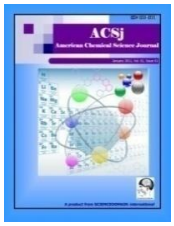

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Batch and Flow Injection Analysis Spectrophotometric Determination of Amoxicillin using N-bromosuccinimide and Indigo Carmine

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Authors' contributions

This work was carried out in collaboration between all authors. Author BMR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author KMM supervised and managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

This paper describes a batch(direct spectrophotometer) and flow injection spectrophotometric for indirect determination of amoxicillin (AMX). The method involves addition of a known excess of N-bromosuccinimde (NBS) to AMX in acidic medium, the residual amount of oxidant (NBS) reacted with indigo carmine which causes bleaching of its blue color. The absorbance was measured at 610 nm. In the batch method Beer's law was obeyed in the concentration range (0.2 - 6.0 µg ml^{-1}) and detection limit of 0.1 µg $ml^{-1}$with a correlation coefficient (r) of 0.9945 and a molar absorptivity of $3.874x10^4$L $mol^{-1}cm^{-1}$. A simple FI-spectrophotometric system was applied for determination of AMX depending on the batch method. A calibration graph gives linear range of (2.0 - 14.0 µg ml^{-1}) AMX, with a detection limit of 1.8 µg ml^{-1}, a correlation coefficient (r) of 0.9965 and a sampling frequency of 48 S h^{-1}. Essential parameters such as accuracy and precision were studied for the two methods by calculation of (RSD %) and (E%) for two different levels of concentration. The method was relatively free from common excipients and it is applied successfully for the determination of AMX in pharmaceutical formulations.

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1. INTRODUCTION

Indigo carmine, or 5, 5'-indigodisulfonic acid sodium salt, also known as indigo tine was a pH indicator, with blue color at (pH = 11.4) and yellow at (pH = 13.0). It was also a redox indicator, turning yellow upon reduction. It has the chemical formula $C_{16}H_8N_2Na_2O_8S_2$. The characteristics of the dye were: molecular weight was (466.35 g mol^{-1}), solubility in water (10 g L^{-1}) appearance (dark blue to purple powder) and maximum wavelength (610 nm). Generally, indigo carmine dye is used in food industry (food colorant) and has the E number E132, cosmetics industries and also for dyeing of denim and polyester fibers. Indigo carmine is a highly toxic which might cause irritations vomiting and diarrhea to human beings[1-2].

N-bromosuccinimide (NBS) acts as a mild oxidizing and brominating agent[3]. The use of NBS as an oxidant is extensive in the determination of a number of organic compounds [4]. It used for the specific purpose of brominating alkenes at the allylicposition [5]. NBS has been used as an oxidizing agent and analytical reagent especially in acid medium. However, the work on oxidation by NBS in alkaline media was scanty [6]. NBS was used for the determination of Pyridoxine and Paracetamol by FI-CL (Flow injection - chemiluminescence] [7], and for determination of promethazine hydrochloride by FI-spectrophotometry [8].

Saleh[9]developed two simple, rapid and selective spectrophotometric procedures for the determination of AMX and cefadroxil. The methods were based on the selective oxidation of the drugs with NBS or N-chlorosuccinimide in an alkaline medium to give an intense yellow product (λ_{max}= 395 nm). The reactions obey Beer's law over the range 1.0 – 20.0 pg ml^{-1} for the two drugs with the two reagents.

A new spectrophotometric method was described for the rapid determination of AMX in bulk drug and dosage forms. The method was based on the oxidation-bromination of AMX by in situ generated bromine followed by estimation of unreacted bromine with indigo carmine and measuring the change in absorbance at 610 nm. In this method quantitation was based on the amount of bromated that has reacted with AMX. The calibration graph was found to be linear over 0.5 - 4.0 µg $ml^{-1}$for AMX, with a molar absorptivity of $5.83x10^4$L $mol^{-1}cm^{-1}$[10].

Kemal et al. were studied a new original UV spectrophotometric method for the determination of amoxicillin in pharmaceutical preparations, while the solution of amoxicillin prepared in 0.1 N NaOH and the absorbance was measured at 247.0 nm [11].

The proposed method describes a batch (direct spectrophotometer)and flow injection spectrophotometric for indirect determination of amoxicillin (AMX). The method involves addition of a known excess of NBS to AMX in acidic medium, the residual amount of oxidant NBS reacted with indigo carmine which causes bleaching of its blue color. The absorbance was measured at 610 nm.

2. EXPERIMENTAL

2.1 Apparatus

Spectral measurements were carried out on (CECIL CE 3021 England) UV-Vis spectrophotometer, equipped with quartz cell of 1.0 cm path length.

FIA-system used in this work with a peristaltic pump (Carter 12/6 cassette pump with five channels, variable speed) to deliver flow streams. The tygon pump tubes with (1.05 mm i.d.(internal diameter)) were used. The rotary valve (Rheodyne U.S.A.) with variable sample volumes was used. The absorbance measurements of the FIA were carried out using spectrophotometer (JENWAY 6405 UV/Vis-Spectrophotometer) with flow cell Sterna-micro (100 µL) with 1.0 cm optical path length. The absorbance was recorded by means of x-t recorder (type PM 825A Philips).

2.2 Reagents and Solutions

Distilled water was used in all preparations. The reagents used were of analytical grade.

2.3 N-bromosuccinimide (NBS) Solution

0.01 M was prepared daily by dissolving 0.1779 g of NBS (Fluka) in a small volume of distilled water, and then diluted to 100 ml in volumetric flask. 0.001 M solution prepared by appropriate

dilution. The solutions were kept in a dark container.

2.4 Indigo Carmine

0.002 M was prepared by dissolving 0.0933 g of indigo carmine (RIEDEL-DEHAEN) (>99%)[12], in a little distilled water, and diluting to volume in a 100 ml volumetric flask. Other solutions were prepared by appropriate dilution.

3. RESULTS AND DISCUSSION

3.1 Spectrophotometric Determination of Amoxicillin Using (NBS) and Indigo Carmine

Preliminary work: To a 25 ml volumetric flask containing 2.0 ml of (25.0 µg ml^{-1}) sample solution, 2.0 ml of 1.0 M HCl, 2.0 ml of (0.001 M) NBS and 2.5 ml of ($5x10^{-4}$M) indigo carmine added, so the final concentration of AMX in 25 ml volumetric flask became (2.0 µg ml^{-1}), for HCl (0.08 M), for NBS ($8x10^{-5}$ M) and for indigo carmine ($5x10^{-5}$ M) in 25ml volumetric flask. The mixture was then diluted to the mark with distilled water. The reagent blank was prepared in the same way without AMX. The absorbance of the prepared solution was measured at 610 nm.

The suggested mechanism of the reaction is showed in Fig. 1, in which AMX oxidizes by a known excess of NBS, then the residual oxidant is estimated by indigo carmine [13].

3.2 Absorption Spectra

Maximum absorption spectrum of indigo carmine was found at λ_{max}610 nm. The conditions of preliminary work were used to obtain the absorption spectra shown in the Fig. 2,where (a) represent the spectrum of indigo carmine only, spectrum (b) for the mixture of AMX-NBS-indigo carmine and spectrum (c) for NBS-indigo carmine against distilled water.

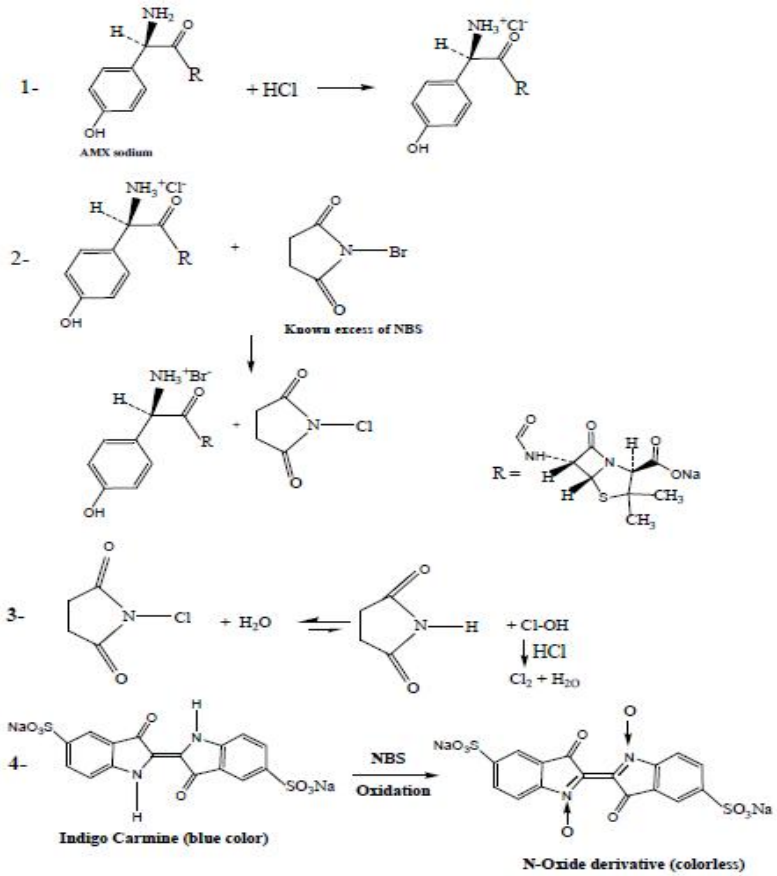

Fig. 1. Suggested reaction mechanism

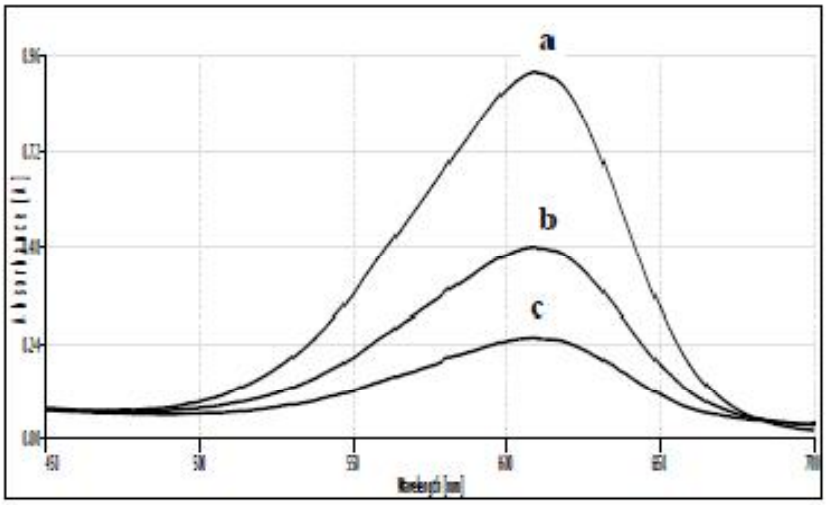

Fig. 2. Absorption spectra of (a) Indigo carmine, (b) AMX-NBS-indigo carmine and (c) NBS-indigo carmine against distilled water

3.3 Effect of Type of Acid

Effects of different types of acid solutions (2.0 ml of 1.0 M) (hydrochloric acid, acetic acid, nitric acid, sulfuric acid) were tested. The results indicate that hydrochloric acid give the best results, therefore it was used in subsequent experiments.

3.4 Effect of Hydrochloric Acid Concentration

The effect of hydrochloric acid in the range of (0.1 – 5.0 ml) of (1.0 M) on the sensitivity of the reaction was investigated. It was found that the best result was obtained with 2.0 ml of 1.0 M hydrochloric acid.

3.5 Effect of N-bromosuccinimide Concentration

The effect of oxidant concentration was studied by adding different volumes of (0.001 M) NBS solution to a constant amount of AMX (25.0 µg ml^{-1}). It was observed that the maximum color intensity was obtained with 1.7ml of NBS, after which further increase in volume resulted in a decrease of absorbance.

3.6 Effect of Indigo CarmineConcentration

The effect of the indigo carmine on the intensity of the color development was studied by measuring the absorbance at different volume of ($5x10^{-4}$M) in the range (2.0 - 3.5 ml) using the optimized volume of hydrochloric acid and NBS. Volume (2.5 ml) gave the best sensitivity, higher than this volume there was no significant change in absorption.

3.7 Order of Addition

The effect of order of addition on the sensitivity of the method was investigated. The results obtained that the order of addition N-bromosuccinimide (O) + hydrochloric acid (A) + AMX (S) +Indigo carmine(R) gave the best sensitivity and more intense color than other probabilities and this order was used in all subsequent experiments.

3.8 Recommended Procedure

2.0 ml of 25.0 µg $ml^{-1}$of the working standard solution of AMX equivalent to 2.0 µg $ml^{-1}$was added into a 25 ml calibrated flask containing 2.0 ml of 1.0 M hydrochloric acid and 1.7 ml of 0.001 M NBS. Then, 2.5 ml of $5x10^{-4}$M indigo carmine was added and the contents were diluted with distilled water to the mark. The blank solution was prepared in the same manner in the absence of AMX. The absorbance was measured againstareagent blank at 610 nm. The unknown concentration derived from the calibration graph or computed from the regression equation derived using Beer's law.

3.9 Calibration Graph and Statistical Data

Under the optimized experimental conditions and applying the recommended procedure, a straight line of a calibration graph was obtained as shown in Fig. 3. Calibration curve was obeyed Beer's law in the concentration ranges of (0.2 - 6.0 µg ml^{-1}) of AMX with a correlation coefficient of (0.9945) and detection limit of 0.1 µg ml^{-1}. Sand ell index and molar absorptivity were found to be 10 µg cm^{-2} and $3.874x10^4$ L mol^{-1} cm^{-1} respectively.

3.10 Precision and Accuracy

To determine the precision and accuracy of the method which depends on the values of SD, RSD% and E%; two different pure AMX solutions (1.0 and 4.0 µg ml^{-1}) were used replicated ten times. The results were shown in Table 1 which indicates a good precision and accuracy of this method.

3.11 Interferences

The possible interferences related to the determination of AMX in pharmaceutical preparations were studied by analyzing a standard solution of 3.0 µg $ml^{-1}$of AMX, a known amount of interferences were added. This study was performed by comparing the absorbance obtained when AMX present alone and in the presence of different concentration of interferences reach to 100 times of the amount of AMX. The results found that a substance was considered not to interfere if the variation in the absorbance of pure AMX and AMX with interference equal or less than ± 5 %. Table 2 illustrated the absorbance after addition of additives by 100 fold excess the amount of AMX.

3.12 Applications

The proposed method was successfully applied for the determination of AMX in pharmaceutical preparations; the same formulations were also analyzed by the BP method (UV-spectrophotometry) [14] as standard method and the results were compared by calculating the relative error.

The accuracy and precision of the present method was comparable to that of the BP(UV-spectrophotometry) [14] method by using t-test and F-test at 95% level of confidence. It was found that all results are in agreement at the 95% confidence level and within an acceptable range of errors ($t_{calculated}$= 0.865 less than t_{table}= 2.571) and ($F_{calculated}$= 1.477 less than F_{table} = 5.05) [15]. The results show no significant differences between them as shown in Table 3.

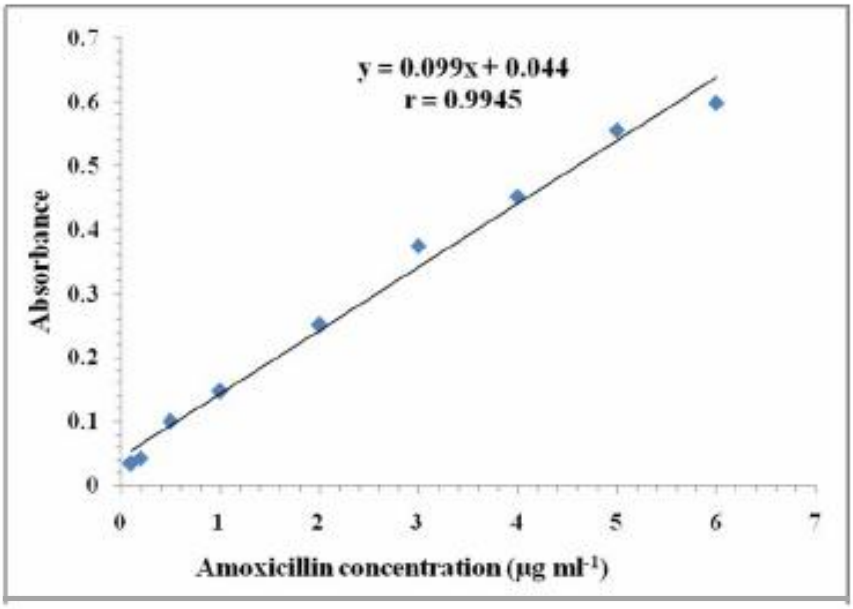

Fig. 3. Calibration graph for the spectrophotometric determination of AMX

Table 1. Precision and accuracy of the proposed spectrophotometric method

Concentration of AMX (µg ml^{-1})	Mean absorbance (n=10)	SD	RSD%*	E%#
1.0	0.1463	0.0039	2.5035	3.3
4.0	0.4515	0.0028	0.58126	2.9

Relative standard deviation; #relative error.

Table 2. Effect of interferences on the absorbance of 3.0 µg ml^{-1}AMX

Interferences	Tolerance level	Absorbance	E%
Glucose	100	0.382	2.14
Galactose	100	0.387	3.48
Fructose	100	0.369	-1.34
Sucrose	100	0.383	2.41
Lactose	100	0.365	-2.41
Starch	100	0.377	0.80
Sodium chloride	100	0.357	-4.55

Table 3. Comparison of present method with standard method[14]for determination of 3.0 µgml^{-1}AMX using t-test and F-test

Formulation		Amount found (µg ml^{-1})		Absolute error	E%
		Present method	Standard method [14]		
Capsule	Amoxicillin S.D.I. (Iraq)	2.915	2.892	0.023	0.795
	Glomox Gobalpharma (U.A.E.)	2.822	2.929	-0.107	-3.653
	Penamox HIKMA (Jordan)	2.912	2.857	0.055	1.925
Tablet	Amoxicillin HEUMANN (Germany)	2.814	2.786	0.028	1.005
Injection	Acamoxil ACAI (Iraq)	3.288	3.214	0.074	2.302
	Largo penBilim (Turkey)	3.141	3.071	0.070	2.279
Mean		2.98	2.96	0.02	
SD		0.19	0.16	0.07	
$t_{calculated}$= 0.865; t_{table}= 2.571					
$F_{calculated}$= 1.477; F_{table}= 5.05					

3.13 FIA-Spectrophotometric Determination of Amoxicillin Using NBS and Indigo Carmine

FIA system in Fig. 4 was operated, a sample volume of 50 µL used to inject 10 µg ml^{-1}of AMX into the flowing stream. Two reaction coils were used in the system with length a = 10 cm and b = 40 cm. Hydrochloric acid was mixed with NBS, then they were mixed and reacted with AMX in the first reaction coil, after that the product was reacted with indigo carmine in the second reaction coil, with a total flow rate 1.0 ml min^{-1}. Then the absorbance of indigo carmine was measured at λ_{max}= 610 nm.

3.14 Optimization of the Experimental Parameters

The physical and chemical parameters that participate in the reaction have been studied to obtain the maximum sensitivity.These optimizations were started using the following experimental physical and chemical variables for the determination of AMX (10 µg ml^{-1}): 1.0 ml min^{-1}flow rate, length of mixing coils (a = 10 cm, b = 40 cm), 50 µL injected volume, 0.1 M HCl, (1x10^{-4}M) NBS and (1x10^{-4}M) indigo carmine.

3.15 Effect of Flow Rate

The flow-rate was conveniently controlled by the peristaltic pump. Effect of flow rate on the sensitivity of the colored product was studied in the range of 0.5 - 2.0 ml min^{-1}. The results obtained showed that the absorbance increased up to a flow rate of 1.0 ml min^{-1}and decreased for greater flow rates, because the reactants were reached to the detector at a shorter time which was not sufficient for completing the reaction. A flow rate of 1.0 ml min^{-1}was chosen for further use and with this flow rate a sampling frequency of 48 S h^{-1} was achieved.

3.16 Effect of Length of Reaction Coils

The effect of reaction coil length on the peak-height was studied. Various reaction coils with increasing lengths were investigated (0.0 - 80.0 cm). Results show that the peak height decrease with increasing the length of coil (a) which was used for the reaction between AMX and NBS, this indicates that the reaction was fast and does not require more time and the dispersion of the sample increases with increasing length of the mixing coil. While a 40cm coil gave the best peak height for coil (b) these values were used in subsequent measurements.

3.17 Effect of Sample Volume

The effect of sample volume was investigated by injection of a volume of different lengths of sample loop; the volume of the injected sample was varied between (40-180 µL). It was found that the peak height increased as the injected sample volume increased up to 70 µL, after that the peak height decreased, because too large a sample volume may give rise to split peaks as a result of only the head and tail of the sampleplug reacting with the carrier-reagent stream and the central portion not even reaching it.

3.18 Effect of hydrochloric Acid Concentration

The effect of the concentration of hydrochloric acid solution was studied in the concentration range 0.04 - 0.5 M. The results showed that the peak height increase with increasing the concentration of hydrochloric acid solution up to 0.2 M and then decrease, therefore, 0.2 M hydrochloric acid was chosen for further use.

3.19 Effect of N-bromosuccinimide Concentration

The method was based on the fact that NBS in an acid medium directly oxidizes AMX, the effect of oxidant concentration was studied by adding different concentration of NBS in the range of ($5.0x10^{-5}$– $2.0x10^{-4}$M). It was observed that the maximum color intensity was obtained with $1.0x10^{-4}$M of NBS, after which further increase in concentration of NBS resulted in a decrease of peak height. Thus, $1.0x10^{-4}$M of the NBS was sufficient to reach with the maximum drug concentration in the Beer's range.

3.20 Effect of Indigo Carmine Concentration

The effect of the dye-concentration on the intensity of the color development was studied in the range of ($6.0x10^{-5}$– $2.0x10^{-4}$M) of indigo carmine. It was shown that $8.0x10^{-5}$M of the dye gave the maximum peak height.

3.21 Calibration Graph and Statistical Data

A calibration curve was obtained by plotting peak height (mV) against concentration of AMX (µg ml^{-1}) under the optimal conditions described before. Beer's law was obeyed in the range 2.0 – 14.0 µg $ml^{-1}$of AMX with correlation coefficient of (0.9965). The calibration graph was described by the equation: y = 7.994X–1.571, where y shows the peak height (mV), 7.994 indicate the slope of the curve, X is the concentration of AMX (µgml^{-1}), and 1.571 shows the intercept of the graph, Fig. 5 illustrate the results. The method has a detection limit of 1.8 µg ml^{-1}.

To evaluate the accuracy (in terms of relative error (E %)) and precision (in terms of relative standard deviation (RSD %)) of the method under optimum experimental conditions, ten replicates of two different concentration levels of AMX solutions were analyzed. The results were shown in Table 4.

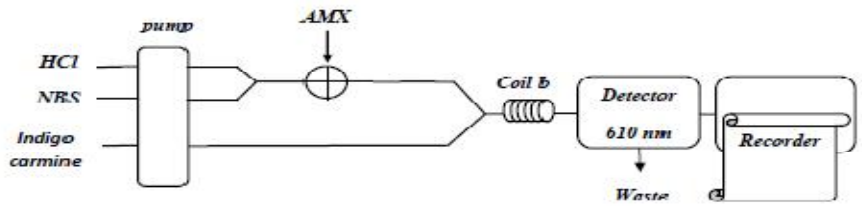

Fig. 4. Schematic diagram of the FIA-spectrophotometric manifold used for the determination of AMX

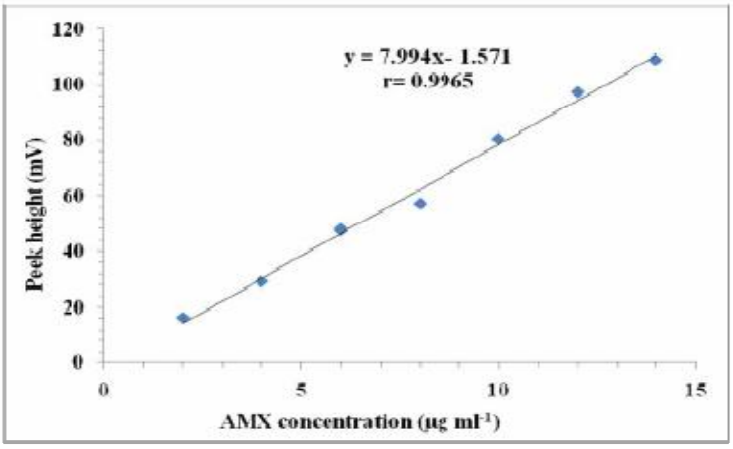

Fig. 5. The calibration graph for determination of AMX using FIA-spectrophotometric method

3.22 Interferences

In order to assess the possible analytical applications of the proposed FIA method, the effect of some common excipients with AMX drugs in pharmaceutical formulations were studied by analyzing synthetic sample solutions containing 8.0 µg ml^{-1} of AMX and excess amounts (100-fold excess) of most excipients except sodium, the results were shown in Table 5 which indicates from the relative error that there was no effect of the seexcipient compounds on the determination of AMX by this method, except sodium chloride which interfere at concentration above 80 fold excess.

3.23 Applications

The present FI-spectrophotometric method was successfully applied for the determination of AMX in pharmaceutical formulations. The samples were also analyzed by the reference method BP(UV-spectrophotometry) [14] as mentioned in Table 6. These results show a good agreement between the two methods.

The results of present method were compared statistically with that obtained by standard method employing t-test and F-test,which indicated that there was no significant difference between accuracy and precision of the two methods at 95% level of confidence ($t_{calculated}$= 1.595<t_{table}= 2.571) and ($F_{calculated}$= 1.036 <F_{table}= 5.05) [15].

Table 4. Accuracy and precision of the method

AMX concentration (µg ml^{-1})	Mean of (10) replicate measurements	E%	SD	RSD%
4.0	29.3	-1.89	0.685	2.33
12.0	97.9	-3.69	1.178	1.20

Table 5. Effect of interferences on the peak height of 8.0 µg ml^{-1}AMX

Interference	Tolerance level	Peak height (mV)	E%
Glucose	100	59.2	3.68
Galactose	100	58.6	2.63
Fructose	100	55.7	-2.45
Sucrose	100	58.1	1.75
Lactose	100	55.8	-2.28
Starch	100	58.2	1.93
Sodium chloride	80	53.9	-4.3

Table 6. Comparison of present method with standard method[14]for determination of 8.0 µg ml^{-1}AMX using t-test and F-test

Formulation		Amount found(µg ml^{-1})		Absolute error	E%
		Present Method(FIA)	Standard method [14]		
Capsule	Amoxicillin S.D.I. (Iraq)	7.863	7.750	0.113	1.458
	Glomox Gobalpharma (U.A.E.)	7.782	7.857	-0.075	-0.955
	Penamox HIKMA (Jordan)	7.887	7.714	0.173	2.243
Tablet	Amoxicillin HEUMANN (Germany)	7.632	7.571	0.061	0.806
Injection	Acamoxil ACAI (Iraq)	8.356	8.393	-0.037	-0.441
	Largo penBilim (Turkey)	8.339	8.143	0.196	2.407
Mean		7.977	7.905	0.072	
SD		0.301	0.306	0.110	
$t_{calculated}$=1.595	t_{table}= 2.571				
$F_{calculated}$=1.036	F_{table}= 5.05				

4. CONCLUSION

Batch (direct spectrophotometric)and FI-spectrophotometric method for indirect determination of AMX have been developed and validated. The methods did not require the removal of excipients, any chemical sample pretreatment, solvent extractionstep, and expensive reagents and solvents. In these methods AMX was oxidized by excess amount of NBS in acidic medium, the excess of oxidant reacted with indigo carmine which bleaches the blue color of it, increasing AMX concentration lead to increase the intensity of the color.

The batch method was considered to be simple, rapid, sensitive, inexpensive and accurate method for the determination of AMX in pharmaceutical formulations. The method was applicable for low concentration of AMX with precision (RSD%; 0.58 - 2.50), high accuracy (E%; 2.9 - 3.3) and reasonable sensitivity in which the molar absorptivity was found to be $3.8740x10^4$L $mol^{-1}cm^{-1}$and sand ell's index 10 µg cm^{-2}. Beer's law was obeyed over the range of AMX concentration (0.2 – 6.0 µg ml^{-1}).

FI-spectrophotometric method can be successfully used for the determination of AMX in capsules, tablets, and injections with advantages simplicity, low cost, high sensitivity, high accuracy and precision. Sensitivity of this method was not less than that obtained for determination of AMX using FIA methods [15-16], that gave the linear ranges between 50.0 – 1200.0 µg $ml^{-1}$and 25.0 – 400.0 µg ml^{-1}respectively.

As comparison in sensitivity between batch and FIA techniques itseems that batch technique with linear range of 0.2 – 6.0 µg $ml^{-1}$and detectionlimit of 0.1 µg $ml^{-1}$was more sensitive than FIA technique with linear range of2.0 – 14.0 µg $ml^{-1}$and detection limit of 1.8 µg ml^{-1}, while FI method givemore precise results.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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