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High Performance Liquid Chromatographic Method for Simultaneous Determination of Cefepime and Sulbactam in Pharmaceutical Formulation (Supime) and Biological Samples

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Abstract: A simple and sensitive assay method was developed for simultaneous determination of cefepime and sulbactam sodium in Supime (a fixed dose combined formulation of cefepime and sulbactam manufactured by Venus Remedies limited, India) with UV detection at 230 nm. Chromatographic separation of two drugs was achieved on a Hypersil ODS C-18 column using a binary mixture of acetonitrile and tetrabutyl ammonium hydroxide as a mobile phase adjusted to pH 5.0 with orthophosphoric acid in ratio 20:80 v/v ratio. The developed liquid chromatographic method offers good linearity, accuracy and precision over the concentration range of 125-750 ppm for cefepime and 62.5-375 ppm for sulbactam sodium. This method was successfully applied for the quality control of formulated products and plasma samples containing Cefepime and sulbactam. Since, Supime, a fixed dose combination of cefepime and sulbactam is a research product of Venus Remedies limited the literature lacks any method of analysis for such combination, the main motive behind this experiment was to develop and validate a method which could be used for the quality control of cefepime and sulbactam in combined dosage form.

Key words: Liquid chromatography, cefepime, sulbactam sodium, tetrabutyl ammonium hydroxide

INTRODUCTION

Cefepime, (2-aminothiazole-4-yl)-2-(Z)-(methoxyiminoacetamido)-3-(methyl-1-pyrrolidino) methyl-ceph3em4-carboxylic acid, is a fourth generation, β -lactamase resistant parenteral cephalosporin with broad spectrum of activity against many Gram-positive and Gram-negative bacteria (Barbhaiya *et al.*, 1991). It differs from the third generation cephalosporins in the quaternary N-methylpyrrolidine ring bonded to C-3 of the cephem nucleus; this provides its zwitterionic character and enables its rapid penetration through the membrane of Gram-negative bacteria (Bellido *et al.*, 1991; Nikaido *et al.*, 1990; Sanders *et al.*, 1996). Cefepime is suitable for treatment of severe infections such as bacterial meningitis, although, data on the penetration of cefepime into human cerebrospinal fluid (CSF) and its use in the treatment of bacterial meningitis are scarce (Modai and Chemother, 1996; Rodenas *et al.*, 1995; Fasching *et al.*, 1986; Elkhaili *et al.*, 1997a).

Sulbactam sodium is a competitive, irreversible beta-lactamase inhibitor and has good inhibitory activities against the clinically important plasmid mediated

beta-lactamases and most frequently responsible for transferred drug resistance. Both cefepime and sulbactam are listed separately in the United States Pharmacopoeia (USP, 2007) and the British Pharmacopoeia (British Pharmacopoeia, 2007). 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 cefepime in different matrixes which includes Capillary Zone Electrophoresis (CZE) (Hao and Sunderland, 2004), Second-derivative spectroscopy (Rodenas *et al.*, 1995) HPLC (Fasching *et al.*, 1986; Elkhaili *et al.*, 1997b; Rabouan-Guyon *et al.*, 1997; Valassis *et al.*, 1999; Breilh *et al.*, 1999; Calahorra *et al.*, 1999; Maddox and Stewart, 1999; Chang *et al.*, 2001; Cherti *et al.*, 2001), polarographic techniques (Ozkan *et al.*, 2002; Jimenez *et al.*, 2000, 2003) and microbiological assay (Kessler *et al.*, 1985). Sulbactam was successfully determined by spectrophotometry (Haginaka *et al.*, 1984b), capillary isotachopheresis (Jelinek *et al.*, 1990), HPLC (Fredj *et al.*, 1986; Bowden and Madsen,

1986; Haginaka *et al.*, 1984a, b) and Gas Chromatography-Mass Spectrometry (GC-MS) (Foulds *et al.*, 1986). Sulbactam along with clavulonic acid (Shah *et al.*, 1990; Fatma *et al.*, 2000), Tazobactam (Guillaume *et al.*, 1995) and Rifampicin (Aparicio *et al.*, 2006) was determined by HPLC. Sulbactam in combination with ampicillin sodium, amoxicillin and piperacillin sodium were determined by UV spectrophotometry and HPLC, respectively (Mahgoub and Aly, 1998; Wang *et al.*, 2004; Qi *et al.*, 2003a, b).

The product Supime* is manufactured by Venus Remedies Limited, India Supime* is a sterile combination of sulbactam sodium and cefepime hydrochloride available as dry powder for injection. Cefepime and sulbactam combination for injection, is supplied for intramuscular or intravenous administration in strengths equivalent to 0.75, 1.5 and 3.0 g along with solvent for injection.

The present communication describes isocratic LC method for simultaneous determination of cefepime hydrochloride and sulbactam sodium, which would be used for the quality control of the formulation developed and other biological applications. The advantage of this method is that by doing one column analysis one can save time and resources. As to the best of our knowledge there is no method present for the simultaneous determination of the combined dosage form. This study achieved satisfactory results in terms of selectivity, linearity, precision and accuracy under simple chromatographic conditions.

MATERIALS AND METHODS

The experiment is part of an inhouse project of Venus Medicine Research Centre which started in April, 2009 and lasted in the month of October in the very same year. The experiment was conducted at Venus Medicine research centre, BADDI, India.

Chemicals and reagents: Cefepime hydrochloride and sulbactam sodium Reference Standards (RS) of United States Pharmacopoeia (USP) were bought from Sigma, United States. Supime*, a Fixed Dose Combination (FDC) was obtained from manufacturer, Venus Remedies Limited, India. Tetrabutyl ammonium hydroxide (TBAH), acetonitrile and phosphoric acid were of chromatographic grade and obtained from Merck, Germany. All other chemicals were of analytical reagent grade unless specified.

Apparatus: Chromatographic separation was performed on Agilent 1200 series liquid chromatographic system

equipped with G1311A quaternary pump, Agilent variable UV/vis detector and a G1329A Auto Injector. EZ Chrome Elite software was employed for data collecting and processing.

Chromatographic conditions: Chromatographic Separation was performed on ODS Hypersil C-18 Stainless Steel column with dimension 250×4.6 mm, 5 μ (Thermo Electron Corporation). The mobile phase consisting of a binary mixture of acetonitrile and TBAH adjusted to pH 5.0 with orthophosphoric acid in 20:80 v/v ratio was delivered at rate of 1.5 mL min⁻¹. The mobile phase was filtered through a 0.45 μm nylon membrane filter (Millipore) and degassed prior to use. Separation was performed at ambient temperature i.e., 25°C and detection was made at 230 nm. The injection volume was 20 μL with a run time of 15 min.

Preparation of standard solution: Dissolved an accurately weighed quantity of Cefepime (RS) 50 mg and sulbactam sodium (RS) 25 mg in water and diluted quantitatively and stepwise, if necessary, with water to obtain a solution having a known concentration of about 500 ppm of Cefepime and 250 ppm sulbactam sodium.

Preparation of sample solution: Transferred about 75 mg of supime, (Cefepime and sulbactam sodium for injection), accurately weighed, to 100 mL volumetric flask. Add water, swirl to dissolve, dilute with water to volume and mix.

Preparation of plasma sample: *In vitro* plasma samples were prepared by spiking stock solutions of three different concentrations of supime into blank plasma. Also, *in vivo* plasma samples are collected at 0 h, 30 min and 8 h to check the limit of detection of the drug injected. The *in vitro* samples were stored for 30 min for drug absorption, then both the samples were deproteinated. Take 0.25 mL of plasma, add 0.75 mL of chilled distilled water and 2.0 mL of acetonitrile to the solution. Now, centrifuge the sample at 7000 rpm for 15 min for protein precipitation. Then the supernatant is collected after centrifugation and an aliquot of 20 μL was injected into the loop of HPLC system. The supernatants were diluted with acetonitrile and diluent if required.

Data analysis: For determination of cefepime and sulbactam sodium separately inject equal volumes of the standard preparation, assay preparation and the plasma samples into the chromatograph, record the chromatograms and measure the responses for the peaks.

RESULTS

To the best of our knowledge there is no HPLC method available for the simultaneous determination of cefepime and sulbactam in combined dosage form. The result of the precision with respect to standard deviation shows a good correlation with the previous simultaneous (Pawar *et al.*, 2008). In terms of recovery study the result of the present study is in agreement with the earlier similar studies (Lakkanatinaporn and Matayatsuk, 2004). The individual linearity range was 125-750 ppm for Cefepime and 62.5-375 ppm for sulbactam sodium. The LOD was calculated and found to be 0.08 ppm (RSD = 7.2 %) for Cefepime and 0.3 ppm (RSD = 8.4 %) for sulbactam sodium. And the LOQ was found to be 0.24 ppm (RSD = 3.7%) for Cefepime and 0.91 ppm (RSD = 4.65 %) for sulbactam sodium.

DISCUSSION

Method development and validation: Several methods are available for the analysis of cefepime and sulbactam individually, but no such method is available for the simultaneous determination of cefepime and sulbactam.

The reverse phase high performance liquid chromatography method was optimized with a view to developed a stability indicating assay method. Pure drugs chromatogram was run in different mobile phases containing methanol, acetonitrile, water and different buffers in different ratios. Different columns (e.g., C8, C18, phenyle) with different dimensions were used. The retention time and tailing factor was calculated for each drugs and for each chromatogram. Finally, Tetrabutylammonium hydroxide along with acetonitrile as organic modifier was selected which gave a sharp and symmetrical peak with minimum tailing.

Thus, we developed a method for the combined analysis of both the drugs not only to save time but other resources too. The TBAH is an ion pairing agent and is commonly used as a base in organic chemistry. It is more soluble in organic solvents. Thus, it is selected as buffer to achieve the separation of two drugs. Since, mild acidic pH favors the retention and separation of two drugs on C-18 column. After some trials pH 5.0 was finally selected for TBAH buffer. Acetonitrile is the most commonly used solvent for LC analysis and often is the first choice for many researchers. Therefore, a binary mixture of acetonitrile and TBAH buffer became the mobile phase for the determination of the two drugs. Firstly, various concentrations of TBAH buffer were tried to find the proper one to achieve our purpose. As a result, 0.005 N TBAH buffer was found to be ideal for our work. Then,

the proportion of acetonitrile and TBAH buffer in mobile phase was determined by varying the proportion of acetonitrile and TBAH buffer from, 10 : 90, 15 : 85, 20 : 80 to 25 : 75. Finally, the 20 : 80 ratio of acetonitrile and TBAH buffer was employed for the simultaneous determination of the two drugs, this system produced symmetric peak shape, good resolution and reasonable retention time for both the drugs. The retention times of Cefepime and sulbactam sodium for six repetition is 2.133 ± 0.02 and 8.971 ± 0.01 min, respectively. The run time is 15 min. A typical chromatogram of a standard solution is shown in Fig. 1.

Since, both cefepime and sulbactam sodium in the mobile phase have no significant UV maximum but end absorption, to ensure the sensitivity of the method, the wavelength of 230 nm was employed for the detection.

Selectivity: Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample, it can be determined by analyzing forced degraded powder samples. Forced degradation studies were performed to provide an indication of stability property of the proposed method by exposing the formulation product to stress condition of UV light, high temperature (105°C), hydrogen peroxide (5%), acid (0.5 N HCl) and base (0.5 N NaOH) in order to test the ability of the proposed method to separate the active component. The samples were degraded to levels where the contents of Cefepime and sulbactam in the samples were lowered to that of the original level. Chromatograms for the oxidation, acid and base degradation shows that under the given stress conditions the two drugs are unstable and significant degraded peaks appear, as shown in Fig. 2. These results suggest that the stability of the two drugs under acidic, basic and oxidation conditions is poor. These results also

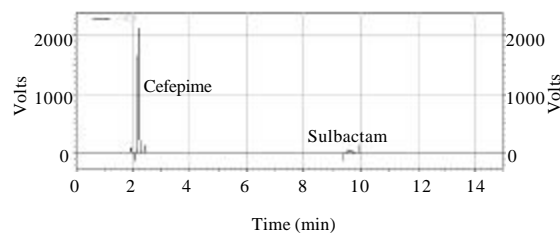


Fig. 1: A typical LC chromatogram of a mixture of cefepime, 500 ppm and sulbactam sodium, 250 ppm. The chromatographic conditions used were: ODS Hypersil C18 Column, mobile phase of acetonitrile and 0.005N TBAH (20:80), flow rate of 1.5 mL min^{-1} , detection wavelength of 230 nm, room temperature 25°C

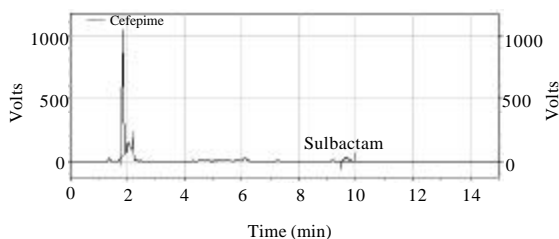


Fig. 2: LC chromatogram of cefepime and sulbactam; oxidation. The chromatographic conditions used were: ODS Hypersil C18 Column, mobile phase of acetonitrile and 0.005N TBAH (20:80), flow rate of 1.5 mL min⁻¹, detection wavelength of 230 nm, room temperature 25°C

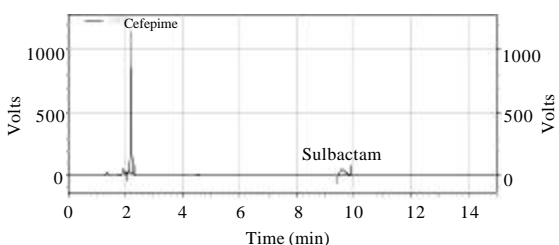


Fig. 3: LC chromatogram of cefepime and sulbactam; Thermal degradation. The chromatographic conditions used were: ODS Hypersil C18 Column, mobile phase of acetonitrile and 0.005N TBAH (20:80), flow rate of 1.5 mL min⁻¹, detection wavelength of 230 nm, room temperature 25°C

suggested that the two drugs under photo (UV) and thermal degradation are found to be stable up to 12 h, as shown in Fig. 3.

Specificity: Specificity is the ability of the method to accurately measure the analyte in the presence of all potential sample components. The analyte peak is evaluated for peak purity from the nearest eluting peak. For this purpose a solution containing 500 ppm of cefepime and 250 ppm sulbactam sodium was injected and peak purity was done. The acceptance criteria for peak purity is that the purity angle should be less than purity threshold. Result of peak purity analysis was found to be satisfactory, total peak purity for cefepime and sulbactam is 0.98 and 1.0, respectively.

System suitability: System performance parameters of the developed HPLC method were determined by analyzing standard working solutions. Chromatographic parameters, such as number of theoretical plates (N), resolution (Rs), capacity factor (k) and selectivity factor (α) were

Table 1: System performance parameters for cefepime hydrochloride and sulbactam sodium (n = 5)

Peak No.	Compounds	t _R (min)	N	K	R _s	α
1	Cefepime	2.127	12132	20.0	--	--
2	Sulbactam	8.980	13918	88.0	34.0	4.4

The chromatographic conditions used were: ODS Hypersil C-18 Column, mobile phase of acetonitrile and 0.005N TBAH (20:80), flow rate of 1.5 mL min⁻¹, detection wavelength of 230 nm, room temperature 25°C. t_R: Retention time, N: Theoretical plates, K: Capacity factor, R_s: Resolution, α : Selectivity factor

determined. The results are shown in Table 1, indicating the good performance of the system. System repeatability was determined by six replicate injections of a working standard solution and the Relative Standard Deviations (RSD) of peak areas of both drugs were calculated to evaluate the repeatability. It was found that RSD for both the drugs was less than 2.0%.

Linearity: Under the experimental conditions described above, linear calibration curves for both cefepime and sulbactam sodium were obtained with six concentration level each. Peak area (A) and concentration (C) of each drug substance was subjected to regression analysis to calculate the regression equation and the correlation coefficients. From the obtained regression line the slope, intercept is calculated, (slope = 0.0000209, intercept = -0.68737 and r = 0.9998, n = 6) for cefepime and (slope = 0.000402, intercept = 0.32563 and r = 0.9996, n = 6) for sulbactam sodium. The individual linearity range was 125-750 ppm for cefepime and 62.5-375 ppm for sulbactam sodium. The results show that within the tested concentration range there was excellent correlation between the peak area and the concentration of each drugs.

Limit of detection and limit of quantification: Limit of Detection (LOD) were established at a signal to noise ratio (S/N) of 3.3. Limit of Quantification (LOQ) was established at a signal to noise ratio (S/N) of 10. The LOD and LOQ were experimentally verified by six injection of cefepime and sulbactam sodium at the LOD and LOQ concentration. The LOD was calculated and found to be 0.08 ppm (RSD = 7.2%) for cefepime and 0.3 ppm (RSD = 8.4%) for sulbactam sodium. And the LOQ was found to be 0.24 ppm (RSD = 3.7%) for cefepime and 0.91 ppm (RSD = 4.65%) for sulbactam sodium.

Accuracy: Accuracy was determined by applying the described method to synthetic mixtures of excipients to which known amount of each drug corresponding to 75, 100 and 125% of test solution had been added. The accuracy was then calculated as the percentage of analyte recovered by the recovery study. The recovery of cefepime and sulbactam in test samples ranged from 80.0 to 120.0%. Mean recoveries (Mean \pm SD) for cefepime and

Table 2: Accuracy and precision of the HPLC method for simultaneous determination of Cefepime and sulbactam sodium

Drug	Method precision		Intermediate precision	
	Mean±SD	RSD (% _{n=6})	Mean±SD	RSD (% _{n=2})
Cefepime	102.44±0.69	0.68	102.27±0.57	0.56
Sulbactam	102.90±0.43	0.42	102.29±0.41	0.39

The chromatographic conditions used were: ODS Hypersil C-18 Column, mobile phase of acetonitrile and 0.005N TBAH (20:80), flow rate of 1.5 mL min⁻¹, detection wavelength of 230 nm, room temperature 25°C

sulbactam sodium from the combination formulation is shown in Table 2 indicating good accuracy of the method for simultaneous determination of the two drugs.

Precision: System precision is the measure of the method variability that can be expected for a given analyst performing the analysis. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precisions may be considered at three levels: repeatability, intermediate precision and reproducibility. Method precision shall be established by determining the assay in six different preparations of a homogenous batch of cefepime-sulbactam for injection. Intermediate precision was determined by studying the variation in assay of a homogeneous sample analyzed by 2 different equipment, analyst and days. The proposed method's reproducibility was checked by different analyst in different laboratory (quality control laboratory of venus remedies limited, unit 2, India). The average assay of three replicate analysis was found to be 100.10% for cefepime and 99.72% for sulbactam with a relative standard deviation of 0.16 and 0.20%, respectively. The average, standard deviation and relative standard deviation shall be calculated. The results for precision are shown in Table 2, indicating that acceptable precision was achieved for cefepime and sulbactam sodium, as revealed by relative standard deviation data (RSD<2.0% in all of the levels of the two drugs).

Robustness: As documented in the ICH guidelines (ICH, 1997) robustness should be considered early in the development of a method. If the results are susceptible to variations in method conditions, these conditions must be adequately controlled. The effect of variations in some experimental conditions was tested. In all the deliberate varied chromatographic conditions such as flow rate, mobile phase variation, pH, temperature variation and different column the result is found to be satisfactory and under acceptable limit for cefepime and sulbactam.

Analytical solution stability: The stability of both the standard and the test was determined by monitoring the

Table 3: Assay results for cefepime and sulbactam sodium sterile powder for injection (Mean±SD)

Batch No.	Cefepime	Sulbactam sodium
1	101.84±0.59	101.88±0.55
2	101.75±0.45	101.51±0.39
3	102.10±0.32	100.92±0.41

The chromatographic conditions used were: ODS Hypersil C-18 Column, mobile phase of acetonitrile and 0.005N TBAH (20:80), flow rate of 1.5 mL min⁻¹, detection wavelength of 230 nm, room temperature 25°C

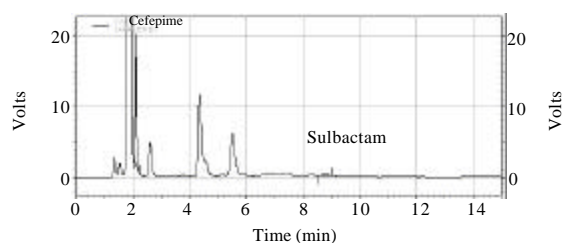


Fig. 4: LC chromatogram plasma samples of cefepime and sulbactam. The chromatographic conditions used were: ODS Hypersil C18 Column, mobile phase of acetonitrile and 0.005N TBAH (20:80), flow rate of 1.5 mL min⁻¹, detection wavelength of 230 nm, room temperature 25°C

peak area responses of the standard solution and a sample solution of cefepime and sulbactam sodium at 0, 6, 12 and 24 h at room temperature and refrigerator. The results showed that there is no significant difference in the area for 24 h. The cumulative RSD at room temperature and refrigerator was found to be 0.14 and 0.21 for cefepime and 0.34 and 0.23 for sulbactam, respectively.

Method application: The validated LC method was applied to the simultaneous determination of cefepime and sulbactam for injection. The three batches of the sample were analyzed and the assay results, expressed as percentage of the label claim are shown in Table 3. The result indicated that the amount of each drugs in the injection corresponds to requirement.

Clinical application: We successfully applied the current method for the analysis of the plasma samples obtained. *In vitro* samples of plasma with three different concentrations of the drugs viz., 80, 120 and 160 mcg mL⁻¹ and *in vivo* samples collected at 0 h, 30 min and 8 h were analyzed. The recovery for three *in vitro* sample concentrations was found to be (95.00±0.30, 95.85±0.37 and 96.22±0.28, for cefepime) and (94.84±0.21, 95.15±0.29 and 96.32±0.18 for sulbactam), respectively. It was found that the detection at 8 h for *in vivo* plasma sample was 310 mcg mL⁻¹ for cefepime and 160 mcg mL⁻¹ for sulbactam. A typical LC chromatogram showing the detection of cefepime and sulbactam is shown in Fig. 4.

These results shows the effectiveness of the method for the analysis of biological samples containing cefepime and sulbactam in combination.

CONCLUSION

The developed HPLC method with UV-Visible detection offers simplicity, selectivity, precision and accuracy. It produces symmetric peak shape, good resolution and reasonable retention time for cefepime and sulbactam sodium. It can be used for the simultaneous determination of cefepime and sulbactam sodium in the pharmaceutical companies and research laboratories for routine and biological samples analysis.

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