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Research Article

Validation of Assay Indicating Method Development of Amoxicillin in Bulk and One of Its Marketed Dosage Form by RP-HPLC

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Abstract

A novel, simple and economic Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method has been developed for the quantification of Amoxicillin (AMX) in bulk and tablet dosage form with greater precision and accuracy. Separation was achieved on Hypersil ODS C18 (250mm×4.6×5micron) column in isocratic mode with mobile phase consisting of Acetonitrile: 0.2M Potassium dihydrogen phosphate buffer (pH 5) (1:99v/v) and conditions optimized with flow rate of 1 ml/minute and wavelength of detection at 254 nm. The retention time of Amoxicillin (AMX) was found to be 6.992 min. The method was validated as per ICH guidelines. Linearity was established for Amoxicillin (AMX) in the range0.6 – 3.4 μg / ml with R^2 value 1. The percentage recovery of Amoxicillin (AMX) was found to be in the range 98.87-99.87 %. The high recovery and low relative standard deviation confirm the suitability of the proposed method for the estimation of the drug in bulk and marketed dosage form. Validation studies demonstrated that the proposed RP-HPLC method is simple, specific, rapid, reliable and reproducible for the determination of Amoxicillin for Quality Control level.

Introduction

Amoxicillin (AMX), shown in Figure 1, is (2S.5R.6R)-6-{[(2R)-2-amino-2-(4-hydroxy phenyl)-acetyl] amino}-3, 3-dimethyl-7-oxo-4-thia-l-azabicyclo [3.2.0] heptane-2-carboxylic acid. Amoxicillin is a moderate-spectrum, bacteriolytic, β-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms. It is usually the drug of choice within the class because it is better absorbed, following oral administration, than other β-lactam antibiotics. It is a semi synthetic antibiotic, an analog of Ampicillin with a broad spectrum of bactericidal activity against many gram- positive and gram- negative microorganisms [1,2]. Amoxicillin is susceptible to degradation by β-lactamase producing bacteria, which are resistant to a broad spectrum of β-lactam antibiotics, such as pencillin. For this reason, it is often combined with clavulanic acid, a β-lactamase inhibitor [3]. Several analytical methods for the determination of amoxicillin by capillary electrophoresis [4], spectrophotometry [5], HPLC [6-11] and HPTLC [12] have been reported. The aim of the present work was to develop and validate a sensitive RP-HPLC method that can be implemented for the quantification of Amoxicillin in bulk as well as in its tablet dosage forms.

Experimental

Materials

Pure Amoxicillin (AMX) used as working standards, was purchased from Yarrow chem. Products, Mumbai, India. Tablets containing 500 mg of Amoxicillin (GERMATIN) was obtained from Apollo Pharmaceuticals Pvt. Ltd, Visakhapatnam, India and used within their shelf life period. Acetonitrile and water (HPLC-grade) were purchased from Merck, India. All other chemicals and reagents employed were of analytical grade, and purchased from Merck, India.

Instrumentation

Shimadzu 1800 UV-visible spectrophotometer (Hyderabad), Ultrasonicator,0.45µm membrane filter, Sartorius Analytical balance, Shimadzu HPLC system, LC Solution soft ware having the configurations, Solvent degasser DCU-20A3 Solvent degasser, Prominence 10 AT vp binary gradient pumps, SPD 10 A VP UV-VIS detector with class VP software, Columns of Hypersil ODS C18 250mm×4.6×5micron were used in this study. A Wenster digital pH meter was used for pH adjustment.

Table 1: Optimization of chromatographic conditions.

Trials	Column	Mobile phase	Flow rate	Wave length	Observation	Results
1	Hypersil ODS, C18 column.	10:90 Acetonitrile and buffer of ph-6	1.5ml	254nm	Peak fronting and Splitting was observed	Rejected
2	Hypersil ODS, C18 column.	10:90 Acetonitrile and buffer of ph-6	1ml	254nm	Tailing was Observed	Rejected
3	Hypersil ODS, C18 column.	1:99 Acetonitrile and buffer of ph-6	1ml	254nm	Tailing was observed	Rejected
4	Hypersil ODS, C18 column.	1:99 Acetonitrile and Phosphate buffer of pH-5	1ml	254nm	Good peak was observed	Accepted

Chromatographic conditions

The selected and optimized mobile phase composed of Acetonitrile: Potassium dihydrogen phosphate buffer (pH 5) (1:99v/v) and conditions optimized were with flow rate of 1 ml/minute, wavelength at 254 nm and Run time of 20 min. Here the peaks were separated and showed better resolution, appreciable theoretical plate counts and good peak symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the present drug Table 1.

Preparation of mobile phase

Mobile phase was prepared by taking Acetonitrile: 0.2 M Potassium di hydrogen phosphate buffer (pH 5) (1:99 v/v). Mobile phase was filtered through 0.45 μm membrane filter and degassed under ultrasonic bath prior to use. The mobile phase was pumped through the column at a flow rate of 1 ml/min.

Preparation of standard solutions

Dissolve 30 mg of amoxicillin working standard in mobile phase and dilute to 50 ml with the same mobile phase. One ml was diluted to 20ml with mobile. Diluted to 1ml of this solution to 50ml with mobile phase. Finally this gave 0.6 ppm solution, then the solution was filtered through the 0.45 μm membrane filter and degassed under ultrasonic bath prior to use. The solution was injected into the HPLC system.

Assay of Amoxicillin from Marketed tablets

Twenty tablets (GERMATIN tablet contains: Amoxicillin 500 mg) was weighed accurately, average weight was calculated. Then powder equivalent to 50 mg was taken in a 50ml volumetric flask. Then 20ml of mobile phase was added and kept for 15mins with occasional shaking. Then 10 ml of Glacial acetic acid was added. Then volume was made to 50ml followed by sonication for 15mins.Then whole of the solution was filtered with 0.45μ filter paper. From the filterate 10 ml was taken and made to 100ml with the mobile phase. Finally 1ml of the above solution was taken in 10ml volumetric flask and made up to volume with mobile phase and injected into RP-HPLC system. Then Assay was carried out for the amount of amoxicillin content.

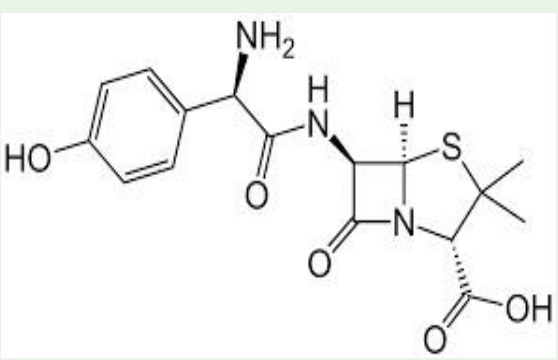

Figure 1: Chemical Structure of Amoxicillin.

Method Validation

The method was validated in accordance with ICH guidelines [13]. The parameters assessed were linearity, accuracy, and precision, reproducibility, robustness and system suitability.

Accuracy

Accuracy was best determined by the standard addition method. Previously analyzed samples of Amoxicillin API were added with standard drug solutions and are analyzed by the proposed method. Recovery (%), RSD (%) and bias (%) were calculated for each concentration.

Accuracy is reported as percentage bias, which is calculated from the expression

$$\%Bias = \frac{(\text{measured value} - \text{true value})}{\text{true value}} \times 100$$

Precision

System precision: Standard solution prepared as per test method and injected six times and the %RSD value was calculated.

Method precision: Six preparations individually using single batch of Amoxicillin drug substance were prepared as per test method and injected each solution induplicate on the same day in to HPLC. % RSD value was calculated to determine intra-day precision.

Ruggedness/Inter day precision: Six preparations individually using single batch of Amoxicillin drug substance as per test method and injected each solution induplicate on the same day in to HPLC using different column, system and analysts on different days. And %RSD value was calculated to determine inter-day precision.

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters". To determine the robustness of the method experimental conditions are purposely altered and chromatographic characters are evaluated. Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), wavelength of detection (±2nm) and acetonitrile content in mobile phase (±2%) were studied to determine the robustness of the method.

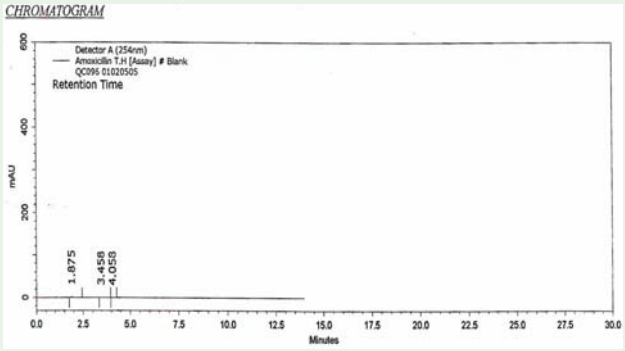

Figure 2: Blank chromatogram for Amoxicillin.

Limit of Detection (LOD)

The Limit of Detection (LOD) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa), which may be related to LOD and the slope of the calibration curve, b, by

LOD = 3 Sa / b

Limit of Quantitation (LOQ)

The LOQ is the concentration that can be quantitated reliably with a specified level of accuracy and precision. The LOQ represent the concentration of analyte that would yield a signal-to-noise ratio of 10.

LOQ = 10 Sa / b

Where, Sa is the standard deviation of the peak area ratio of analyte to IS (6 injections) of the drugs and b is slope of the corresponding calibration curve.

Linearity and Range

Linearity indicates the ability of analytical procedures to produce results that are directly proportional to the concentration of analyte in the given sample. A series of solutions of drug substance standard were prepared in the concentration range from (0.6μg/ml) to (3.4μg/ml). These solutions were injected into the HPLC and linearity was

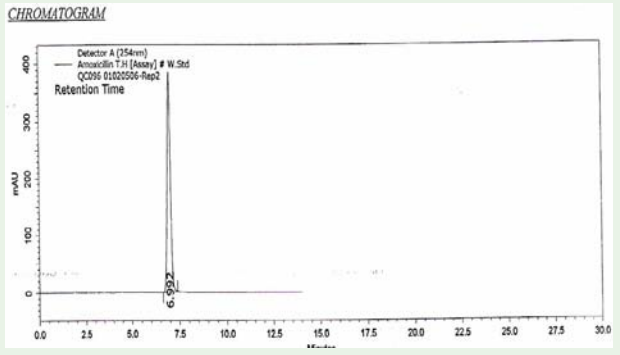

Figure 3: Standard chromatogram for Amoxicillin.

Table 2: Data of recovery for Amoxicillin.

Concentration	Amount added (mg)	Amount Found (mg)	% Recovery	Mean	SD	%RSD
80%- Sample 1	24.81	24.79	99.92	99.41	0.33	0.33%
80%-Sample 2	24.89	24.75	99.44			
80%-Sample 3	24.51	24.33	99.27			
100%-Sample 1	30.22	30.21	99.97	99.76	0.18	0. 18%
100%-Sample 2	30.02	29.91	99.63			
100%- Sample 3	30.05	29.90	99.69			
120% Sample 1	36.01	35.68	99.08	98.87	0.30	0.30%
120% - Sample 2	35.30	34.78	98.53			
120% - Sample 3	36.00	35.67	99.08			

determined by observing peak areas. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

Results and Discussion

The blank chromatogram and optimized chromatogram for amoxicillin is shown in Figure 2 and Figure 3 respectively.

Accuracy: Recovery study

Acceptance criteria: The percentage recovery should be within 98.0% to 102.0%. % RSD should not be more than 1.0%. The recovery of the method, determined by adding a previously analyzed test solution with additional drug standard solution at three levels of concentration, was 98.87- 99.76 %. The values of recovery (%) and RSD (%) listed in Table 2 indicate the method is accurate.

Linearity & Range

The calibration curve showed good linearity in the range of 0.6-3.4 μg/ml, for Amoxicillin (API) with correlation coefficient (r^2) of 1. The slope and intercept of the calibration graph was calculated by using linear regression analysis. The regression equation of the calibration curve was: y = 76594x- 24947; r^2 = 1. A correlation coefficient suggests that the developed HPLC method had an excellent linearity over the investigated range. The results for linearity are shown in Figure 4 and Table 3.

Table 3: Data for linearity.

Conc.(μg/ml)	AUC
0.6	4542042
1	6056056
1.4	7570070
1.8	9084084
2.2	10598098
2.6	12112112
3	13626126
3.4	15140140

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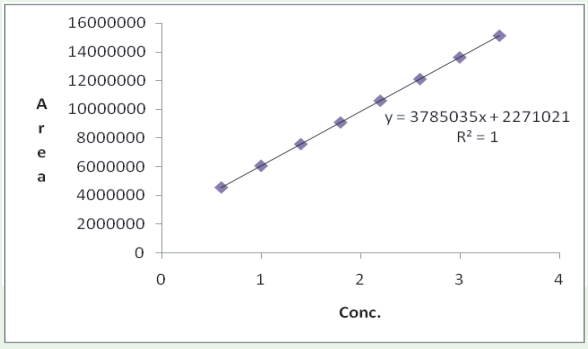

Figure 4: Calibration curve for Amoxicillin.

Table 4: Data of system precision for Amoxicillin.

Injection ID	Area
1	4542042
2	4533188
3	4535021
4	4530680
5	4536185
6	4532243
Mean	4534893
SD	4012
% RSD	0.09

Precision: Intra-assay & inter-assay

System precision

Aceptance criteria: RSD for area should not be more than 1%.

The intra & inter day variation of the method was carried out and the high values of mean assay and low values of standard deviation and % RSD (% RSD < 2%) within a day and day to day variations for Meloxicam revealed that the proposed method is precise (Table 4).

Method Precision

Acceptance criteria: RSD values should not be more than 1% and the results are well within the limits (Table 5).

Table 5: Data of method precision for Amoxicillin.

Injection ID	Area
1	4542042
2	4533188
3	4535021
4	4530680
5	4536185
6	4532243
Mean	4534893
SD	4012
RSD	0.09

Table 6: Data of Intermediate precision for Amoxicillin.

Sample	Assay %(w/w)	
	Set- I	Set-II
1	98.90	98.83
2	98.86	98.83
3	98.96	98.77
4	98.98	98.85
5	98.87	98.80
6	99.03	98.86
Mean	98.93	98.82
SD	0.1	0.03
%RSD	0.1	0.03
CI	0.1	0.1
Over all Mean	98.88	
Over all SD	0.1	
Overall RSD (%)	0.1	

Robustness

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favor of (Table 5, % RSD < 2%) the developed RP-HPLC method for the analysis of Amoxicillin API.

Acceptance criteria: RSD value should not be more than 1% and the results obtained are well within limits (Table 6).

Stability

From the below data it was concluded that: The test sample was stable for atleast 9 hours at room temperature (about 25°c).The test sample was stable for at least 15 hours of the temperature and the results are shown in Table 7.

Estimation of Amoxicllin in Tablet Dosage Form

Assay was performed by using the regression equation ($y = 76594x - 24947$; $r^2 = 1$) obtained from the standard curve of Amoxicllin API. Results obtained are given in table 8 and Figure 5.

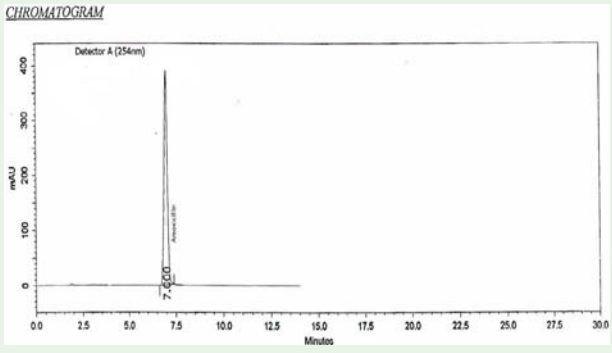

Figure 5: Chromatogram for Amoxicillin marketed tablet.

Table 7: Data of stability for Amoxicillin.

Time in hours	Room temperature25 ˚c		Room temperature 6˚ c	
Initial	4559861		4559861	
After 1 hr	4540232	0.3	4552531	0.1
After 2 hr	4542807	0.2	4522082	0.4
After 3 hr	4555984	0.2	4527089	0.4
After 4 hr	4579426	0.2	4541420	0.4
After 5 hr	4598693	0.5	4554183	0.3
After 6 hr	4515495	0.6	4536440	0.3
After 7 hr	4503377	0.7	4471191	0.6
After 8 hr	4464949	0.9	4582647	0.7
After 9 hr	4460204	1	4564295	0.7
After 10 hr	4456992	1.1	4549355	0.6
After 11 hr	4458707	1.1	4545623	0.6
After 12 hr	4445107	1.2	4544017	0.6
After 13 hr			4538216	0.6
After 14 hr			4577662	0.6
After 15 hr			4571867	0.6

Recovery Data for estimation Amoxicillin in GERMATIN tablets:

Conclusion

A New RP-HPLC method indicating assay of AMX in bulk and in pharmaceutical dosage forms is established. This method is simple, reliable, linear, accurate, sensitive and reproducible as well as cost effective for the effective quantitative analysis of AMX in bulk and tablet formulations. The method was completely validated showing satisfactory data for all the method validation parameters tested and method is free from interference of the other active ingredients and additives used in the formulations. Therefore the method is suitable for use of the routine quality control analysis of AMX in API or in pharmaceutical dosage forms.

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