Colorimetric determination of Amoxicillin using 4-Aminoantipyrine and the effects of different parameters

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Abstract. The wide utilize of antibiotics has led to spreading of antibiotics resistance among bacterial populations and reducing the effectiveness of antibiotics. However, most of the administrated antibiotics enter the environmental matrixes through direct runoff and excretion of feces or urine as unchanged original drugs, active metabolites and/or degradation products. Amoxicillin is one of the most valuable and highly consumed antibiotics. Thus, amoxicillin removal from pharmaceutical wastes offers many economic and environmental benefits. Therefore, the aim of current study was to characterize an identification method for amoxicillin and to study the effects of different parameter such as order of addition, nature of the complex, temperature and effect of concentration and type of the base.

A spectrophotometric, selective and sensitive method has been proposed for the determination of Amoxicillin. The method is based on the oxidation of potassium ferricyanide and coupling of the oxidized product with the reagent, 4-aminoantipyrine, to give intensely colored complex that absorbs light strongly in the spectral region of 509nm at room temperature. The results found that the order of addition, drug (Amoxicillin)-potassium ferricyanide-base-4-aminoantipyrine, gave highest absorbance and sensitivity, the best base was NaOH (0.1N) needful for developing the colored product and increase its stability. That complex, at 25°C, gave a preferable sensitivity and was selected for further use in this study. Under the proposed optimum condition, Beer’s law was conform in the concentration range of (5-100 mg L⁻¹). Good linearity (r²=0.9994). Calculation of standard error, limit of detection (LOD) (signal/noise = 3), accuracy and precision (RSD%) better than 1%. The utilized method seemed simple, rapid, reproducible and accurate. In addition, it is applicable for the assay of the drug under investigation in various dosage forms and the results are in very good concord with those obtained in previous studies.

Keywords: Amoxicillin, 4-aminoantipyrine, spectrophotometric, Pharmaceuticals, Colorimetric, potassium ferricyanide.

Introduction.

Pharmaceuticals constitute a wide group of human and veterinary medicinal compounds which have been used for long times throughout the world. The most important pharmaceuticals found in the waters are antibiotics, hormonal drugs, analgesics, and painkillers. Antibiotics are probably the most successful family of drugs so far developed for improving human health and the most widely used drugs for the prevention or treatment of bacterial infections in humans, animals and plants.
The excreted antibiotics can further enter the aqueous environment from soil and sediments by surface runoff, leaching and desorption, \cite{1}. These chemicals find their way into the water via sewage systems of drug manufacturing plants, hospitals \cite{1} and eventually enter drinking water due to their high stability towards conventional biodegradation \cite{1}, and chlorination disinfection treatment. Antibiotics are difficult to be degraded by biodegradation due to the stable naphthol ring within their structure \cite{2}.

Amoxicillin is a broad-spectrum β-lactam antibiotic that belongs to penicillin class \cite{2}. It consists of two fundamentals parts, inner that contains β-lactam and the side chain called d-hydroxiphenilglicin (Figure 1). It is also commonly employed for human prescription medicine as a therapeutic agent due to its broad spectrum against bacteria \cite{2}. Thus, amoxicillin removal from pharmaceutical wastes offers many economical and environmental benefits. There are many methods for AMX removal from sewage water such as biodegradation, electrocoagulation, membrane processes, Nano filtration membrane, ozonation, reverse osmosis, catalytic degradation, Fenton oxidation, Photocatalytic oxidation/degradation, electrochemical oxidation/degradation and adsorption \cite{2}.

On the other hand, 4-Aminoantipyrine (AAP) 4-amino-1,5-dimethyl-2 phenyl-1,2-dihydro-3H-pyrazol-3-one \cite{2} a yellow crystalline powder that possesses anti-inflammatory and analgesic, antipyretic properties. The formula is C_{11}H_{13}N_{3}O, relative molecular mass is 203.24 g/mol and , molar volume is 168.3 L/mol \cite{2} It has been utilized as a useful chromogenic reagent for the determination of different phenols in aqueous solutions \cite{2}. The determination is based on the oxidative coupling of phenols with 4-aminoantipyrine by oxidants like potassium ferricyanide to yield highly colored diagnostic quinoneimine dyes \cite{2}. In addition, coupling reaction of phenols with 4-AAP is a well-established method for the determination of oxidants \cite{2,4}.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{ChemicalStructure.png}
\caption{The chemical structure of amoxicillin (AMX).}
\end{figure}

**Material and Methods**

**General procedure**

Aliquots of amoxicillin ranging from 5–100 mg L\(^{-1}\) were transferred into a series of 10ml volumetric flasks. To each flask 2ml of an aqueous solution of potassium ferricyanide (0.3gm/100ml) were added followed by 1ml from 0.1N NaOH., 2 ml aqueous solution of 4AAP (0.3gm/100ml). The final volume was made up to 10ml with distilled water \cite{2}. The absorbance of the panic-colored species formed was measured at 509 nm against reagent blank and beer’s law was conformed in concentration range of 5–100 mg L\(^{-1}\). The amount of amoxicillin existent in the sample was computed from calibration curve (Figure 2).
Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

The linearity of the developed method was investigated by replicate analysis ($n = 6$) at ten concentration levels (5, 10, 20, 30, 40, 50, 60, 70, 80 and 100 mg L$^{-1}$) of reference standard amoxicillin. The results (Table 1) showed an excellent linearity ($r^2 = 0.9994$) as the obtained absorbance was plotted against the concentration of amoxicillin and the linear regression equation was evaluated by statistical treatment of calibration data [2].

The LOD for the proposed method was calculated using the following equation (1). The signal-to-noise ratio (S/N) was obtained by standard addition quantification and subsequent extrapolation to an S/N of 3 [2].

LOD = 3 SD / k          \hspace{1cm} (1)

The LOQ was experimentally calculated as the lowest spiked concentration level of the calibration curve defined as calculated using the following equation (2) [5, 6].

LOQ =10 SD / k          \hspace{1cm} (2)

Where SD is the standard deviation of replicate determination values under the same conditions as for the sample analysis in the absence of analyte and $k$ is the sensitivity, namely the slope of the calibration graph.

| Parameter                          | Proposed value          |
|-----------------------------------|-------------------------|
| Regression equation               | $Y= 0.00565 X + 0.01162$|
| Slope                             | 0.00565                 |
| Linear range ( g ml$^{-1}$)       | 5-100                   |
| Detection limit LOD ( g ml$^{-1}$) | $9.507 \times 10^{-5}$  |
| limit of Quantitation LOQ ( g ml$^{-1}$) | $3.17 \times 10^{-4}$  |
| Sandals Sensitivity ( g cm$^{-2}$) | $1.77 \times 10^{-7}$  |
| Correlation coefficient           | 0.9994                  |
| Molar absorptivity coefficient (L. mol$^{-1}$,cm$^{-1}$) | 2064.51                 |
Effects of different parameters

Order of addition

The effect of the order of addition was studied by preparing solutions with different orders of the concentration of amoxicillin 100 mg L\(^{-1}\), potassium ferricyanide 0.3gm/100 ml, 4-AAP 0.3gm/100 and 1ml NaOH 0.1N.

| NO. | Aspiration order                  |
|-----|-----------------------------------|
| 1   | drug–ferricyanide–base–4AAP       |
| 2   | drug-base–ferricyanide–4AAP       |
| 3   | Ferricyanide–drug–base–4AAP       |
| 4   | Ferricyanide-base–drug–4AAP       |
| 5   | Ferricyanide-4AAP-drug–base       |
| 6   | 4AAP–base–ferricyanide–drug       |
| 7   | 4AAP–ferricyanide–base–drug       |
| 8   | 4AAP–ferricyanide–drug–base       |

Nature of the base

The effects of different concentrations of the base NaOH (0.1, 0.2, 0.3 and 0.4 N) and several other types of bases (NaOH, NaHCO\(_3\) and Na\(_2\)CO\(_3\)), at a concentration of 0.1N were investigated. Also, AMX (5-100 mg L\(^{-1}\)), potassium ferricyanide 0.3gm/100ml and 4-AAP 0.3gm/100ml were used.

Effect of temperature

The effects of the different temperatures (15, 25, 35, 45, 55 and 65°C) were investigated using water bath (hot or cold) for 10min. The system was operated at the initial mentioned concentrations of AMX (100mg L\(^{-1}\)), potassium ferricyanide (0.3gm/100ml), 4-AAP (0.3gm/100 ml) and NaOH (0.1N).

Nature of the complex

Effect of the molar ratio method beneath the optimized conditions AMX (100 mg L\(^{-1}\)) using (0.5, 1, 1.5, 2, 2.5, 3, 3.5ml), 4- AAP (0.3gm/100ml) using (3.5, 3, 2.5, 2, 1.5, 1, 0.5ml) to give a volume of 4ml and completing the volume with 2ml potassium ferricyanide and 1ml NaOH (0.1N) in 10-ml volumetric flasks.

Interference studies

In order to assess the potential analytical applications of the proposed method, the effect of some common excipients frequently found with AMX in pharmaceutical formulations such as glucose, lactose, sucrose, starch, fructose, talc, magnesium stearate and sodium chloride were studied by analyzing synthetic sample solutions containing 50mg L\(^{-1}\) of AMX.
Pharmaceutical preparations

Procedure for syrups

The contents of 4 bottles of Syrups were mixed and the average volume of one bottle was determined. An precise volume equivalent to include 125, 250,457 mg of the studied drug was dissolved in 100ml water. This solution was further diluted stepwise to the requisite concentration with water and analyzed.

Procedure for the capsules

The contents of ten capsules of the drug AMX were weighed and grounded into a fine powder. Amount of powder containing about 250, 500 mg of the drug was weighed accurately, dissolved in 50ml of water, followed by 10min in an ultrasonic bath, filtered through filter paper and washed with water. Then, the filtrate plus washings were diluted to 100ml with water in a calibrated flask. An aliquot was used for the determination of each drug.

Results and discussion

Absorption spectra

The spectral characteristics of the systems (amoxicillin under optimum conditions) are shown in Figure 3. Under the same conditions, the reagent blank showed very small absorbance quantity (A=0.003) in the region of interest, so all the absorbance measurements were carried out against a reagent blank. Panic-colored compound was produced with maximum absorbance in the visible range at 509nm [7].The factors which were found to affect the formation of the colored species included time, order of addition ,reagent concentration and temperature.

![Absorption spectra](image)

Figure 3 Absorption spectra of 100mg L⁻¹ of amoxicillin treated as described under procedure and measured against reagent blank.
Effect of the order of addition

This factor was studied by preparing a solution with different arrangement (Table 2). The order No. 1: drug (Amoxicillin)- potassium ferricyanide –base-4-AAP. Maximum absorbance was obtained (Figure 4) and was selected because it gave highest absorbance and sensitivity [7-9].

![Figure 4](image1)

Figure 4 Effect of the order of addition of Amoxicillin.

Effect of nature of base

In order to determine the best base, we used different types of bases (NaOH, Na₂CO₃ and NaHCO₃; Figure 5a). Because color formation, best sensitivity, high intensity and the highest absorbance value are dependent upon type of base and amount of base, therefore, the best base, NaOH, was used as the alkaline agent. The latter gave the highest absorbance completion. On the other hand, Na₂CO₃ was unstable and gave shift absorbance values. Also, NaHCO₃ gave suffer low absorbance values. Therefore, these bases can be arranged in ascending order; NaOH < NaHCO₃ < Na₂CO₃ [10, 11]. Also, the effect of NaOH was studied in the concentration range of 0.1-0.4N to find the concentration necessary for developing the colored product and increasing its stability. The results showed that the greatest absorbance intensity was obtained with 0.1N [7, 12]. At 0.2, 0.3 and 0.4N precipitation occurred and absorbance decreased (Figure 5b).

![Figure 5](image2)
Effect of temperature

The effect of temperature on the color intensity of the dye was studied. In practice, high absorbance was a minimum blank value, preferable sensitivity. When the color was developed at room temperature (25°C) and was selected for further use in this study [9, 13], but when the color was developed in an ice-bath (15°C) or in a water bath (65°C) a loss in color intensity and stability were observed. It is therefore recommended that the color reaction should be carried out at room temperature (25°C) [14]. It was obtained when the calibrated flasks were placed in a water bath at (65°C) or in an ice-bath at (15°C) (Table 3).

| Temperature (°C) | Absorbance |
|------------------|------------|
| 15               | 0.413      |
| 25               | 0.586      |
| 35               | 0.342      |
| 45               | 0.239      |
| 55               | 0.21       |
| 65               | 0.118      |
Nature of the complex

The stoichiometry of the reaction was investigated by changing the molar ratio of the reagents \[^{5,8}\]. The results gained (Figure 6a) showed a 1:1 of AMX to 4.AAP product which was formed at 509nm. The formation of the dye may have possibly occurred as given in Figure 6b).

![Figure 6a Study of the molar ratio of the reaction between of amoxicillin (AMX) and 4-AAP for the colored product formed.](image)

Analytical applications

The proposed method was applied successfully to the analysis of some pharmaceutical formulations containing amoxicillin. The results in Table 4 indicated that those obtained by the official spectrophotometric method using 4-AAP reagent \[^{12}\], had high accuracy and selectivity. The
determination was carried out for six times with different concentrations. The proposed method is suitable for the determination of amoxicillin formulations without interferences from excipients such as glucose, lactose, and starch, excess of each excipient or from degradation products. None of these substances interfered seriously. Under the optimum conditions, the precision in the determination of AMX was studied. Table 4 showed the values of the relative standard deviation (%RSD) for each of the six reading for AMX standard solution containing 20, 50, and 100mg L⁻¹. While the accuracy of the proposed method was performed by reading 50mg L⁻¹ six times. The results obtained indicated that the average recovery is 99.2-100.2% of the developed method [13] (Table 5).

Table 4 Determination of amoxicillin in different formulations using the proposed and official methods

| Pharmaceutical preparation | Conc. Of AMX (mg L⁻¹) | E% | Rec% | RSD% |
|-----------------------------|-----------------------|----|------|------|
| Amoxicillin Capsules 250mg (SDI), Iraq | 20 20.0833 | 0.4166 | 100.4166 | 0.1855 |
| | 50 50.1166 | 0.2333 | 100.2333 | 0.1041 |
| | 100 100.125 | 0.125 | 100.125 | 0.0558 |
| Amoxmark Capsules 500mg(Aurobindo Pharma), India | 20 19.9 | -0.5 | 99.5 | 0.2247 |
| | 50 49.8 | -0.4 | 99.6 | 0.1796 |
| | 100 99.9 | -0.1 | 99.9 | 0.0447 |
| Amoxicillin Capsules 500mg, (Cosar)Iran | 20 19.9166 | -0.4166 | 99.5833 | 0.1871 |
| | 50 49.65 | -0.7 | 99.3 | 0.3152 |
| | 100 99.3833 | -0.6166 | 99.3833 | 0.2774 |
| Neomox Syrup 250mg Neopharma (Abu Dhabi, UAE) | 20 20.2333 | 1.1666 | 101.1666 | 0.5157 |
| | 50 49.3833 | -1.2333 | 98.7666 | 0.5584 |
| | 100 99.1666 | -0.8333 | 99.1666 | 0.3758 |
| Amoxicillin Largopen Syrup125mg Bilim pharmaceuticals (Istanbul) | 20 19.9 | -0.5 | 99.5 | 0.2247 |
| | 50 49.65 | -0.7 | 99.3 | 0.3152 |
| | 100 99 | -1 | 99 | 0.4517 |
| Co Amoxil Syrup457mg (Acino) | 20 19.9833 | -0.0833 | 99.9166 | 0.0372 |
| | 50 49.4833 | -1.0333 | 98.9666 | 0.4669 |
| | 100 98.8333 | -1.1666 | 98.8333 | 0.5279 |
| Amoxil Vial Injection 250mg , India | 20 19.9 | -0.5 | 99.5 | 0.224 |
| | 50 49.4166 | -1.1666 | 98.8333 | 0.5279 |
| | 100 99 | -1 | 99 | 0.4517 |
| Amoxil Vial Injection 500mg(Cosar)Iran | 20 20.03 | 0.16 | 100.1 | 0.074 |
| | 50 49.4166 | -1.1666 | 98.8333 | 0.5279 |
| | 100 99.3833 | -0.6166 | 101.1666 | 0.5157 |

Table 5 Determination of amoxicillin in different excipients using the proposed and official methods

| Excipients | Conc. Of AMX (mg L⁻¹) | E% | Rec% |
|------------|-----------------------|----|------|
| Lactose | Present | 50 | 49.6 | -0.8 | 99.2 |
| | Found | 50 | 49.6 | -0.8 | 99.2 |
| Starch | 50 | 50.2 | 0.4 | 100.4 |
| | 50 | 50.1 | 0.2 | 100.2 |
| Fructose | 50 | 50.2 | 0.4 | 100.4 |
| | 50 | 49.6 | -0.8 | 99.2 |
| Sodium chloride | 50 | 50.1 | 0.2 | 100.2 |
| | 50 | 49.8 | -0.4 | 99.6 |

Conclusions

The aim of this method-validation study was to document successful implementation of the method for determination of antibiotic drugs, such as Amoxicillin (AMX), under study in their pharmaceutical preparations for stability and higher sensitivity. Current study showed that maximum absorbance was attained at 509nm by using UV-Visible spectrophotometer. The Sandals Sensitivity
(S) was found to be $1.77 \times 10^{-7}$ g cm$^{-2}$ for AMX. The developed method gave a better linear range. The detection limit was found to be $9.507 \times 10^{-5}$ μg ml$^{-1}$ for AMX. The recovery (accuracy) was in the range (95.833–100.416), AMX. Therefore, this method could be applied with high satisfaction in the determination of AMX in pure and dosage forms with a high accuracy and precision.

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