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A Validated RP-HPLC Method Development for Amoxicillin in Pharmaceutical Dosage Forms

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Abstract

A rapid and simple Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method has been developed for the quantification of Amoxicillin in tablet dosage form. Separation was achieved on Chromatopak-C18 (250mm×4.6×5micron) column in isocratic mode with mobile phase consisting of Acetonitrile: 0.2M Potassium dihydrogen phosphate buffer (pH 3) (22:78v/v) and conditions optimized with flow rate of 1 ml/minute and wavelength of detection at 283 nm. The retention time of Amoxicillin was found to be 6.4 min. Linearity was established for Amoxicillin in the range 10 – 100 μg / ml with R^2 value 0.999. This method was validated in accordance with ICH guidelines, the linearity, accuracy, precision, specificity, robustness, ruggedness, and system suitability results were within the acceptance criteria. 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.

Keywords: RP-HPLC, Amoxicillin, Method Development, Validation.

1. Introduction

1.1 Amoxicillin

Amoxicillin is an extended spectrum penicillin group of antibiotic. Amoxicillin is active against many gram positive and gram negative bacteria.

Chemical structure

The molecular formula is $C_{16}H_{19}N_3O_5S•3H_2O$ and the molecular weight is 419.45. The chemical structure is:

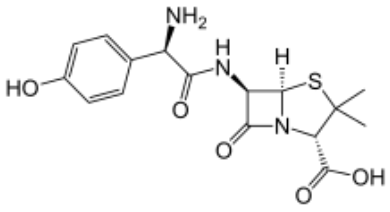

Figure 1: Structure of Amoxicillin

1.2 Mechanism of action

Amoxicillin acts by inhibiting bacterial cell wall synthesis. Lack of bacterial cell wall results in death due to lysis of bacteria. So amoxicillin is useful only for actively growing and cell wall synthesizing bacteria. Amoxicillin is an extended spectrum, penicillinase-susceptible, semi-synthetic amino- penicillin. It is a close chemical and pharmacological congener of ampicillin (amino-p-hydroxy-benzyl penicillin).

Amoxicillin like other penicillins, inhibit the penicillin binding proteins (PBPs {specifically PBP-1A}), which are transmembrane surface enzymes that catalyse the cross linking (transpeptidation) between the peptidoglycans in the bacterial cell wall. Amoxicillin is able to bind to the PBPs by geometrically mimicking the natural D-alanyl-D-alanine substrate that is generally bound to the enzyme. By substituting itself for D-alanyl-D-alanine, the appropriate

strands needed for cross-linking are not available and the cell wall biosynthesis process is left incomplete.

It also acylates the penicillin-sensitive transpeptidase C-terminal domain by opening the lactam ring. This inactivation of the enzyme prevents the formation of a cross-link of two linear peptidoglycan strands, inhibiting bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins or murein hydrolases; it is possible that amoxicillin interferes with an autolysin inhibitor. [1-6]

2. Materials and Methods

2.1 Experimental

2.1.1 Materials

Tablets containing 125 mg of Amoxicillin was obtained from Apollo Pharmaceuticals Pvt. Ltd, Tirupati, India and used within their shelf life period. Acetonitrile and water (HPLC-grade) were purchased from SD Fine Chem. Pvt. Ltd, India. All other chemicals and reagents employed were of analytical grade, and purchased from SD Fine Chem. Pvt. Ltd, India.

2.1.2 Instrumentation

Ultrasonicator,0.45µm membrane filter, (PCI Analytical, Model Number 1.5L50) Analytical balance (Wenser), Analytical Technologies HPLC system, LC Solution soft ware having the configurations, Solvent degasser DCU-20A3 Solvent degasser, UV-VIS detector with class VP software, Columns of Chromatopak-C18 (250mm×4.6×5micron) were used in this study. A (Eutect) digital pH meter was used for pH adjustment.

2.1.3 Chromatographic conditions

The selected and optimized mobile phase composed of Acetonitrile: Potassium dihydrogen phosphate buffer (pH 3) (22:78v/v) and conditions optimized were with flow rate of 1 ml/minute, wavelength at 283 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

2.1.4 Preparation of mobile phase

Mobile phase was prepared by taking Acetonitrile: 0.1 M Potassium di hydrogen phosphate buffer (pH 3) (22:78v/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.

2.1.5 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.

2.1.6 Sample Preparation

Amoxicillin is available as tablets containing 125mg of Amoxicillin. Amoxicillin is available in the local market with brand names AMOX 125. Twenty tablets of Amoxicillin were taken into a fine powder of the tablets and the powder equivalent to 100mg of Amoxicillin was weighed accurately and transferred into a 100ml standard volumetric flask. The contents were dissolved in buffer and sonicated for 30 Minutes. This entire solution was filtered through 0.45 micron Whatmann filter paper (No. 41) and the final solution was made with mobile phase to get the solution of 1000µg/ ml. 1ml of this solution was transferred to 10ml volumetric flask, volume was made with acetonitrile. It gives 100 µg/ml. 5ml of the solution were pipetted out separately into 10ml volumetric flask to give 10µg/ml concentration. The sample solution 20µl was injected and chromatographed.

3. Results and Discussions

The goal of the present study is to develop and validate a simple, accurate and precise ultraviolet spectrophotometric method and reversed-phase high-performance liquid chromatographic (RP-HPLC) for the estimation of Amoxicillin in bulk and pharmaceutical dosage form.

3.1 Experimental trails

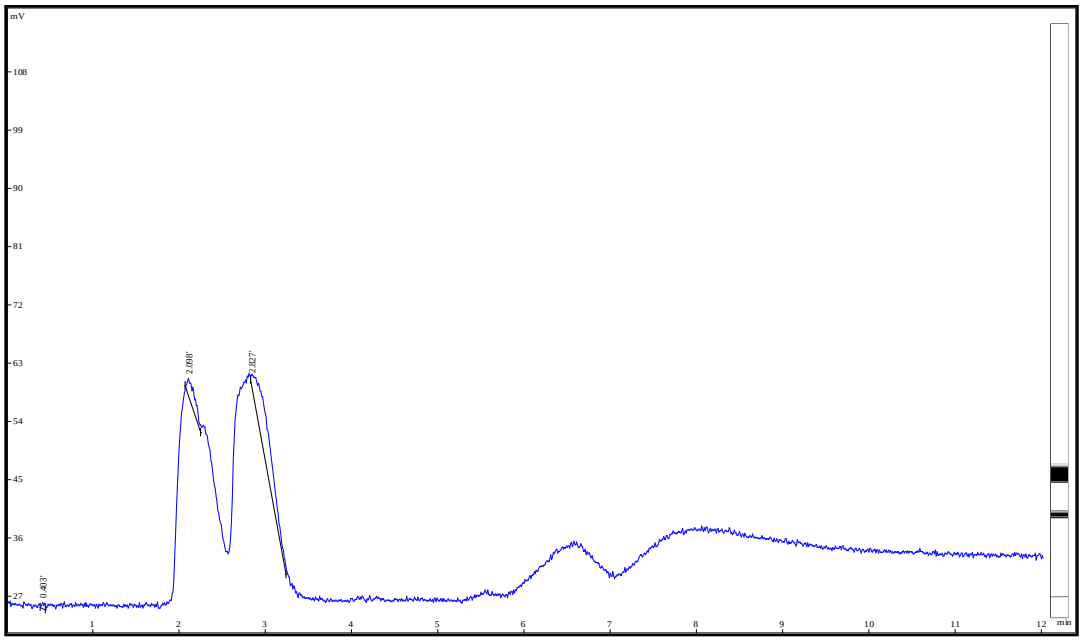

Figure 2: Chromatogram of Trial – 1

Trial – 1 was performed on LC, Chromatopak-C18 (250mm×4.6×5micron) column using mobile phase of methanol: water in the ratio of 50:50v/v at U.V detection at 267ηm.

Result: Broad peaks with some extra peaks were appeared so this was not satisfactory.

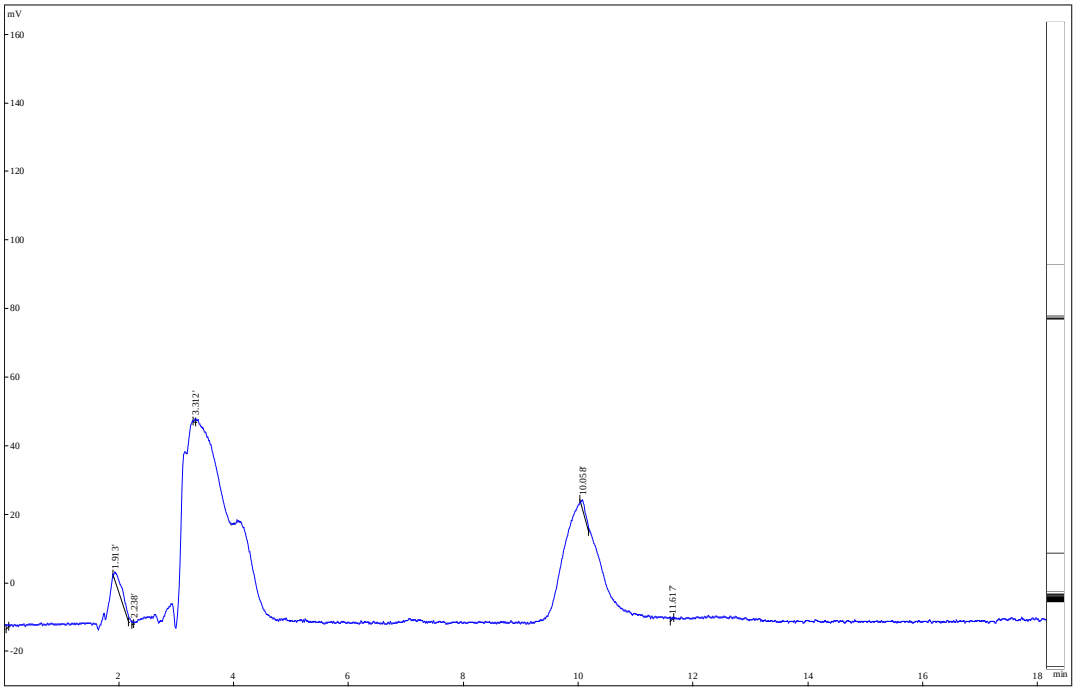

Figure 3: Chromatogram of Trial – 2

- Trial – 2 was performed on Chromatopak-C18 (250mm×4.6×5micron) column using mobile phase of Acetonitrile: phosphate buffer in the ratio of 95:05 v/v at U.V detection at 283ηm.
- Fronting with broad peaks and some extra peaks were appeared so this was not satisfactory.

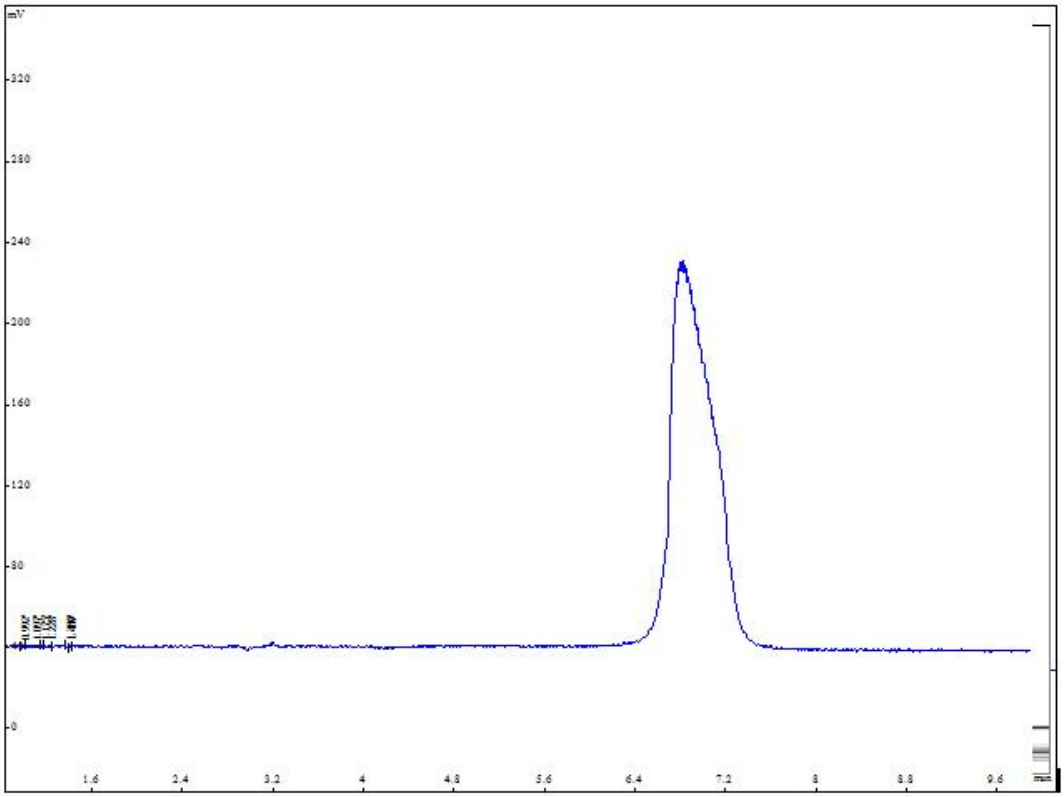

Figure 4: Chromatogram of Trial – 3

- Mobile phase of Acetonitrile: Potassium dihydrogen phosphate buffer adjusted to pH 3 with orthophosphoric acid in the ratio of 22:78 v/v at U.V detectection at 283ηm
- A sharp peak with a retension time of 6.461.

The trail 3 was selected for the futher work in HPLC as the result was satisfactory with a sharp peak with the retension time of 6.461mins

3.2 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.

3.2.1 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 = (measured value - true value) true value X 100

3.2.2 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.

3.3 Repeatability

Table 1: Precision

Concentration [µg/ml]	Peak area
50	6381711
50	6272822
50	6363603
50	6384644
50	6385755
50	6490823
Area Mean	6379893
S.D	69419.4
%R.S.D	**1.088**

3.4 Linearity

Various standards in the range 10-100µg/ml of Amoxicillin were injected onto the chromatographic system and the peak area was measured. A graph of peak area (on Y-axis) versus concentration (on X-axis) is plotted and the correlation coefficient was calculated.Table19 gives information about the linearity data. From the results, the correlation coefficient for Amoxicillin is 0.999 and, it can be concluded that the linearity is well within the limit. Hence the method is linear.

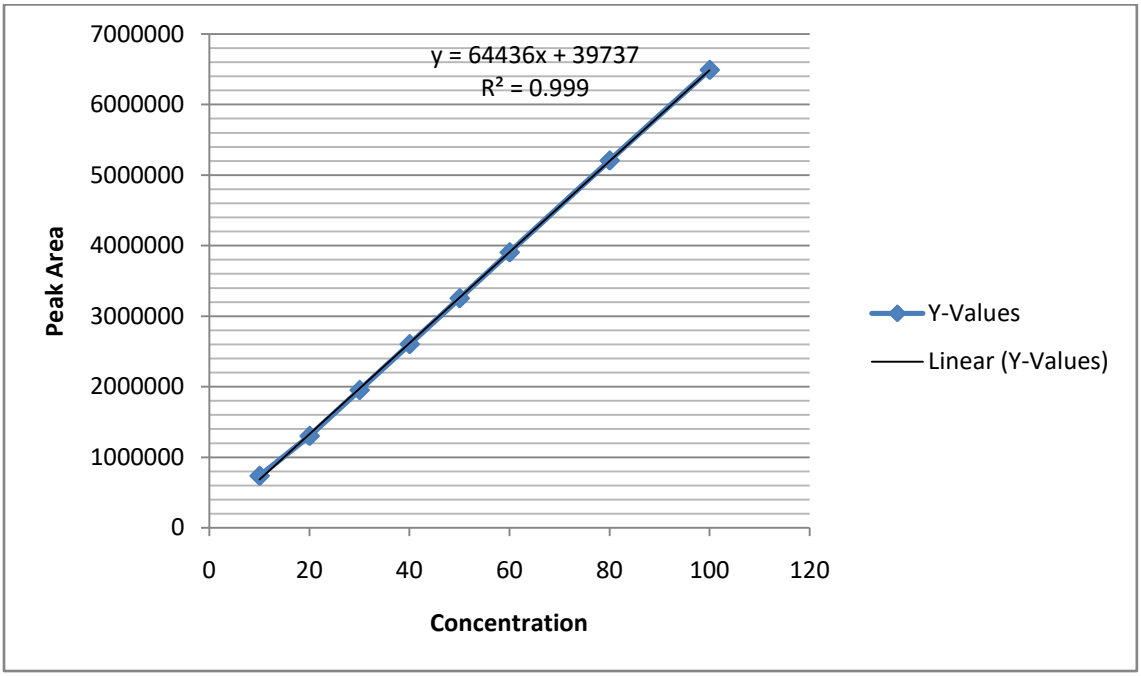

Figure 5: Linearity graph of Amoxicillin

Table 2: Summary of Linearity

Concentration (µg/ml)	Peak Area
10	735698
20	1301541
30	1952312
40	2603083
50	3253854
60	3904625
80	5206167
100	6490823

3.5 Accuracy studies

Table 3: Summary of Accuracy studies

Spiked level (%)	Formulation Conc. (µg/ml)	Amount Found	% Recovery	% Mean recovery	SD	%RSD
50	50	79.55	99.45	99.66	0.599	0.601
	50	79.41	99.2			
	50	80.27	100.34			
100	50	81.14	101.4	101.3	0.602	0.594
	50	81.57	101.9			
	50	80.62	100.7			
150	50	79.94	99.92	100.17	0.296	0.296
	50	80.1	100.1			
	50	80.42	100.5			

The accuracy of the method, recovery studies were carried out by adding different amounts (50%, 100% and 150%) of bulk samples of Amoxicillin within the linearity range were taken and added to the pre-analyzed formulation of concentration 20µg/ml. From that percentage recovery values were calculated. The results were within the range and were found to be highly accurate which was shown in table 3.

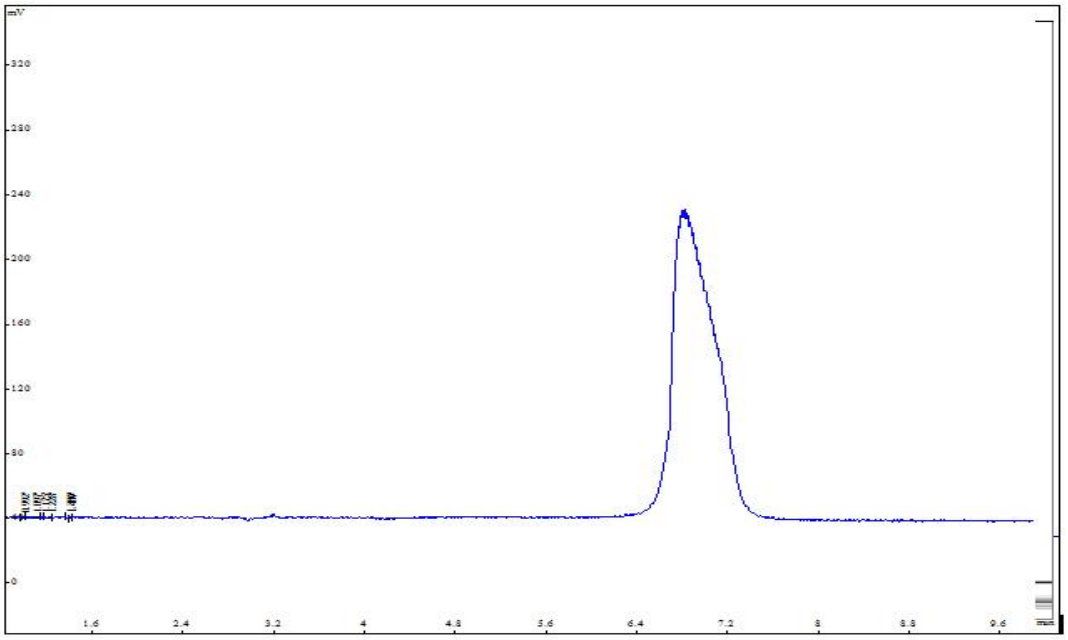

Figure 6: Chromatogram of 50%

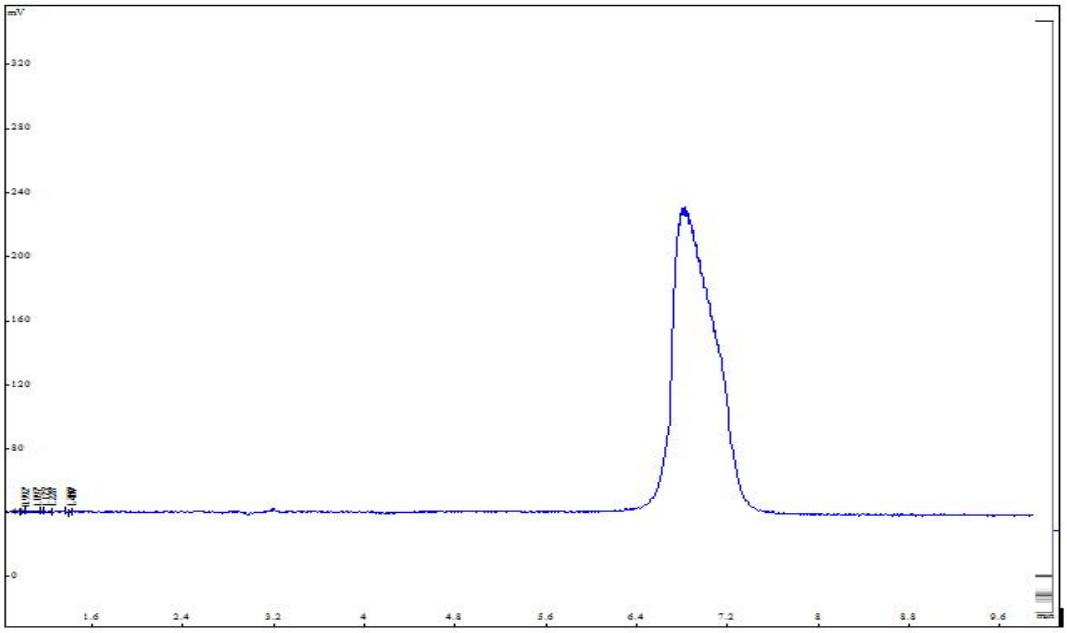

Figure 7: Chromatogram of 100%

3. 6 Limit of Detection and Limit of Quantification

The LOD and LOQ were calculated based on the standard deviation of the response and the slope of the constructed calibration curve, as described in International Conference on Harmonization guidelines Q2 (R1) was shown in table 4.

Table 4: Summaries of LOD and LOQ

Parameter	Concentration (µg/ml)
LOD	0.168
LOQ	0.512

3.7 Robustness

The Robustness of the method was determined by making slight changes in the experimental conditions such as change in the flow rate, mobile phase and wave length.

Table 5: Summary of Robustness

Parameters	Condition	%R.S.D	R_T
Flow rate	0.9ml/min	0.379	6.450
Actual flow rate	**1ml/min**	**0.236**	6.461
Flow rate	1.1ml/min	0.22	6.470
Wavelength	**267nm**	0.218	6.421
Wavelength	**283nm**	**0.236**	6.461
Wavelength	**276nm**	0.258	6.455

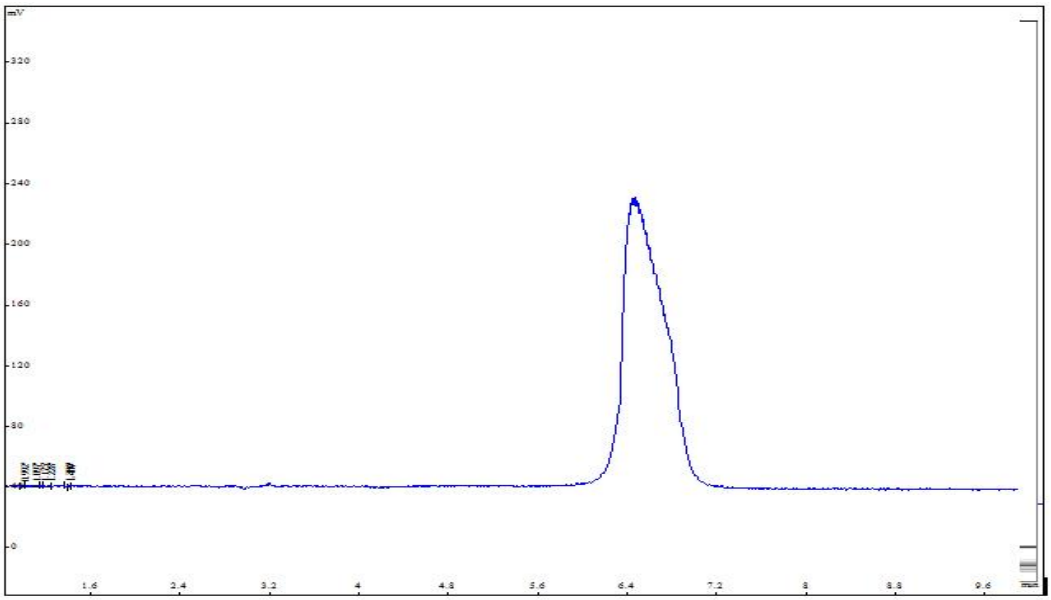

Figure 8: Chromatogram of Flow rate: 0.9ml/min

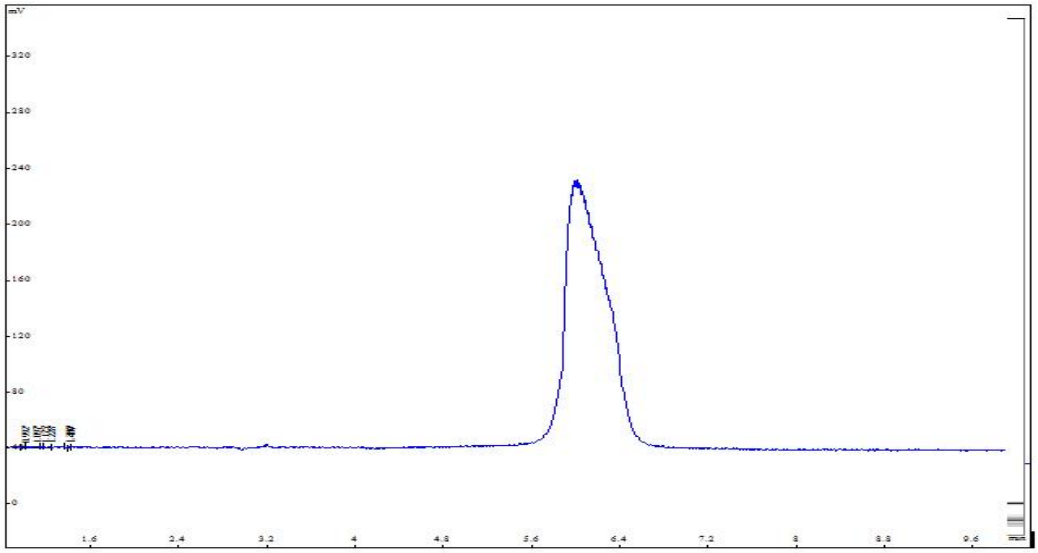

Figure 9: Chromatogram of Flow rate: 1.1ml/min

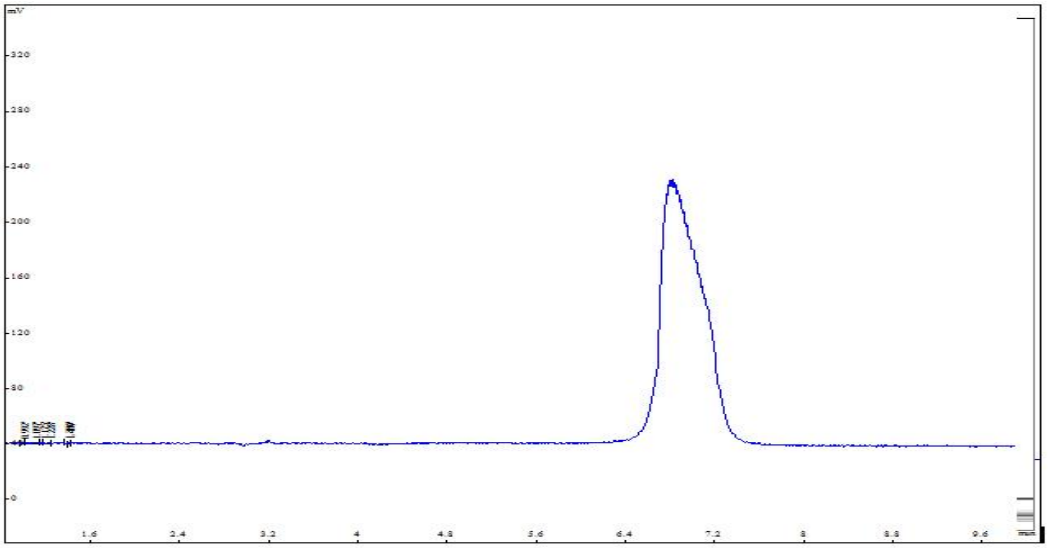

Figure 10: Chromatogram of Flow rate: 1ml/min

3.8 System Suitability

The result of system suitability parameters for Amoxicillin is represented in Table 6 which indicates that all the system suitability parameters pass the criteria.

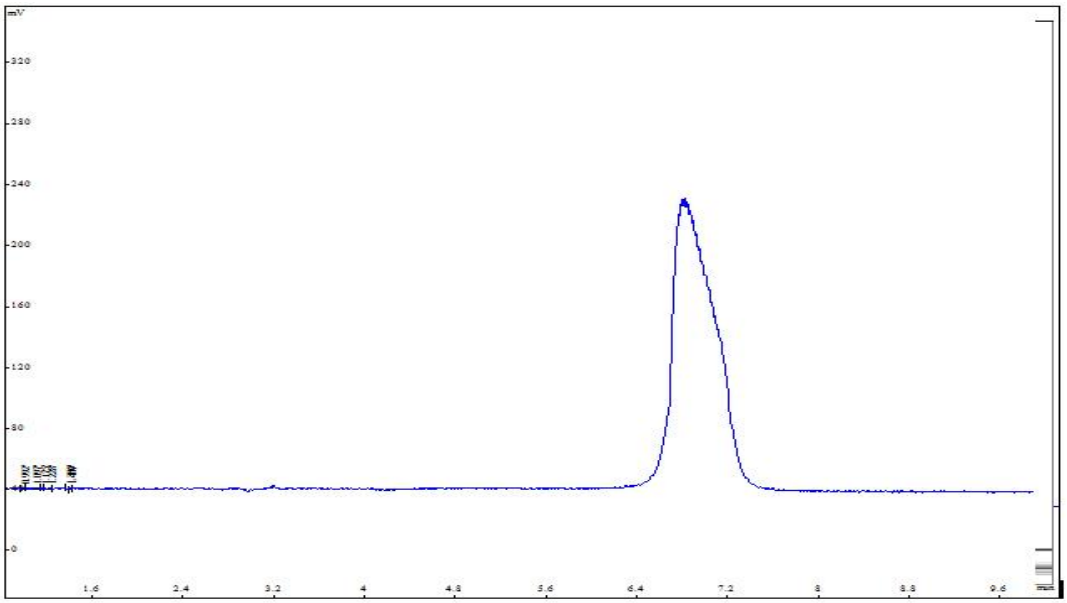

Figure 11: Chromatogram for system suitability

Table 6: Summary of system suitability

Parameters	Amoxicillin
Retention time (min)	6.461
Theoretical plates	1199
HETP	0.014
Asymmetry	1.1

4. Conclusion

A simple reverse phase HPLC method was developed for the determination of Amoxicillin present in pharmaceutical dosage forms. An LC Chromatopak-C18 (250mm×4.6×5micron) column UV Detector in isocratic mode, with mobile phase Acetonitrile: phosphate buffer, pH adjusted to 3 with orthophosphoric acid (22:78 v/v) was used. The flow rate was 1.0ml/min and effluent was monitored at 283ηm. The retention times were 6.4 min for Amoxicillin pharmaceutical dosage form. The linearity range was found to be 10-100µg/ml. The proposed method was also validated. The proposed study describes a new RP-HPLC method for the Estimation of Amoxicillin in pharmaceutical dosage form. The method gave good results within a short analysis time. The developed method was validated in accordance with ICH guidelines and all of the results were within the limits. The HPLC method for the Estimation of Amoxicillin in tablet dosage form was also found to be simple, rapid, precise, accurate and sensitive. A good agreement was observed with HPLC method. The validated HPLC method can be used for the routine analysis of quality control samples. Since the developed method has been applied only to a single brand (Amoxicillin 125) the same method is applicable to different brands.

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