Original article

Development and Validation of a Simple HPLC-UV Method for Determination of Amoxicillin trihydrate in Bulk Drug and Pharmaceutical Dosage Forms

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Abstract

Background

Many analytical methods for testing amoxicillin trihydrate from different monographs such as United States and British pharmacopoeia, use acetonitrile HPLC grade as an organic solvent in the mobile phase; however, this solvent is expensive and not environmentally friendly

Objectives

Developing and validating a simple, affordable, accurate, precise and environmentally friendly HPLC-UV method, for determining amoxicillin in formulations by using methanol HPLC grade as an organic solvent in the mobile phase

Methods

An HPLC system was used for developing and validation of laboratory test method which is less expensive and uses environmentally-friendly mixture of mobile phase solutions. Specificity, linearity, precision, repeatability, and accuracy were studied.

Results

The retention time (RT) for amoxicillin was 3.53 ± 0.020 min, and no interfering peaks were recorded with the blank, standard and sample at RT, ensuring specificity. Calibration curve of 20 to 160µg/ml was used. The correlation coefficient (r^2) =0.9998, which indicates that the method has the linearity to this range of 20 to 160µg/ml. Intra- and between days' repeatability were assessed by injecting solutions three times a day and within three days. The %RSD of 0.3% and 0.7% within and between days respectively, were recorded. The %RSD was ≤ 2%, which indicates a precision of the method. An average percent recovery of 100.5±3.6% was recorded.

Conclusion

An environmentally-friendly, simple, affordable, selective, specific, rapid, sensitive, repeatable, with precision and accurate HPLC-UV method, has been developed and validated for the estimation of amoxicillin trihydrate in pharmaceutical dosage forms

and can be adopted for the purpose of quality control.
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Introduction

Amoxicillin is a broad-spectrum beta-lactam antibiotic against gram-positive and gram-negative bacteria. Amoxicillin is a crystalline powder with a white or almost white appearance. Amoxicillin is very slightly soluble in ethanol (96%) and soluble in water, practically insoluble in fatty oils. The chemical structure of amoxicillin is detailed on figure1.[1]

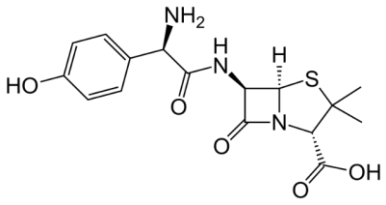

Figure 1. **Amoxicillin trihydrate Chemical Structure**

It is usually the drug of choice within the class because it is better absorbed following oral administration. Amoxicillin acts by inhibiting the synthesis of the bacterial cell wall. Literature survey reveals that only a few spectrophotometric methods and few analytical methods have been reported for the quantitative estimation of amoxicillin in bulk drug and pharmaceutical formulation. Hence an attempt has been made to develop new HPLC methods for its estimation in bulk and pharmaceutical formulation with good precision, accuracy, linearity and reproducibility.[1]

Falsified medical products may contain no active ingredient, the wrong active ingredient or the wrong amount of the correct active ingredient.[2] For amoxicillin, many analytical methods from different monographs such as United State and British pharmacopoeia, use acetonitrile HPLC grade as an organic solvent in the mobile phase; however, this solvent is expensive and not environment friendly.[3] The current study was undertaken to develop and validate a simple, less expensive, an environmentally friendly, accurate, and precise HPLC-UV based analytical method for testing amoxicillin in pharmaceutical formulations. The method was assessed for its specificity, range, linearity, precision and accuracy.[6]

Methods

Chemicals and reagents

All amoxicillin samples (capsules and tablets) used in the assay of active ingredient were purchased locally in different retail pharmacies in Rwanda. The following reagents of analytical grade were purchased from Chemlab N.V: Sodium Hydroxide, distilled water, monobasic potassium phosphate

(KH_2PO_4) and methanol HPLC grade. USP amoxicillin trihydrate reference standard with 86.6% purity was used.

Instrumentation

The HPLC system consisted of a quaternary low-pressure pump CE 4104 with Auto Quest autosampler 4800-100, coupled with solvent degassers CE 4020 / CE 4040 and an oven CE 4600. A Hypersil R-P, C-18 column 250 mm x 4.6 mm, 5µm particle was also used. Detection was recorded using a UV-Visible Detector CE 4201. The system was equipped with a power stream software package for chromatographs. A fast, clean sonicator was used for degassing and homogenising the mobile phases.

Analytical conditions

After evaluation of the different ratio of selected solvents, a mobile phase composed of a monobasic potassium phosphate (KH_2PO_4) buffer with methanol where the ratio of 95:05 V/V and a flow rate of 1.5 ml/min were validated as the most appropriate for elution of amoxicillin trihydrate. The wavelength was set at 230 nm, the total run time was 5 min and was enough for the test. To have a chromatogram with good resolution, linearity and accuracy, the injection volume of 20µl was fixed. At initial stage, before starting injection of all solutions, the column was equilibrated for 60 min with KH_2PO_4 buffer and methanol as mobile phase for the ratio mentioned (95:05 V/V). All analyses were conducted at 25^0 C as the column temperature which was equivalent also to the room temperature.

Preparation of buffer solution

The monobasic potassium phosphate was prepared by dissolving 13.6 g of KH_2PO_4 in 2000ml of distilled water and adjusting the pH at 5.0 ± 0.1 by using an aqueous solution of KOH with the concentration of 45% (w/v).[4]

Preparation of reference standard solutions

The amoxicillin standard stock solution was prepared by weighing 100mg of USP amoxicillin trihydrate reference standard in100ml flask that contains 70 ml of the mobile phase solutions, the obtained solution was sonicated for 20 min then the obtained solution was filtered using a membrane filter and top up the solution to the marks with the mobile phase solutions to obtain a solution of 1000µg/ml of amoxicillin trihydrate. Different volumes of the standard stock solution were put in 10ml flasks and then diluted with the mobile phase to volume to constitute a calibration curve range between 20-160µg/ml.

Preparation of the sample solution

To prepare the sample solution from capsule samples, twenty capsules each containing 500 mg of amoxicillin trihydrate were emptied, and the content was accurately weighed. An aliquot equivalent to 100mg of amoxicillin was taken and poured in a 100 ml flask containing 70 ml of the mobile phase solutions. The sonication of the solution was done for 20 min and topped up to the mark with the mobile phase solutions. After this, the obtained solution was filtered using a membrane filter, then dilution with the mobile phase solutions in order to get the solution 1000µg/ml of amoxicillin.

For tablet samples, five tablets were added in glass blender jar containing a measured volume of the mobile phase sufficient to give a solution of 1 mg/ml. Samples were blended for 4 ± 1 minutes, allowed to stand for 5 min, and then centrifugation of the mixture was implemented. A volume of the mobile phase equivalent to 3/4 of the capacity of the flask was poured, and sonication of 5 min was done, dilution with the mobile phase to volume. The solution was stirred for about 30 minutes then a portion of the sample was transferred to a centrifuge, and a portion of the clear supernatant was filtered through a 1µm pores diameter filter. The filtrate was used as the assay preparation within 6 hours from its preparation.[4]

Method validation

The validation of this method was implemented according to the International Conference on Harmonization guidelines, for specificity /selectivity, precision, linearity, accuracy, repeatability.[5–7]

Specificity

This method was evaluated with its specificity by analysing chromatograms using the blank solution composed of the mobile phase, by analysing the standard solution of amoxicillin and finally by evaluating chromatograms of the sample solution.There should be no interference with the active pharmaceutical ingredient peak at the expected retention time.[5–7]

Linearity and range

Linearity was assessed by injection of amoxicillin reference standard solution in a range consisting of 20, 40, 80, 100 and 160µg/ml. The slope, the correlation coefficient (R^2) and the intercept were calculated.

Precision

The precision was assessed by evaluating within and between days' repeatability. The average % RSD was calculated upon injection of the calibration curve concentrations three times a day and on different days, by the same

analyst under the same conditions.

Within day's repeatability: intra-day precision of the validated method was done by evaluating the solution of (20µg/ml) as low concentration, medium (40, 80 and 100µg/ml) and high (160µg/ml) of the standard solution. These solutions were injected in triplicate within a day. The precision was calculated as % RSD at each concentration level.

Between day's repeatability: This precision on different days of the method was evaluated by using an injection of standard solutions in three replicates on the first day and on three consecutive days. Hence the precision was evaluated using percentage relative standard deviation of measured solutions.

The % RSD for the assay results should not be more than 2%, and % RSD for injected calibration levels should not be more than 2%.[5–7]

Accuracy

We were using the standard addition method, by spiking sample solution using 80, 100 and 120% of the reference standard solution. The accuracy was calculated as per cent recovery. The mean percent recovery of amoxicillin trihydrate at each level should not be less than (NLT) 90.0% and not more than(NMT) 105.0%.[5–7]

Results

Method Specificity

As shown in Figures 2, 3 and 4, there was no interfering peak in the region where amoxicillin peak was recorded. Additionally, the retention time of the sample solution corresponds to that of the standard solution at 3.5 minutes.

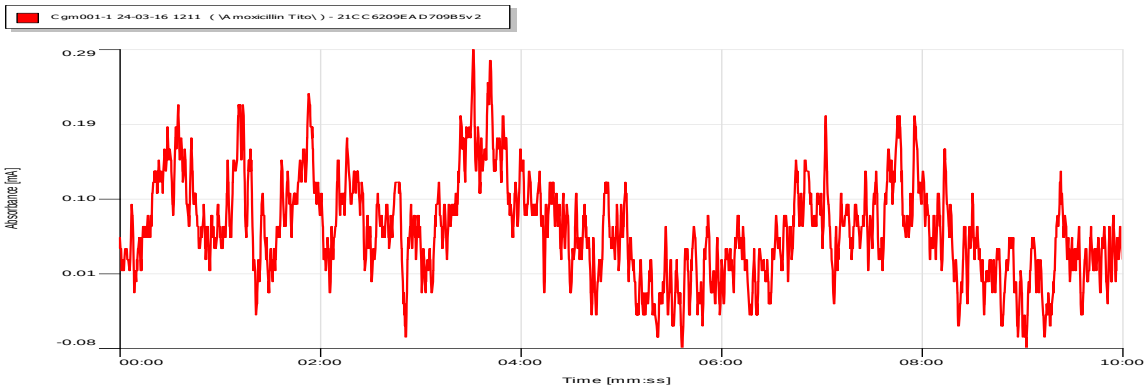

Figure 2. Chromatogram showing noises after injections of blank solution

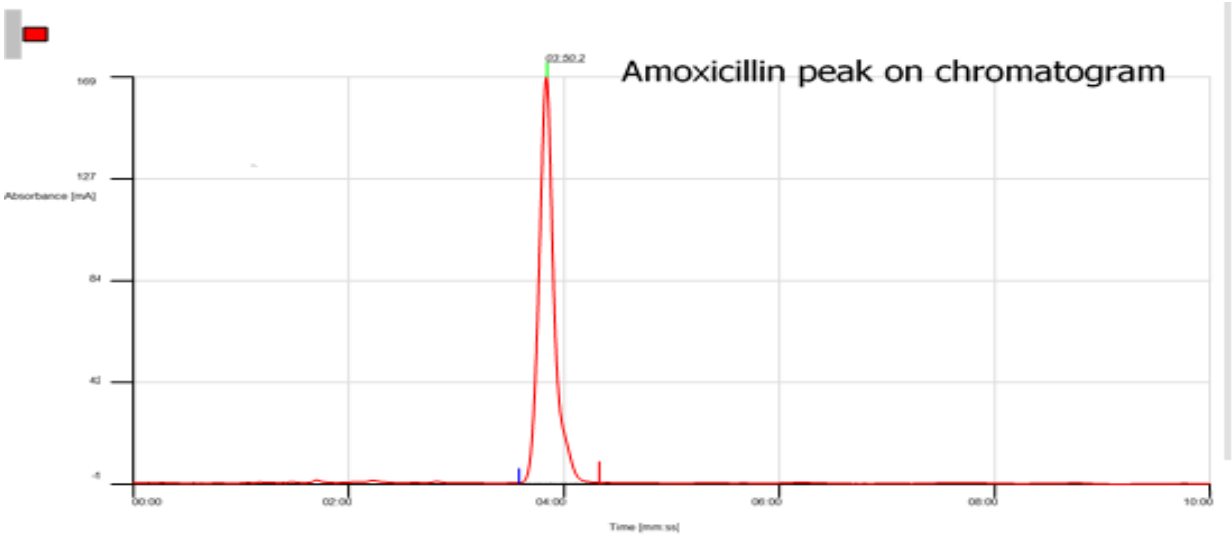

Figure 3. Chromatogram showing Amoxicillin peak after injection of Reference Standard Solution

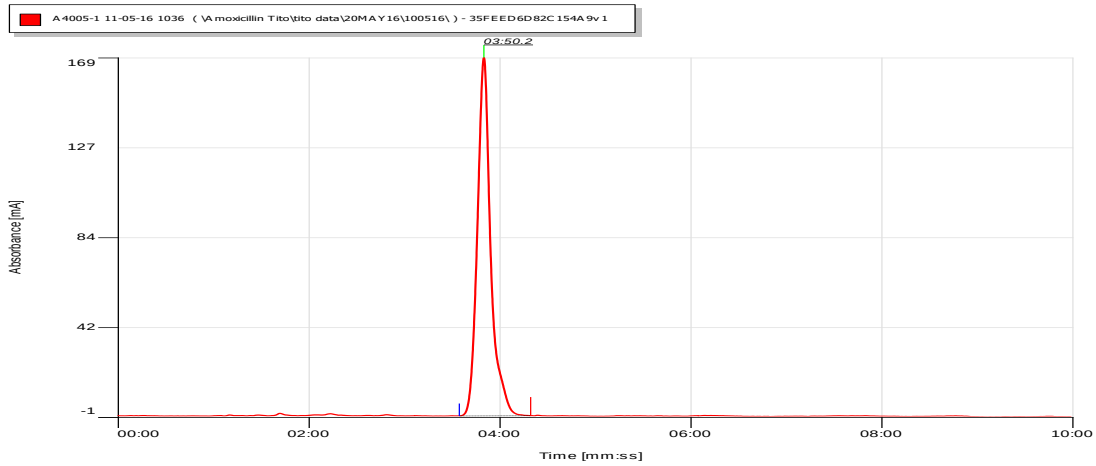

Figure 4. Chromatogram showing Amoxicillin peak after injection Sample Solution

Method linearity

The linearity of this method was assessed by injecting a calibration range consisting of 20, 40, 80, 100 and 160µg/ml in 3 replicates a day. Within 3 different days, peak areas were recorded and a regression equation Y = 1.6517X+5.8667, where Y and X represent responses in terms of peak areas and concentrations, respectively. A correlation coefficient (R^2) equal to 0.9998 was obtained, which indicates an excellent linear relationship between detector response and amoxicillin concentration in the injected solutions. The calibration curve is showing the linearity of the methods detailed in figure 5.

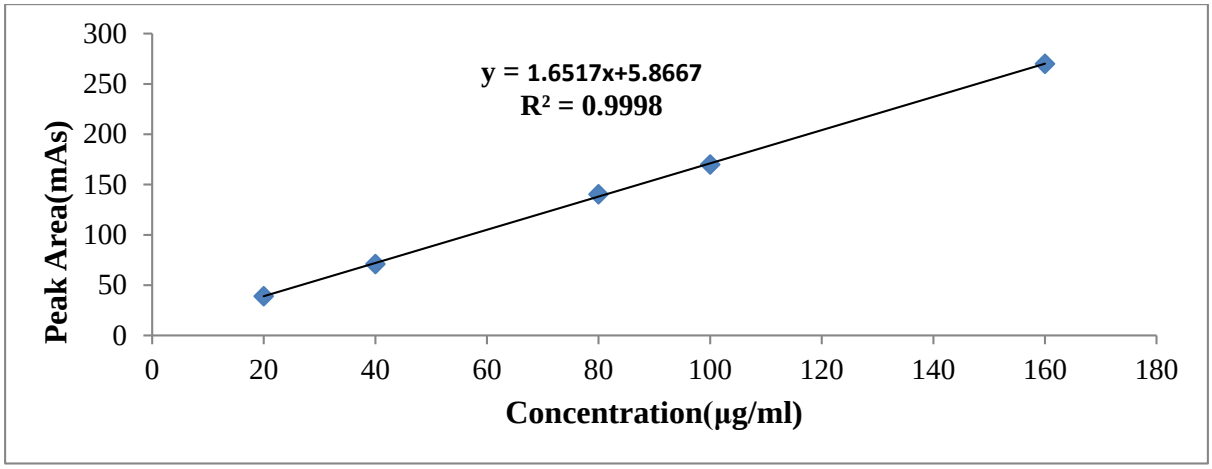

Figure 5. Calibration curve showing the linearity of the method

Precision

The precision within the same day and interday's results are summarized in Table 1 and Table 2. This method was precise as the %RSD was less than 2% for both retention time and peak area within and between days.

Table 1. The precision of the method within a day (Intra-day Repeatability)

Concentrations µg/ml	Retention time(RT)	%RSD (RT)	Peak area/Response	%RSD Response
20	3.5 (±0.005)	0.2	39.7 (±0.80)	0.6
40	3.5 (±0.006)	0.2	71.2 (±0.91)	1.3
80	3.5 (±0.006)	0.2	140.7 (±2.30)	1.6
100	3.5 (±0.060)	0.2	165.2 (±2.80)	1.7
160	3.5 (±0.010)	0.2	269.1 (±0.84)	0.3

Table 2. Between days or (Inter-day repeatability)

Concentration (µg/ml)	Retention time (±SD)	%RSD	Peak area (±SD)	%RSD (Peak Response)
20	3.5 (±0.010)	0.4	38.5 (±1.90)	1.8
40	3.5 (±0.020)	0.6	70.7 (±1.93)	2.8
80	3.5 (±0.020)	0.8	140.4 (±1.94)	1.4
100	3.5 (±0.030)	0.9	170.0 (±1.63)	0.4
160	3.5 (±0.030)	0.7	269.5 (±1.25)	0.7

Accuracy

Method accuracy results are presented in Table 3 as a percentage of drug recovered upon treating samples according to our method. The recovery percentage ranged between 97.5 (±0.6) and 105.5 (±0.1) % with a mean percent recovery of 100.5 (±3.6%); this indicates a good accuracy of our method.[5–7]

Table 3. Percent recovery as an indication of the method accuracy

Concentration Sample (µg/ml)	Spiked level (%)	Concentration after spiking (µg/ml)	% Recovery ± SD
1088.8	80	1167.6	98.5 (± 0.3)
1030.0	100	1135.5	105.5 (± 0.1)
1030.0	120	1147.0	97.5 (± 0.6)
Mean Recovery			100.5 ± 3.6

The Table below summarizes validation data obtained with their acceptance criteria.[4,8]

Table 4. The summary report of validation data

Essay Validation Sheet	Value	Acceptance Criteria
Accuracy=Mean±Sd(Drug Product)	100.5 ± 3.6	98-102% at 95% CI of the mean
Slope	1.6517	Not Applicale
Intercept	5.8667	Not Applicale
Linearity Range	**20-160ppm**	**Not Applicale**
Specificity	Method is Specific and selective	Absence of interference
Precision		
a) Repeatability Intraday(%RSD N=6)	0.2%	2.0%
b) Repeatability Inter-Day(%RSD N=6)	0.4%	2.0%
Correlation Coefficient(R) of linearity range	0.9998	0.995
Retention Time(Min)	3.5 ± 0.020	Not Applicale
System Suitability Testing(SST)		
Tailing Factor	0.36±0.40	Not More Than 2.5
Capacity Factor(K')	0.85±0.03	1.2 -2.8
Theoretical Plate (N)	6757±43.2	Not Less Than 2000

Discussion

A number of methods are available for Amoxicillin trihydrate determination.[1,4] but many of them are used for certain specific purposes and no one can be generalized for Amoxicillin trihydrate determination in its different forms or different sample matrix. Most of the methods available are highly expensive either due to the use of expensive solvents in mobile phase preparation, instruments or such chemical reagents that are not easily accessible. In addition, most of those solvents are not environmentally friendly. Therefore, there was a great need to develop

and validate a method which is not only economical but also environmentally friendly. The Validation was done following the International Conference on harmonization[6] and other references.[10] Optimization was done using mobile phase solutions consisting of different chemical composition where methanol and buffer(economical and environmentally friendly) replaced acetonitrile and buffer which are expensive and not environmentally friendly.[3] At initial stage, before starting injection of all solutions, the column was equilibrated for 60 min. Chromatographic conditions were C-18 column 250 mm x 4.6 mm, 5µm particle maintained at ambient temperature 25°C, with mobile phase composed by monobasic potassium phosphate (KH_2PO_4) and methanol in the ratio of 95:05 V/V (pH at 5.0 ± 0.1) .The flow rate of 1.5 ml/min and the injection volume of 20µl were used. The run time for each injection was set to 5 min, injections were done in three replicates and the detection was recorded using a UV-Visible detector at a wavelength of 230 nm.

The peak of the Amoxicillin trihydrate was found at 3.5 ± 0.020 min, which is an advantage for our method compared with other researches where retention time was 6.3 min,[8] which indicated that their method was expensive in terms of mobile phase consumption and other costs related to electricity, equipment and time. The specificity and selectivity were evaluated by checking the interferences, where no interfering peak in the region where amoxicillin trihydrate peak was recorded. Evaluation of linearity was done at the range of 20, 40, 80, 100 and 160µg/ml, where a correlation coefficient (R^2) equal to 0.9998 was found, which indicated an excellent linear relationship between peak areas and amoxicillin trihydrate concentration in the injected solutions. The precision of the method was evaluated for intra-day and inter-day repeatability where the percent RSD of 0.3% and 0.7% within and between days respectively were obtained, which indicated a precision of the method. The accuracy was shown with good percent recovery, with a mean percent recovery of 100.5 (±3.6%); this indicated a good accuracy of our method. All evaluated parameters were in accordance with.[1,9,10]The method was found to be sensitive, specific, precise and accurate , economical and environmentally friendly as.[3,8]

Conclusion and Recommendation

An environmentally friendly, simple, less expensive, selective/specific, linear, precise and accurate HPLC-UV method for determination of amoxicillin in bulk and in pharmaceutical dosage forms has been developed and validated. The method which instead of acetonitrile, it uses methanol which is more affordable

and environmentally friendly. This method is also associated with a shorter run time, which makes it fast and economical. The study therefore recommends that the method is suitable for use in routine quality control of amoxicillin trihydrate in bulk drug or pharmaceutical dosage forms.

Conflict of interests

The authors declare that there is no conflict of interest related to this manuscript.

Acknowledgements

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Authors' contributions

TU contributed to all aspects of the work and was responsible for the study conception, design, and data analysis as the principal Investigator, TB contributed in data discussion, in reviewing comments, contributed to the drafting, reviewed the manuscript and provided their intellectual inputs.

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