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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF CEFTAZIDIME AND AVIBACTAM IN BULK DRUG AND FORMULATION

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Abstract

A precise and robust method was developed method for the estimation of Ceftazidime and Avibactamin bulk and pharmaceutical dosage form.The Method used Agilent 1260 Infinity II model HPLC with DAD detector and Agilent Zorbax Bonus RP Column with dimension 250 x 4.6 mm, 5 µm. The Mobile phase combination used was 0.1% Perchloric acid and Acetonitrile (80:20). Flow rate at 0.5 ml/min and wavelength at 230 nm with run time of 20 minutes. The retention time of Ceftazidime (CEF) and Avibactam (AVB) peaks was at 4.42 and 6.05 minutes, respectively. The developed method was validated according to ICH Q2 (R1) guidelines. The instrument precision for CEF& AVB had a %RSD of 0.24% and 0.12%, respectively. The Intra & Inter day precision for CEF & AVB had a %RSD of 0.39% and 0.19%, respectively.Method was linear and accurate for concentration range of 160-240µg/ml and 40-60µg/ml for CEF & AVB respectively, with regression coefficient of 0.999 for both CEF & AVB and % RSD for accuracy for CEF at 80%, 100% and 120% was found to be 0.89%, 0.11% and 0.36%, respectively; and for AVB at 80%, 100% and 120% was found to be 0.14%, 0.09% and 0.15% respectively. The LOD & LOQ for CEF are 3.69µg/ml and 11.19µg/ml respectively and the LOD & LOQ for AVB are 1.52µg/ml and 4.60 µg/ml respectively.

Keywords: Ceftazidime, Avibactam, antibiotic, RP-HPLC, Robustness, %RSD, Precision, LOD, LOQ, Accuracy.

1. Introduction

Traditional medicines, such as cephalosporins of the second or third generation in conjunction with metronidazole, are less effective in treating complex urinary tract infections (cUTI) and difficult intra-abdominal infections (cIAI) due to the presence of multidrug-resistant bacteria, which are frequently the cause of these diseases [1,2]Use of new antibiotics, Ceftazidime& Avibactam (Avycaz), were developed as part of the QIDP initiative in order to meet the demand for more effective therapies than the ones that were already available. Both are recommended for the treatment of cUTI and cIAI, which are both life-threatening medical conditions [3,4].

Ceftazidime-avibactam, is a combination of the third-generation cephalosporin ceftazidime and the innovative non-β-lactam β-lactamase inhibitor avibactam. This combination is administered intravenously. In the European Union, ceftazidime-avibactam has been granted approval for the treatment of adults who are suffering from complicated urinary tract infections (cUTIs), which may include pyelonephritis, complicated intra-abdominal infections (cIAIs), hospital-acquired pneumonia (HAP), which may include ventilator-associated pneumonia (VAP), and other infections caused by aerobic Gram-negative organisms as well as patients who have limited treatment. Ceftazidime-avibactam exhibits remarkable in vitro activity against a wide range of Gram-negative pathogens, such as numerous extended-spectrum β-lactamase-, AmpC-, Klebsiellapneumoniaecarbapenemase-, and OXA-48-producing Enterobacteriaceae, as well as drug-resistant Pseudomonas aeruginosa isolates [5]. However, it does not exhibit any activity against metallo-β-lactamase-producing strains. The clinical efficacy of ceftazidime-avibactam in the treatment of cUTI, cIAI, and HAP (including VAP) in adults was proven in pivotal phase III non-inferiority trials with carbapenem comparators [6].

The IUPAC name of Ceftazidime is (6R,7R)-7-[[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-(2-carboxypropan-2-yloxyimino)acetyl]amino]-8-oxo-3-(pyridin-1-ium-1-ylmethyl)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (Figure 1)[7].The IUPAC name of Avibactam is [(2S,5R)-2-carbamoyl-7-oxo-1,6-diazabicyclo[3.2.1]octan-6-yl] hydrogen sulphate (Figure 2) [8].

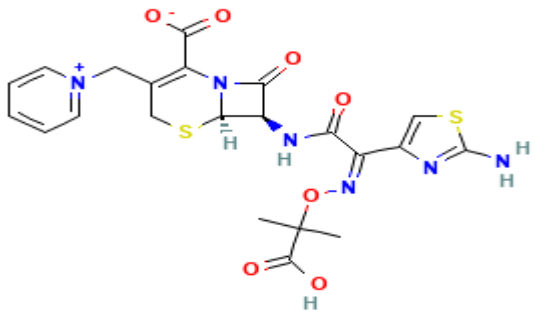


Figure 1: Structure of Ceftazidime

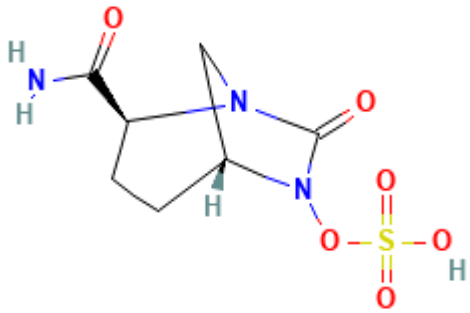


Figure 2: Structure of Avibactam

According to the literature review [9-26], there was few Liquid Chromatography analysis for Simultaneous estimation of CEF & AVB in Combination pharmaceutical dosage form. So, current study was planned for development and validation of method developed for Ceftazidime& Avibactam.

Table 1: Quality Target Profile for HPLC Method development

Parameter	Limits
Theoretical Plates	Not less than 2000
Asymmetry	Not More than 2.0 (Fairly at 1.0)
Tailing Factor	Not More than 2.0 (Fairly at 1.0)
Run time	Not More than 20 minutes
Resolution	Not Less than 2.0

2. Material and Method

2.1. Chemicals and Reagents

A complimentary sample of ceftazidime and avibactam was made available by Aadhaar Life Sciences Pvt. Ltd. In India, Qualigens was the supplier of the HPLC-grade acetonitrile that was purchased. A grade of perchloric acid that was of AR quality was acquired from Merck in India. A water supply was provided via the internal Milli-Q system. All of the weighing was carried out on NABL scales that had been calibrated. The production of the samples was carried out with the use of the analytical balance and Type A glassware.

2.2. Instrumentation

The instrument used for development and validation was an Agilent 1260 Infinity II equipped with a quaternary pump and DAD detector. Software from Agilent called OpenlabEzchrom was used. Wet chemistry was conducted using the Labmanultrasonicator and the Aczet analytical balance.

2.3. HPLC Method Development

2.3.1. The table 2 and 3 describes trials done during the development phase with the results and observations.

Table 2: Method Development for Ceftazidime& Avibactam HPLC

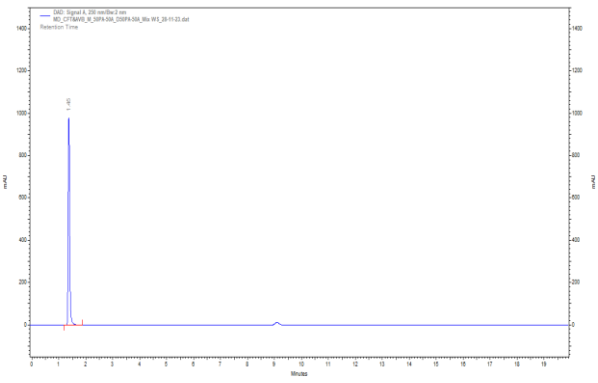
Trial No.	Mobile Phase	Ratio	Diluent	Column	Flow Rate (ml/min)	Wavelength (nm)
1.	0.1% Perchloric Acid : Acetonitrile	50-50	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax SB-Aq (250 x 4.6 mm, 5µ)	1.0	250
2.	0.1% Perchloric Acid : Acetonitrile	60-40	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax SB-Aq (250 x 4.6 mm, 5µ)	1.0	250
3.	0.1% Perchloric Acid : Acetonitrile	70-30	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax SB-Aq (250 x 4.6 mm, 5µ)	1.0	230
4.	0.1% Perchloric Acid : Acetonitrile	80-20	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax SB-Aq (250 x 4.6 mm, 5µ)	1.0	230
5.	0.1% Perchloric Acid : Acetonitrile	80-20	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)	1.0	230
6.	0.1% Perchloric Acid : Acetonitrile	90-10	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)	1.0	230
7.	0.1% Perchloric Acid : Acetonitrile	80-20	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)	0.8	230
8.	0.1% Perchloric Acid : Acetonitrile	80-20	0.1% Perchloric Acid : Acetonitrile (50:50)	Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)	0.5	230

Table 3: Method development results of Ceftazidime and Avibactam

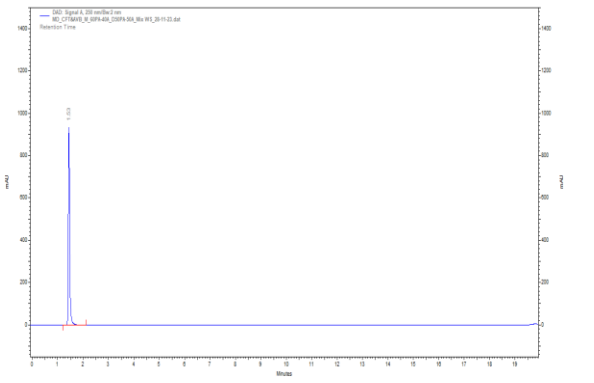
Trial No.	Ceftazidime				Avibactam			
	RT	**TP**	**Asymmetry**	**Resolution**	**RT**	**TP**	**Asymmetry**	**Resolution**
1.	1.45	3803	1.25	0.00	Not Detected			
2.	1.53	4067	1.29	0.00	Not Detected			
3.	1.41	2811	1.14	0.00	Not Detected			
4.	1.71	1741	3.42	0.00	2.27	3146	0.00	3.43
5.	2.21	8734	1.10	0.00	3.03	14454	1.17	8.32
6.	2.36	9084	1.20	0.00	3.19	12934	1.66	7.83
7.	2.76	10657	1.18	0.00	3.78	17651	1.20	9.24
8.	4.42	14402	1.17	0.00	6.07	23995	1.13	10.83

Following all of the aforementioned tests, it was discovered that the peak of highest absorption occurred at a wavelength of 230 nm. The diluent was maintained at a constant ratio of 50-50 0.1% perchloric acid to acetonitrile throughout all of the trials. Throughout all of the tests, the Agilent Zorbax Bonus RP column (250 x 4.6 mm, 5 micron) was consistently utilized. In accordance with the quality target profile that had been established in advance for the development work, the conditions for trial 8 were finalized, and individual Standard was executed in order to validate the retention times. As can be seen in figure 3, the chromatograms of the method development were displayed.

Trial 1



Trial 2



Trial 3

Trial 4

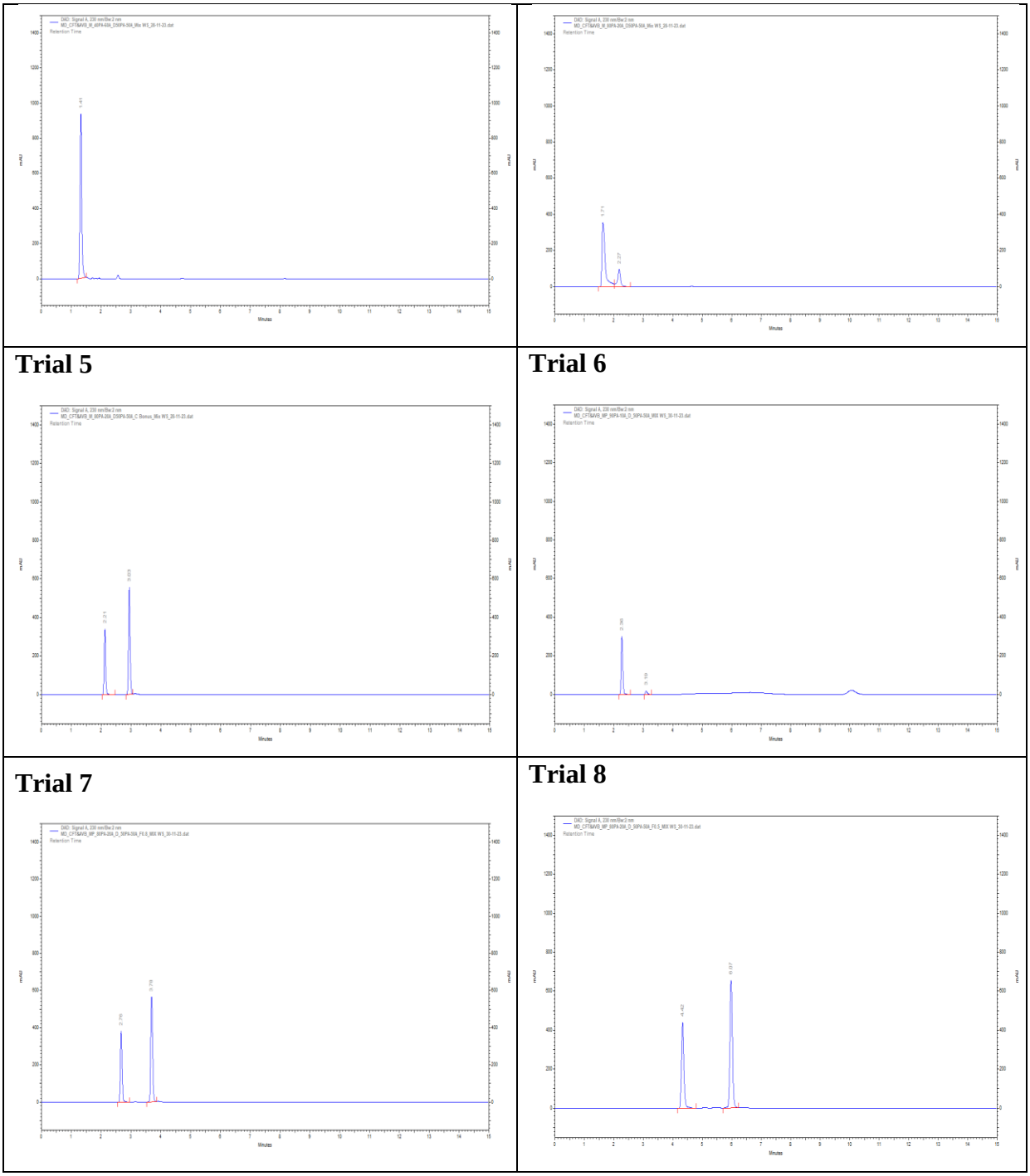


Figure 3: Method Development Trials

2.3.2. Final Chromatographic Conditions:

Table 4: Final Chromatographic Condition

Parameter	Condition
HPLC Instrument	Agilent 1260 Infinity II
Column	Agilent ZorbaxBonus RP (250 mm x 4.60 mm5µm)
Wavelength	230 nm
Mobile Phase	Mobile Phase A –0.1% Perchloric acid: 80% Mobile Phase B – Acetonitrile : 20%
Diluent	0.1% Perchloric acid : Acetonitrile (50:50) v/v

Run time	20 minutes
Injection Volume	10 micro liters
Flow Rate	0.5 ml/min
Column oven Temperature	30°C (± 2°C allowed by Robustness)

2.3.3. Preparation of Mobile Phase

Preparation of 0.1% Perchloric acid

Take 800 ml of water using graduated cylinder. Pipette out 1 ml of Perchloric acid and add this to measured water, mix well then adjust the volume to 1000 ml using water .

Mobile Phase: 80%- 0.1% Perchloric acid:20% Acetonitrile

Mix separately measured 800 ml of 0.1% Perchloric acid and 200 ml of Acetonitrile into a suitable container. Filter the mobile phase through 0.45 µm nylon membrane filter. Briefly sonicate to degas.

2.3.4. Preparation of Diluent

Mix separately measured 500 ml of Perchloric acid and 500 ml of Acetonitrile into a suitable container and mix well. Mixture is to be filtered through 0.45 µm nylon membrane filter. Briefly sonicate to degas.

2.3.5. Preparation of Standard Solution

A. Working Standard:

1. Ceftazidime Standard Stock Solution-I (CSSS-I):
    i. Initially Prepare a Standard Stock Solution (SSS-I) of by adding10 mg of Ceftazidime in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. Of Ceftazidime= 1000 µg/ml).
2. Avibactam Standard Stock Solution-I(ASSS-I):
    i. Then prepare a Standard Stock Solution (SSS-II) of Avibactam by adding 10mg in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent.(Conc. of Avibactam= 1000µg/ml).

3. Then add 2.0 ml of CSSS-I & 0.5 ml ASSS-I in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Ceftazidime = 200µg/ml &Avibactam= 50µg/ml).

B. Preparation of Sample for Assay

1. Zebistin 2.5 Injection were used as marketed product .
2. Weigh powder equivalent to 20 mg of Ceftazidime and 5 mg of Avibactam and transfer to 10 ml volumetric flask & add 5-7 ml diluent, mix for 5 minutes and make the volume to 10 ml with diluent. (Conc. of Ceftazidime = 2000 µg/ml and Avibactam = 50 µg/ml).
3. Then add 1.0 ml of above stock solution in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent (Conc. of Ceftazidime= 200 µg/ml & Avibactam = 50 µg/ml).

2.4. Method validation

2.4.1. Specificity

The preparation of individual injections of Ceftazidime and Avibactam, with concentrations of 200 µg/ml and 50 µg/ml, respectively, was carried out, and by analyzing the Retention Time, peaks were observed. Injection of blank was performed to guarantee that there would be no interference from the blank peak with the primary analyte peaks.

2.4.2. System Suitability

For the purpose of determining whether or not the system was suitable, a series of tests was carried out first. According to the ICH guideline system, the theoretical plate count, tailing factor, and resolution are all found to be within the acceptable parameters specified by the system.

2.4.3. Accuracy

To determine the accuracy of a technique, one must examine how closely its test findings correspond to the actual value. In the recovery studies, three distinct concentration levers were evaluated. At each level, three replicate injections were performed and the amount of drug present, the percentage of recovery, and the related standard deviation were calculated.

2.4.4. Repeatability

The degree of concordance that exists between the findings of individual tests is something that determines the analytical precision. An examination was performed on multiple samples of a uniform sample. After preparing a single sample in accordance with the instructions, six injections were done from the same sample and checked to ensure that the system was suitable. Instrument precision was done as Instrument precision (how good the instrument execute back to back replicate injection of similar concentration).

2.4.5. Linearity

The capacity of an analytical method to produce results that are proportionate to analyte concentrations within a certain range is referred to as the methodological linearity of the method. When determining linearity, there were five different sets of standard solutions that were utilized. The regression equation was established by plotting the peak area against the concentration of the standard solution on the calibration curve. This allowed for the development of the equation. For the purpose of determining the slope, intercept, and correlation coefficient, the least-squares method was utilized.

2.4.6. LOD and LOQ

The limit of detection (LOD) and the limit of quantification (LOQ) are terms that indicate the capability of the method to detect and quantify the smallest amount of analyte, respectively. Calculating the LOD and LOQ required the use of the standard deviation and the slope of the regression line, which were both determined by the following equations.

2.4.7. Robustness

The Robustness was performed changing the column temperature by ± 2˚C and Wavelength by ± 2 nm.

Table 5: Robustness Trials

Condition	Increased	Normal	Decreased
Column Oven Temperature	32˚C	30˚C	28˚C
Wavelength	232 nm	230 nm	228 nm

2.4.8. Inter-day & Intraday Precision:

To determine the stability of the solution for intraday precision, the prepared working standard was analyzed in the morning and in the evening, and the percentage of relative standard deviation (RSD) was computed. The identical solution was injected on the second day, and the results of the intraday precision and percent relative standard deviation were compared with the data from the morning.

3. Results and Discussion

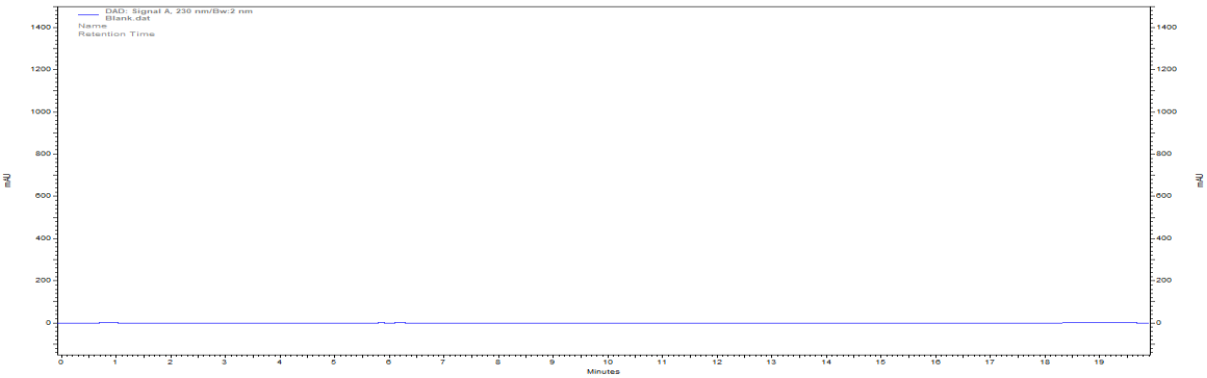
3.1. Specificity

Specificity was performed to check if there was any interaction between the peaks from blank or the APIs.

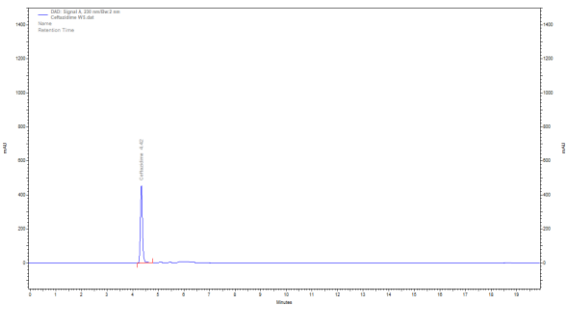
Table 6: Specificity results of Ceftazidime and Avibactam

Sample	Ceftazidime			Avibactam		
	RT	Area	% Assay	RT	Area	% Assay
Ceftazidime	4.42	5387845	-	-	-	-
Avibactam	-	-	-	6.05	8019787	-
MIX WS	4.42	5388803	-	6.05	8024243	-
Drug Product	4.42	5374421	99.73	6.05	7948896	99.06

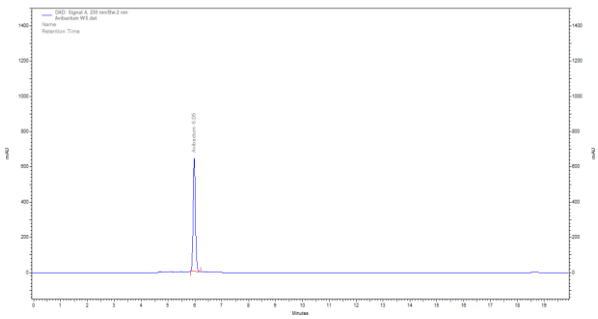
a. Diluent



b. Ceftazidime WS



c. Avibactam WS



d. Mix WS

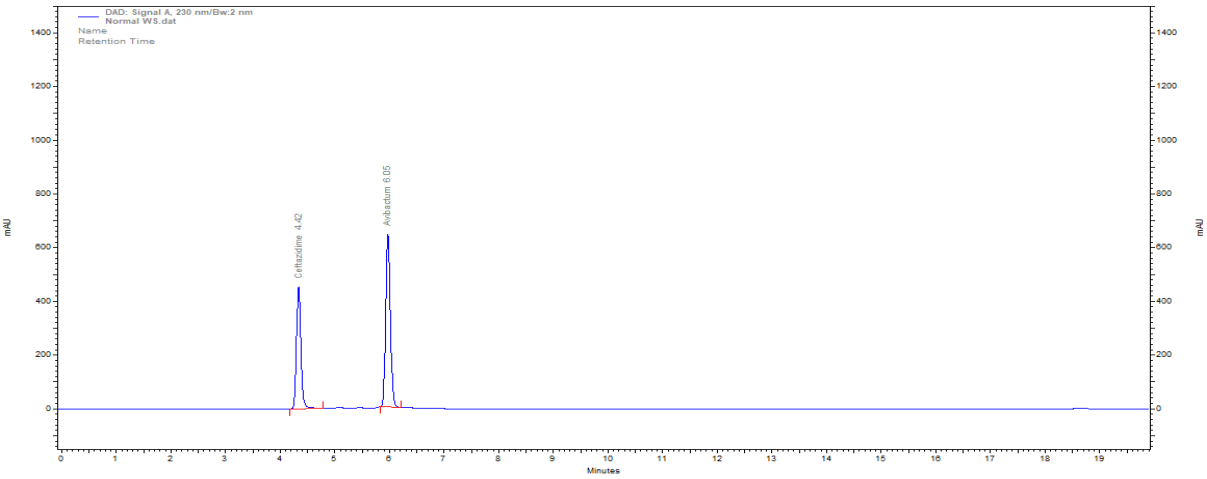


Figure 4: Chromatogram ID a]Diluent, b] Ceftazidime c] Avibactam, d] Mixture Working Standard of CEF & AVB.

3.2. Instrument Precision and System suitability

The HPLC Instrument was tested for its suitability to perform the validation. Based on the limits mentioned in table 1, the equipment was found to be suitable for continuing the validations. Instrument precisions of both the drugs were performed after system suitability and the reported data in below shows the relative standard deviation for Instrument precision of CEF & AVB are 0.24% and 0.12% respectively. This %RSD shows the method is very much precise with respect to multiple sample preparation for same concentration. The data is shown in table 7-9.

Table 7: System suitability for Ceftazidime

Ceftazidime				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	4.42	1.13	14699	0.00
Rep 2	4.42	1.11	14521	0.00

Rep 3	4.42	1.12	14854	0.00
Rep 4	4.42	1.10	14446	0.00
Rep 5	4.42	1.12	14785	0.00
Rep 6	4.42	1.11	14565	0.00
Avg	4.42			
STDEV	0.00			
RSD	0.00			

Table 8: System suitability for Avibactam

Avibactam				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	6.05	1.13	23647	10.77
Rep 2	6.05	1.12	23754	10.77
Rep 3	6.05	1.14	23775	10.77
Rep 4	6.05	1.10	23545	10.77
Rep 5	6.05	1.11	23674	10.77
Rep 6	6.05	1.13	23236	10.77
Avg	6.05			
STDEV	0.00			
RSD	0.00			

Table 9: Instrument precision of Ceftazidime and Avibactam

Repeatability		
Sample ID	**Peak area**	
	Ceftazidime	**Avibactam**
100% Rep 1	5388803	8024243
100% Rep 2	5386512	8034541
100% Rep 3	5377854	8021145
100% Rep 4	5384611	8042241
100% Rep 5	5379525	8042232
100% Rep 6	5354113	8022445
AVG	5378570	8031141
STDEV	12679.534	9807.172
%RSD	0.24	0.12

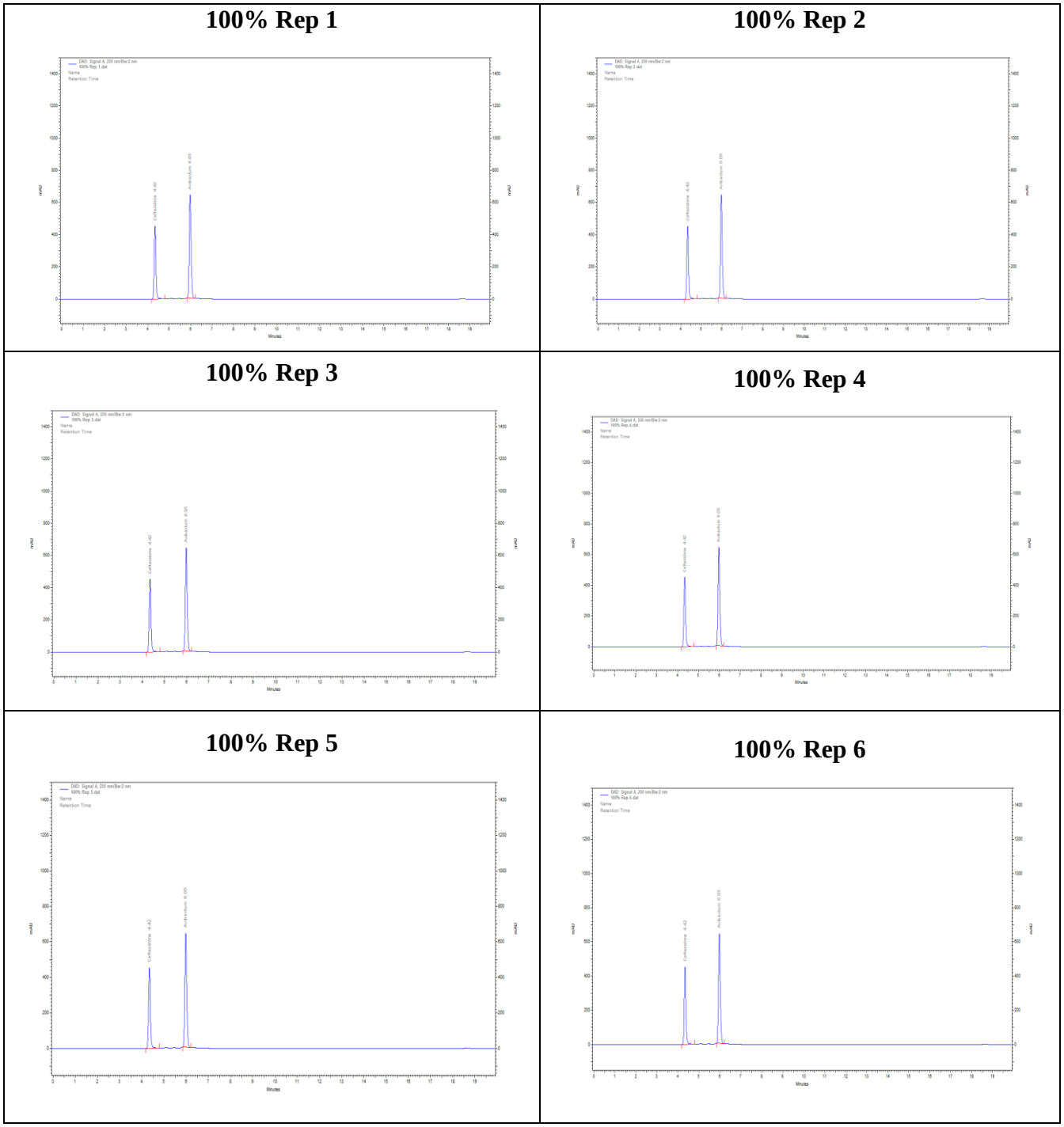


Figure 5: Instrument Precision Ceftazidime& Avibactam

3.3. Linearity of Ceftazidime& Avibactam

Linearity was performed at different levels. The graph plotted between peak area and concentration showed linearity with correlation coefficient as shown in table below. The linearity data in shown in table 10 and graph in figure 6.

Table 10: Linearity data of CEF & AVB

Ceftazidime			Avibactam		
% Level	**Conc (ug/ml)**	**Area**	**% Level**	**Conc (ug/ml)**	**Area**
80	160	4278883	80	40	6394306
90	180	4841158	90	45	7212675
100	200	5388803	100	50	8024243
110	220	5951895	110	55	8916397
120	240	6482629	120	60	9736687
R^2= 0.999			R^2= 0.999		

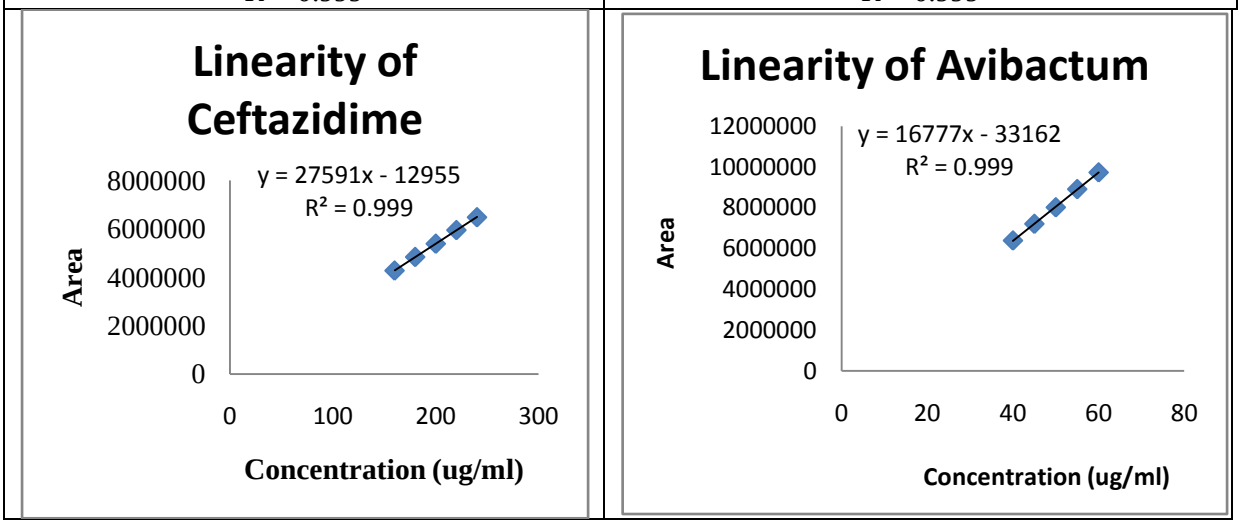


Figure 6: Linearity graph of Ceftazidime and Avibactam

3.4. LOD and LOQ for Ceftazidime and Avibactam

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined for CEF & AVB. The results of analysis are shown in table 11.

Table 11:LOD and LOQ for CEF & AVB

Name	LOD (µg/ml)	LOQ (µg/ml)
Ceftazidime	3.69	11.19
Avibactam	1.52	4.60

The LOD and LOQ were significantly low, implying the method to be very efficient in determining low concentration of drug. This value of LOD and LOQ can be used during cleaning validation in industry which can help companies know if the manufactured vessel or equipment is free from APIs stains.

3.5. Accuracy

Accuracy for CEF was performed in triplicates and it was observed that the method was accurate for the range 80%, 100% and 120%. The relative standard deviation for 80%, 100% and 120% were 0.89%, 0.11% and 0.36% respectively. The accuracy determined the methods

ability to analyses different concentration of drug in solution accurately. The accuracy data is shown in table 12.

Table 12: Accuracy data for Ceftazidime

Sample ID	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	%RSD
80%	Rep 1	159.52	4278883	158.63	99.44	100.40	0.896402	0.89
	Rep 2		4325551	160.36	100.53			
	Rep 3		4355412	161.47	101.22			
100%	Rep 1	199.40	5388803	199.78	100.19	100.11	0.107367	0.11
	Rep 2		5386512	199.69	100.15			
	Rep 3		5377854	199.37	99.99			
120%	Rep 1	239.28	6482629	240.33	100.44	100.03	0.363071	0.36
	Rep 2		6437562	238.66	99.74			
	Rep 3		6448954	239.08	99.92			

Accuracy for AVB was performed in triplicates and it was observed that the method was accurate for the range 80%, 100% and 120%. The relative standard deviation for 80%, 100% and 120% were 0.14%, 0.09% and 0.15% respectively. The accuracy determined the methods ability to analyses different concentration of drug in solution accurately. The accuracy data is shown in table 13.

Table 13: Accuracy data for Avibactam

Sample ID	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	%RSD
80%	Rep 1	39.88	6394306	39.69	99.52	99.37	0.136774	0.14
	Rep 2		6378555	39.59	99.28			
	Rep 3		6379678	39.60	99.30			
100%	Rep 1	49.85	8024243	49.81	99.91	99.94	0.087324	0.09
	Rep 2		8034541	49.87	100.04			
	Rep 3		8021145	49.79	99.88			
120%	Rep 1	59.82	9736687	60.44	101.03	101.16	0.153024	0.15
	Rep 2		9745878	60.49	101.13			
	Rep 3		9765554	60.62	101.33			

3.5. Inter and Intraday Precision

Intra and inter day precision study was performed and reported the % RSD change in peak area of the APIs at different time points. The acceptance criteria is to have %RSD of peak area <2%. The Results are given in Table 14.

Table 14: Intra &Interday Precision of CEF & AVB

Intra Day precision					
Day 1	**Sample ID**	**Ceftazidime**		**Avibactam**	
		Area	**Assay**	**Area**	**Assay**
Morning	**WS**	5388803	-	8024243	-
	DP	5374421	99.73	7948896	99.06
Evening	**WS**	5329741	-	8019457	-
	DP	5319447	99.81	7951254	99.15
Inter Day precision					
Day	**Sample ID**	**Ceftazidime**		**Avibactam**	
		Area	**Assay**	**Area**	**Assay**
Day 2	**WS**	5334586	-	8011364	-
	DP	5286544	99.10	7965542	99.43
%RSD		0.39		0.19	

3.6. Robustness

Robustness is done to check how deviating the method is with respect to its critical parameters. All over the world, the equipment is calibrated before use, but to know if the method is robust, changes were done in column temperature and Wavelength as shown in table 15 and 16.

Table 15: Robustness study - Change in Column temperature

Column Oven Temp Change					
Condition	**Sample**	**Ceftazidime**		**Avibactam**	
		Area	**Assay**	**Area**	**Assay**
28˚C	**WS**	5297855	-	8011475	-
	DP	5245654	99.01	7933241	99.02
30˚C	**WS**	5388803	-	8024243	-
	DP	5374421	99.73	7948896	99.06
32˚C	**WS**	5325745	-	7954551	-
	DP	5319554	99.88	7931222	99.71

Table 16: Robustness study - Change in Wavelength

Wavelength (nm)			
Condition	**Sample**	**Ceftazidime**	**Avibactam**

		Area	Assay	Area	Assay
228	**WS**	5287452	-	8019475	-
	DP	5297545	100.19	7855456	97.95
230	**WS**	5388803	-	8024243	-
	DP	5374421	99.73	7948896	99.06
232	**WS**	5378996	-	7966384	-
	DP	5286451	98.28	7925642	99.49

Hence, the method was found to be robust with a small change in column temperature and change in wavelength. There was no significant change in Retention time, or Area of replicate injection.

Conclusion

In this research article, a precise and accurate method was developed based on method developed technique for estimation of CEF & AVB in bulk drugs and formulation by RP-HPLC technique. The developed method was validated for accuracy, precision and robustness. The proposed methods were found to be appropriate due to its simplicity, reliability, sensitivity, rapidness and selectivity for detection at very low concentrations. Validation data demonstrates that, these methods are accurate, precise, simple and economic and can be used in the routine analysis of Ceftazidime and Avibactam in various formulations.

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