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Research Article

SIMULTANEOUS ESTIMATION OF AMOXICLLIN AND SULBACTAM IN A PARENTERAL FORMULATION BY REVERSE PHASE HPLC METHOD

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ABSTRACT

A method for determining the concentration of Amoxicillin and Sulbactam simultaneously in Amoxirum forte injection vial, based on a easy-to-use, high-performance liquid chromatography has been developed. Investigated drugs were resolved on Merck ODS inertsil silica C18 (5 μ m, 25 mm x 4.6mm ID) reverse phase column, with a mobile phase of Buffer:Acetonitrile (10:1) at the pH of 5, with injection volume being 20μL. The mobile phase supplied at 1 mL/min while the detection was carried out at 230 nm. The method yielded separations at retention times of 4.2 and 6.4 minutes for amoxycillin sodium and sulbactam sodium respectively. The peak area-concentration data were linear with correlation coefficient of 0.99 in the concentration range of 56.85 to 82.87 μg/mL for amoxycillin sodium and 28.43-41.44 μg/mL for Sulbactam sodium respectively. We have successfully applied the developed method for the determination of these drugs in mixtures and in injection formulations.

Keywords: Amoxycillin sodium, Sulbactam sodium, Reverse phase HPLC, Retention time, Linearity.

INTRODUCTION

Penicillin was the first antibiotic to be used clinically. Though it was originally obtained from the fungus Penicillium notatum, the source of yielding mutant currently used is P. Chrysogenum[1]. The penicillin nucleus consists of fused thiazolidine structure and betalactam rings to which side chains are attached through an amide linkage. Penicillin G having a benzyl side chain has the most desirable properties and is used clinically[1].

Amino penicillin's are groups of penicillins that have broader spectrum of antibacterial activity than that of penicillin G [1]. They are active against gram-negative microorganisms. The presence of α-amino group provides ability to cross cell wall barriers, which are otherwise inaccessible by other penicillins[1]. These drugs are readily hydrolysed by broad-spectrum β-lactamases and produce allergic reactions [1]. Amoxycillin Sodium is sodium (6*R*)-6-(α -D-4-hydroxy-phenylglycylamino) penicillanate[2]. It is amino penicillin, which has a phenolic functional group. It has a broader spectrum of activity than benzyl penicillin, especially against gram-negative bacilli.[2]

Sulbactam is an irreversible inhibitor of β–lactamase which binds the enzyme and does not allow it to interact with the antibiotic. Hence it is given in combination with β–lactam antibiotics. Both these drugs have been listed in Indian Pharmacopoeia[2] and United States Pharmacopoeia[3]. β-lactamases are a family of enzymes that inactivate β-lactam antibiotics by opening the β-lactam ring. Different β-lactamase differ in their substrate affinities[3]. Sulbactam Sodium is Sodium (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicycloheptane-2-carboxylate 4,4-dioxide[3]. Sulbactam is a semisynthetic β–lactamase inhibitor. Sulbactam is penicillin acid Sulphone with β–lactamase inhibitory properties. It has a β–lactam ring but does not possess any antibacterial activity on its own, but inhibits a wide variety of β–lactamase[4].

The simultaneous estimation of sulbactam sodium and ampicillin sodium has been widely reported[5,6]. A liquid Chromatographic method has been reported for the simultaneous estimation of amoxicillin sodium (retention time ~ 5.5 min) and sulbactam sodium (retention time ~ 8.1 min) in a combination formulation[7]. The present work discusses a Reverse Phase HPLC method development and validation that can be used to estimate the concentrations of amoxicillin sodium and sulbactam sodium simultaneously in a parenteral formulation, along with its advantages over the earlier developed methods.

The development of a method of analysis is usually based on prior art or existing literature, using the same or quite similar instrumentation. It is indeed very rare nowadays for developing a HPLC based method where comparison with an existing literature is not made. Hence, the new method is usually a fine tuning of existing methods to the analyte in question to achieve satisfactory precision, accuracy, specificity, ruggedness and robustness.

MATERIALS AND METHODS

Working standards of Amoxycillin sodium and Sulbactam sodium were obtained from Karnataka Antibiotics & Pharmaceuticals Ltd., Bangalore. These were equivalent to 89.52% of amoxycillin and 91.0% of sulbactam. Amoxirum forte injection (3000 mg) was procured from Karnataka Antibiotics & Pharmaceuticals Ltd., Bangalore. As per the label claim, each vial contained Amoxycillin Sodium IP and Sulbactam Sodium USP which were equivalent to 2000 mg and 1000 mg of Amoxycillin and Sulbactam 1000 mg respectively. HPLC grade reagents have been used during the method development.

Preparation of standard stock solutions

An accurately weighed quantity of the drug equivalent to 80 and 40 mg respectively of amoxycillin and sulbactam was transferred to 100 mL volumetric flask. The mixture was dissolved in double distilled water and treated in an ultrasonic bath for 10 minutes at room temperature. The final volume was made up to 100 mL. 5 mL of this stock solution was diluted 10 times using the mobile phase.

Preparation of sample stock solutions

1.2 g of the sample blend was dissolved in double distilled water, sonicated and volume made up to 100 mL. Upon filtration using Whatman filter paper no. 1, the solution was diluted with the mobile phase to obtain concentration of 0.12mg/mL.

Procedure

A binary gradient HPLC with UV-detector (Shimadzu, Japan) with Merck ODS Inertsil C18 Column (No: 233248; particle size ~5 μm, 250 mm x 4.6 mm I.D.) has been used. A buffer was prepared by dissolving 13.609g of Potassium Dihydrogen Phosphate in 1000 mL of double distilled water. The suitability of system was confirmed using freshly prepared standard stock solution of amoxycillin sodium and sulbactam sodium. For this purpose, injections of 20μL of the standard and sample solutions were made and absorbance measured at 230 nm.

RESULTS AND DISCUSSIONS

Method Development

The first step in the method development is identification of an appropriate mobile phase. For this purpose, several commonly used

mobile phases such as methanol-phosphate buffer, Acetonitrile-phosphate buffer and Tetra Butyl Ammonium Hydroxide-Acetonitrile were tested with pH being maintained between 3 and 7. Use of this pH range is due to the susceptibility of analytes to degradation outside this pH range. After several experiments, a mixture of acetonitrile and Potassium Dihydrogen Phosphate buffer was chosen as the mobile phase for estimation of these two drugs. Through experimental optimization, the concentration of KH_2PO_4 buffer was found to be 0.1 N. Similarly acetonitrile: KH_2PO_4 ratio was found to be 1:10. Hence all the subsequent experiments were carried out using 1:10 ratio of acetonitrile: 0.1 N KH_2PO_4 as the mobile phase. A typical chromatogram of standard stock solution of amoxicillin sodium and sulbactam sodium obtained using this mobile phase is shown in Fig.1.

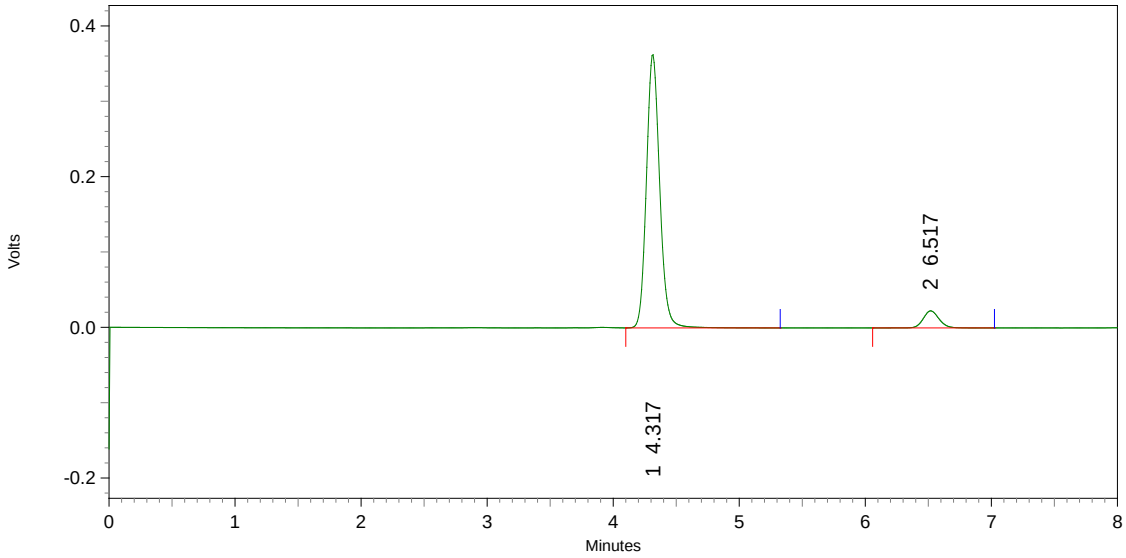

Fig. 1: Chromatogram of the standard solution. The retention times of 4.317 min and 6.517 min correspond to amoxycillin sodium and sulbactam sodium respectively.

It may be observed from Fig. 1 that the chromatogram exhibits two well-defined, well-resolved, sharp peaks. The peaks are at retention times of 4.3 and 6.5 min. The peak area corresponding to the retention time of 4.3 min is 2653904, while that corresponding to retention time of 6.5 min is 197813.

Upon ascertaining the capability of the developed method to resolve the two components present in the standard satisfactorily, solutions of amoxirum forte injection vial were also analyzed using this method. Figure 2 shows the chromatogram of a sample solution using the method developed in the present work.

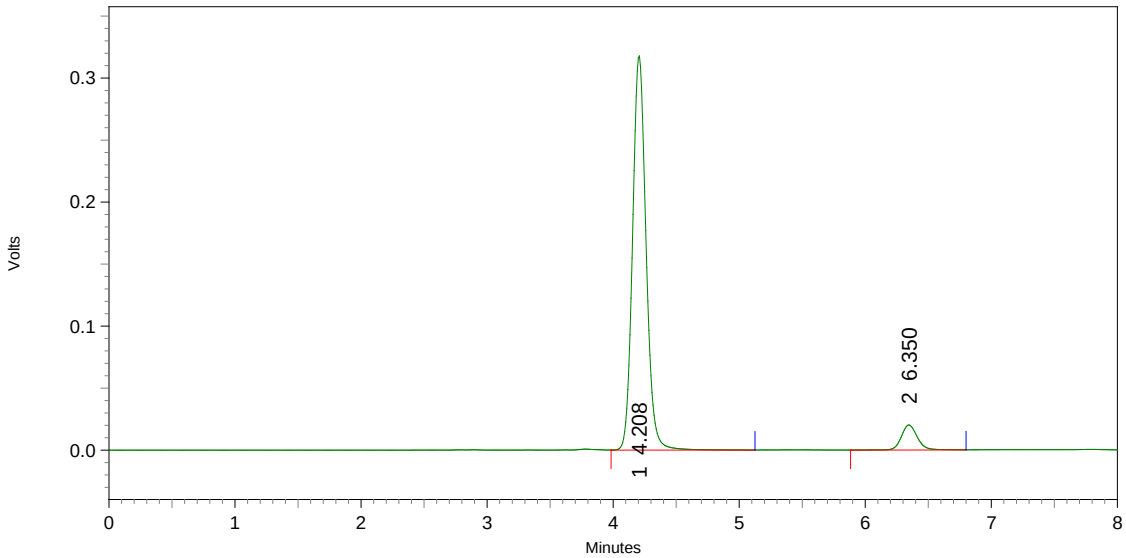

Fig. 2: Chromatogram of sample solution of amoxirum forte injection vial.

It may be observed from Figure 2 that two distinct, well-defined, sharp peaks appear at the retention times of 4.208 min and 6.350 min respectively. These retention times compare well with the retention times observed for amoxicillin sodium and sulbactam sodium in the standard. The composition of amoxycillin sodium and sulbactam sodium was estimated using the respective peak areas obtained for the sample and that for the standard using the following formula:

$$W_s = \frac{A_t}{A_s}\frac{M_s}{M_t}\left(\frac{std\ \ purity}{10}\right)\left(Avg\ \ fill\ mass\ per\ vial\right) \quad (1)$$

Method Validation

System Suitability Parameters

Six replicate injections of a standard solution were carried out to determine number of theoretical plates, percentage relative standard deviation and resolution of the respective analyte peak areas. The results of the validation are shown in Table 1. The relative standard deviations of the peak areas were found to be less than 1 % for both amoxycillin sodium and sulbactam sodium, with average number of theoretical plates being 8381.97 and 12568.90 respectively (Table 2). The resolution was found to be greater than 10.

Table 1: Relative standard deviation for six repetitions of the standard

Trial No	Amoxycillin Sodium Area in standard	RT of amoxycillin sodium peak	Sulbactam sodium Area in standard	RT of sulbactam sodium peak
1	2641047	4.292	197607	6.483
2	2633604	4.267	196927	6.450
3	2629808	4.258	196824	6.442
4	2616438	4.233	195997	6.40
5	2605661	4.217	194472	6.367
6	2653904	4.317	197813	6.517
Average	2630077	4.264	196606.67	6.443
RSD	0.654755828	0.8653	0.624362415	0.8430

Table 2: System suitability parameters

Serial Number	Parameters	Obtained values for amoxicillin sodium	Obtained values for sulbactam sodium
1.	Theoretical plates	8381.97	12568.90
2.	Tailing factor	0.97	1.01
3.	Assymmetry	1.2	1
4.	% RSD of peak retention time	0.87	0.84

Linearity

With the method developed here, linear calibration curves were obtained for both amoxicillin sodium and sulbactam sodium for five concentration levels ranging from 80% to 120 %.

Linear equations were obtained relating peak area (A) with concentration (C, µg/mL) for the two analytes using regression analysis as follows:

For amoxicillin sodium: $A = 32683.47C$; $R^2 = 0.99$(2)

For sulbactam sodium: $A = 4893.93C$; $R^2 = 0.99$(3)

Figures 3 and 4 show the linearity of the developed method for the range of concentrations investigated (56.85 to 82.87 µg/mL for amoxicillin and 28.43 to 41.44 µg/mL for sulbactam). The excellent correlation between peak area and concentration is also evident from figures 3 and 4. Table 3 show the data that testify the linearity and range for the method.

Accuracy

To determine the accuracy, synthetic mixtures of drug components were spiked in three concentration levels of 80%, 100% and 120% with known quantity of analytes and analyzed performed thrice for each concentration level. The mean recoveries were 100.3% ± 1.3% (n = 9) and 99.8 % ± 1.5% (n = 9), respectively for amoxicillin and sulbactam, highlighting the method's accuracy.

Precision

To determine precision, six determinations of samples of 100% test concentration were performed by the same analyst at the same working conditions. From the data (not shown here for brevity), the relative standard deviations obtained for amoxicyllin and sulbactam were 0.71% and 0.69%, respectively. The values of relative standard deviations less than 1 % indicate high repeatability of the method.

Specificity

To ascertain the specificity of the developed method, the sample blend was spiked with 40mg of Ampicillin Sodium as the impurity and analyzed for which the chromatogram is shown in Figure 5. The retention time and peak area for ampicillin sodium (impurity) was found to be 1.76 minutes and 309842 respectively (Figure 5). The presence of ampicllin sodium in the sample did not affect the assay values of amoxycillin and sulbactam, with the assay values obtained lying within ±1% of relative standard deviation.

Table 3: Linearity & Range for amoxicillin sodium and sulbactam sodium

S. No.	Condition	No of injections	Conc. Of amoxycillin sodium in µg/mL	Area of Amoxycillin sodium peak in sample	Conc of sulbactam sodium in µg/mL	Area of Sulbactam sodium peak in sample
1	80%	1		1908796		137618
		2		1908380		136956
		Avg	56.85	1908588	28.43	137287
2	90%	1		2113354		161201
		2		2116798		161539
		Avg	64.66	2115076	32.33	161370
3	100%	1		2367835		180010
		2		2367791		171179
		Avg	71.67	2367813	35.83	175594.5
4	110%	1		2554738		194093
		2		2556075		193038
		Avg	78.67	2555407	39.33	193565.5
5	120%	1		2680336		202324
		2		2650625		198423
		Avg	82.87	2665481	41.44	200373
% curve fitting				99.89		99.79
Correlation coefficient				0.9989		0.9977

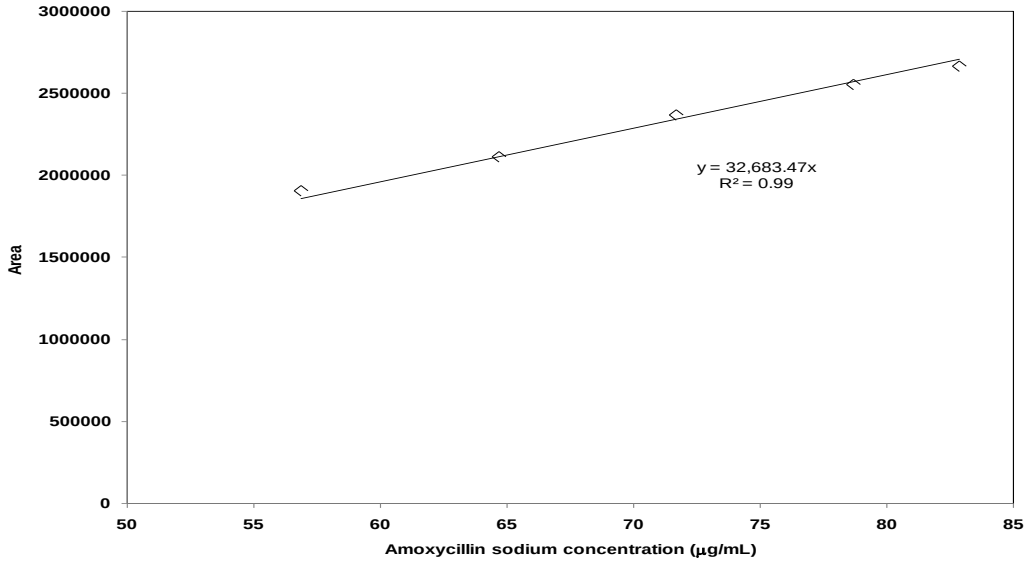

Fig. 3: Graph depicting the linearity of developed method for estimation of amoxcyillin sodium

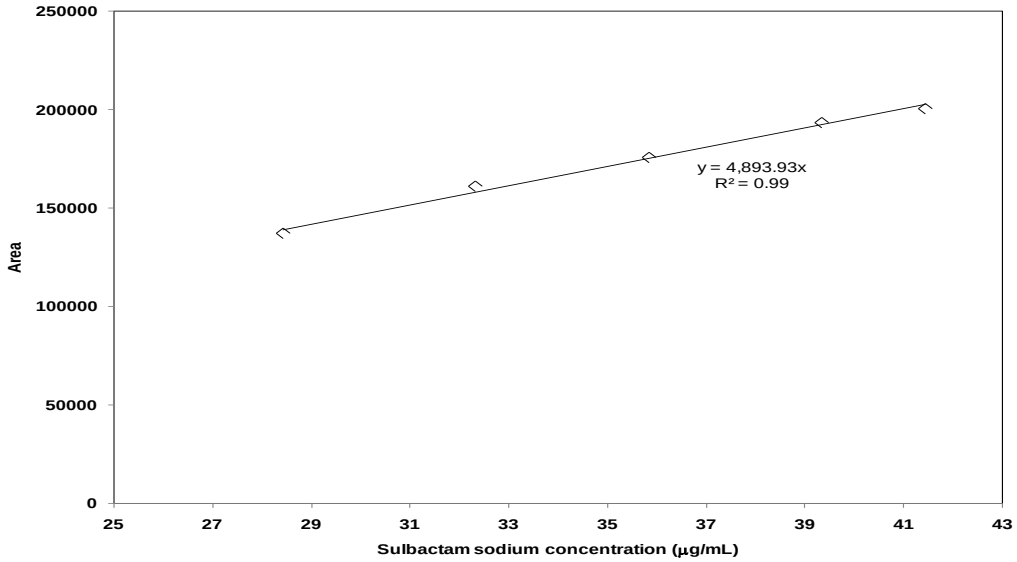

Fig. 4: Graph depicting the linearity of developed method for estimation of sulbactam sodium

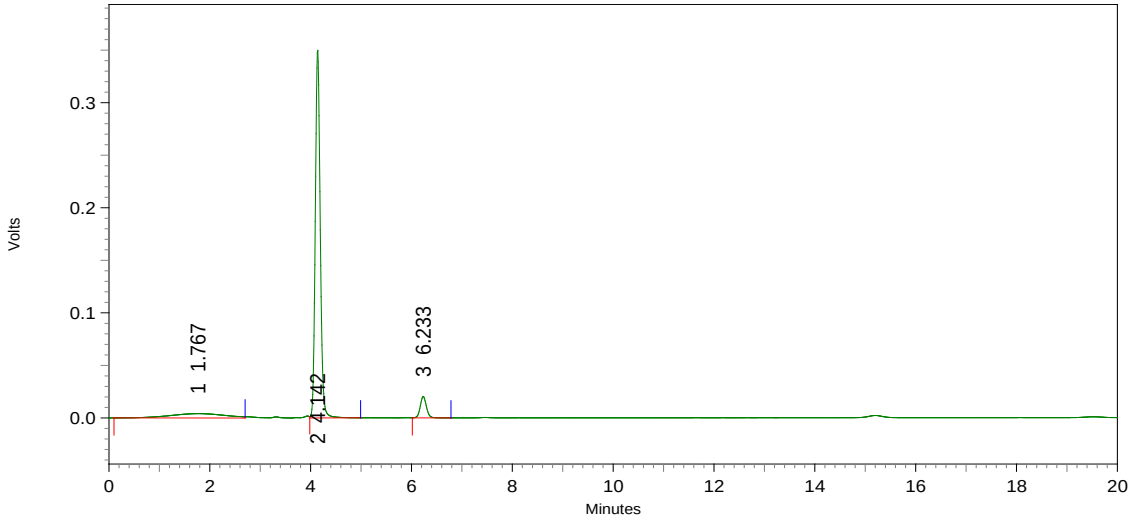

Fig. 5: Chromatogram obtained by spiking ampicllin sodium in the sample blend indicating the specificity of the method

Limit of Quantification

To determine Limit of Quantification, a predetermined sample solution was diluted repeatedly and analyzed to obtain the chromatogram. The LOQ was established as the minimum drug concentration for which the estimated drug concentration closely matched with that of pre-determined concentration. This was found to be 8µg/mL and 4µg/mL respectively for amoxicillin sodium and sulbactam sodium.

Limit of Detection (LOD)

Limit of Detection depends both on the analysis procedure and the instrument of analysis. LOD was determined by dilution of the samples utilized for LOQ and analyzed. Figure 7 shows the chromatogram of the sample blend analyzed at LOD at which peaks corresponding to amoxicillin sodium and sulbactam sodium were detected but could not be quantified. The LOD for amoxicillin sodium and sulbactam sodium were 0.8µg/mL and 0.4µg/mL respectively.

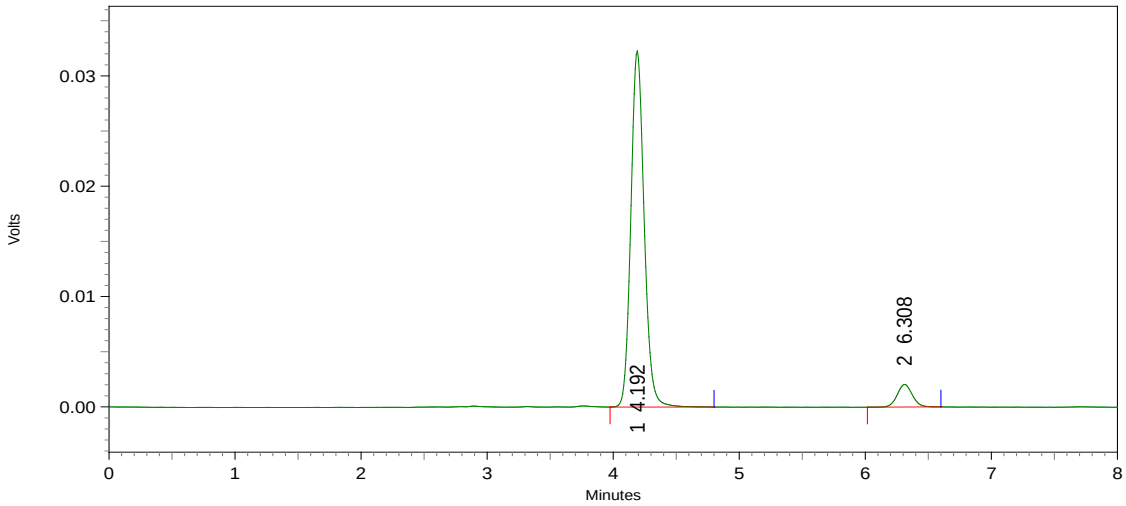

Fig. 6: Chromatogram of the blend at the Limit of Quantification (LOQ)

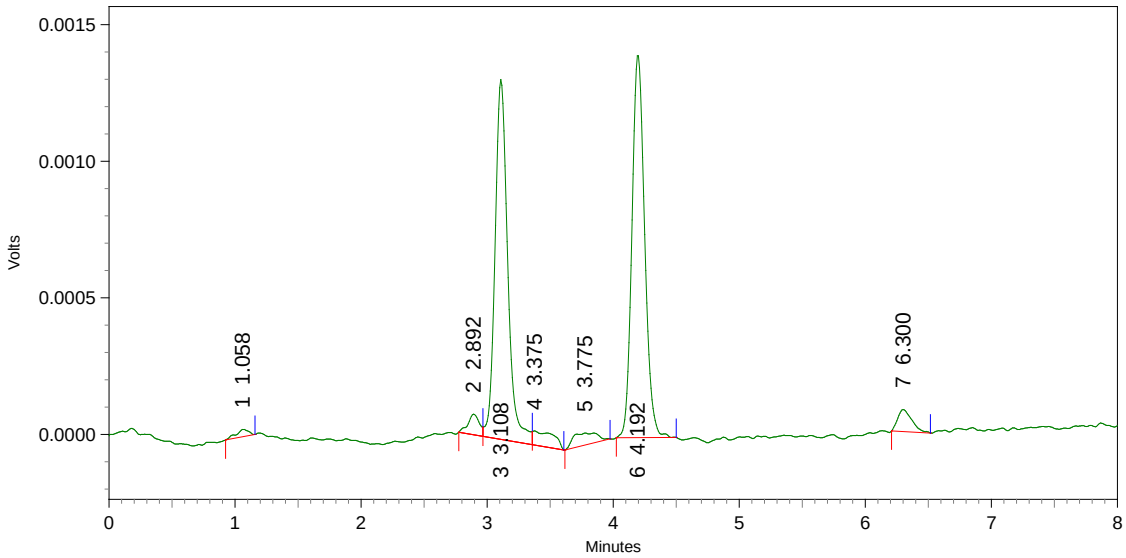

Fig. 7: Chromatogram of the blend analyzed at LOD

Ruggedness

Normally during the method development, the method is to be tested for its ruggedness. Typically to ascertain the ruggedness, the analysis is to be carried out by different analysts using different instruments on different days. Figure 8 shows the chromatogram obtained when the blend was run in another HPLC unit fitted with PDA detector, instead of UV detector utilized for the method development.

The results of the study for ruggedness are given in table 4. The calculated percentage relative standard deviations were 0.48% and 0.64% respectively, for amoxicillin sodium and sulbactam sodium. Hence the method developed in the present study is rugged, as per the conditions outlined in the ICH guidelines.

Robustness

Robustness is the measurement of small but deliberate variations in developed method, to check the reliability of the method under normal working conditions. In the present work, test for robustness was carried by pH of the mobile phase (by ±0.3%) and the flow rate of the mobile phase (0.9-1.1 mL/min), apart from changing the mobile phase composition to 0.95:10 ratio from the original 1:10 ratio. There were no significant changes in the retention time and assay values with respect to the composition of analytes, confirming the robustness of the method.

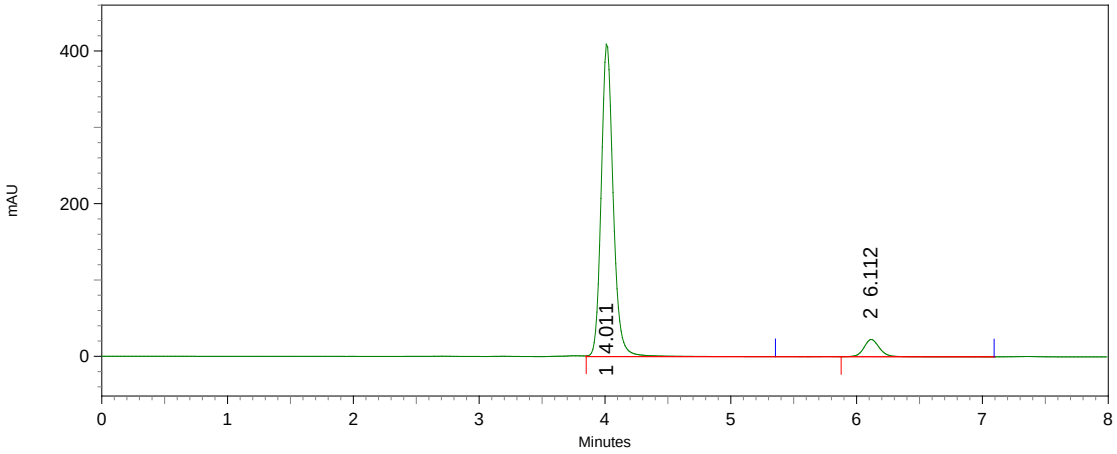

Fig. 8: Chromatogram of the blend obtained using different instrument of HPLC with PDA detector instead of UV detector to ascertain the ruggedness of the method

Table 4: Results of test for ruggedness

Condition	Run No	Amoxycillin sodium area in standard	Amoxycillin sodium area in sample	% Amoxycillin	Sulbactam sodium area in the standard	Sulbactam sodium area in the sample	% Sulbactam
Different	1	2728933	2517210	102.51	198091	163558	101.14
Instrument	2	2736838	2487428		197121	160876	
	Avg	2732885.5	2502319		197606	162217	
Different	1	2731570	2377398	101.44	204257	180060	102.16
Column	2	2703992	2374788		201636	180973	
	Avg	2717781	2376093		202946.5	180516.5	
Different	1	2712991	2507325	102.13	193014	193435	102.37
Analyst	2	2708634	2489535		190562	181290	
	Avg	2710812.5	2498430		191788	187362.5	

Table 5: Results of assay for determination of amoxicillin and sulbactam in the parenteral formulation

Batch number	Label claim for content of amoxicillin in mg per vial	Content of amoxicillin found in mg per vial ± S.D	Label claim for content of sulbactam per vial	Content of sulbactam found in mg per vial ± S.D	% of amoxicillin in amoxirum forte injection vial±S.D	% of sulbactam in amoxirum forte injection vial±S.D
1	2000 mg	2.0042±0.05	1000mg	1020±0.08	100.3± 0.8	99.8±1.3

Table 6: Snapshot of validation parameters

Parameters Validated	HPLC Method Using C-18 column
Accuracy	100.3% ± 1.3% for amoxicillin sodium and 99.8 % ± 1.5% for sulbactam sodium
Precision	RSD less than 2.0%
Specificity	The presence of ampicillin sodium in the sample did not affect the assay values of amoxicillin and sulbactam
Quantification Limit	The assay could be performed even at 10 times dilution i.e., to say 12 to 120 µg/mL
Linearity & Range	Conc. range of 56.85 to 82.87 µg/mL with R^2 =0.9989 for amoxicillin sodium and 28.43 to 41.44 µg/mL for sulbactum sodium with R^2 =0.9979
Ruggedness	RSD is less than ± 1.0

Method application

The method developed here was utilized for simultaneous estimation of amoxicillin sodium and sulbactam sodium in parenteral formulations. Samples from three different batches were analysed. The mean assay results are tabulated in Table 5. Table 6 provides a snapshot of validation parameters.

CONCLUSIONS

A reverse phase HPLC method was developed, which was subsequently validated (USP and ICH guidelines) for the assay of amoxicillin and sulbactam in combination formulation. Linear relationship between peak area and concentration was obtained for both the analytes. The % RSD for accuracy, precision, specificity were found to be less than 2 while it was less than 1% for ruggedness. The method was found to satisfy the guidelines of USP and ICH for validation of a new analytical method.

The developed method requires a short runtime of less than seven minutes utilizing inexpensive mobile phase and is linear over a wide range of analyte concentration. Hence this is a suitable method for the analysis of amoxicillin and sulbactam in parenteral formulations and in other formulations containing amoxicillin sodium and sulbactam sodium.

ACKNOWLEGEMENT

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