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Development and Validation of Stability Indicating Method for Simultaneous Estimation of Ceftriaxone and Sulbactam Injection using RP-UPLC Method

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Abstract:- **This research manuscript describes simple, sensitive, accurate, precise and repeatable RP-UPLC method for the simultaneous determination of Ceftriaxone (CEF) and Sulbactam (SUL) Injection in combine dosage form. The sample was analyzed by reverse phase C18 column (Purospher Star 100 × 2.1 mm ID, 2 µm) as stationary phase and 0.05M Sodium di-hydrogen ortho-phosphate dihydrate: Acetonitrile (86:14) as a mobile phase and pH 4.5 was adjusted by 0.1M ortho-Phosporic acid or 0.1M Sodium hydroxide at a flow rate of 0.4 ml/min. Quantification was achieved of Ceftriaxone at 254 nm and of Sulbactam at 195 nm with PDA detector. The retention time for Ceftriaxone and Sulbactam was found to be 1.002 and 0.784 minute respectively. The linearity for Ceftriaxone and Sulbactam was obtained in the concentration range of 10-70 µg/ml and 5-35 µg/ml respectively. The method was successfully applied to pharmaceutical formulation because no chromatographic interferences from excipients were found. The precision (intraday, inter day and repeatability) of method was found within limits. The method was validated as per ICH guidelines. Ceftriaxone and Sulbactam API and market formulation were subjected to acid and alkali hydrolysis, oxidation, thermal and photolytic forced degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values. Besides, the peak purity of drug substance and drug product peak also confirmed the specificity of the methods with respect to the degradation products. In the forced degradation study Ceftriaxone and Sulbactam showed maximum degradation in base hydrolysis stress study followed by less degradation in thermal degradation. The developed method was simple, specific, sensitive, rapid, and economic and can be used for estimation of Ceftriaxone and Sulbactam in bulk and their combined dosage form for routine analysis and stability studies.**

Keywords: *Ceftriaxone, Sulbactam, Method validation, RP-UPLC, Forced degradation*

I. INTRODUCTION

Ceftriaxone (Figure 1) is (6R, 7R)-3[(acetyl-oxy)methyl]-7-[[2Z)-2amino-4-thiazolyl) (methoxy-amino)-acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4,2,0]oct-2-ene-2carboxylic acid. Ceftriaxone is a cephalosporin beta-lactam antibiotics used in the treatment of bacterial infections usually caused by susceptible, gram-positive organism. Sulbactam (Figure 2) is chemically(2R,5R)-3,3-dimethyl-4,47-trioxo-4,6thia-1-azabicyclo[3-2-0]heptane-2 carboxylic acid. It is a competitive, irreversible beta lactamase inhibitor and has good inhibitor activities against the clinically important plasmid mediated beta-lactamase and most frequently responsible for transferred drug resistance [1, 2]. Both Ceftriaxone and Sulbactam are listed in the USP, BP and IP. To meet the clinical needs, a new combination was developed and consequently for the quality control of the formulation an analytical method was required. A literature survey revealed that several methods have been used for determination of Ceftriaxone sodium which includes High performance Thin Layer Chromatography (HPTLC) [3], High performance Liquid Chromatography (HPLC) [4, 5] and spectrometry [6, 7]. Sulbactam was successfully determined by Capillary Isotachophoresis [8]. However, there is no work was reported for the simultaneous estimation of these drugs by RP-UPLC method. Hence, in the present study an attempt has been made to develop simple, and accurate, sensitive, precise and repeatable RP-UPLC method, for the simultaneous estimation of both drugs in dry powder for injection dosage form.

II. MATERIALS & METHODS

2.1 Apparatus

The chromatography was performed on a Waters (Acquity) RP-UPLC instrument equipped with PDA detector and Em-power 2 software, Purospher Star C18 column (100 mm × 2.1 mm ID, 2 µm) was used as stationary phase. Mettler Toledo analytical balance (Germany), an ultrasonic cleaner (Frontline FS 4, Mumbai, India) and Whatmann filter paper No. 41 (Whatman International Ltd., England) were used in the study.

2.2 Reagents and materials

Ceftriaxone and sulbactam bulk powder was obtained from Nirlife, Healthcare division of Nirma Ltd. Ahmedabad, India. The commercial fixed dose combination product was procured from the Nirlife. Acetonitrile (HPLC grade, Finar Reagent, Ahemedabad, India), Sodium di-hydrogen ortho-phosphate dihydrate (AR, Finar Reagent, Ahemedabad, India), Sodium hydroxide (AR, Finar Reagent, Ahemedabad, India), ortho-Phosphoric acid (AR, Finar Reagent, Ahemedabad, India), used were of HPLC grade was used in the study.

2.3 Chromatographic condition

In this work we used reverse phase C18 UPLC column (Purospher Star 100×2.1 mm ID, 2 µm, Merck Specialities) as stationary phase and using a mobile phase consisting of 0.05M Sodium dihydrogen ortho-phosphate dihydrate: acetonitrile (86:14 % v/v) adjusted to pH 4.5with 0.1M Ortho-phosphoric or 0.1M Sodium hydroxide, in the flow rate of 0.4 ml/min.

2.4 Preparation of mobile phase

Accurately Weigh 7.8 gm of Sodium dihydrogen ortho-phosphate dihydrate (M.W. 156.01) was transferred into 1000 ml volumetric flask. Approximately 800 ml of water was added into the volumetric flask and sonicated. Volume was made up to 1000 ml with water. From this buffer solution 860 ml of solution was withdrawn and mixed with 140 ml of Acetonitrile into separated 1000 ml volumetric flask to make a mobile phase ratio buffer: Acetonitrile (86:14 % v/v). pH of 4.5 was adjusted by using 0.1M ortho-phosphoric acid or 0.1M Sodium hydroxide of mobile phase. This mobile phase used as diluents also throughout study.

2.5 Preparation of standard stock solutions

An accurately weighed Ceftriaxone (10 mg) and Sulbactam (5 mg) were transferred to 100 ml volumetric flask, dissolved in 50 ml water for injection (W.F.I) and diluted up to mark with water for injection (W.F.I.) to get 100 µg/ml solution of Ceftriaxone and 50 µg/ml solution of Sulbactam

2.6 Method Validation

The method was validated in compliance with ICH guidelines [9].

2.6.1 Preparation of calibration curve

Aliquots (of 1,2,3,4,5,6,7 ml) of mixed standard working solutions (equivalent to 10,20,30,40,50,60,70 ppm of Ceftriaxone and 5,10,15,20,25,30,35 ppm of Sulbactam) were transferred in a series of 10 ml volumetric flasks, and the volume was made up to the mark with water for injection (W.F.I.). Each solution was injected under the operating chromatographic condition as described above and responses were recorded. Calibration curves were constructed by plotting the peak areas versus the concentration, and the regression equations were calculated (Table 1 and Table 2) and (Figure 3 and Figure 4). Each response was average of three determinations

2.6.2 Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of Ceftriaxone and Sulbactam by the standard addition method. Known amounts of standard solutions of Ceftriaxone and Sulbactam were at added at 80, 100 and 120 % level to pre-quantified sample solutions of Ceftriaxone sodium equivalent to Ceftriaxone 40 µg/ml and Sulbactam sodium equivalent to Sulbactam 20 µg/ml. The amounts of Ceftriaxone and Sulbactam were estimated by applying obtained values to the respective regression line equations.

2.6.3 Method precision (repeatability)

The precision of the instrument was checked by repeatedly injecting (n=6) solutions of Ceftriaxone and Sulbactam (40 µg/ml and 20 µg/ml respectively) without changing the parameters.

2.6.4 Intermediate precision (reproducibility)

The intraday and inter day precisions of the proposed method was determined by estimating the corresponding responses 3 times on the same day and on 3 different days over a period of one week for 3 different concentrations of standard solutions of Ceftriaxone sodium equivalent to Ceftriaxone (20, 40, and 60 µg/ml) and Sulbactam sodium equivalent to Sulbactam (10, 20 and 30 µg/ml). The results were reported in terms of relative standard deviation (% RSD).

2.6.5 System suitability

The parameters used in system suitability test were asymmetry of the chromatographic peak, peak resolution and theoretical plates, as % RSD of peak area for replicate injections (Table 4)

2.6.6 Preparation of Marketed sample solution for Assay

For determination of the content of Ceftriaxone and Sulbactam in dry powder for injection; Take about 18 mg (Ceftriaxone sodium equivalent to Ceftriaxone 10 mg and Sulbactam sodium equivalent to Sulbactam 5 mg) of powder and transferred to 100 ml volumetric flask, dissolved in W.F.I. (50 ml) sonicated for 30 min and dilute up to the mark with W.F.I. The solution was filtered through Whatmann filter paper No. 41 and residue was washed with W.F.I. The solution was diluted up to the mark with W.F.I. Accurately measured 4.0 ml of solution was transferred to 10 ml volumetric flask, diluted up to the mark with W.F.I to get final working concentration of Ceftriaxone sodium equivalent to Ceftriaxone (40 µg/ml) and Sulbactam sodium equivalent to Sulbactam (20 µg/ml). A sample solution was injected under the operating chromatographic condition as described above and responses were recorded (Figure 5) and (Table 5). The analysis procedure was repeated three times with dry powder for injection formulation.

III. RESULTS AND DISCUSSION

To optimize the RP-UPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Ceftriaxone and Sulbactam were obtained with a mobile phase comprising of 0.05M Sodium di-hydrogen ortho-phosphate dihydrate: Acetonitrile (86:14, %v/v) and pH of 4.5 adjusted by 0.1M Sodium hydroxide or 0.1M ortho-Phosphoric acid at a flow rate of 0.4 ml/min to get better reproducibility and repeatability. Quantification was achieved with PDA detection of Ceftriaxone at 254 nm and Sulbactam at 195 nm based on peak area. The retention time for Ceftriaxone and Sulbactam were found to be 1.002 and 0.784 min, respectively (Figure 5). Linear correlation was obtained between peak area versus concentrations of Ceftriaxone and Sulbactam in the concentration ranges of concentration range of 10-70 µg/ml and 5-35 µg/ml are r^2=0.9999 and r^2=0.9999 and mean accuracies 99.41 ± 0.040 % and 99.98 ± 0.060 % for Ceftriaxone and Sulbactam (Table 5), which indicates accuracy of the proposed method. The % RSD values for Ceftriaxone and Sulbactam were found to be < 2 %, which indicates that the proposed method is repeatable. The low % RSD values of repeatability of assay (0.35-0.39 %), inter day (0.52-0.203 % and 0.033-0.153 %) and intraday (0.038-0.319 % and 0.088-0.307 %) variations for Ceftriaxone and Sulbactam, respectively, reveal that the proposed method is precise. LOD values for Ceftriaxone and Sulbactam were found to be 0.008 µg/ml and 0.025 µg/ml, respectively and LOQ values for Ceftriaxone and Sulbactam were found to be 0.027 µg/ml and 0.083 µg/ml, respectively (Table 3). These data show that the proposed method is sensitive for the determination of Ceftriaxone and Sulbactam. The results of system suitability testing are given in (Table 4).

3.1 Degradation study of Ceftriaxone and Sulbactam in 0.1N HCl at 70°C for 4 hours in reflux condition.

It showed a peak of degradation product. Degradation peak was found at 1.669 min in drug product. Ceftriaxone and Sulbactam peak was observed at retention time 1.008 min and 0.783 min respectively. (Figure 6) The % drug degradation observed of Ceftriaxone and Sulbactam was 17.71 % and 13.72 % respectively.

3.2 Degradation study of Ceftriaxone and Sulbactam in 0.1N NaOH at 70°C for 4 hours in reflux condition.

It showed a peak of degradation product. Degradation peak was found at 0.591 min in drug product. Ceftriaxone and Sulbactam peak was observed at retention time 1.004 min and 0.782 min respectively. (Figure 7) The % drug degradation observed of Ceftriaxone and Sulbactam was 11.51 % and 19.31 % respectively. From this it is observed that Sulbactam showed maximum degradation in base hydrolysis degradation condition.

3.3 Oxidation degradation study of Ceftriaxone and Sulbactam in 3 % H_2O_2 at 70°C for about 1 hour in reflux condition.

Sample and drug substances were treated with 3 % solution of hydrogen peroxide and kept in water bath at 70°C in reflux condition for about 1 hour. There were three degradation peak was found at 1.865, 2.233 and 2.526 min for Ceftriaxone in drug product. Degradation of Sulbactam was found out at 0.541 min in both

Sulbactam-API and drug product. The % degradation observed of Ceftriaxone and Sulbactam was 14.96 % and 10.82 % respectively (Figure 8).

3.4 Thermal Degradation study of Ceftriaxone and Sulbactam at 60°C for about 24 hrs

Thermal degradation of Ceftriaxone and Sulbactam at 60°C for about 24 hrs in hot air oven was carried out. There was no degradation peak found in thermal degradation chromatogram because there was lower degradation found in thermal degradation study. % Degradation of Ceftriaxone and Sulbactam was found to be 0.53 % and 0.46 % respectively (Figure 9).

3.5 Photolytic Degradation study of Ceftriaxone and Sulbactam

Sample and drug substances were exposed to energy of 1.2 million lux hrs fluorescent light and 200 w/m^2 of UV for about 7 days. There were minor degradation peaks found at 3.121 min, 3.314 min and 4.223 min for Ceftriaxone in drug substance and drug product. Degradation peak for Sulbactam was found at 0.377 min. % degradation of Ceftriaxone and Sulbactam was found at 5.24 % for Ceftriaxone and 2.31 % for Sulbactam. Ceftriaxone showed least degradation in photolytic condition (Figure 10).

IV. CONCLUSION

Stability indicating RP-UPLC methods for estimation of Ceftriaxone and Sulbactam in their combine dosage form was established and validated as per the ICH guidelines. The forced degradation study and peak purity data confirmed that there was no merging between peaks of active ingredients and any other degradation products as well as other additives. Hence the specificity of the proposed method was established. The linearity of developed method was achieved in the range of 10-70 µg/ml for Ceftriaxone (r^2=0.9999) and 5-35 µg/ml for Sulbactam (r^2=0.9999). The percentage recovery of drug was achieved in the range of 98-101 % which was within the acceptance criteria. The percentage RSD was NMT 2 % which proved the precision of the developed method. Different degradation products were found for drug substance and drug product in acidic, alkaline, oxidative, thermal and photolytic force degradation. Peak of Degraded products were not interfering with the main drug peak of Ceftriaxone and Sulbactam. Thus these degradation products have not been identified. The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust. Hence it can be used for the routine analysis of Ceftriaxone and Sulbactam in their bulk and combine dosage form in quality control laboratory and stability studies.

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Table 1: Linearity of Ceftriaxone

Concentration (ppm)	Average Area	SD	% RSD
10	164998	285.7	0.173
20	385963	768.5	0.199
30	625789	1135.2	0.181
40	865548	3545.0	0.386
50	1096629	2478.0	0.226
60	1338421	1983.6	0.148
70	1580215	4151.1	0.263

Table 2: Linearity of Sulbactam

Concentration (ppm)	Average Area	SD	% RSD
5	50286	70.6	0.140
10	100732	398.6	0.396
15	151127	422.7	0.280
20	202428	256.4	0.127
25	252005	462.8	0.184
30	300850	427.9	0.142
35	349691	450.8	0.129

Table 3: Summary of validation parameter for CEF and SUL

Parameters	RP-UPLC method	
	Ceftriaxone	Sulbactam
Concentration range (ppm)	10-70	5-35
Slope	23648	9995.2
Intercept	80549	1112.9
Correlation coefficient	0.9999	0.9999
LOD[a] (µg/ml)	0.008	0.025
LOQ[b] (µg/ml)	0.027	0.083
Repeatability (% RSD[d], n = 6)	0.35	0.39
Precision (% RSD)		
Inter day (*n* = 3)	0.52-0.203	0.33-0.153
Intraday (*n* = 3)	0.038-0.319	0.088-0.307
Accuracy (% RSD[d])	0.032-0.088	0.116-0.145

a = Limit of detection b = Limit of quantification n = number of determinations
d = Relative standard deviation

Table 4: System suitability test parameters for CEF and SUL

Parameters	CEF ± % RSD	SUL ± % RSD
Retention time (min)	1.002 ± 0.417	0.784 ± 0.251
Tailing factor	1.03 ± 0.500	1.08 ± 0.586
Theoretical plates	10670 ± 0.136	9859 ± 0.082
Resolution	6.24 ± 0.187	

Table 5: Analysis of marketed formulation of CEF and SUL

Injection	Label claim (1000 mg/Vial)		Amount found (500 mg/Vial)		% Label claim ± %RSD (n=3)	
	CEF	SUL	CEF	SUL	CEF	SUL
I	1000	500	994.1	499.9	99.41 ± 0.040	99.98 ± 0.060

Table 6: %Degradation of Ceftriaxone and Sulbactam in different conditions

Degradation condition	Area		Concentration In mcg/ml		% Potency		% Degradation	
	CEF	SUL	CEF	SUL	CEF	SUL	CEF	SUL
Acidic/ 0.1 N HCl/ 70°C/Reflux/ 4hr/ Solution	865322	202219	39.99	19.98	99.98	99.90	17.71	13.72
	712082	174451	32.91	17.24	82.27	86.18		
Alkaline/ 0.1N NaOH/ Reflux/70°C/ 4 hr/ Solution	865106	202331	39.98	19.99	99.95	99.95	11.51	19.31
	765478	163238	35.38	16.13	88.44	80.64		
Oxidative/ 3% H2O2/ Reflux/1 hr/ Solution	864890	202228	39.97	19.98	99.93	99.90	14.96	10.82
	735452	180319	33.99	17.82	84.97	89.08		
Thermal / 60°C/ 24 hr/ Solid	865539	202430	40.00	20.00	100.00	100.00	0.53	0.46
	860952	201499	39.79	19.91	99.47	99.54		
Photo/1.2 million lux hrs fluorescent light/200w/m^2 of UV/7 days	865543	202425	40.00	20.00	100.00	100.00	5.27	2.31
	819919	197758	37.89	19.54	94.73	97.69		

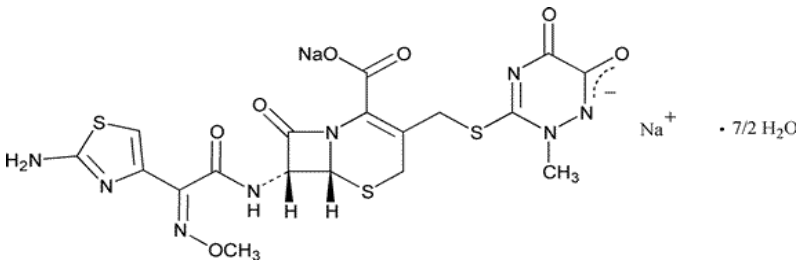

Figure 1: Structure of Ceftriaxone sodium

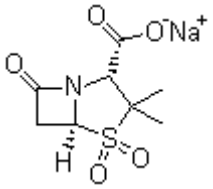

Figure 2: Structure of Sulbactam sodium

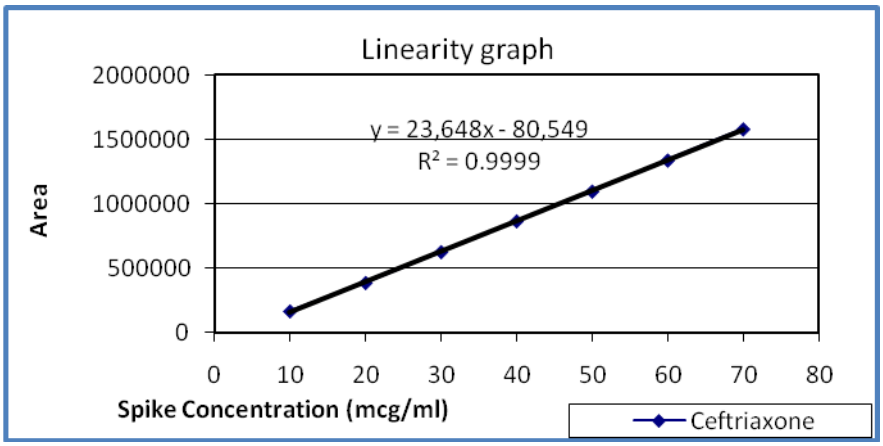

Figure 3: Linearity of Ceftriaxone

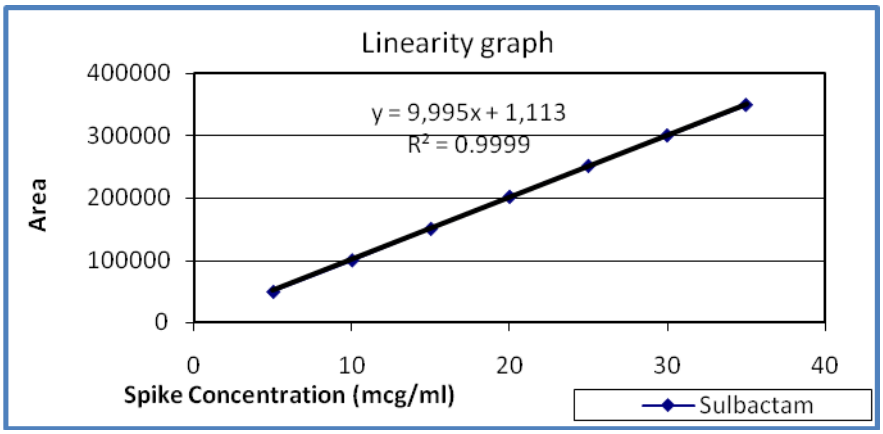

Figure 4: Linearity of Sulbactam

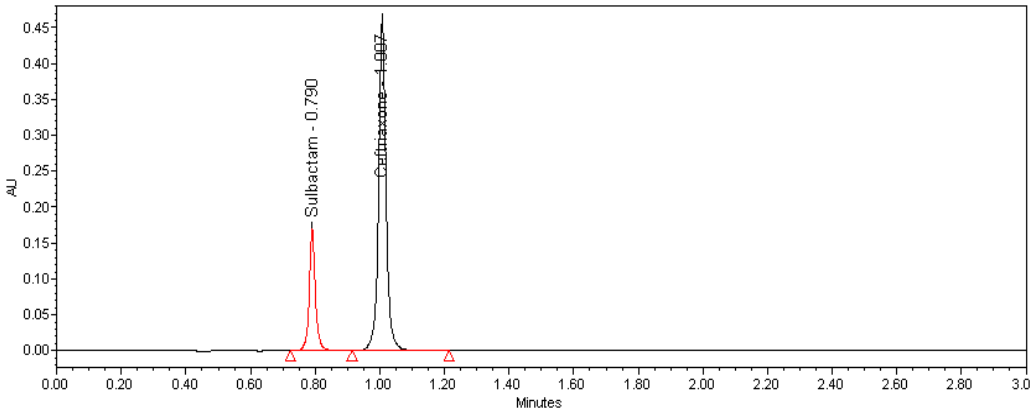

Figure 5: Optimized condition chromatogram of Assay of Drug

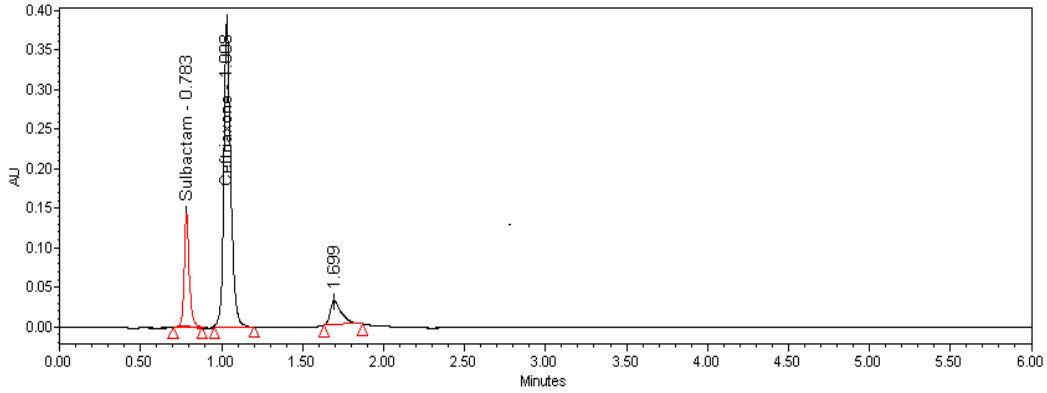

Figure 6: Acid hydrolysis of Ceftriaxone and Sulbactam

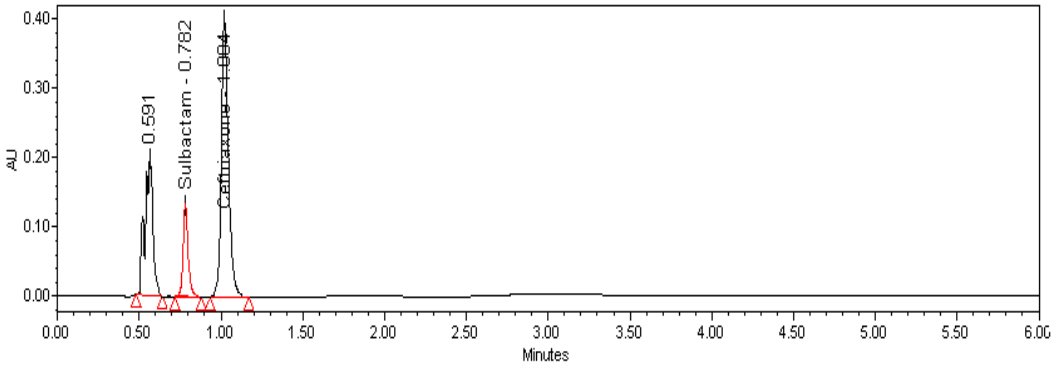

Figure 7: Base hydrolysis of Ceftriaxone and Sulbactam

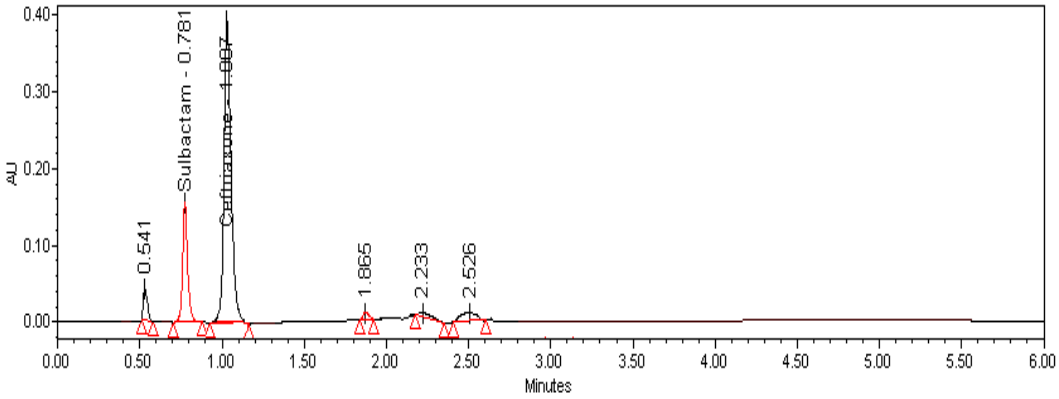

Figure 8: Oxidation of Ceftriaxone and Sulbactam

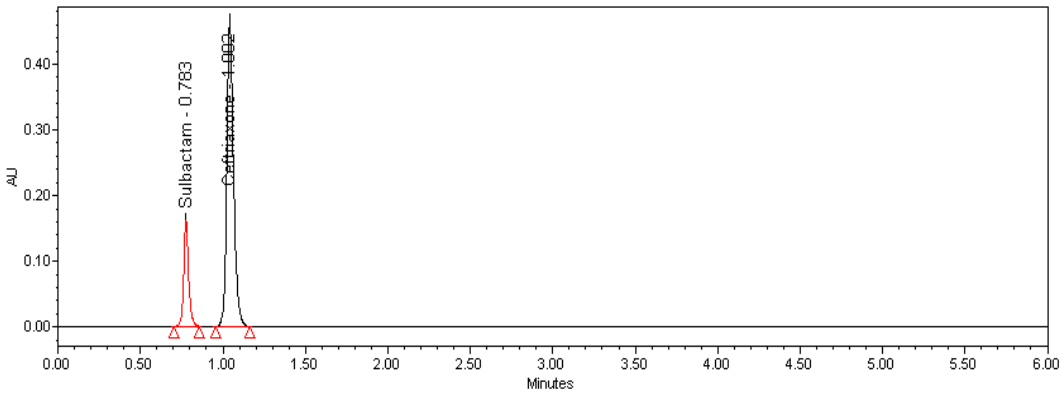

Figure 9: Thermal degradation of Ceftriaxone and Sulbactam

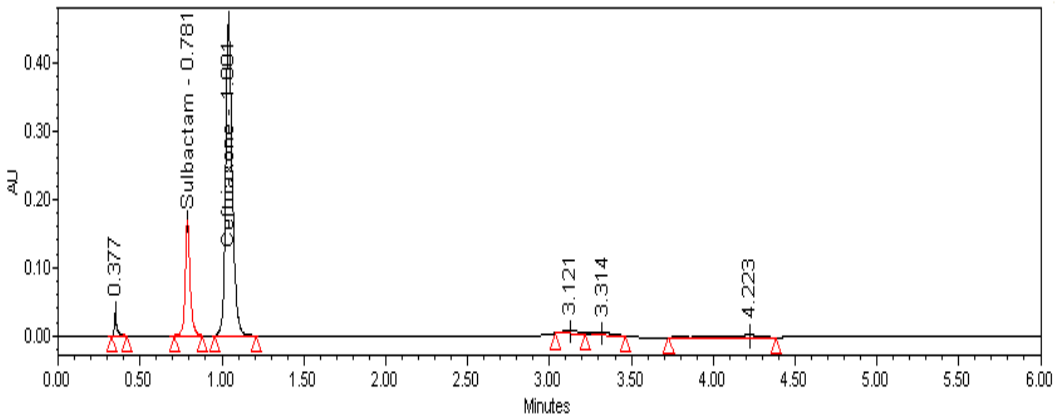

Figure 10: Photo stability of Ceftriaxone and Sulbactam