ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 4, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

QUANTITATIVE DETERMINATION OF AMOXICILLIN FROM FORMULATED DOSAGE FORM BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION TECHNIQUE AND A NEW METHOD VALIDATION

RAJU CHANDRA[1]*, DEEPAK KUMAR[2], MITHUN KUMAR[2]

[1]Department of Chemistry, Dolphin Institute of Biomedical and Natural sciences, Dehradun - 248 001, Uttarakhand, India. [2]Department of Pharmaceutical Chemistry, Dolphin Institute of Biomedical and Natural Sciences, Dehradun - 248 001, Uttarakhand, India.
Email: rajakschh@gmail.com

Received: 14 April 2016, Revised and Accepted: 19 April 2016

ABSTRACT

Objective: The main purpose of this study is to develop and method validation for the determination of active constituent amoxicillin from the formulated dosage form according to International Council for Harmonization Guidelines.

Methods: To method validation a reversed phase high-performance liquid chromatography (liquid-liquid chromatography) technique in isocratic mode has applied.

Results: The wavelength of the amoxicillin was identifying 273 nm with ultraviolet-visible spectrophotometer. The retention time (R_t) of amoxicillin was identify 4.66 minutes at the flow rate of mobile phase 1.0 ml/minute. The correlation coefficient (R^2) was calculated 0.99 with correlation range 5-100 µg/ml. The limit of detection and limit of quantification values for amoxicillin were calculated 0.03 and 0.09 µg/ml, respectively. The recoveries for amoxicillin were calculated 96.80, 98.0 and 102.72%.

Conclusions: The developed method is suitable for assured the quality of amoxicillin from formulated dosage form. This method can be successfully used for a routine bases analysis in a quality control laboratory.

Keywords: Reversed phase high-performance liquid chromatography, Amoxicillin, Dosage form.

INTRODUCTION

Amoxicillin is a semisynthetic antibiotic. It is an analog of ampicillin with a broad spectrum of bactericidal activity against many Gram-positive and Gram-negative micro-organisms. The IUPAC (Fig. 1) name of the amoxicillin is (2S, 5R, 6R)-6-[(R)-(-)-2-amino-2-(p-hydroxyphenyl) acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid trihydrate. The presence of a benzyl ring in the side chain extends the antibacterial activity to Gram-negative bacteria [1-5]. The purpose of our work is to develop a validation protocol using reverse phase high-performance liquid chromatography (RP-HPLC) to the qualitative and quantitative study of amoxicillin. Its purpose is to provide some guidance and recommendations on how to consider the various validation characteristics for each analytical procedure in some cases. The overall capabilities of a number of analytical procedures in combination may be investigated to ensure the quality of the drug substance or drug product. The main purpose of validation of an analytical procedure is to demonstrate that the procedure is suitable for its intended purpose. Due to their complex nature, analytical procedures for biological and biotechnological products in the some cases may be approached differently to validate a simple and sensitive assay methodology.

METHODS

Instruments

The following instruments are used for the amoxicillin analysis:

1. HPLC system: Cyberlab™ HPLC
2. Analytical balance: Vibra
3. Ultrasonic bath: Toshcon
4. Syringe 25 µL: Eosge, made in Australia
5. Filter paper 0.45 µm: Pall-Life science
6. Filter assembly: Pall-Corporation.

Chromatographic conditions

The analysis of amoxicillin has been done in the following suitable conditions:

1. Column: Capcell Pak C_{18} (15 mm × 250 mm)
2. Mobile phase: Methanol: Water (35:65)
3. Flow rate: 1 mL/minute
4. Injection volume: 25 µL
5. Wavelength (λ max): 272 nm
6. Retention time of amoxicillin: 4.66 minutes
7. Run time: 7.0 minutes
8. Column temperature: 15-17°C.

Reagents and chemicals

Table 1 shows reagents and chemical used in the method validation.

Preparation of mobile phase

A methanol/water mobile phase was prepared in the ratio of 35:65 volume by volume. The mobile phase was filtered through a 0.45 µm nylon membrane and degassed by sonication.

Preparation of amoxicillin stock solution

A 100 µg/ml stock solution was prepared from the pure form of amoxicillin drug content. Transfer 10 mg amoxicillin in a 100 ml volumetric flask and mixed with methanol after solvation the amoxicillin volumetric flask maintained to the meniscus. The prepared stock solution was filtered through 0.45 µm nylon filter membrane.

Preparation of sample solution

Prepare a sample solution from the 10 tablets of amoxicillin. Amoxicillin tablets were weight and crushed by mortar pestle. The crushed tablets were mixed well, and then an equivalent amount of 10 mg was transferred into a small conical flask and extracted with methanol/water (35:65, v/v) mobile phase. The extract was filtered

into a 100 ml volumetric flask and the volume make up to the meniscus. Achieved aliquots were covered the working concentration range 100 µg/ml.

Preparation of calibration curve

A calibration curve was constructed for evaluation the linearity of the method. The calibration curve was plotted using average peak area and different drug concentration levels (µg/ml). For the calibration curve, the serial dilutions of stock solution of amoxicillin were prepared in the series of 5, 10, 20, 25, 50 and 100 µg/ml. The volume of each dilution 10 ml was maintained with the mobile phase (methanol:water). These different serial dilutions were filtered through a 0.45 µm nylon membrane. From the each dilution, 25 µl was injected into the column in thrice replication. The calibration curve was constructed by plotting average peak area (of three chromatograms) versus concentrations of dilutions.

METHOD VALIDATION

The describe method was validated according to International Council for Harmonisation (ICH) guidelines [6-8] with a respect to linearity, specificity, precision, limit of detection (LOD), and limit of quantification (LOQ).

Linearity

Linearity plays an important role in the method validation. It is provide a well-defined protocol for the application to know the unknown quantity of the active constituent from the any comprise system. The linearity can be calculated using the following equation:

$$y=mx+b$$

In the above the equation "m" and "b" are constants parameters, the constant "m" determines the slope or gradient of that line, and the constant term "b" determines the point at which the line crosses the y-axis, otherwise known as the y-intercept.

Specificity

The specificity was determined with the excipient and active constituent of amoxicillin. An equivalent weight of amoxicillin and excipients was taken and prepared a solution similarly to the sample solution. The prepared solution was determined as per the described method. Specificity can be calculated using the following equation as per ICH guidelines:

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Number of true negatives+number of false positives}}$$

Accuracy

The accuracy of the method was determined by recovery method. The recovery was checked at the three theoretical concentration levels 5, 10 and 25 µg. These three samples were injected in thrice replication and recorded the chromatograms for calculation the recoveries percentage.

System suitability test (SST)

The reproducibility of the system was checked to the measurement of peak area. This was carried out using three replicates of the same concentration of standard and sample, respectively.

LOD and LOQ

According to the ICH guidelines the detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD can be calculated using the following equation as per ICH guidelines.

$$\text{Limit of detection} = 3.3 \times \frac{\text{Standard deviation of the peak area of the drug}}{\text{Slope of the corresponding calibration curve}}$$

The quantization limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantization limit is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products. LOQ can be calculated using the following equation as per ICH guidelines.

$$\text{Limit of quantification} = 10 \times \frac{\text{Standard deviation of the peak area of the drug}}{\text{Slope of the corresponding calbration curve}}$$

To determine the LOD and LOQ, to prepare three replications of low concentrations serial dilutions of mixed standard of amoxicillin from the standard stock solution.

RESULTS AND DISCUSSION

Selection of mobile phase

It is a basic need of liquid chromatography technique. To selection the mobile phase, the various composition of mobile phases were checked with methanol and water (25:75, 35:65, 45:55 and 20:80, 30:70, 40:60, volume by volume) on reverse phase C_{18}-column at wavelength 273 nm. During the selection of mobile phase, the flow rate of mobile was 1.0 ml/minute. The solvent methanol and water in the ratio 35:65 v/v was selected as mobile

Table 1: Reagents and chemical used in method validation

Serial number	Chemicals	Grade	Purity (%)
1	Amoxicillin	Analytical grade	99.99
2	Methanol	HPLC grade	99.70
3	Water	HPLC grade	99.80

HPLC: High performance liquid chromatography

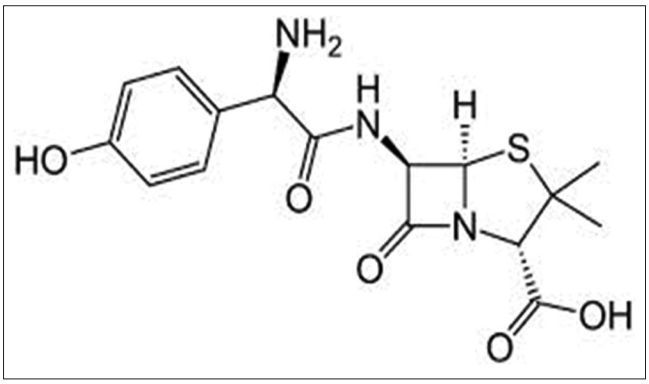

Fig. 1: Amoxicillin

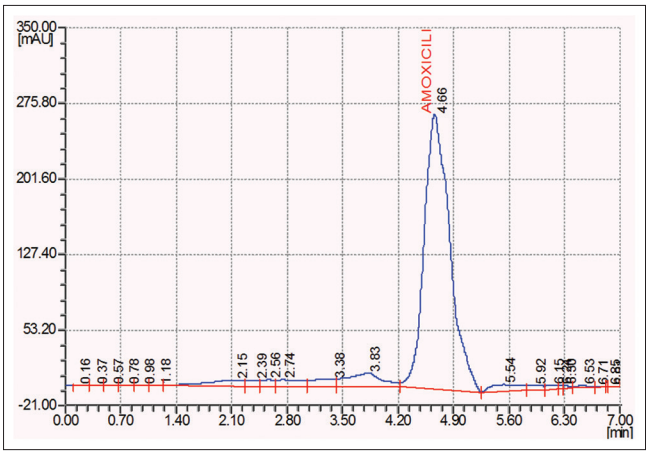

Fig. 2: Reference chromatogram of amoxicilin

phase. This ratio of solvent methanol and water has been given a suitable retention time and peak area of amoxicillin active constituent.

Chromatographic conditions

A well-defined chromatogram (Fig. 2) of amoxicillin was obtained in 4.66 minutes with a flow rate of mobile phase 1.0 ml/minute. The wavelength of amoxicillin was identified 273 nm from the ultraviolet-visible spectrophotometer. The methanol and water solvent was taken in the ratio of 35:65 volume by volume.

SST

To establish the chromatographic conditions were performed SST during the development and optimization of the method. The test was performed by injecting the standard mixture in thrice replication and the various parameters such as retention time (R_t), tailing factor (T_f), resolution factor (R_f), and theoretical plates (T_p) were computed as reported by USP and International conference harmonized guidelines. System suitability parameters were shown in following Table 2. The slandered error in all parameters retention time (R_t), tailing factor (T_f), resolution factor (R_f), and theoretical plates (T_p) were calculated <1.0, which indicate that the conditions of the applied method are better for HPLC analysis [9,10].

Linearity

Detector response for the proposed method determined to be linear over the range of five concentration level prepared and injected, 5-100 µg/ml for amoxicillin. The calibration curve was plotted between concentrations of the respective drug and obtained average peak area. The linearity of the method was evaluated by linear regression analysis. The linear regression equation of proposed method representing slope and intercept for amoxicillin. The statistical data calculated for amoxicillin found to be accurate and was given in Table 3. The correlation coefficient (R^2) was shown the better relationship of the applied method [11].

Table 2: Summary of validation system suitability parameter

Serial number	Parameters	Mean	SEM
1	R_t	4.66 minutes	±0.36
2	T_f	0.82	±0.06
3	R_f	1.423	±0.07
4	T_p	475.34	±0.44

SEM: Standard error (±) of mean, R_t: Retention time, T_f: Tailing factor, R_f: Resolution factor, T_p: Theoretical plates, SEM: Standard error of mean

Table 3: Results for linearity of the method

Serial number	Parameters	RP-HPLC results
1	Correlation range	5-100 µg/ml
2	Regression equation	y=711.6x−183.1
3	R^2	0.999

Table 4: Results for LOD and LOQ

Serial number	Parameters	RP-HPLC results
4	LOD	0.03 µg/ml
5	LOQ	0.09 µg/ml

LOD: Limit of detection, LOQ: Limit of quantification, RP-HPLC: Reversed phase high performance liquid chromatography

Table 5: Recoveries results of the applied method

Serial number	Added in µg	Recovery in µg	Recovery in %
1	5	4.84	96.80
2	10	9.8	98.0
3	25	25.68	102.72

LOD and LOQ

The LOD and LOQ of method were determined by calculating the signal to noise for amoxicillin is 3 and 10, respectively. The LOD and LOQ values for amoxicillin were calculated 0.03 and 0.09 µg/ml as shown in Table 4.

Specificity

The specificity of the method was determined by checking the interference with the components from placebo. As shown in the chromatogram of amoxicillin (Fig. 2), there is no interference between amoxicillin and placebo. Hence, the method is specific for HPLC analysis [12].

Accuracy

The accuracy of the method was computed by the determination of recoveries at three concentrations levels 5, 10 and 25 µg. The amount of amoxicillin recovered and calculated as per the ICH guidelines. The recoveries of amoxicillin were calculated from the obtained data by RP-HPLC technique 96.80% for 5 µg, 98.0% for 10 µg and 102.72% for 25 µg (Table 5). These all results shows that the drug content in dosage form few variable but acceptable because the percentage of drug content in dosage form more than 95% [13].

The above results showed that the experiment successful for the development and method validation for the simultaneous analysis of amoxicillin from formulated tablets.

CONCLUSION

The developed method is suitable for assure the quality of amoxicillin from formulated tablets. This method can be successfully used for a routine bases analysis. The result also shows that the method could find practical application quality control tool for the simultaneous estimation of amoxicillin from their dosage form in a quality control laboratory.

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