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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AMOXYCILLIN AND SULBACTAM IN BINARY MIXTURE

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ABSTRACT

A simple, precise, rapid and accurate RP- HPLC method was developed for simultaneous estimation of Amoxycillin (AMX) and Sulbactam (SUL) in Pharmaceutical dosage forms using Inertsil ODS 4.6x250mm, with 5 µm particle size. The mobile phase composed of acetonitrile: phosphate buffer (50mM KH_2PO_4) in the ratio of 10:90v/v (pH adjusted to 4.6 with orthophosphoric acid) was used for separation. The flow rate was maintained at 1.2 ml/min and the effluents were monitored at 238 nm. The retention times observed for Amoxycillin and Sulbactam were 3.297min and 5.157 respectively. The detector response was linear in the concentration of 336 - 784 µg/ml for AMX and 144-336 µg/ml for SUL. The Limit of Detection (LOD) is 399.45µg for AMX and 1481.967µg for SUL. The Limit of Quantification (LOQ) is 1210.441µg for AMX and 4490.81µg for SUL. The percentage assay of AMX and SUL were found to be 99.16%, 98.19% respectively. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of AMX and SUL in bulk drug and in its pharmaceutical dosage forms.

Key words: Amoxycillin sodium, Sulbactam sodium, High performance liquid chromatography, Validation, Simultaneous estimation.

INTRODUCTION

Amoxicillin is a semi-synthetic β-lactam antibiotic belonging to the group of penicillins. The chemical structure of amoxicillin consists of d-4-hydroxyphenylglycine side chain attached to 6-aminopenicillanic acid (6-APA) moiety. Chemically it is [(2S, 5R, 6R)-6- [(2R)-2-Amino-2-(4-hydroxyphenyl) acetyl] amino]-3, 3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid trihydrate (Fig.1). The molecular formula is $C_{16}H_{19}N_3O_5S$ and molecular weight is 419.45 gm/mol [1]. Because of its broad spectrum of bactericidal activity it is widely used in medicines, various preparations of this drug alone including capsules, tablets, powder for oral suspension, and injections as well as in combination with other ingredients, e.g. amoxicillin/ clavulanate, amoxicillin/Sulbactam. β-lactam antibiotics are used in health and medical circles due to their high efficiency in eradication of many infectious diseases [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. 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. Sulbactam Sodium chemically is Sodium (2, 5)-3, 3-dimethyl-7-oxo-4-thia-1-azabicycloheptane-2-carbo xylate 4, 4-dioxide (Fig.2). The molecular formula is $C_8H_{11}NO_5S$ and molecular weight is 233.24.gms/mol [3, 4]. 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 [5, 6].

Although, the simultaneous estimation of sulbactam sodium and ampicillin sodium has been reported, [7] the key merits of this method is shorter run times, economy and simplicity. All of these properties are very important in practice, particularly when a large number of samples are to be analyzed in various applications. The non-interference of the common

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excipients as evident by the absence of additional peaks in the chromatograms offers wider applicability. Two liquid Chromatographic methods have been reported for the simultaneous estimation of amoxicillin sodium and sulbactam sodium [8]. The present work discusses a simple Reverse Phase HPLC to estimate the concentrations of amoxicillin sodium and sulbactam sodium simultaneously in a pharmaceutical dosage form.

EXPERIMENTAL

Materials / Chemicals and Reagents

AMX and SUL were obtained as a gift samples from Chandra laboratories, Hyderabad, Telangana. acetonitrile, methanol and water used were of HPLC grade (Qualigens). Potassium dihydrogen orthophosphate and ortho-phosphoric acid used were of AR grade. Commercially available tablets (AMPHY IBL, Profic organic Ltd.) were procured from local market.

Chromatography Instrument

Quantitative HPLC was performed on liquid Chromatography, SHIMADZU LC-20 AT, UV detector module equipped with manual injector with injection volume 20 µl, and 2693 pump. An Inertsil ODS 3V, RP-C18 Column (250x4.6 mm i.d; particle size 5 µ) was used. The HPLC system was equipped with spinchrome software. The column was maintained at 25^0C and eluted under isocratic conditions over 7 min at a flow rate of 1.2 ml/min.

HPLC Conditions

The contents of the mobile phase consisting of 6.8 gms of KH_2PO_4 in 1000 ml of water adjusting the pH upto 4.6 with dilute orthophosphoric acid and acetonitrile both were mixed in the ratio of 90 : 10 v/v (50mM KH_2PO_4: acetonitrile). This was filtered before use, through a 0.45 µm membrane filter, and pumped from the respective solvent reservoirs to the column at a flow rate of 1.2 ml/min. The run time was set at 10.0 min and the column temperature was ambient. Prior to the injection (20 µl) of the drug solution, the column was equilibrated for at least 30 min with the mobile phases flowing through the system. The eluents were monitored at 238 nm [9,10].

Preparation of the Primary Standard/Stock Drug Solution

A standard stock solution of the drug was prepared by dissolving 280 mg of AMX, 150 mg of SUL in 50 ml volumetric flask containing 15 ml of diluent (buffer: acetonitrile 90:10 v/v), sonicated for about 10 min and then made up to 50 ml with the mobile phase to get standard stock solution of 5600 µg/ml of AMX, 2400 µg/ml of SUL.

Preparation of the Working Standard Drug Solution

5ml of the above stock solution was taken in 50 ml volumetric flask and made up to 50 ml with diluents (buffer: acetonitrile - 90:10 v/v) to get a concentration of each 560 µg/ml of AMX, 240 µg/ml of SUL respectively.

Preparation of Sample solution

Twenty tablets (AMPHY IBL - Profic organic Ltd.) were weighed, and then powdered. A sample of the powdered tablets, equivalent to mixture containing concentration of each 5600 µg/ml of AMX, 2400 µg/ml of SUL active ingredients, were mixed with 15 ml of diluent (buffer : acetonitrile 90:10 v/v) in 50 ml volumetric flask. The mixture was allowed to stand for 1 hr with intermittent sonication to ensure complete solubility of the drugs, and then filtered through a 0.35 µm membrane filter, followed by adding diluent up to 50 ml to obtain a stock solution. 5ml of the above sample stock solution was taken in 50 ml volumetric flask and made up to 50 ml with diluent to get a concentration of each 560 µg/ml of AMX, 240 µg/ml of SUL respectively.

Method Validation Tests

The method was validated in terms of the following parameters; linearity, specificity, accuracy, precision, and system suitability parameters as per the ICH guidelines.

Accuracy

Accuracy is the closeness in agreement between the accepted true value or a reference value and the actual results obtained. Accuracy studies are usually evaluated by determining the recovery of a spiked sample of the analyte into the mixture of the sample to be analysed.A known amount of pure drug at three different levels i.e. 80%, 100%, and 120% was added to preanalyzed sample solutions and total concentration was determined by the proposed HPLC method. Recovery of the individual substances at 80%, 100%, and 120% of specified concentrations were between 99.37% -101.95%, which proves the accuracy of the method. Results of the recovery studies are tabulated in Table no. 1. From the data obtained, it is obvious that the method is remarkably accurate, which ensures that this method produces reliable results.

Precision

Method precision was determined by injecting six replicates of the drug sample solution. The retention times and peak areas of six replicates are recorded. The precision expresses as the %RSD of peak areas and it should not be more than 2%. The low value (<1%) of RSD indicates the repeatability of the method. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs (Table no. 2).

Linearity

The linearity of the method was determined by constructing calibration curves. Sample solutions of AMX and SUL at different concentration levels (60%, 80%, 100%, 120%, and 140%) were used. Before injection of the solution, the column was equilibrated for at least 30 min with the mobile phase. The peak areas of the chromatograms were plotted against the concentrations of AMX and SUL to obtain the calibration curves. Aliquots

of standard AMX, SUL stock solutions were taken in different 10 ml volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of AMX, SUL were in the range of 336-784 µg/ml and 144-336 µg/ml respectively. Each of these drug solutions (20µL) was injected three times into the column, and the peak areas and retention times were recorded. Calibration graphs were obtained by plotting peak area versus concentration of AMX, SUL (Fig. 3&4). The plot of peak areas of each sample against respective concentration of AMX, SUL were found to be linear in the range of 336-784 µg/ml and 144-336 µg/ml with correlation coefficient of 0.99. The regression characteristics, such as slope, intercept, and %RSD were calculated for this method. The results obtained were presented in Table no. 4.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variation in method parameters and provides an indication of its reliability during normal usage. There was no change in system suitability parameters. The result of robustness studies along with its different parameters are tabulated in Table no. 3.

Ruggedness

Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory, and from analyst to analyst. There was no marked difference obtained in results. The results are tabulated in Table no .7.

Limit of Detection and Quantification

Limit of Detection (LOD) of the method was determined as the lowest concentrations of active pharmaceutical ingredients producing a signal-to-noise (S/N) ratio of about 3. The Limit of Quantitation (LOQ) was determined as the lowest concentrations of active pharmaceutical ingredients capable of being quantified with acceptable accuracy and precision producing signal-to-noise (S/N) ratio of about 10. The Limit of Detection (LOD) was found to be 399.45, 1481.9 µg for AMX and SUL. The Limit of Quantification (LOQ) analyzed was 1210.4, 4490.8 µg for AMX, SUL respectively. These values reflect the sensitivity of the method, which is of great importance in most studies and also indicating the method can be used for detection and quantification of analytes in a very wide concentration range.

System suitability

The peak resolution, theoretical plates, tailing factor, peak symmetry were calculated for the standard solutions. Results shown in Table no. 6. The results obtained indicate the suitability of the system for the analysis of the drug combination and the system suitability parameters are within the range during method. The system suitability report tabulated in Table no.5.

Assay

10 µl of sample solution (AMPHY IBL - Profic organic Ltd.) was injected into the injector of liquid chromatography. The retention times were found to be 3.303 min for AMX and 5.177 min for SUL successively. The amount of drug present per tablet was calculated by comparing the peak area of the sample solution with that of the standard solution. The data are presented in Table no. 8.

RESULTS AND DISCUSSION

HPLC Method Development and Optimization

In response to lack of simple, reliable and easy-to-use method for the determination of AMX and SUL concentrations in pharmaceutical matrices, an isocratic Reversed-Phase HPLC method was developed for quantification of above mentioned, API. We examined several HPLC method variables with respect to their corresponding effects on the result of analysis. To optimize the chromatographic conditions, different combinations of methanol-water, and sodium acetate buffer-acetonitrile and acetonitrile-di-potassium phosphate buffer were tested. Acetonitrile with Phosphate buffer system [pH 4.6] was promisingly preferred, because it resulted in greater resolution of API after several preliminary investigatory runs, compared with other mobile phases. The other parameters in this factorial design were temperature, flow rate, detection wavelength and volume of injection. Buffer molarity was changed and optimum buffer strength was selected as 50mM on the basis of theoretical plate number. At 238 nm, UV responses of two active pharmaceutical analytes were good and free from interferences. Under these conditions, the analyte peaks were well defined and free from tailing. Considering the whole body of the data obtained from this extensive study, the set of conditions indicated earlier in this article was selected for further validation. Typical chromatogram of AMX, SUL (Standard and Sample) has been shown in Fig: 3 & 4.

The system suitability tests were carried out on freshly prepared working stock solutions of AMX and SUL. Parameters that were studied to evaluate the suitability of the system were discussed and presented in Table 5&6.

Figure 1. Structure of Amoxycillin

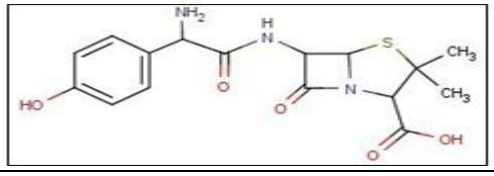

Figure 2. Structure of Sulbactam

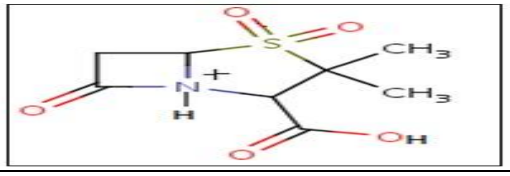

Figure 3.Calibration curve of Amoxycillin sodium

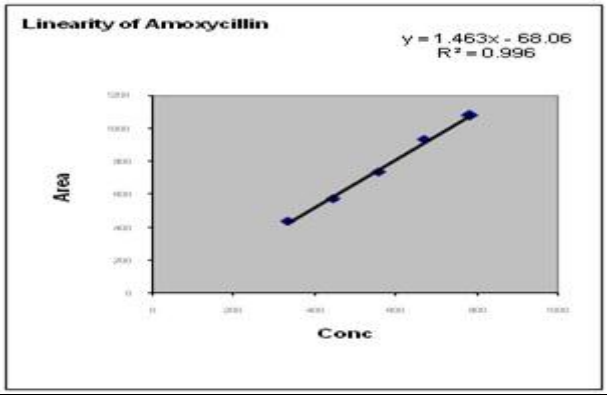

Figure 4. Calibration curve of Sulbactam sodium

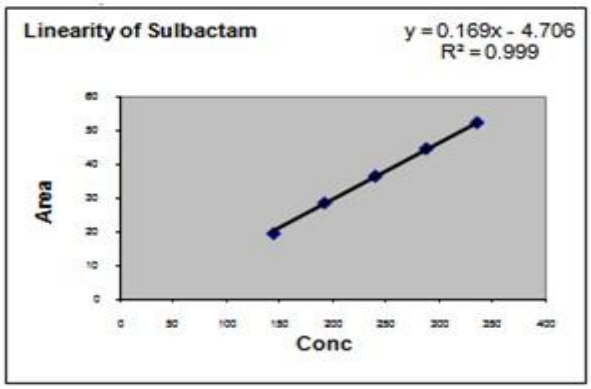

Figure 5. Chromatogram of Standard

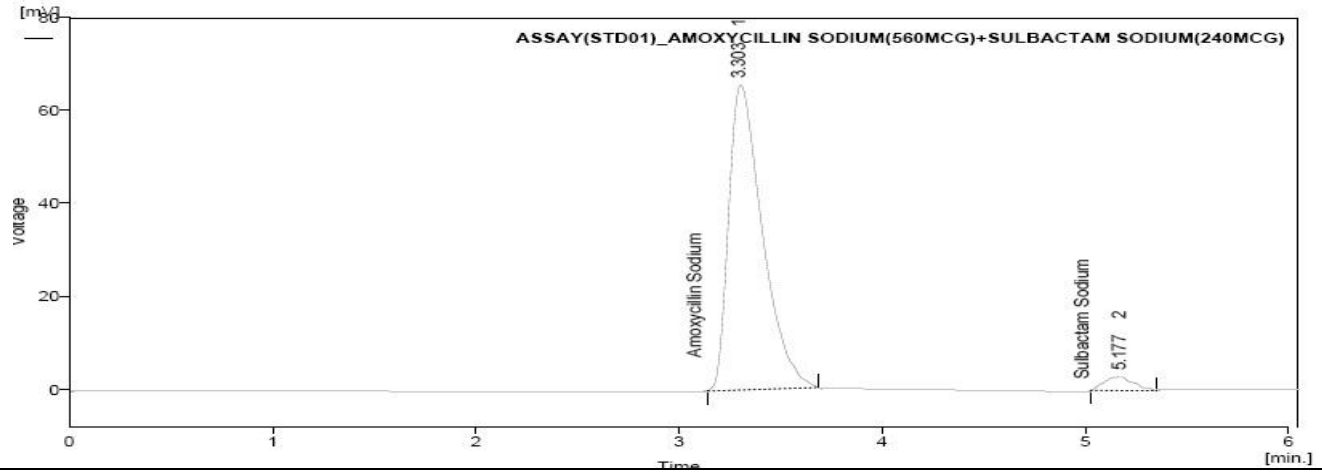

Figure 6. chromatogram of sample

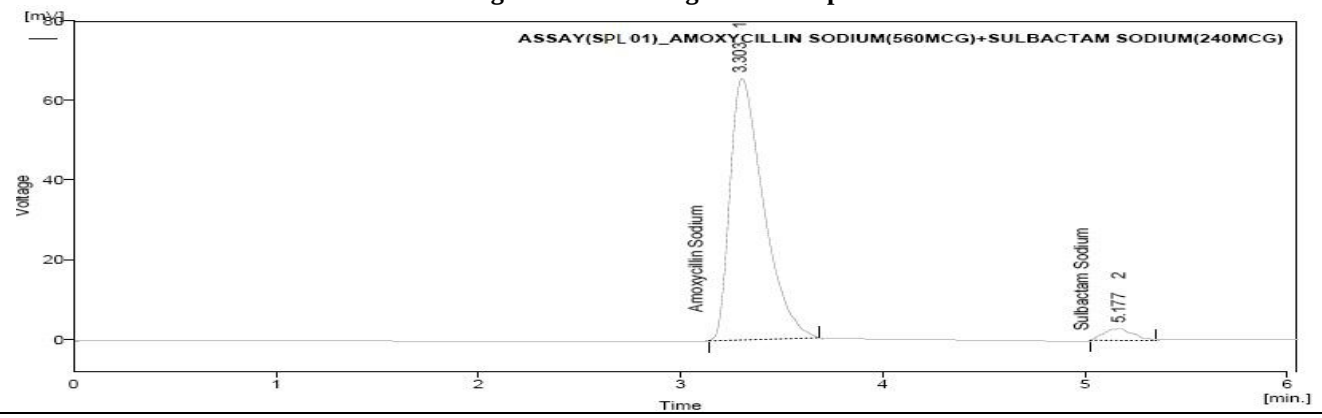

Table 1. Results for Accuracy

Levels of recovery	Amount Present (µg/ml)		Added conc. (µg/ml)		Total amount recovered (µg/ml)		% recovery	
DRUG	AMX	SUL	AMX	SUL	AMX	SUL	AMX	SUL
80	448	192	112	48	563.91	242.22	100.70	100.93
100	448	192	224	96	685.07	286.19	101.95	99.37
120	448	192	336	144	791.87	340.13	101.00	101.23

Table 2. Results for Precision

S.No	Amoxycillin sodium		Sulbactam sodium	
	Retention time	Peak area	Retention time	Peak area
1	3.280	774.259	5.183	25.671
2	3.283	765.594	5.173	26.565
3	3.307	772.851	5.193	26.172

4	3.317	782.589	5.190	26.138
5	3.310	771.374	5.177	26.255
6	3.300	775.855	5.203	26.463
Average	3.2995	773.754	5.187	26.211
%RSD	0.45	0.72	0.21	1.19

Table 3. System Suitability

Flow Rate (ml/min)		System Suitability Results		
		USP Plate Count	USP Tailing	Retention time(min)
Low	1.0	3063	1.228	3.923
Actual*	1.2	3866	1.252	3.303
High	1.4	3866	1.2	2.937

Table 4. Results for Linearity

Concentration Amoxycillin sodium (µg/ml)	Peak area of Amoxycillin sodium	Concentration of Sulbactam sodium (µg/ml)	Peak area of Sulbactam sodium
336	437.719	144	19.295
448	573.656	192	28.328
560	735.102	240	36.228
672	931.600	288	44.331
784	1078.031	336	52.045

Table 5. System suitability report

S.No	Amoxycillin sodium		Sulbcatm sodium	
	Retention time	Peak area	Retention time	Peak area
1	3.283	774.259	5.183	25.671
2	3.317	765.594	5.173	26.565
3	3.307	772.851	5.193	26.172
4	3.315	782.589	5.190	26.138
5	3.310	771.374	5.177	26.255
6	3.300	775.855	5.203	26.463
Avg	3.299	773.754	5.187	26.211
%RSD	0.45	0.72	0.21	1.19

Table 6. System suitability parameters

Parameters	Amoxycillin sodium	Sulbactam sodium
Linearity range	336-784 µg/ml	144-336µg/ml
Correlation coefficient	0.996	0.999
Slope(m)	1.463	0.169
Intercept	68.06	4.706
Theoretical plates	3879	4121
Tailing factor	1.28	1.167
Retention time	3.253	5.000

Table 7. Results for Ruggedness

Analyst	Area of Amoxycillin sodium	Area of Sulbactam sodium
Analyst 1	737.606	29.181
Analyst 2	756.494	28.265

Table 8. Results for Assay

Drug	Label claim	%Assay	Amount present
Amoxycillin sodium	350 mg	99.16	501.4
Sulbactam sodium	150mg	98.19	50.35

CONCLUSION

A simple, precise, accurate, robust, rugged and economical RP-HPLC method was developed in this study for the quantification of AMX and SUL in bulk and pharmaceutical dosage forms. One of the key advantages of this method is its considerably shorter run times, easy-

to-use and its simplicity. All of these properties are very important in practice, particularly when a large number of samples are to be analyzed in various applications. The absence of additional peaks in the chromatogram indicates non-interference of the common excipients used in the tablets, giving the benefit of wider applicability. The results of validation tests were collectively indicative for a method with a relatively wide linear range, acceptable precision.

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