Development and validation of RP-HPLC-UV method for Simultaneous Determination of Ceftriaxone and Sulbactum in Rat Plasma

ABSTRACT:

A reverse phase-liquid chromatographic method with UV detection is described for simultaneous determination 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 (250mm X 4.6mm, i.d, 5µm) using a mobile phase consisting of 10mM potassium dihydrogen orthophosphate buffer(pH- 5) and acetonitrile (90:10 % v/v). The liquid chromatographic method developed offers symmetric peak shape, good resolution, and reasonable retention time for both drugs. Linearity, accuracy, and precision were found to be acceptable over the concentration ranges 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: Liquid chromatography, Ceftriaxone sodium, Sulbactam sodium, Protein precipitation method.

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 used as a beta-lactamase inhibitor. Structural formulas of CFX and SBM were given in Fig.1. These drugs are frequently associated in pharmaceutical formulations against meningitis, typhoid, gonorrhoea and urinary track infections[2].

Sulbactomax is a commericially 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, 500mg+250 mg, 1gm+0.5gm, 2gm+1gm) of CFX and SBM respectively. It is administered as intramuscular and intravenous injection after reconstitution with solvent supplied with the pack.

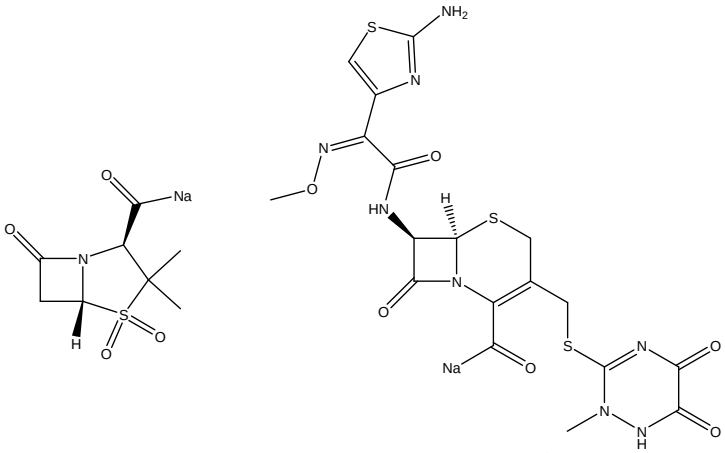


Fig.1.Chemical structure of CFX and SBM

Sulbactomax is a synergistic antimicrobial combination with marked *invitro* antibacterial activity against a broad spectrum of organisms. SBM not only potentiates the antibacterial activity of CFX but also exhibits a moderate antibacterial activity by forming a protein complex with beta-lactamas. SBM irreversibly blocks their destructive hydrolytic activity. Thus, SBM addition extends the spectrum of activity of CFX. As SBM also binds with some penicillin binding proteins, sensitive strains are often rendered more susceptible to the Sulbactomax than CFX alone. In bacterial strains that produce either low amounts of Beta- lactamase, or none at all, a synergistic effect is observed when SBM is associated with CFX that has a complementary affinity for the target sites.

Sulbactomax is active against all the organisms sensitive/resistant to CFX. In addition, it demonstrates synergistic activity (reduction in minimum inhibitory concentrations for the combination versus those of each component) in a variety of organisms. Improved Efficacy as compared to CFX alone, lesser side effects, broader spectrum coverage and improved results of bacterial MIC makes this product unique in the world.

A literature survey revealed that their exist few spectrophotometric[3], spectrofluorimetric[4], HPLC[5,6,7,8] methods for the estimation of Ceftriaxone Sodium and Sulbactam Sodium individually and in combined forms.

However, there is no work was 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.

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 local market. Blank rat plasma was obtained from JSS medical college and Hospital, Mysore, India.

2.2. Instrumentation and Analytical Conditions

A HPLC equipped with UV detector was used for the present research work. The separation was achieved using C-18 column. The mobile phase was a mixture of phosphate Buffer (pH adjusted to 5 with potassium hydroxide) and acetonitrile (90:10) v/v. The contents of mobile phase was filtered before use through 0.45 µ membrane filter, degassed with a helium sparge for 15min was used at flow rate of 1.0 mL min-1. The column temperature was maintained at 20 ± 10^{0}C. 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.

2.4. Preparation of mobile phase:

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

2.5. Preparation of standard and sample solution

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

For the preparation of sample solution, accurately weighed (100mg) Cetriax-S 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.

2.6. Preparation of calibration curve:

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

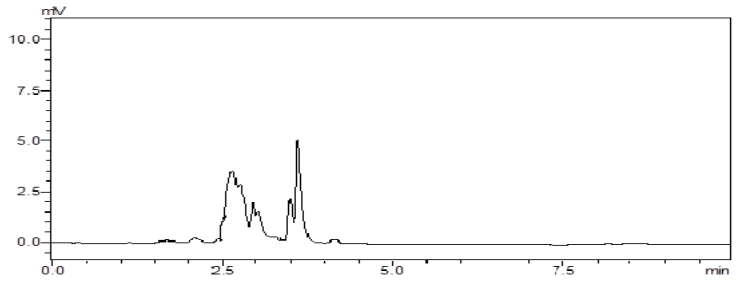
2.7. Preparation of plasma samples:

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

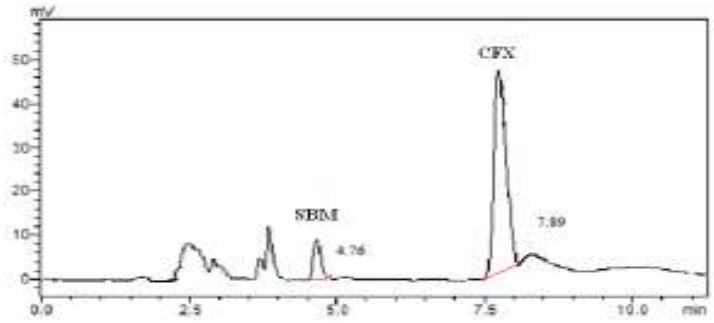
3. RESULTS AND DISCUSSION

3.1 Method Development

Taking into consideration, the instability of CFX and SBM in strong alkaline and strong acidic condition, the pH value of the mobile phase should be limited within the range of 3-7. Since mild acidic pH favours the retention and separation of two drugs on C-18 column. After some trials, phosphate buffer with pH 5 was finally selected. The method development started with the methanol and phosphate buffer. In this mobile phase, the drugs were not of eluted, so the organic phase was changed from methanol to acetonitrile. Since both CFX and SBM in the mobile phase have no significant UV maximum, the wavelength of 230 nm was employed for the detection After a number of preliminary trails, 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 produced symmetric peak shape, good resolution and reasonable retention time for both the drugs (Table 1). The retention times of CFX and SBM for six repetitions 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
Mobile Phase	Acetonitrile and pH-5 buffer in the ratio of 10:90(v/v)
Flow rate	1.00 mL min^{-1}
Detection	230nm
Injection volume	10 µL
Temperature column	Room temperature

3.2. Method validation

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics [9]. The method was validated for different parameters like linearity, accuracy, precision, recovery, selectivity and sensitivity [10].

3.2.1 Selectivity

Selectivity is defined as, "the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The definition of selectivity is quite similar to the definition of specificity: "the ability to assess unequivocally the analyte in the presence of components which might be expected to be present. Selectivity is evaluated by injecting extracted blank plasma and comparing with the response of extracted LLOQ samples. There should be no endogenous peak present within 10% window of the retention time of analyte. If any peak is present at the retention time of analyte, its response should be ≤ 20% of response of an extracted Lower calibration standard i.e. LLOQ standard.

3.2.2 Sensitivity

Sensitivity is measured using Lower Limit of Quantification (LLOQ) is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples independent of standards and determining the co-efficient of variation and appropriate confidence interval. The LLOQ should serve the lowest concentration on the standard curve and should not be confused with limit of detection and low QC sample.

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 made 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 Fig. 3 and Fig. 4 of both CFX and SBM respectively.

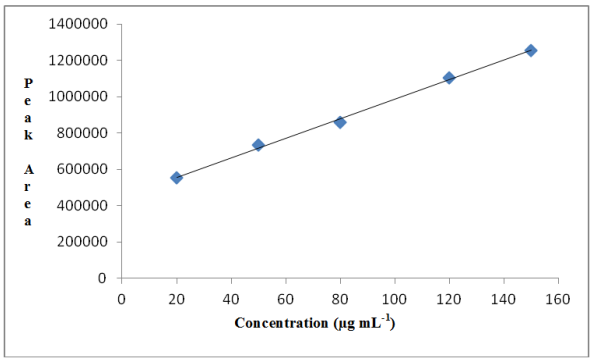


Fig.3. Calibration graph of CFX

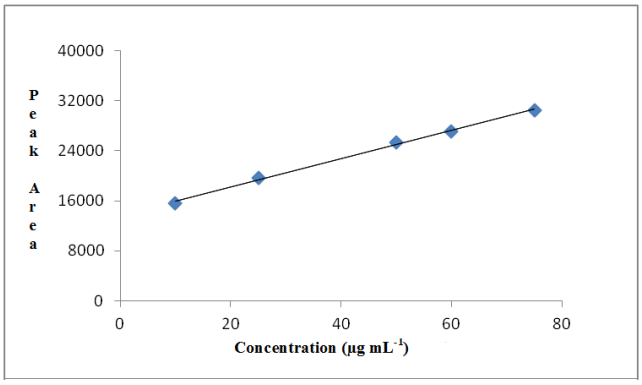


Fig.4. Calibration graph of SBM

Table 2. Linearity of CFX and SBM

Parameters	**CFX**	**SBM**
Retention time (min)	7.3	4.6
Linear range (ppm) [n=5] (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

3.2.4. Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery of the analyte need not be 100%. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery. Results are sumerised in Table 3.

$$100 \times \frac{Mean\ responce\ of\ extracted\ sample}{Mean\ responce\ of\ unextracted\ sample}$$

Table 3. Recovery studies of CFX sodium and SBM

Concentration of CFX and sulbactam	Amount recoverd% for CFX	Amount recoverd% for SBM
Low	98.7%	99.9%
Medium	96.8%	98.9%
High	99.3%	98.6%

3.2.5. Precision

For validation of bioanalytical method, precision should be determined 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 precision around the mean value should not exceed 15% of the CV except for LLOQ, where it should not exceed 20% of the CV.

The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (CV). The statistical method for estimation of the precision should be predefined and calculated according standard practise. Precision should be demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs and results are sumerised in Table 4 & 5.

% CV (precision) =100 x Standard deviation/Mean

Table 4. Precision of CFX

CFX	Intra day Validation	Inter day Validation

Level	Spiked plasma Conc. µg mL^{-1}	Mean Conc. µg mL^{-1}	Coefficent variation %	Spiked plasma Conc. µg mL^{-1}	Mean conc. µg mL^{-1}	Coefficent variation %
Low	20	19.4	0.98	20	18.3	1.42
Medium	100	99.5	0.76	100	98.8	1.32
High	150	148.3	1.34	150	147.8	1.7

Table 5. Precision of CFX

Sulbactam Level	Intra day Validation			Inter day Validation		
	Spiked plasma µg mL^{-1}	Mean determined µg mL^{-1}	Coefficent variation %	Spiked plasma µg mL^{-1}	Mean µg mL^{-1}	Coefficient variation %
Low	10	9.3	0.96	10	9.7	0.76
Medium	50	49	1.00	50	48	1.2
High	75	74.3	1.02	75	73.9	1.04

3.2.6. Stability studies

Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low, medium and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same

conditions. The freeze–thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70^{0}C during the three freeze and thaw cycles.

Short-Term Temperature Stability

Three aliquots of each of the low,medium and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low, medium and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

Stock Solution Stability

The stability of stock solutions of drug should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions Results are sumerised in Table 6.

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	84.5	93.3	94.9	88.5	96.3	97.9

Auto sampler	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

In the present work, a simple, accurate and stability indicating HPLC method for the simultaneous determination of ceftriaxone and sulbactam in their pharmaceutical formulation was developed. The method was validated according to 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 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 <2.

5. CONCLUSION

The method involves simple and precise method for simultaneous determination of CFX sodium and SBM. It produces symmetric peak shape, good resolution and reasonable retention time for both drugs. So this method can be applicable for the simultaneous estimation of CFX sodium and SBM in quality control studies for routine analysis.

6. AKNOWLEDGMENT

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