Research Article

Development and Validation of HPLC Method for Simultaneous Estimation of Ceftriaxone and Sulbactam in Pharmaceutical Dosage Form

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ABSTRACT

A simple, precise, sensitive and accurate High Performance Liquid Chromatography method was developed for the estimation of Ceftriaxone and Sulbactam in pharmaceutical dosage form to create an affordable and reliable method. The sample was analyzed with BDS hypersil C_{18} column (250*4.6mm and 5 µm) thermo scientific in isocratic mode, using a mobile phase containing Phosphate buffer (pH3.5): Methanol in the ratio of 60:40 v/v at a flow rate of 1.0ml/min. The analyte were detected at 277 nm. The retention times were found to be 3.960 min and 6.220 min for Ceftriaxone and Sulbactam respectively. As per ICH guideline the method was validated. Linearity of Ceftriaxone was found in the range 10-30 µg/ml (R^2=0.999) and that for the Sulbactam was found to be 5-15 µg/ml (R^2=0.998).The accuracy of the present method was evaluated at 80%, 100% and120%. Recovery was found to be in the range 99.56-99.61% and 99.52-99.93% for Ceftriaxone and Sulbactam respectively. Intermediate precision studies were carried out and the % RSD values were less than two. The method is found to be robust since the retention times and tailing factors were within the limits even after little deliberate variations in pH, flow rate and mobile phase ratio. The values of LOD and LOQ were found to be 0.694µg/ml and2.105µg/ml for Ceftriaxone and 0.828 µg/ml and2.510µg/ml for Sulbactam. This demonstrates that the developed method is simple, precise, sensitive, accurate and robust for simultaneous estimation of Ceftriaxone and Sulbactam in pharmaceutical dosage form.

Keywords: Ceftriaxone sodium, HPLC, method development, sulbactam sodium, validation

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INTRODUCTION

Ceftriaxone is a third generation, long acting, broad spectrum parenteral cephalosporin and chemically it is Disodium (6R,7R) - 3[(acetyloxy) methyl] -7-[(2Z) – (2-amino-4-thiazolyl) (methoxy amino) – acetyl] amino] -8-oxo-5-thia-1-azabicyclo [4.2.0.] oct-2-ene- 2-carboxylic acid.It is useful for severe infections prophylactically and post-surgery. The bactericidal activity of CFTX results from inhibition of cell wall synthesis. CFTX has a molecular weight of 661.6 gm/mol. It is a sterile, semi-synthetic, white color powder, freely soluble in water and sparingly soluble in methanol [1, 2, 3].

Sulbactam is anirreversible β-lactamase inhibitor. Chemically it is(2S,5R)-3,3-Dimethyl-7-oxo-4-thia-1- azabicyclo[3.2.0] heptane-2-carboxylic acid 4,4-dioxide. It binds to the enzyme and does not allow it to degrade the antibiotic. SLB is used to treat infections caused by bacteria resistant to beta-lactamase resistant inhibitors. It blocks the enzyme which breaks down beta-lactams and thereby allows them to kill the bacteria. Its molecular weight is 233.243gm/mol, soluble in methanol and water [4].

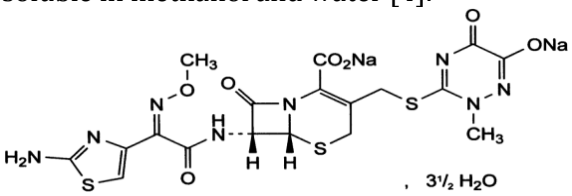

Figure 1: Structure of Ceftriaxone (CFTX)

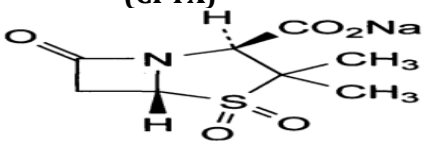

Figure 2: Structure of Sulbactam

Troxin-S injection(contain Ceftriaxone 1000mg and Sulbactam 500mg) is a synergistic anti-microbial combination with marked in-vitro antibacterial activity against a broad spectrum of organisms. The combine formulation has been approved by Central Drug Standard Control Organization (CDSCO) in 21/09/2011[5].From literature review it has been revealed that for estimation of CFTX and SLB as an individual and in combination with other drugs, research work has been carried out [6-8]. Very few analytical methods including liquid chromatography and stability indicating HPLC methods have been reported for the estimation of CFTX and SLB in combined pharmaceutical formulation [9,10]. Current study has been carried out for development and validation of a new assay method for the analysis of CFTX along with SLB in pharmaceutical dosage form as an alternative and reliable method.

MATERIALS AND METHODS

Instrumentation:

The present work was executed on a Shimadzu HPLC system with SPD-20AT detector. A HPLC column BDS hypersil C_{18} column (250 mm, 4.6mm and 5 µm**)** thermo scientificwas used for elution. The signal output was recorded and interpreted through Spinchrom software.

Chemicals and Solvents:

Pharmaceutical grade CFTX sodium, SLB sodium and its commercial formulation Troxin-S injection containing CFTX sodium 1gm and SLB sodium 0.5 gm were procured from Intracin Pharmaceuticals pvt Ltd. Nadiad, Gujarat. HPLC grade methanol, acetonitrile and water were procured from Finar Chemicals Ltd., Mumbai, India. AR grade Ortho-phosphoric acid, Tri ethyl amine and Potassium dihydrogen phosphate were procured from Merck (India) Ltd.

Preparation of Standard solutions:

Reference standard of CFTX 20 mg and SLB 10 mg was transferred to 100 ml volumetric flask and dissolved in methanol. The flask was shaken for 30 min and the volume was made up to the mark with methanol to obtain standard stock solution of CFTX (200 µg/ml) and SLB (100 µg/ml). Stock solution was filtered through a 0.45 µm glass fiber filter. It was further diluted to get 20 µg/ml of CFTX and 10µg/ml of SLB. The working standard solutions of CFTX and SLB were prepared from suitable aliquots of stock solution.

Preparation of Sample Solution:

Equivalent weight of powder 20 mg of CFTX and 10 mg of SLB was taken and transferred in to a 100 ml volumetric flask. Then solution was filtered through wattman filter paper and the volume was adjusted up to the mark with methanol. From this 1 ml solution was taken in to a 10 ml volumetric flask and the volume was adjusted up to mark with methanol to get a concentration of CFTX (20 µg/ml) and SLB (10 µg/ml).

Buffer preparation:

Accurately weighed quantity of 6.8 grams of Potassium di-hydrogen phosphate was transferred to 1000ml of water and mixed. The pH was adjusted to 3.5 with 0.05M ortho-phosphoric acid solution. The solution was filtered through 0.45µm membrane filter.

Determination of maximum wave length (λ_{max}):

Solutions of 20µg/ml and 10µg/ml of CFTX and SLB were prepared separately and scanned from 190-400nm in UV-Visible spectrophotometer. The optimum response of both the analyte was observed at 277 nm. Hence, this wavelength was selected for further analysis.

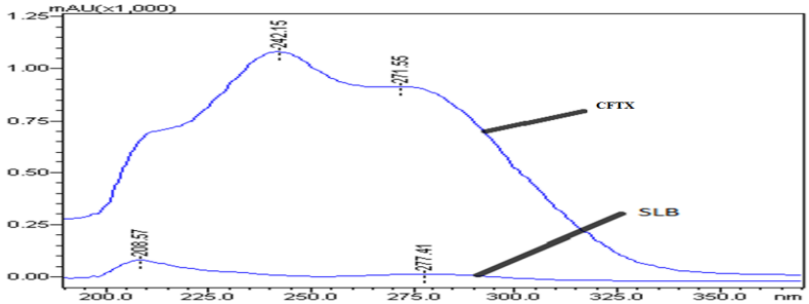

Figure 3: UV- Overlay spectra of Ceftriaxone and Sulbactam

Chromatographic conditions:
HPLC column BDS hypersil C_{18} column (250 mm, 4.6mm and 5 µm**)** thermo scientificwas used for elution. The mobile phase was pumped through the column at a flow rate of 1ml/min. The sample injection volume was 20µl. The detector was set to a wavelength of 277 nm and the chromatographic run time was set to 20 minutes.

Method Development:
The method development was started with initial chromatographic conditions as stated above. Various compositions of water, methanol, acetonitrile and phosphate buffer were tested for better separation of the analytes. Different mobile phases were applied still satisfactory result not obtained. Finally the method was optimized with the following condition given in (**Table 1**). The chromatogram is shown in (**Fig. 4**).

Table 1: Optimized Chromatographic conditions

Parameters	Conditions
Mobile phase	Phosphate buffer (pH: 3.5): Methanol (60:40 v/v)
Stationary phase	BDS hypersil C_{18}, 250mm × 4.6mm, 5µ (particle size), Thermo scientific
Flow rate (ml/min.)	1
Run time (min.)	10
Volume of Injection (µL)	20
Detection wavelength (nm)	277
Retention time (min.)	CFTX: 3.96,SLB: 6.32

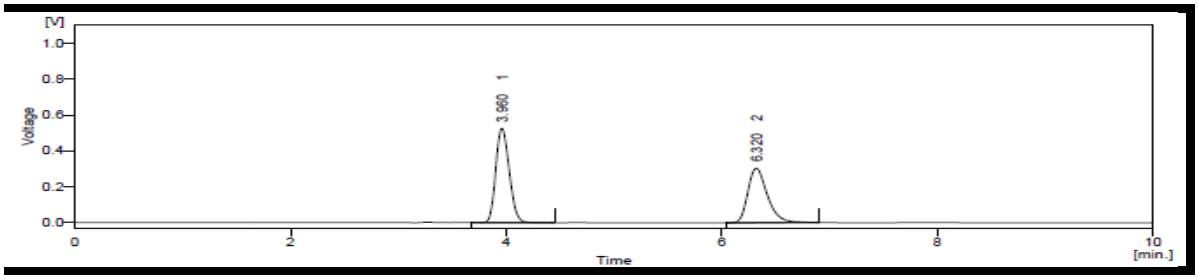

Figure 4: Chromatogram of CFTX (20µg/ml) and SLB (10µg/ml) by HPLC method

Method Validation:
The proposed method is validated as per International Conference on Harmonization [ICH (Q2B)] guidelines by the following parameters.

Linearity:
Several aliquots of standard stock solutions of CFTX and SLB were transferred into 10ml volumetric flasks and diluted up to the mark by diluents to achieve the concentrations of 10 to 30µg/ml for CFTX and 5 to 15µg/ml for SLB. Each sample solution was injected into HPLC system and the peak areas were measured. A graph of peak areas vs. concentrations was plotted and the correlation coefficient was calculated. The results are shown in Table –2 & 3 and in (**Fig. 5 & 6**).

Table 2: Result of Linearity

CFTX		SLB	
Concentration (µg/ml)	**Mean Peak Area (n=3)**	**Concentration (µg/ml)**	**Mean Peak Area (n=3)**
10	2240.161	5	1878.693
15	3307.08	7.5	2773.397
20	4521.21	10	3791.58
25	5566.676	12.5	4668.28
30	6772.74	15	5879.762
Correlation coefficient	0.99973	**Correlation coefficient**	0.99849

n = numbers of replicate injection

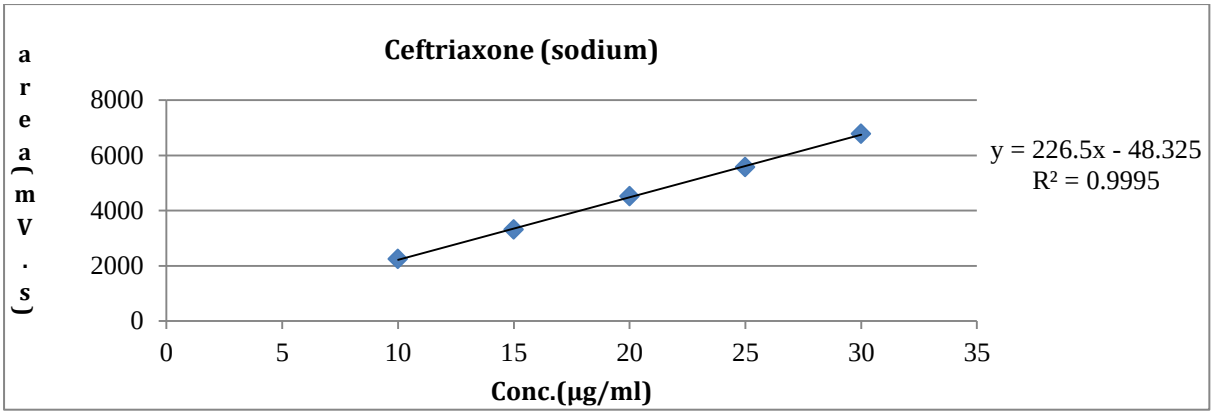

Figure 5: Calibration curve of CFTX

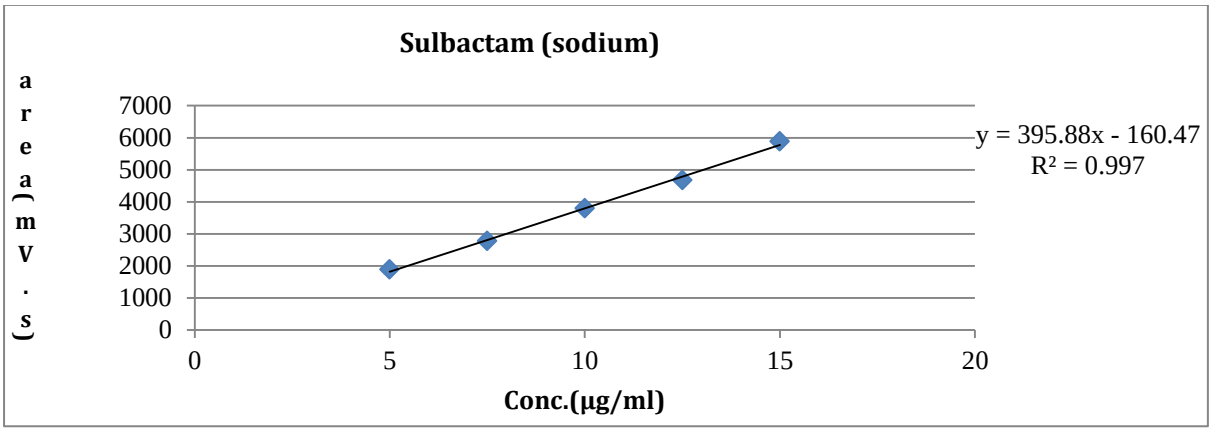

Figure 6: Calibration curve of SLB

Table 3: Regression Analysis Data

Parameters	CFTX	SLB
Linear Range (n=3) (µg/ml)	10-30	5-15
Regression Equation	Y=226.5x - 48.325	Y=395.88x - 160.47
Slope	226.5	395.88
Intercept	48.325	160.47
Correlation Coefficient (R^2)	0.999	0.998

n = number of replicate injections

System Suitability:

Sample solutions of CFTX and SLB were injected in triplicates as per the procedure. From the standard chromatogram system suitability parameters like theoretical plates, tailing factor and resolution are evaluated. The results are given in (**Table 4**).

Table 4: System Suitability Studies

Parameters	CFTX	SLB	Acceptance criteria
Theoretical plates	4432	5920	Not less than 2000
USP tailing factor	1.313	1.523	Not more than 2
Resolution	8.332		Not less than 2

Accuracy:

The accuracy of the proposed method was evaluated in triplicates by recovery studies at various concentrations of CFTX and SLB equivalent to 80%, 100% and 120% by addition of known amounts of standard to pre-analyzed sample preparation. For each concentration, three sets were prepared and injected. The percentage recovery values are calculated in (**Table 5**).

Table 5: Recovery studies for Ceftriaxone and Sulbactam

Drug	% Level	Amount of sample taken (µg/ml)	Amount of standard spiked (µg /ml)	Total conc. (µg/ml)	Amount of standard recover (µg /ml)	Peak Area	% Recovery
CFTX	80	10	8	18	7.96	4067.2	99.61
	100	10	10	20	9.95	4541.5	99.56
	120	10	12	22	11.89	5008.0	99.58
SLB	80	5	4	9	3.99	3480.7	99.92
	100	5	5	10	4.98	3853.2	99.63
	120	5	6	11	5.97	4231.1	99.63

The percentage recovery for each level should be between 98.0-102.0% as per ICH guideline. The percentage recovery of CFTX and SLB are found to be within the limits i.e. 99.56-99.61% and 99.63-99.92% respectively.

Precision:

Repeatability, intraday and interday precision were evaluated by injecting the sample solutions in to the HPLC system in triplicate on the same day (repeatability) and on different days (intermediate precision) at 20 µg/ml of CFTX and 10 µg/ml of SLB. The results were reported in terms of % RSD. The results are given in (**Table 6 & 7**).

Table 6: Results of Repeatability

Sr. No.	Peak area		Retention time (min)		Tailing factor	
	CFTX	SLB	CFTX	SLB	CFTX	SLB
1	4503.2	3776.44	3.960	6.270	1.313	1.558
2	4457.78	3784.02	3.963	6.277	1.355	1.558
3	4521.204	3708.85	3.970	6.283	1.313	1.535
4	4530.25	3799.16	3.973	6.290	1.313	1.523
5	4507.76	3780.24	3.960	6.270	1.313	1.558
6	4516.61	3787.81	3.963	6.277	1.344	1.558
Mean	4506.13	3772.75	3.964	6.277	1.325	1.548
SD	23.337	32.2621	Limit: %RSD for area NMT 2.0%			
%RSD	0.5179	0.8551				

Table 7: Results of Intermediate Precision

Drug	Concentration (µg/ml)	Inter-day area mean (n=3) ± SD	%RSD	Intra-day area mean (n=3) ± SD	%RSD
CFTX	10	2218.93 ± 17.49	0.788	2223.61 ± 15.41	0.92
	20	4480.94 ± 30.84	0.689	4475.64 ± 51.86	1.15
	30	6727.83 ± 20.15	0.299	6728.42 ± 32.63	0.48
SLB	5	1850.81 ± 28.77	1.55	1858.56 ± 20.77	1.11
	10	3732.17 ± 63.54	1.70	3745.32 ± 51.48	1.37
	15	5619.56 ± 45.29	0.80	5617.5 ± 69.47	1.23

Robustness:

It is the capacity of the analytical method to remain unaffected by small but deliberate variations in method parameters. The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like pH, flow rate and mobile phase ratio etc.

Effect of variation of pH: The pH is varied between 3.3-3.7 and the chromatograms are recorded.

Effect of variation of flow rate: The flow rate is varied between 0.8-1.2ml/min and the chromatograms are recorded.

Effect of variation of mobile phase: Standard solution was prepared and injected into HPLC system and the chromatograms were recorded at two different mobile phase ratio. The results are summarized in (**Table 8**).

Table 8: Results of robustness

Variation		CFTX		SLB	
		RT	Peak Area	RT	Peak Area
pH (3.5)	3.7	3.573	4310.77	6.374	3594.36
	3.3	3.456	4627.47	6.276	3894.15
% RSD		0.837		0.961	
Flow Rate (1ml/min)	1.2	3.716	4406.35	6.347	3667.40
	0.8	3.542	4674.56	6.425	3896.22
% RSD		0.918		1.598	
Mobile Phase Ratio (60:40 v/v)	62:42	3.734	4394.21	6.365	3698.03
	58:38	3.465	4622.27	6.629	3854.11
% RSD		1.053		1.276	

RT= Retention time

The method is robust since the retention times and tailing factors were within the limits even after little deliberate variations in pH(±0.2), flow rate (±0.2ml/min) and mobile phase (±2 ml).

Limit of Detection and Limit of Quantification:

The detection limit of an analytical method is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated.

Limit of quantification is the lowest concentration of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy.

$$DL = \frac{3.3\,\sigma}{S} \qquad QL = \frac{10\,\sigma}{S}$$

σ = standard deviation of the response

S= slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte. The results are given in (**Table 9**).

Table 9: Results of LOD and LOQ

Sr. No	Parameters	CFTX	SLB
1	LOD(µg/ml)	0.69474	0.82830
2	LOQ(µg/ml)	2.10528	2.51001

Specificity:

It is the ability of the method to measure the analyte of interest specifically in presence of matrix and other components. Samples of blank and placebo were injected as per the test procedure. The chromatograms of blank and placebo are represented as (**Fig. 7 & 8**).

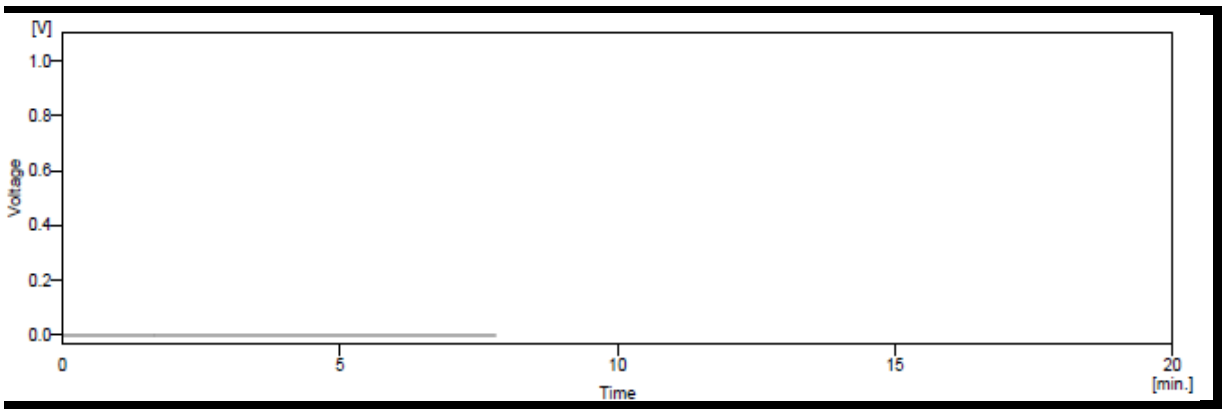

Figure 7: Chromatogram of Blank

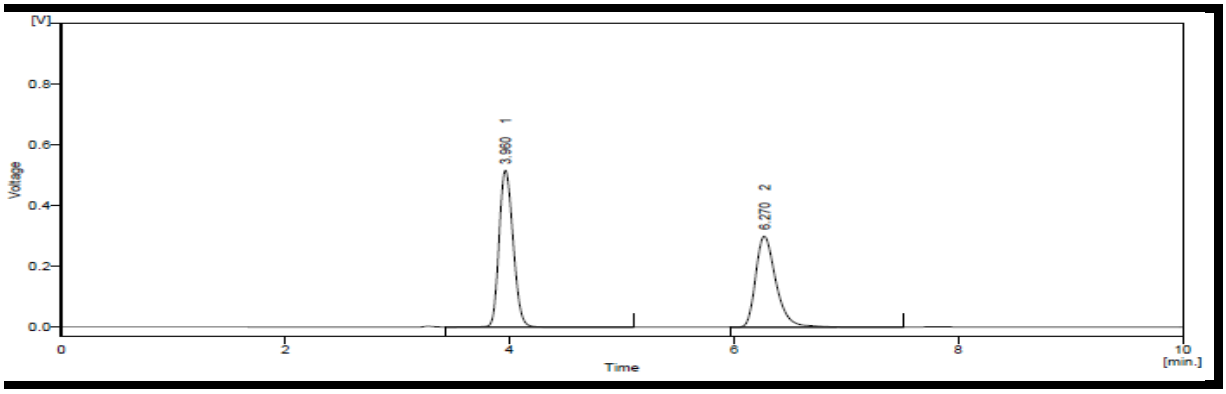

Figure 8: Chromatogram of placebo

No peaks were observed near the retention times of CFTX and SLB in the chromatogram of blank indicating no interference from mobile phase. Therefore the method is found to be specific.

Quantitative estimation of marketed pharmaceutical formulation

The proposed validated method was successfully applied to determine CFTX and SLB injection formulation. 1.5 gm powder mixture of CFTX and SLB was transferred in to a 10 ml volumetric flask and dissolved in methanol, it was shaken for 30 min and the volume was made up to the mark with mobile phase. The content was ultra sonicated for 20 min. It contains 1000 µg CFTX and 500 µg of SLB. Again taken 0.2 ml of solution in 100ml volumetric flask and make up to 100 ml in volumetric flask. It contains CFTX (20 µg/ml) and SLB (10 µg/ml). The solution was filtered through a 0.45 µm glass filter paper. This solution was further diluted with mobile phase to obtain mixed sample solutions in the Beer's and Lamberts range. The assay result obtained for CFTX and SLB was comparable with the corresponding labeled amounts which are given in (**Table 10**).

Table 10: Results of Quantitative estimation

Sr. No.	Actual concentration CFTX (mg/5 ml)	Actual concentration SLB (mg/5 ml)	Concentration found CFTX (µg/ml)	Concentration found SLB (µg/ml)	% Assay (90-115%) CFTX (%)	% Assay (90-115%) SLB (%)
1	1000	500	977.71	507.05	97.77	101.41
2	1000	500	988.78	498.10	98.87	99.62
3	1000	500	991.77	509.59	99.17	101.91
		Mean			98.609	100.98
		SD			0.740929	1.2073
		% RSD (Limit: NMT 2.0%)			0.751379	1.1955

RESULTS AND DISCUSSION

Several mobile phase compositions were tried to resolve the peak of CFTX and SLB. The mobile phase containing Phosphate buffer: Methanol in proportion of 60:40 v/v was found ideal to resolve the peak of CFTX and SLB. Retention time of CFTX and SLB were 3.96 and 6.32 min respectively (**Figure 4**). The proposed method was found to be linear in concentration range10-30µg/ml for CFTX and 5-15 µg/ml for SLB. The data was shown in (**Table 2 & 3**) and (**Figure 5 & 6**). System suitability parameters were evaluated and results shown in (**Table 4**), which were within acceptance criteria. The mean percentage recovery for CFTX and SLB were found to be 99.58% and 99.72% respectively, which are well within the limit and hence the method was found to be accurate (**Table 5**). Results of repeatability were shown in (**Table 6**) and results of intermediate precision were shown in (**Table 7**). The robustness of the method was investigated by varying experimental conditions such as changes in pH, flow rate and mobile phase ratio. The result obtained implies method is robust for routine

qualitative analysis (**Table 8**). LOD and LOQ values were 0.694µg/mL and 2.105µg/mL for CFTX and 0.828µg/mL and 2.510µg/mL for SLB (**Table 9**)**.** Result of assay is shown in (**Table 10**).

CONCLUSION

The proposed High performance liquid chromatographic method is simple, sensitive, rapid and economical. The accountability of the proposed method has been established by evaluating validation parameters as per ICH (Q2B) guidelines. The results were in good agreement with acceptable limits. Therefore the method has been proven to be linear, accurate, precise, specific and robust. This method can be adopted for the estimation of CFTX and SLB in bulk and also in combined pharmaceutical dosage forms as a part of regular quality control analysis.

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REFERENCES

1. Indian pharmacopoeia. 6th Ed. Ministry of health and family welfare, Indian Pharmacopoeial Commission, Ghaziabad, India, 2010. 1024-1025.
2. British pharmacopoeia. 6th Ed. The Stationary Office, London Medicines and Healthcare Product Regulatory Agency, 2009. 408-409.
3. United State Pharmacopoeia- 34 and National Formulary-29. Asian Ed. United State Pharmacopoeia Convention, Rockville, MD, USA, 2011. 1697.
4. British pharmacopoeia. 6th Ed. The Stationary Office, London Medicines and Healthcare Product Regulatory Agency, 2009. 1938-1939.
5. Approval of Central Drug Standard Control Organization [Online]. 2011October;Available from: URL:cdsco.nic.in/new%20drug%20update.doc
6. Shanta Adiki, Baisakhi Dey, Nureddin Arebi, Fauzi Melad, Prakash Katakam and Babu Rao Chandu.Development and validation of UV and RP-HPLC methods for the estimation of Ceftriaxone sodium in pharmaceutical formulations. Journal of Pharmaceutical Science and Innovation. 2014;Jan-Feb, 3(1):87-90.
7. Panak Patel, Vidisha Patel and Gautam Chauhan. Development and validation of high performance thin layer chromatographic method for simultaneous estimation of Ceftriaxone sodium and Tazobactam sodium in their combined pharmaceutical formulation. 2014; May, 6(5):623-629.
8. R Nanda, V Bhagwat, S Potawale, N Vidyasagar and R Mishra.Spectrophotometric method for simultaneous estimation of Cefotaxim sodium and Sulbactum sodium in pharmaceutical dosage form.International Journal of Pharmaceutical Science and Research. 2010;Sept,2(3):1612-1617.
9. Nirav Patel, Alpesh Arvadiya, Sneha Jansari, Vipul Mistry and Hemant Desai. Development and validation of stability indicating method for simultaneous estimation of Ceftriaxone and Sulbactam injection using RP-UPLC method. IOSR Journal of Pharmacy. 2012;Dec,2(6):29-37.
10. B Shrestha, N Bhuyan and B Sinha. Development and validation of a stability indicating HPLC method for estimation of Ceftriaxone and Sulbactam in sterile powder forinjection. International Journal of PharmTech Research. 2012; Dec, 4(4):1660-1666.