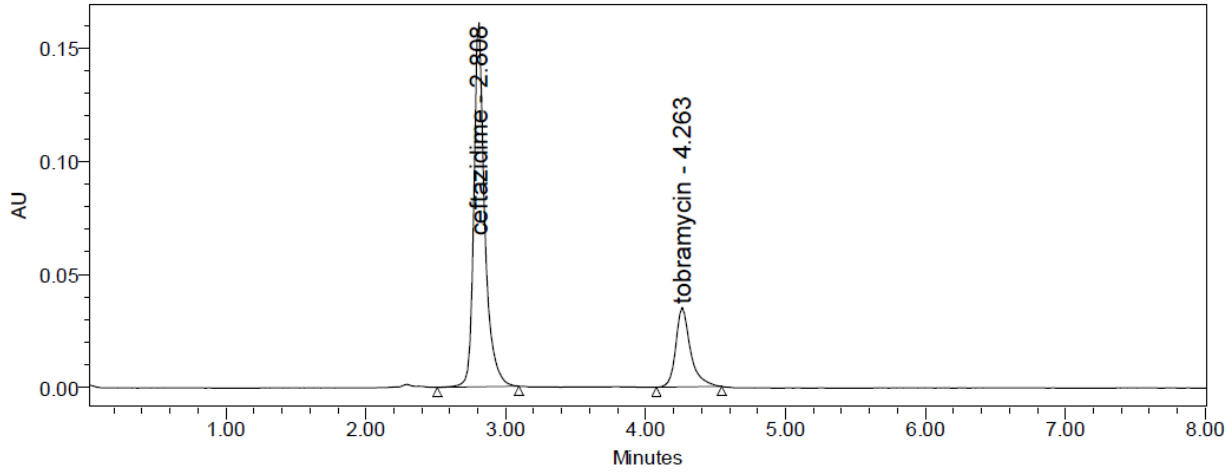

Fig 3: A typical chromatogram of Tobramycin and Ceftazidime in injection dosage form

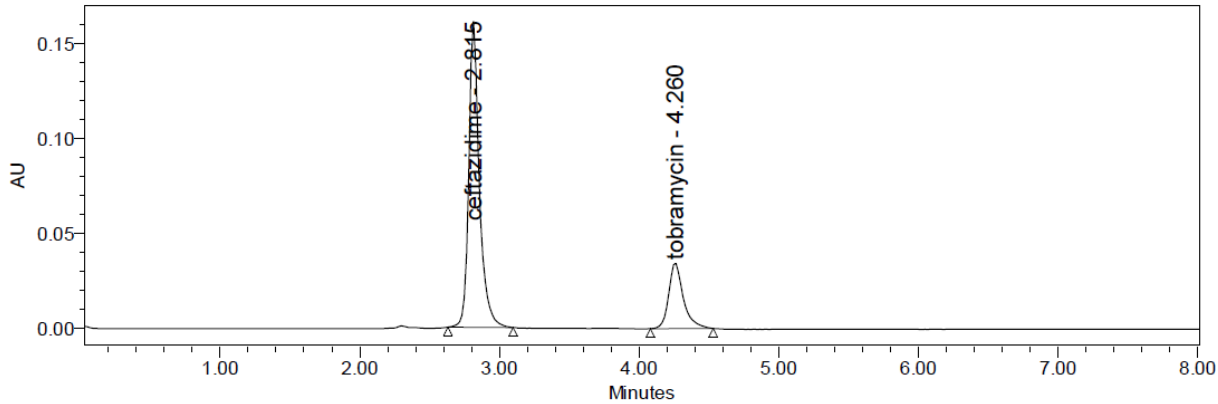

Fig 4: Standard Chromatogram of Tobramycin and Ceftazidime

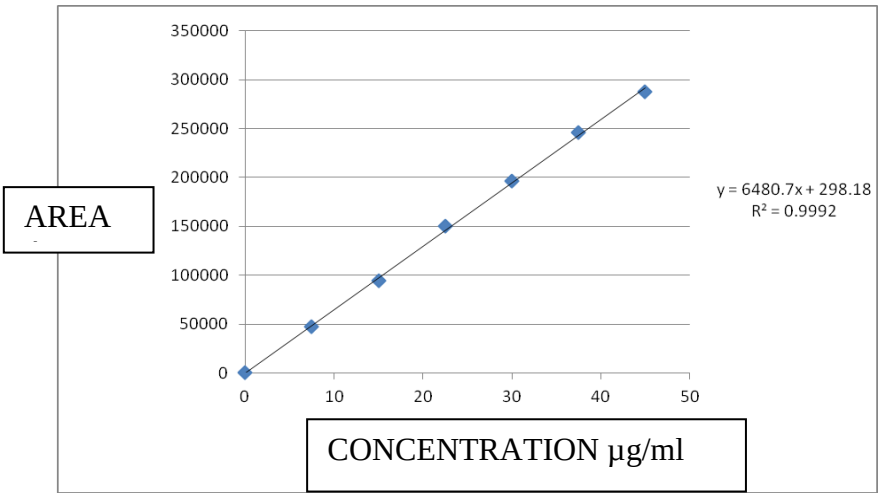

Fig 5: Linearity curve of Tobramycin

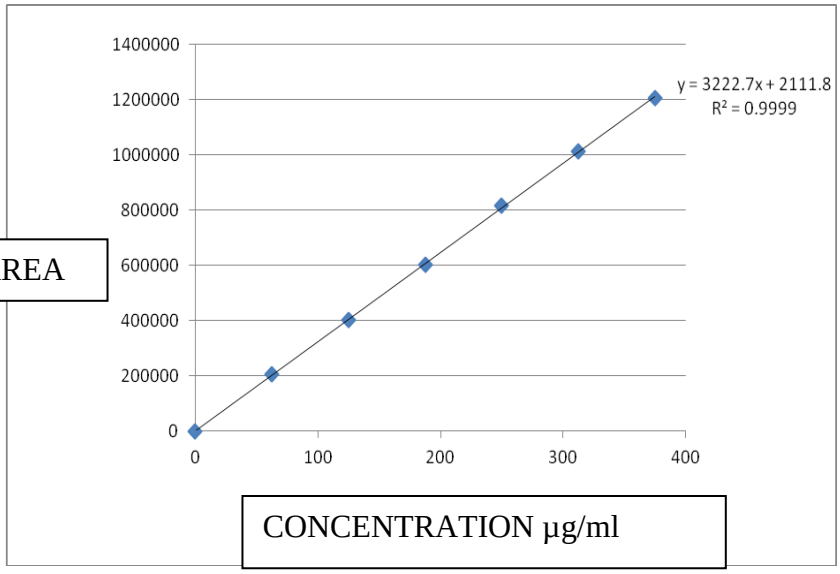

Fig 6: Linearity curve of Ceftazidime

Table-1: Optimized Chromatographic conditions

Parameter	Condition
Mobile Phase	Phosphate Buffer: Acetonitrile (50:50 % V/V) pH adjusted to 3.6
Column	Hypersil BDS C18, 250 x 4.6 mm, 5µ.
Wave length	260nm
Flow rate	1.0 mL/min
Injection volume	10µL
Run time	8 min
Diluent	Water: Acetonitrile (50:50)

Table 2: Results of Forced Degradation Studies

S.No	Injection	Tobramycin		Ceftazidime	
		% Assay	% Degradation	% Assay	% Degradation
1	Acid Degradation	95.72	4.28	95.82	4.18
2.	Base Degradation	95.17	4.83	97.75	2.25
3.	Peroxide	94.85	5.15	95.14	4.86
4.	Thermal Degradation	99.96	0.04	98.98	1.02
5.	UV Degradation	99.45	0.55	99.70	0.30
6.	Neutral degradation	99.41	0.59	99.89	0.11

Table 3: Precision method of proposed RP-HPLC method

Drug	Mean Area	% RSD
Tobramycin	251160	0.3
Ceftazidime	848954	0.5

Table 4: % Recovery Data for Tobramycin and Ceftazidime

Drug	Spiked Level %	% Recovery	% RSD
Tobramycin	50	100.02	1.87
	100	101.47	1.30
	150	100.90	1.13
Ceftazidime	50	99.83	0.19
	100	99.69	0.93
	150	99.76	0.45

Table 5: Results of Linearity

S.No	Tobramycin		Ceftazidime	
	Conc. (µg/ml)	Peak Area	Conc. (µg/ml)	Peak Area
1	7.5	47707	62.5	205997
2	15	94765	125	403940
3	22.5	150560	187.5	601267
4	30	196062	250	815773
5	37.5	246041	312.5	1012899
6	45	287659	375	1204694

Table 6: Robustness Data

Parameters	Changed Condition	Mean Peak Area		USP plate count	
		TOBRA	CEFTA	TOBRA	CEFTA
Flow rate (mL/min)	0.9ml	271898	884671	9392	7609
	1.0ml	252144	847081	9128	7195
	1.1ml	254951	860289	8509	6663
Temperature (±5)	25^0C	253716	862336	8652	6847
	30^0C	252144	847081	9128	7195
	35^0C	259994	880322	9392	7625
Mobile phase (±5%)	45:55 %v/v	256531	865161	8568	6847
	50:50 %v/v	252144	847081	9128	7195
	55:45 %v/v	243097	836250	8937	6871

TOBRA-Tobramycin **CEFTA-**Ceftazidime

Table 7: Results of LOD and LOQ

Drug	LOD (µg/ml)	LOQ (µg/ml)
Tobramycin	0.020	0.061
Ceftazidime	0.246	0.746

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