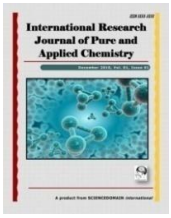

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A Validated RP-HPLC-UV Method for Simultaneous Estimation of Ceftriaxone and Sulbactum in Rat Plasma

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

A reverse phase-liquid chromatographic method with UV detection is developed for simultaneous estimation of ceftriaxone sodium and sulbactam sodium in rat plasma. A simple protein precipitation technique was employed for the extraction of drugs from blank plasma. Chromatographic separation of the two drugs was achieved on C18 column (250 mm X 4.6 mm, i.d, 5 µm) using a mobile phase consisting of 10mM potassium dihydrogen orthophosphate buffer (pH- 5) and acetonitrile (90:10% v/v). The developed liquid chromatographic method eluted both the drugs with a reasonable retention time, acceptable symmetric peak shape and good resolution. The developed bio-analytical method was linear, accurate, and precise over the concentration range of 20-150 µg mL^{-1} for ceftriaxone and 10-75 µg mL^{-1} for sulbactam. The developed method was successfully applied to monitor ceftriaxone and sulbactam sodium concentrations in rat plasma.

Keywords: Ceftriaxone sodium; sulbactam sodium; liquid chromatography; rat plasma.

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1. INTRODUCTION

Ceftriaxone [1] (CFX) is a third generation cephalosporin. Chemically it is (6R,7R)-7-{2-(2-amino-4-thiazolyl)-(Z)-2- [methoxyiminuteo-acetamido]-3{[(2,5-dihydro-6-hydroxy-2-methyl-5-oxo-as-triazin-3-yl)thio]methyl}-8-oxo-5-thia-l-azobicyclo [4,2,0] oct-2-ene-2-carboxylic acid. Sulbactam (SBM) chemically (2S,5R)-3,3-Dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane -2-carboxylic acid 4,4-dioxide is used as a beta-lactamase inhibitor. Structural formulae of CFX and SBM are given in Fig. 1. These drugs are frequently associated in pharmaceutical formulations against meningitis, typhoid, gonorrhoea and urinary tract infections [2].

Sulbactomax is a commercially available pharmaceutical product containing SBM and CFX. The product is available as a dry powder for injection. The product is supplied in different strengths (250 mg+125 mg, 500 mg+250 mg, 1 gm+0.5 gm, 2 gm+1 gm) of CFX and SBM respectively.

Sulbactomax is a synergistic antimicrobial formulation with marked *in vitro* antibacterial action against a broad spectrum of organisms. This formulation not only potentiates the antibacterial activity of CFX but also shows a moderate antibacterial activity by forming a protein complex with beta-lactamas. SBM permanently blocks their destructive hydrolytic activity. So, SBM extends the spectrum of activity of CFX. As SBM also binds with some penicillin binding proteins, sensitive strains are often rendered added susceptible to the Sulbactomax than CFX. In bacterial strains that make also low amounts of beta- lactamase, or none at all, a synergistic effect is observed when SBM is related with CFX that has a corresponding affinity for the target sites. Sulbactomax is active against all the organisms sensitive to CFX. In addition, it shows synergistic activity (reduction in minimum inhibitory concentrations for the combination against those of each component) in a variety of organisms. Improved efficiency as compared to CFX alone, smaller side effects, broader spectrum coverage and improved results of bacterial MIC (minimum inhibitory concentration) makes this product distinctive in the world. A literature survey revealed a spectrophotometric [3], spectrofluorimetric in human plasma [4], HPLC for the estimation of marketed formulations [5,6], in human plasma [7] and for the determination of pharmacokinetics in dogs [8], capillary electrophoresis [9] and Gas –Mass spectroscopy [10] methods for the estimation of CFX and SBM individually and in combined forms. However, there is no method development reported for the simultaneous estimation of CFX and SBM by HPLC in rat plasma. The present communication describes an isocratic liquid chromatography (LC) method for simultaneous determination of CFX sodium and SBM, which would be used for the quality control of the formulation developed and other biological applications.

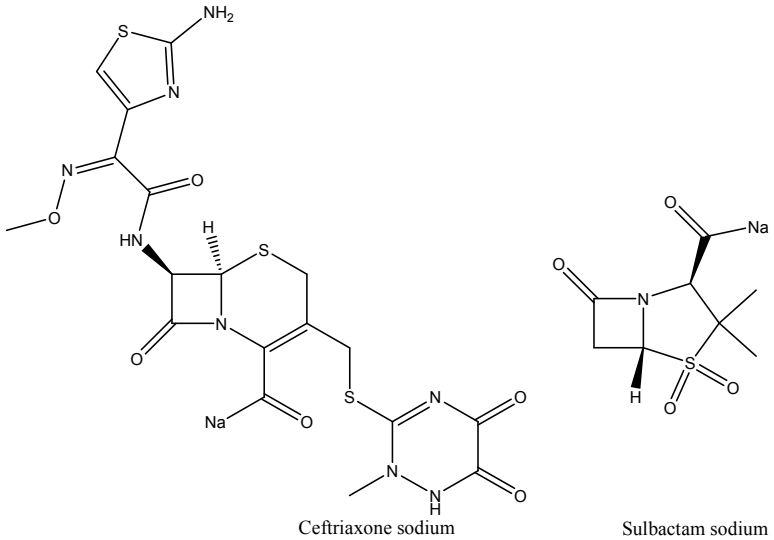

Fig. 1. Chemical structure of CFX and SBM

2. EXPERIMENTAL

2.1 Chemicals and Reagents

All chemicals and reagents used were of analytical grade only. Milli-Q-water was used throughout the process and acetonitrile of HPLC grade were procured from Merck Chemical Laboratories, Bangalore, India. Commercial formulation, CetriaxS injection containing ceftriaxone sodium 1gm and sulbactam sodium 0.5 gm were obtained from the local market. Blank rat plasma was obtained from Department of Pharmacology, JSS Pharmacy College Mysore, India.

2.2 Instrumentation and Analytical Conditions

A HPLC connected with UV detector was used for the current research work. The separation was obtained using C-18 column. The mobile phase was a combination of phosphate buffer (pH adjusted to 5 with potassium hydroxide) and acetonitrile (90:10) v/v. The mobile phase was filtered before use through 0.45 µ membrane filter, degassed with a helium sparge for 15 min at flow rate of 1.0 mL min^{-1}. The column temperature was kept at 20±10°C and the injection volume of samples was 10 µL. The analyte was monitored at wavelength of 230 nm and optimized chromatographic conditions are shown in Table 1.

2.3 Preparation of Mobile Phase

Phosphate buffer was prepared by dissolving 1.36gm of Potassium dihydrogen orthophosphate in 1000 mL of water and sonicated using sonicator for 5 minutes, then the pH was attuned to 5 using potassium hydroxide solution. Then the solution was filtered by vacuum filtration. The mobile phase was prepared by mixing phosphate buffer and acetonitrile in the ratio 90:10v/v.

2.4 Preparation of Standard and Sample Solution

Separately weighed quantity of CFX sodium (10 mg) and SBM sodium (10 mg) was transferred into a 100 mL volumetric flask and made up to 100 mL with water to get 100 µg mL^{-1} of CFX sodium and 100 µg mL^{-1} of SBM. From this, different solutions containing the mixture of CFX sodium (20-150 µg mL^{-1}) and SBM sodium (10-75 µg mL^{-1}) were prepared.

For the preparation of sample solution, Cetriax-S powder for injection (containing 1gm of CFX and 0.5 gm of SBM) was transferred to a 100 mL volumetric flask. Distilled water was added, and then swirled to dissolve it, diluted to 100 mL with the same solvent.

2.5 Preparation of Calibration Curve

Five different concentrated solutions containing mixture of CFX (20-150 µg mL^{-1}) and SBM (10-75 µg mL^{-1}) were injected onto HPLC. A calibration curve was prepared taking concentrations on X-axis and Peak Area on Y-Axis.

2.6 Preparation of Plasma Samples

Plasma samples of CFX and SBM was prepared by the protein precipitation method. A blank was prepared by taking 0.1 mL of rat plasma and to this 1.9 mL of acetonitrile was added and sample was prepared by taking 0.1 mL of combination of CFX and SBM (which were mixed in equal volumes) and 0.1 mL of rat plasma was added to the 2 mL Eppendorf tubes containing 1.8 mL of acetonitrile. These samples were centrifuged for 10 min at 10,000 rpm. The supernatant solution filtered through 0.45 µ syringe filter and transferred to HPLC vials.

3. RESULTS AND DISCUSSION

3.1 Method Development

Taking into consideration, the instability of CFX and SBM in strong basic and strong acidic condition, the pH value of the mobile phase should be limited within the range of 3-7, since low acidic pH favors the retention and separation of two drugs on C-18 column. After some changes in the composition, phosphate buffer with pH 5 was finally selected. The method development selected with the combination of methanol and phosphate buffer. In this mobile trial phase, the drugs were not eluted effectively, so the organic phase was changed from methanol to acetonitrile as solvent. Both CFX and SBM in the mobile phase with the wavelength of 230 nm was selected for the analysis. After a number of preliminary trials for selection of column, a Phenomenex C-18 column and binary mixture of phosphate buffer (pH 5) and acetonitrile (90:10% v/v) was optimized as mobile phase which formed symmetric peak shape, good resolution and reasonable retention time for both the drugs (Table 1). The separation times of CFX and SBM for six injections were found to be 7.8±0.02 min and 4.7±0.006 respectively (Fig. 2). (a) (b).

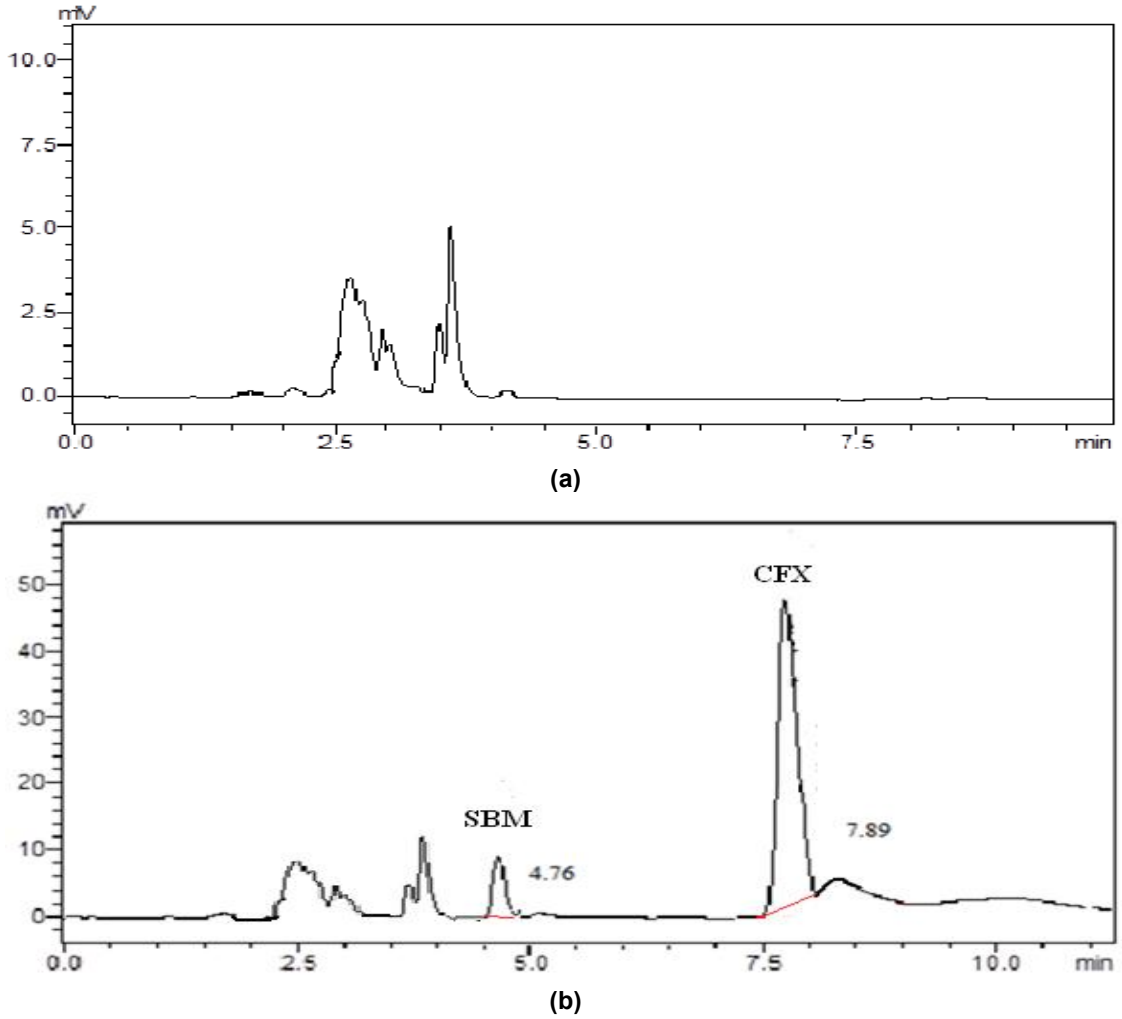

Fig. 2. LC chromatogram of rat blank plasma (a) plasma spiked with standard CFX and SBM(b)

Table 1. Optimized chromatographic conditions

Parameter	Optimized condition
Chromatograph	HPLC with UV- detector
Column	C18 Column with 5 µm particle size and 150 mm x 4.6 mm in length
Mobile phase	Acetonitrile and pH-5 buffer in the ratio of 10:90(v/v)
Flow rate	1.00 mL min^{-1}
Detection	230 nm
Injection volume	10 µL
Temperature column	Room temperature

3.2 Method Validation

Method validation is a process of establishing documented evidence, which provides a high extent of assurance that a specific activity will always produce a desired result or product gathering its predetermined specifications and quality characteristics [11]. The method was validated for different parameters like linearity, accuracy, precision, recovery, selectivity and sensitivity [12].

3.2.1 Selectivity

Selectivity is defined as, "the competence of an analytical method to discriminate and determine

the analyte in the presence of other components in the sample [12]". Selectivity is obtained by injecting extracted blank plasma and comparing with the results of extracted LLOQ samples. Both the peaks of Ceftriaxone and Sulbactum did not interfere with any additive components.

3.2.2 Sensitivity

Sensitivity is measured using Lower Limit of Quantification (LLOQ). LLOQ is the smallest concentration of the standard curve that can be determined with suitable accuracy and precision [12]". The LLOQ was established using five samples independent of standards and determined the co-efficient of difference and appropriate confidence interval.

3.2.3 Linearity of response

To demonstrate the linearity of response, series of solutions ranging from (20-150 µg mL^{-1}) of CFX and SBM of (10-75 µg mL^{-1}) were prepared and injected onto the HPLC system following the described conditions. The graph was constructed between concentration vs. peak area and it was found that correlation co-efficient and regression analysis were within the limits and the results are summarized in the Table 2, and the calibration graphs are shown in Figs. 3 and 4 for CFX and SBM respectively.

3.2.4 Recovery

"The recovery of an sample to be analysed is the response obtained from an amount of the analyte added to and extracted from the biological component, compared to the detector response obtained for the real concentration of the pure authentic standard" [12]. The recovery of the analyte may not be 100% result [12]. Recovery experiments were carried out by comparing the methodical results for extracted samples at three concentrations (low, medium, and high) with without extracted standards that represent 100% recovery. Results are summarised in Table 3.

$$\% \text{ Recovery} = \frac{\text{Mean response of extracted sample}}{\text{Mean response of unextracted sample}} 100$$

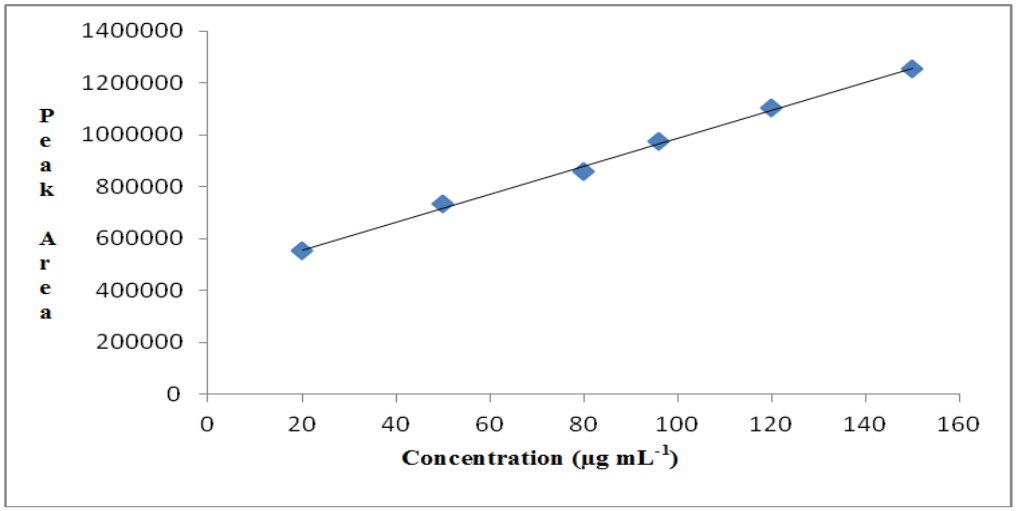

Fig. 3. Calibration graph of CFX

Table 2. Linearity of CFX and SBM

Parameters	CFX	SBM
Retention time (min)	7.3	4.6
Linear range (ppm) [n=6] (µg mL^{-1})	20-150	10-75
Correlation coefficient (r^2)	0.996	0.997
Slope	1513.1	155.58
Intercept	272333	61596
Lowest limit of quatification LLOQ (µg mL^{-1})	0.87	0.96

3.2.5 Accuracy and precision

For validation of bioanalytical method, accuracy and precision should be measured using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within ± 15% of the theoretical value, except at LLOQ, where it should not deviate by more than ± 20%. The accuracy and precision in the region of the mean value should not exceed 15% of the CV except for LLOQ, where it should not exceed 20% of the CV. The accuracy of the analytical method describes the nearness of agreement between the experiment value and the reference value. The precision of the analytical method provides the closeness of repeated individual measures of analyte. Accuracy is expressed as % obtained. Precision is expressed as the coefficient of variation (CV). The arithmetic method for estimation of the accuracy and precision should be predefined and designed according standard practice. Accuracy and Precision should be demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs and results are summarised in Tables 4 & 5.

% CV (Precision) =100 x Standard deviation/Mean.

3.2.6 Stability studies

3.2.6.1 Freeze and thaw stability

Analyte stability was verified after three freeze and thaw cycles. All the three aliquots at each of the low, medium and high concentrations were stored at the proposed storage temperature for 24 hours and placed unassisted at room temperature. When completely placed, the samples were refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle was repeated two more times, and then estimated after the third cycle.

3.2.6.2 Short-term temperature stability

Three portions of each of the low, medium and high concentrations were thawed at room temperature and kept at this temperature from 4 to 24 hours and analyzed.

3.2.6.3 Long-term stability

The sample placed time in a long-term stability assessment should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability was assessed by storing three aliquots of each of the low, medium and high concentrations under the same situation as the study samples. The concentrations of all the stability samples were compared to the mean of back-calculated values for the standards at the suitable concentrations from the first day of long-term stability testing.

3.2.6.4 Stock solution stability

The stability of stock solutions of drugs was revealed at room temperature for 6 hours. After completion of the preferred storage time, the stability was tested by comparing the instrument response with that of freshly prepared solutions Results are summarized in Table 6.

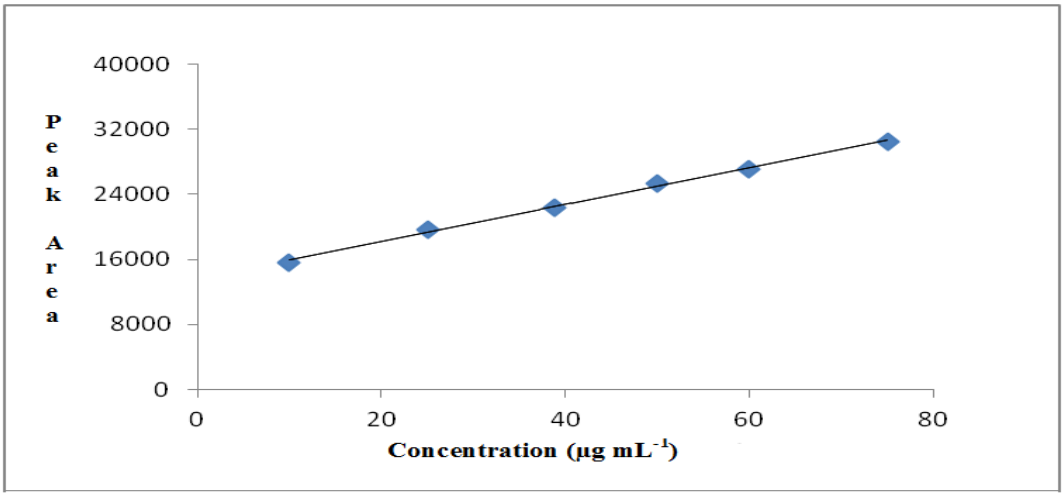

Fig. 4. Calibration graph of SBM

Table 3. Recovery studies of CFX sodium and SBM

	Concentration of CFX µg	Amount recovered % for CFX	Concentration of sulbactam µg	Amount recovered % for SBM
Low	20	98.7%	10	99.9%
Medium	100	96.8%	50	98.9%
High	150	99.3%	75	98.6%

Table 4. Accuracy and precision of CFX

Theoretical concentration µg mL^{-1}	Intra-day		Inter-day	
	%CV	Accuracy (%)	%CV	Accuracy (%)
20	0.98	98.4	1.42	96.1
100	0.76	103.7	1.32	102.3
150	1.34	99.5	1.7	98.7

Table 5. Accuracy and precision of SLB

Theoretical concentration µg mL^{-1}	Intra-day		Inter-day	
	%CV	Accuracy (%)	%CV	Accuracy (%)
10	0.96	101.7	0.76	95.6
50	1.00	99.8	1.2	103.4
75	1.02	97.3	1.04	97.4

Table 6. Stability studies of CFX and SBM

Stability	µg mL^{-1} (error %) CFX			µg mL^{-1} (error %) SBM		
	20	100	150	10	50	75
Freeze-thaw (cycle 3)	84.5	93.3	94.9	88.5	96.3	97.9
Long term (45 days)	100.5	100.6	100.8	100.5	101.6	100.8
Short term	93.9	97.6	101.4	93.9	93.6	103.4
Stock Solution	95.6	97.6	93.2	95.3	96.8	98.5

4. SUMMARY

The present work, a simple, accurate, precise and stability indicating HPLC method for the simultaneous determination of ceftriaxone and sulbactam in rat plasma was developed. The method was validated according to standard FDA guidelines. CFX and SBM were eluted at 7.3 min and 4.6 min respectively. The correlation coefficient (r^2) for CFX and SBM analysis were found to be 0.996 and 0.9976 respectively. Lower Limit of quantification (LLOQ) was found to be 0.87 µg mL^{-1} for ceftriaxone and 0.96 µg mL^{-1} for sulbactam. The %CV for the intraday and interday precision were found to be less than 2.

5. CONCLUSION

The method involves uncomplicated and precise method for simultaneous determination of CFX sodium and SBM. It produces symmetric peak shape, good resolution and reasonable retention time or both drugs. The selectivity, sensitivity, precision, and accuracy recorded with this method make it appropriate for the purpose of the present study. So this method can be applicable for the simultaneous estimation of CFX sodium and SBM for bioequivalence, pharmacokinetic and toxicokinetic studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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