The Validation and Development of Analytical Technique for the Fast and Economical Evaluation of Amoxicillin in Solid Dosage form Through UV/Visible Spectroscopy

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ABSTRACT

Objective: The key objective of the study is to explore the sensitive, rapid, simple, accurate and economic UV/Vis spectrophotometric method to determine the amount of Amoxicillin trihydrate (AMTR) in bulk pharmaceuticals and in various formulations including dry powder (syrup), tablets and capsules by employing Copper sulphate buffer solution.

Methodology: The current spectroscopic analysis performed with 12.5 µg/ml concentration of AMTR at 320 nm follows the Beer’s Lambert Law. The linearity range (10-15µg/ml) and regression data presented a significant correlation coefficient (r² =0.999). The appropriate level of accuracy, precision, linearity, and mean percentage recovery of AMTR were found adequate relating to the % age error and standard deviations.

Results: Amoxicillin trihydrate and its known strengths were scanned and analyzed by our validated method such as; 10.0 µg/ml Amoxicillin (80%), 11.25 µg/ml Amoxicillin (90%), 12.5 µg/ml Amoxicillin (100%), 13.75 µg/ml Amoxicillin (110%) and 15.0 µg/ml Amoxicillin (120%).

Conclusions: As the recommended protocol of amoxicillin determination (spectrophotometry) is accurate, precise and statistically evaluated, therefore it could readily be employed for qualitative purposes of either for the raw material and pharmaceutical preparations.

Keywords: UV/Vis Spectroscopy, validation, amoxicillin trihydrate, Pharmaceutical, formulations.

INTRODUCTION

Amoxicillin is associated to β lactam antibiotics and effectively respond to Gram +ve and Gram –ve strains of bacteria. The infectious diseases in animals, humans and plants are effectively control with Amoxicillin [1]. It is hydrolyzed by the staphylococcal penicillinase [2]. Amoxicillin is found in three hydrated forms such as; monohydrate, dehydrate and trihydrate, while, the trihydrated form is the supreme stable [3]. Chemical name of Amoxicillin trihydrate (AMTR) 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 [4]. Its molecular weight and molecular formula are 419.4 and
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C$_{16}$H$_{18}$N$_3$O$_5$S.3H$_2$O respectively as well as structural-formula [5] is given in Figure 1.

Figure 1. Structure of Amoxicillin-trihydrate.

AMTR is a white crystalline-powder. It is slightly-soluble in water and ethanol (96 %), practically-insoluble in oils of fatty acid. In dilute acids and dilute alkali hydroxides it dissolves in between 15°C - 25°C. It melts at about 183°C with decomposition characteristics. Drug is officially listed in British Pharmacopoeia, United States Pharmacopoeia and Martindale [6]. Because of therapeutic significance of Amoxicillin, biological and quality control labs required rapid, sensitive and reliable method to monitor it clinically [1]. Therefore, numerous analytical methods have been reported for the estimation of Amoxicillin in bulk and pharmaceutical dosage forms like spectrophotometric [6,7], HPLC [9,10], derivative-spectrometric and titrimetric techniques [3,11]. The current study is focused on the validation of simple, precise and economical UV/Vis spectrophotometer method to evaluate the Amoxicillin trihydrate in pure and formulations.

**EXPERIMENTAL**

Preparation of Copper Sulphate buffer solution

Di-potassium hydrogen phosphate was weighed (15.22g) and dissolved in 900ml distilled water. Citric acid monohydrate (9.744g) and copper sulphate pentahydrate (0.117g) were added to the solution and sonicated. The final 1000 mL volume was made with distilled-water. [12].

Reagents and Solvents

The standard (reference) Amoxicillin trihydrate analytical grade (99.88%) and the dry powder (oral suspension) containing excipients were acquired from Munawar Pharma Lahore and stored at room temperature. All other chemicals were of analytical grades and water was freshly dual distilled.

Instrumentation and Conditions

All analytical works were performed with double-beam UV/Vis spectrophotometer (Shimadzu UV-1700) fitted out with dual lamp as energy source and UV-Visible detector. Analytical balance (Sartorius model TE214S) was used for weighing solid chemicals.

Development of UV-Vis Spectrophotometer Method

**Standard Solution preparation for Linearity**

Solution of Amoxicillin trihydrate equivalent to 62.5 mg was prepared by dissolving 72 mg of standard Amoxicillin in 180 ml distilled water and diluted to 200 ml with water. Filtered after proper shaking, the 1st portion of filtrate was discarded and remaining filtrate was stored as stock solution. Then 02 ml of stock solution was diluted to 50 ml with Copper sulphate buffer. We heated (60 - 70ºC) mixture in water-bath for 30 minutes and cooled in ice-bath to 25°C. The strength of resulting solution was 12.5µg/ml, it scanned at 250 - 350 nm with UV/Visible spectrophotometer using copper sulphate buffer as a blank. The $\lambda_{\text{max}}$ we perceived was 320 nm [13].

We perceived linearity and precision of Amoxicillin in ranges of 10 - 15 µg/mL after preparation of 05 dilutions (80%, 90%, 100%, 110% and 120%) from the stock solution of reference standard [14].

Preparation of Standard/Sample Solution

The standard solution of sample Amoxcillin powder was prepared by dissolving 72 mg of powder equivalent to 62.5 mg of ‘Amoxicillin’ in 200 ml distilled water. After filtration, we discarded first portion of filtrate and remaining amount was collected as stock solution. 02 ml of stock solution was mixed with 48 ml copper sulphate buffer. Then mixture was heated in water-bath for 30 min and cooled to 25°C with ice-bath. The strength of resulting solution was 12.5 µg/mL [15].

Preparation of Sample solution of some Commercial Brands

CLOCIL, AMOXICAP and LOMOXY Dry powder for Oral suspension 125mg/5ml

The sample solution of dry powder of samples we made by liquefying appropriate amount of drugs equivalent to 62.5 mg of Amoxicillin in 200 ml distilled
water. Then filtered and required strength of commercially available drugs was prepared with buffer solution in similar fashion as done with standards i.e., 12.5 μg/mL [15]. The optimized situations of the proposed protocol and regression calibration were accessible in Table 1 and Table 2.

**Table 1. Optimized Conditions for the Proposed Method.**

| S/N | Parameters          | Optimized condition                                      |
|-----|---------------------|----------------------------------------------------------|
| 1   | Quartz Cell         | Quartz Cell 1cm                                          |
| 2   | Solvent (s)         | Double distilled water; Copper sulphate buffer solution   |
| 3   | Concentration used  | 12.5 μg/ml (0.00125%)                                     |
| 4   | Cell volume         | 1ml                                                      |
| 5   | Detection wavelength (Å) | 320 nm  UV/Vis Spectrophotometer |
| 6   | Temperature         | ambient                                                  |

**Table 2. Regression Study for the Calibration.**

| Sr. No. | Considerations          | Optimized condition |
|---------|-------------------------|---------------------|
| 1       | Linearity-range (µg/mL)| 10-15µg/ml          |
| 2       | Correlation coefficient (r²) | 0.999              |

**RESULTS**

**Instrument Precision**

**Table 3. Instrument Precision**

| Sr. No. | Absorption of Standards (Amoxicillin trihydrate) | Statistical Calculations                                      |
|---------|--------------------------------------------------|---------------------------------------------------------------|
| 1.      | 0.364                                            | Mean = \(0.364 + 0.366 + 0.363 + 0.364 + 0.365\) = 0.364       |
| 2.      | 0.366                                            | 5                                                             |
| 3.      | 0.363                                            | Std Deviation = 0.19                                          |
| 4.      | 0.364                                            | RSD = \(\pm 0.198\)%                                         |
| 5.      | 0.365                                            |                                                               |

The precision was determined using Amoxicillin standards which established mean value 0.364, standard deviation 0.19 and RDS value 0.198% as given in Table 3. The scan of standard Amoxicillin and its target set value is given in Figures 2A and 2B.

**Validation protocol**

Establishing documented evidence that a system does what it purports to do. Analytical validation essentially means the examination of six basic attributes such as; Linearity and range, Precision, Accuracy, Robustness, Sensitivity and Specificity.
Linearity

Results of a trial proportional to the concentrations of analytes (sample) is called linearity. The linear relationship amid the concentration and absorbance at wavelength 320 nm was confirmed by plotting the graph between these two parameters in the range of 80, 90, 100, 110 and 120% of the target value (12.5µg/ml Amoxicillin) [17].

The calibration graph is shown below, indicated the linear association observed between concentration versus absorbance of solution measured at ʎ 320 nm. The correlation coefficient of calibration data was calculated to 0.999.

This indicates that the test procedure obeys the Beer’s law.

Standard Preparation for Linearity

The stock solution (12.5 µg/ml) of standard Amoxicillin was prepared similar to prepared earlier. For the preparation of series of solution, pipetted out 1.6ml, 1.8ml, 2.0ml, 2.2ml and 2.4ml respectively from organized stock solution in secluded 50ml measuring flasks, then added Copper sulphate (buffer) solution to each flask and made up-to the volume (50 ml). The resulting dilutions related to standard stock were made as 80%, 90%, 100%, 110% and 120% of drug respectively [18]. The scan of standards Amoxicillin solutions and their % age of target values were presented in Figures 2B, 2C, 2D, 2G and 2F.

Figure 2. A: Scan of standard (12.5 µg/ml Amoxicillin solution), B: Scan of standard (12.5 µg/ml Amoxicillin solution) target set value, C: Scan of standard (10.0 µg/ml Amoxicillin) 80% of target set value, D: Scan of standard (11.25 µg/ml Amoxicillin) 90% of target set value, E: Scan of standard (12.5 µg/ml Amoxicillin) 100% of target set value, F: Scan of standard (13.75 µg/ml Amoxicillin) 110% of target set value, G: Scan of standard...
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(15.0 µg/ml Amoxicillin) 120% of target set value, H: Linear Regression Line of Amoxicillin trihydrate (Concentration vs absorbance).

Acceptance Criteria
Correlation Coefficient (r²) = -1 to +1

Table 4. Amoxicillin Trihydrate Concentration and Absorbance Relationship in Linear Regression.

| Sr.# | Volume of the Solution (taken) | %age Conc. of Target Value | Concentration (µg/ml) | Absorbance of the test solution. |
|------|-------------------------------|----------------------------|-----------------------|---------------------------------|
| 1    | 1.6ml                         | 80%                        | 10.0 µg /ml           | 0.290                           |
| 2    | 1.8ml                         | 90%                        | 11.25 µg/ml           | 0.326                           |
| 3    | 2.0ml                         | 100%                       | 12.5 µg/ml            | 0.362                           |
| 4    | 2.2ml                         | 110%                       | 13.75 µg/ml           | 0.398                           |
| 5    | 2.4ml                         | 120%                       | 15.0 µg/ml            | 0.434                           |

It is cleared from the calibration chart that linear relationship existed and followed the Beer’s Law between absorbance and the concentration of analyte presented in Table 4 and illustrated in Figure 2H.

Test sample from Local Market – CLOCIL Scanned

The scanning of different concentrations of commercially available dry powder of Amoxicillin (Clocil suspension) was done and satisfactory results were attained which shown in Figures 3A, 3B, 3C, 3D and 3E.

Figure 3. A: Scan of Test Sample (10.0 µg/ml Amoxicillin solution), B: Scan of Test Sample (11.25 µg/ml Amoxicillin solution), C: Scan of Test Sample (12.5 µg/ml Amoxicillin solution), D: Scan of Test Sample (13.75 µg/ml Amoxicillin solution), E: Scan of Test Sample (15.0 µg/ml Amoxicillin solution).
Acceptance Criteria
The interval between upper and lower concentration of analyte in analytical procedure has appropriate level of accuracy, precision, and linearity [19]. The % age relative SD of triplicates obtained at each level (80 %, 100 %, 120 %). NMT 2.0 % ± RSD.

Procedure
The range 80 % to 120 % is when the accuracy, precision and linearity 80% and 120% are established, % age RSD of triplicates at each level (80 %, 100 %, 120 %) is < 2.0 % and average of triplicate values obtained at each level (80 %, 100 %, 120 %).
Hence, the range from 80% to 120% is established.

Precision
Precision is the degree of how close the individual test results to the replicates of same analyte sample under identical conditions [20].
Precision is commonly expressed as Relative Standard-Deviation (Coefficient of variation).
Precision is performed through;

a) Repeatability
b) Reproducibility.

Sample Stock Solution (12.5 µg/ml)
Weighed accurately 1.0417 g of sample powder equivalent to 62.5 mg of Amoxicillin in 200 ml measuring-flask and dissolved in 180 mL of double distilled water then diluted to 200 ml by means of same solvent and shaken well to mix. Filtered and discarded first portion of filtrate while remaining part was collected as stock solution. Diluted 2.0 ml stock-solution up-to 50 ml with Copper sulphate buffer. Heated in waterbath for half an hour (70˚C) and then cooled to room temperature.

Repeatability
Assay was performed on three separate samples in 02 replicates.
Concentration of Reference: 12.5 µg/ml
Wavelength: 320nm
Absorbance of the Reference: 0.363

Table 5. Results of Repeatability.

| Samples | Concentration of analyte (µg/ml) | Absorbance | %age Results | Variation from Theoretical Results |
|---------|---------------------------------|------------|--------------|-----------------------------------|
| I       | 12.5                            | 0.364      | 100.27%      | 0.27%                             |
|         | 12.5                            | 0.362      | 99.72%       | 0.28%                             |
|         | 12.5                            | 0.365      | 100.55%      | 0.55%                             |
| II      | 12.5                            | 0.361      | 99.44%       | 0.66%                             |
|         | 12.5                            | 0.366      | 100.82%      | 0.82%                             |
|         | 12.5                            | 0.364      | 100.27%      | 0.27%                             |

Average of 6 Results: 100.17%
Standard Deviation: 0.469
%RSD: 0.47%
RSD of 6 Results is: 100.17 ± 0.47% (Limit: NMT 2%). The results in the tabulation form presented in Table 5.

Reproducibility
The utilization of analytical procedure by different analyst within the same laboratory is referred reproducibility. Assay is performed on three separate samples in each case. The significant reproducibility facts revealed in Table 6.

a) Reproducibility
Concentration of Reference: 12.5 µg/ml
Wavelength: 320 nm
Absorbance of the Reference: 0.363
Table 6. The Response of Reproducibility.

| Samples | Concentration of analyte (µg/ml) | Absorbance | %age Results | Variation from Theoretical Results |
|---------|----------------------------------|------------|--------------|-----------------------------------|
| 1       | 12.5                             | 0.362      | 99.72%       | 0.28%                             |
| 2       | 12.5                             | 0.364      | 100.27%      | 0.27%                             |
| 3       | 12.5                             | 0.365      | 100.55%      | 0.55%                             |

Average of 3 Results: 100.18%
Standard Deviation: 0.344
% RSD: 0.34%
% RSD of 3 Results is: 100.18 ± 0.34% (Limit: NMT 2%)

Accuracy

It is measure of faithfulness of the analytical technique and its result value is close to the true value. The method is said to be accurate if on the average the method provides the true answer. Accuracy implies there is no inherent systematic error or bias - (bias is the deviation from true value). By using the reference standard the accuracy of a method can be measured and extent of bias that may exist can also be determined.

Actually accuracy of an analytical process is the closeness of test results attained specified protocol to the factual value. It is evaluated by the addition of known amounts of an analyte to the Placebo (sample) with different quantities (3 x 3 replicates) and is designed as the %age recovery of known added amount of analyte to the Placebo.

Placebo

For this purpose three samples of about 200 g were organized in the laboratory, as per to the manufacturing route for the product, and quantities equivalent to 80%, 100% and 120% of the labeled amount of analyte were added to each placebo. Test solutions were prepared of each concentration (i.e. 80%, 100% and 120%) assayed in duplicate and tested according to the test procedure of the product and results are to be tabulated in Table 7.

Concentrations of standards: 10 µg/mL, 12.5 µg/mL, 15 µg/mL
Wavelength: 320 nm
Absorbance of the reference standard 80% = 0.290
Absorbance of the reference standard 100% = 0.363
Absorbance of the reference standard 120%: 0.436

Table 7. The Variation of Theoretical and Claimed Placebo Concentration.

| Contents of Active added in Placebo (% of Label claim) | 80% | 100% | 120% |
|------------------------------------------------------|-----|------|------|
|                                                      | Sample1 | Sample2 | Sample1 | Sample2 | Sample1 | Sample2 |
| Abs. of the test sample                               | 0.292 | 0.289 | 0.364 | 0.362 | 0.437 | 0.438 |
| %age of label claim                                   | 100.68% | 99.65% | 100.27% | 99.72% | 100.22% | 100.45% |
| Variation from theoretical Results or difference      | 0.68% | 0.35% | 0.27% | 0.28% | 0.22% | 0.45% |
| Average or %age Recovery                              | 100.16% | 99.99% | 100.33% |
| Standard Deviation of Variation from Theoretical Results | ± 0.51 | ± 0.27 | ± 0.11 |
Robustness
Robustness is a measurement of the capacity of an analytical procedure to remain unaffected by minor but deliberate deviations in parameters of protocol and offers an indication of its reliability in usual practice. The resulting changes are made deliberately in testing procedure. The test solutions are to be prepared according to the test procedure and kept the concentration of analyte same but the volume of solvent is changed and assayed according to the test procedure. The results are compiled with initial results and tabulated in Table 8.

Concentrations of standards: 12.5 µg/ml
Wavelength (λ): 320 nm
Absorbance of the standard solution = 0.364

Table 8. Determination of Label Claimed Versus Amount of Samples.

| Volume Variation | 50 ml | 100 ml | 200 ml |
|------------------|-------|-------|--------|
|                  | Sample-I | Sample-II | Sample-I | Sample-II | Sample-I | Sample-II |
| Sample concentration | 12.5µg/ml | 12.5µg/ml | 12.5µg/ml | 12.5µg/ml | 12.5µg/ml | 12.5µg/ml |
| Abs. of Test Solution | 0.363 | 0.365 | 0.364 | 0.365 | 0.362 | 0.364 |
| %age of Label Claim | 99.72 | 100.27 | 100.00 | 100.27 | 99.45 | 100.00 |
| Average | 99.99 | 100.13 | 99.72 | Average | 99.99 | 100.13 |
| Standard Deviation | ± 0.19 | ± 0.09 | ± 0.19 | Standard Deviation | ± 0.19 | ± 0.09 |

Specificity
The specificity is defined as the ability to dis-unite the analyte in the presence of other components like matrix components. Specificity shows that the procedure is unaffected by the presence of excipients or impurities. Specificity is performed by running a standard solution (as identification test) comparing with a placebo run.

Placebo Preparation
Prepare placebo by taking excipients of preparation, without active ingredient (Amoxicillin trihydrate), mixed to made 200 g in following ratio.
Colloidal silicon dioxide (3.322 g), Sodium benzoage (0.246 g), Sodium citrate (0.246 g), Carboxy methyl cellulose sodium (0.984 g), Bubble gum (dry essence) (0.615 g), Methylparaben (0.203 g), Propylparaben (0.203 g), Sucrose (192.86 g) and saccharin sodium (0.123 g).

Limit of Detection (LOD)
The least amount of an analyte could be determined, but it is not necessarily to evaluate it quantitatively in prescribed experimental situations is termed as LOD. It is important for the determination of little amount of drugs besides impurities and placebo. The LOD is usually cited as the concentration giving a signal to noise ratio of 2:1 which is confirmed near this value by examining a number of samples.

As per following equation, “signal to noise” ratio is determined:

\[ s = \frac{H}{h} \]

H correspond to peak’s height relating to constituent h is absolute value of highest noise-fluctuation in the chromatogram initiation from the baseline of a blank (solution).
Remarks: There is no any impurity detected under the conc. 2.5 µg/ml. The absorbance peak wavelength (λ) is same and no “signal to noise” ratio found.

Limit of Quantification (LOQ)
The determination of the least amount of an analyte in a sample with acceptable accuracy and precision is called LOQ. Lowest concentration of analyte (Amoxicillin trihydrate) was determined up-to 5 µg/ml. The inter-/intra-day response on the Amoxicillin concentration was scanned (concentration and absorbance) and found significant results that was given in Figures 4A, 4B, 4C and 4D.
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DISCUSSION

The data presented the linear regression line between concentration and absorbance of analyte followed the Beer's Lambert law [21]. According to the Linear regression the \( Y = 0.002000 = 3.014e^{-008} \) and correlation coefficient of \( r = 1.000 \). This revealed that linear relationship between absorbance and concentration of Amoxicillin trihydrate has the significant configuration as given in Figure 2H and Table 4. The validation of repeatability and reproducibility were performed with 03 samples in 2 replicates having concentration of 12.5 µg/ml of Amoxicillin trihydrate. Each result showed the reliable and substantial variation when compared with theoretical results as given in Table 5 [22]. In addition accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness were also evaluated by employing the proposed spectrophotometric methods and found reliable values on comparison with standard outcomes [23, 24] presented in Tables 7 and 8.

CONCLUSION

Our study demonstrated the authentication the evaluation of Amoxicillin trihydrate in various pharmaceutical preparations and bulk that newly designed with UV/Visible spectroscopic method. The proposed spectrophotometric technique is quite simple, sensitive, reproducible, economical and fast when compared with other stated methods employed for the determination of Amoxicillin. In conclusion, this validation mode will be a worth for the monotonous examination of Amoxicillin trihydrate either in the bulk and solid pharmaceutical dosage form.

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