



## SPECTROPHOTOMETRIC DETERMINATION OF AMOXICILLIN IN PHARMACEUTICAL PREPARATIONS THROUGH DIAZOTIZATION AND COUPLING REACTION

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### Abstract

Two simple, rapid and sensitive spectrophotometric methods have been developed for the determination of amoxicillin (AMX) in pure form and pharmaceutical preparations. The proposed methods are based on a coupling reaction between AMX and diazotized p-amino benzoic acid or diazotized procain in alkaline medium to form an intense yellow, water-soluble dyes that are stable and have a maximum absorption at 435 nm using diazotized p- amino benzoic acid and 450 nm on using diazotized procain. The calibration graphs were linear over the concentration ranges of 0.4 to 10  $\mu\text{g mL}^{-1}$  and 0.4 to 14  $\mu\text{g mL}^{-1}$  with a limit of detection (LOD) of 0.1877  $\mu\text{g mL}^{-1}$  and 0.1916  $\mu\text{g mL}^{-1}$ , molar absorptivity of  $1.914 \times 10^4 \text{ L mol}^{-1}\text{cm}^{-1}$  and  $2.544 \times 10^4 \text{ L mol}^{-1}\text{cm}^{-1}$ , Sandell's sensitivity of  $21.912 \times 10^{-3} \mu\text{g ml}^{-1}$  and  $16.486 \times 10^{-3} \mu\text{g ml}^{-1}$  for diazotized p-amino benzoic acid and diazotized procain, respectively. The proposed methods were successfully applied to the determination of AMX in capsules and injections.

**Keywords:** spectrophotometry; diazotized p-amino benzoic acid; diazotized procain; amoxicillin; pharmaceutical analysis.

التقدير الطيفي للاموكسيسيلين في المستحضرات الصيدلانية بوساطة تفاعل الازوتة و الازدواج

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### الخلاصة

تم تطوير طريقتين تحليليتين للتقدير البسيط و السريع و الحساس للاموكسيسيلين بصورته النقية وفي المستحضرات الصيدلانية. اعتمدت الطريقة الاولى على تفاعل الازدواج بين الاموكسيسيلين مع كاشف بارا امينو حامض البنزويك المؤزوت والثانية على كاشف البروكاين المؤزوت في الوسط القاعدي لتكوين اصباغ صفراء

مستقرة وذائبة في الماء اعطت أقصى امتصاص عند طول موجي ٤٣٥ نانومتر للكاشف بارا امينو حامض البنزويك المؤزوت و ٤٥٠ نانومتر للكاشف بروكائين المؤزوت. يشير الرسم البياني للامتصاص مقابل التركيز بان قانون بير ينطبق ضمن المدى ٠,٤ - ١٠ مايكروغرام مل<sup>-1</sup> و ٠,٤-١٤ مايكروغرام مل<sup>-1</sup> ويحد كشف ٠,١٨٧٧ مايكروغرام مل<sup>-1</sup> و ٠,١٩١٦ مايكروغرام مل<sup>-1</sup> للكاشفين بارا امينو حامض البنزويك و البروكائين المؤزوتين على التوالي. تم تطبيق الطرق المقترحة بنجاح في تقدير الاموكسي المستحضرات الصيدلانية المغلفة (كبسول) والحقن

## Introduction

Amoxicillin is a semi-synthetic  $\beta$ -lactam antibiotic belonging to the group of penicillins. The chemical structure of amoxicillin consists of d-4-hydroxyphenylglycine side chain attached to 6-aminopenicillanic acid (6-APA) moiety. Because of its broad spectrum of bactericidal activity and therefore widespread use in medicines, various preparations of this drug alone including capsules, tablets, powder for oral suspension, and injections as well as in combination with other ingredients, e.g. amoxicillin/clavulanate tablets are commercially available<sup>(1)</sup>. Currently, several analytical methods for the quantitation of amoxicillin in pharmaceutical formulations have been reported. Examples of these methods are iodometric titration<sup>(2)</sup>, fluorometry<sup>(3)</sup>, chemiluminescence<sup>(4-7)</sup>, voltammetry<sup>(8-10)</sup>, spectrophotometry<sup>(11-15)</sup>, atomic absorption spectrometry<sup>(16)</sup>, liquid chromatography<sup>(17-20)</sup>, electrophoresis<sup>(21, 22)</sup>, colorimetry<sup>(23, 24)</sup>, and high-performance liquid chromatography (HPLC)<sup>(2, 25-27)</sup>. Though the latter method is specified in the pharmacopoeias, it requires instruments with high cost compared to the spectrophotometric assay. In addition, some chromatographic systems use a large amount of high purity organic solvents as a mobile phases and take a long system stabilization time. In this work, two rapid and sensitive methods using spectrophotometric detection at 435 and 450nm were proposed for the determination of AMX in pharmaceutical preparations. The methods are based on the diazotization reaction of p-amino benzoic acid and procaine with sodium nitrite in acid medium,<sup>(28)</sup> the formed diazonium salts are then coupled with AMX in alkaline medium to form a yellow water-soluble azo dyes. The proposed methods have been successfully applied to the determination of AMX in pharmaceutical preparations. The

methods are safe, simple, sensitive, selective and accurate.

## Experimental

### Apparatus

All spectral and absorbance measurements were performed on an Optima Spectrophotometer UV - VIS ( Japan) double-beam and using 1 cm quartz cells.

### Preparation of solutions

1-Amoxicillin trihydrate (AMX) stock standard solution ( $1000 \mu\text{g mL}^{-1}$ ) ( $2.38 \times 10^{-3}\text{M}$ ) was prepared by dissolving 0.100 g of pure AMX (SDI) in 5 mL of ethanol and made up to 100 mL volumetric flask with distilled water. Working standard solutions were prepared by suitable dilution of the stock standard solution with distilled water.

2- Sodium nitrite solution ( $1 \times 10^{-3}\text{M}$ ) was prepared by dissolving 0.0173 g of sodium nitrite (Merck) in distilled water and diluting to the mark in 250 mL volumetric flask.

3- Hydrochloric acid solution (1 M) was prepared by diluting 43 mL of 11.64 M of concentrated hydrochloric acid (BDH) with distilled water in 500 mL volumetric flask.

4- P-amino benzoic acid ( $1 \times 10^{-3}\text{M}$ ) solution was prepared by dissolving 0.0137 g of p-amino benzoic acid (BDH) in 2 mL of ethanol (BDH) and diluting to the marked with distilled water in 100 mL volumetric flask.

5-Procaine HCl:( pure standard drug {SDI} ) ( $1 \times 10^{-3}\text{M}$ ) was prepared by dissolving 0.0273 g of procaine HCl (SDI) in distilled water and diluting

to the mark in 100 mL volumetric flask.

6-Ammonium hydroxide solution (2 M) was prepared by diluting 74.9mL of 13.36M (Fluka) of concentrated ammonium hydroxide

with distilled water in 500 mL volumetric flask.

### Pharmaceutical preparations of Amoxicillin

Pharmaceutical preparations were obtained from commercial sources.

1-Amoline injections (Oubari pharma-Syria):500mg Amoxicillin.

2- Amoxicillin injections (Pan pharma-France):500mg Amoxicillin.

3-Amoxicillin capsules (Ajanta Limited-India): 500mg Amoxicillin.

4- Amoxicillin capsules (SDI-Iraq): 500mg Amoxicillin.

### General procedure for calibration

#### Method A

In method (A), a 1mL of ( $1 \times 10^{-3}$  M) p-amino benzoic acid was transferred into a series of 25mL calibrated flask. To this solution equimolar of sodium nitrite solution ( $1 \times 10^{-3}$  M) was added and the acidity was adjusted with 0.5 mL of 1 M hydrochloric acid solution. The solution was shaken thoroughly. Then, An aliquot of a standard solution( $100 \mu\text{g mL}^{-1}$ ) ( $2.38 \times 10^{-4}$ M) containing 0.1 – 2.5 mL ( $9.53 \times 10^{-7}$  -  $2.38 \times 10^{-5}$ M) of AMX was transferred into this series of 25 mL calibrated flasks and 1 mL of 2 M ammonium hydroxide solutions was added and the contents were diluted to the mark with distilled water and mixed well. After 15min, the absorbance of the colored azo dye was measured at 435nm against the corresponding reagent blank.

#### Method B

In method (B), a 1mL of ( $1 \times 10^{-3}$  M) procaine hydrochloride was transferred into a series of 25mL calibrated flask. To this solution equimolar of sodium nitrite solution ( $1 \times 10^{-3}$  M) was added and the acidity was adjusted with 0.5 mL of 1 M hydrochloric acid solution. The solution was shaken thoroughly. Then, An aliquot of a standard solution ( $100 \mu\text{g mL}^{-1}$ ) ( $2.38 \times 10^{-4}$ M) containing 0.1 – 3.5 mL ( $9.53 \times 10^{-7}$  -  $3.34 \times 10^{-5}$ M) of AMX was transferred into this series of 25 mL calibrated flasks and 0.6 mL of 2 M ammonium hydroxide solution was added and the contents were diluted to the mark with distilled water and mixed well. After 15min, the absorbance of the colored azo

dye was measured at 450nm against the corresponding reagent blank.

### Determination of the composition of the azo dyes in solution Mole-ratio method

In method A, a 2mL of ( $1 \times 10^{-3}$  M) amoxicillin solution was transferred into a series of 25mL volumetric flask. To this solution 0.5,1,1.5,----5.5mL of ( $1 \times 10^{-3}$  M) diazotized p-amino benzoic acid and 1mL of 2 M ammonium hydroxide solutions were added, the contents were diluted to the mark with distilled water and mixed well. After 15min, the absorbance of the colored azo dye was measured at 435nm against the corresponding reagent blanks.

In method B, a 2mL of ( $3.665 \times 10^{-4}$  M) amoxicillin solution was transferred into a series of 25mL volumetric flask. To this solution 0.5,1,1.5,----5.5mL of ( $3.665 \times 10^{-4}$  M) diazotized procaine and 0.6 mL of 2 M ammonium hydroxide solutions were added, the contents were diluted to the mark with distilled water and mixed well. After 15min, the absorbance of the colored azo dye was measured at 450nm against the corresponding reagent blank.

### Continuous variations method

In method A, different volumes (1-6mL) of amoxicillin solution ( $1 \times 10^{-3}$  M) were transferred to separate 25mL volumetric flask. To each flask, respectively was added 5,4,3,2,1,0 mL of the ( $1 \times 10^{-3}$  M) diazotized p-amino benzoic acid and 1mL of (2 M) ammonium hydroxide solutions, the contents were diluted to the mark with distilled water and mixed well. After 15min, the absorbance of the colored azo dye was measured at 435nm against the corresponding reagent blank. In method B, the procedure is the same as above but just the concentration of amoxicillin and diazotized procaine were  $3.665 \times 10^{-4}$  M and 0.6mL of ammonium hydroxide was added. The absorbance was measured at 450nm.

### Procedure for the assay of pharmaceutical preparations

1. Tablets solution ( $500 \mu\text{g mL}^{-1}$ )  
Weigh and finally powdered of 10 tablets, dissolve an accurately weighed portion of the powder equivalent to about 50 mg of AMX in 2.5 mL of ethanol and transferred into a 100 mL volumetric flask, and completed to the mark with

distilled water. Further appropriate solution ( $100 \mu\text{g mL}^{-1}$ ) was made up by simple dilution with distilled water. Two different concentrations ( $4 \mu\text{g mL}^{-1}$  and  $6 \mu\text{g mL}^{-1}$ ) of this tablets solution were analyzed in four replicate by analytical spectrophotometric procedure.

2- Vials ( $500 \mu\text{g mL}^{-1}$ )

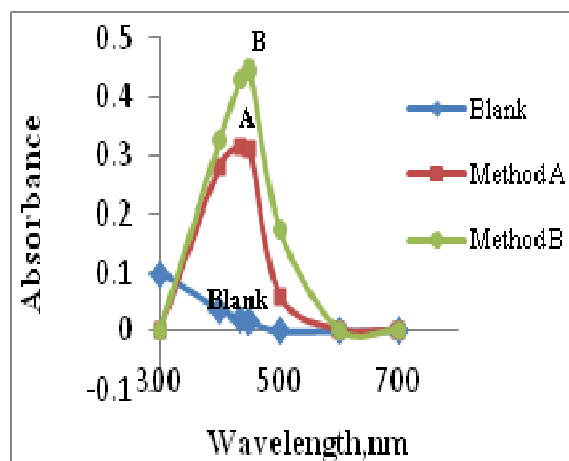
An accurate weighed amount of mixed content of 5 vials equivalent to 100mg of the pure drug was dissolved in 2.5 ml of ethanol. The solution was transferred into 200 ml volumetric flask; and diluted to the marked with distilled water.

Further appropriate solutions of pharmaceutical preparations were made up by simple dilution with distilled water.

## Results and discussion

### Absorption spectra

When a diluted aqueous solution of AMX was mixed with diazotized p-amino benzoic acid(method A) and diazotized procaine (method B) in alkaline medium, an intense yellow azo dyes formed immediately, which became stable after 15min. The yellow products have a maximum absorption at 435nm and 450nm for method A and B respectively. (Figure-1) shows the spectra of the products formed and the reagent blank, the maximum absorption at 435nm(method A) which produce from mixing [1mL of p-amino benzoic acid ( $1 \times 10^{-3}$  M), 1mL of sodium nitrite solution ( $1 \times 10^{-3}$  M), 0.5 mL of hydrochloric acid solution(1 M), 2.5mL of AMX ( $100 \mu\text{g mL}^{-1} = 2.38 \times 10^{-4}$ M) and 1mL of ammonium hydroxide(2M) and diluted to 25mL with distilled water] measured versus reagent blank[it contains all components above except of AMX] which has negligible absorbance at this wavelength. The maximum absorption at 450nm (method B) which produce by mixing[ 1mL of procaine ( $1 \times 10^{-3}$  M), 1mL of sodium nitrite solution ( $1 \times 10^{-3}$  M), 0.5 mL of hydrochloric acid solution(1 M), 2.5mL of AMX ( $100 \mu\text{g mL}^{-1} = 2.38 \times 10^{-4}$ M) and 0.6mL of ammonium hydroxide(2M) and diluted to 25mL with distilled water] measured versus reagent blank [it contains all components above except of AMX] which has negligible absorbance at this wavelength.



(Figure.-1): Absorbance spectra of AMX ( $10 \mu\text{g mL}^{-1}$ ) treated as described under procedure above (method A and B) and measured against blank, The reagent blank measured against distilled water

### Optimization of the experimental conditions

The effects of various parameters on the absorption intensity of the formed products were optimized.

The diazotization reaction of AMX was formed in acidic medium. Therefore the effects of different acids solutions (1 M) were studied such as hydrochloric acid, sulfuric acid, phosphoric acid and acetic acid. It was found that hydrochloric acid was the most suitable acidic medium for a maximum absorbance and was used in all subsequent experiments.

The coupling reaction of diazotized reagents with AMX was formed in alkaline medium. Therefore, the effects of different alkaline solutions (2 M) were studied such as sodium hydroxide, sodium carbonate, potassium hydroxide and ammonium hydroxide. It was found that ammonium hydroxide was the most suitable alkaline medium for a maximum absorbance for methods (A and B) and was used in all subsequent experiments.

The effect of different volumes of hydrochloric acid (1 M) were studied on the maximum absorbance by varying the volume of HCl between (0.05-2mL) and fixing the other parameters:( p-amino benzoic acid ( $1 \times 10^{-3}$  M) (method A), procain( $1 \times 10^{-3}$  M)(method B) and ammonium hydroxide (2 M).It was found that 0.5mL of HCL(1M) in both methods gave the

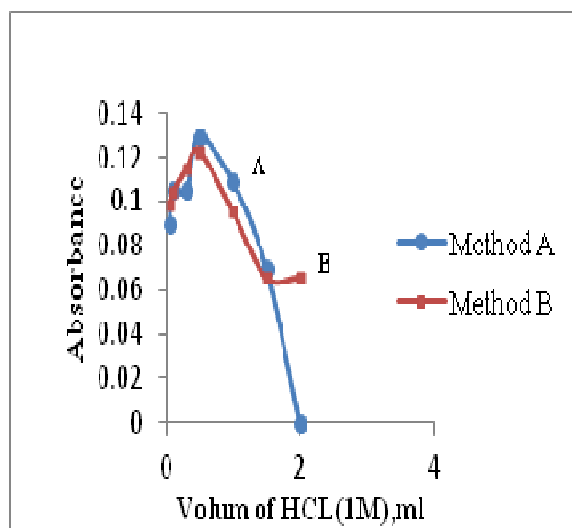
highest absorbance and was chosen for further use (Figure.-2).

Similarly, the effect of different volumes of ammonium hydroxide (2 M) was studied on the maximum absorbance by varying the volume of ammonium hydroxide solution between (0.1-4mL) with fixing the other parameters:( p-amino benzoic acid ( $1 \times 10^{-3}$  M) (method A), procain( $1 \times 10^{-3}$  M)(method B) and HCl(1M).A volume of 1mL, 0.6mL of ammonium hydroxide (2 M) for methods A and B respectively were enough to obtain the maximum absorbance.(Figure.-3).

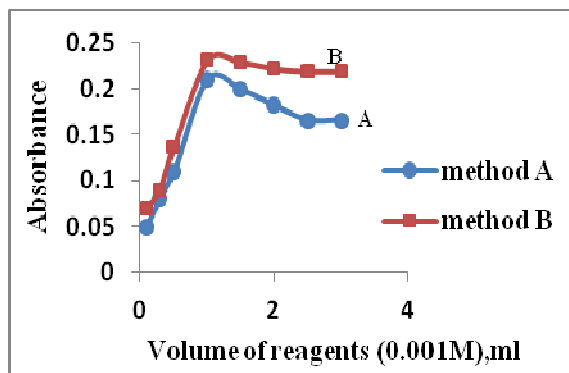
Effect of reagents (p-amino benzoic acid ( $1 \times 10^{-3}$  M) (method A) and procain ( $1 \times 10^{-3}$  M) (method B) were studied in the range of (0.1-3mL) with fixing the volume of HCl and  $\text{NH}_4\text{OH}$ .The greatest absorbance intensity were obtained with 1mL for both reagents.(Figure.-4).

#### Effect of order of addition

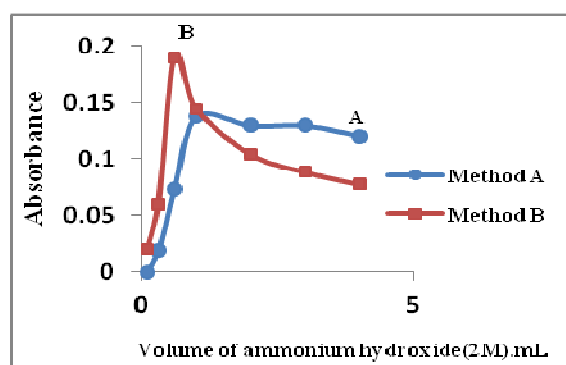
Different orders of addition of reagents were examined and it was found that the order of addition of reagents by mixing p-amino benzoic acid (method A) or procaine (method B) with sodium nitrite then HCl, AMX and ammonium hydroxide gave the highest absorbance and were used in all subsequent experiments.



(Figure.-2):Effect of the volume of HCl(mL).  
\*Obtained by varying HCl volume and fixing the volumes of  $\text{NH}_4\text{OH}$  and reagents.



(Figure.-3):Effect of the volume of reagents(mL). \*Obtained by varying the volumes of the reagents and fixing the volumes of  $\text{NH}_4\text{OH}$  and HCl.



(Figure.-4):Effect the volume of ammonium hydroxide(mL). \*Obtained by varying the volumes of ammonium hydroxide and fixing the volumes of HCl and the reagents.  
\*Method A using p-amino benzoic acid reagent, Method B using procain reagent.

#### Effect of reaction time

In spite of the rapid color development (formed immediately) the color intensity reached a maximum after AMX solution had been reacted with diazotized p-amino benzoic acid(method A), diazotized procain (method B) and ammonium hydroxide for 15min, therefore a 15 min development time was selected as optimum in the general procedures. The color obtained was stable for 2hr.

#### Structures of the products

The stoichiometry of the reaction between AMX and diazotized p-amino benzoic acid (method A) or diazotized procain (method B) were investigated under the recommended



talc, starch and poly vinyl pirrolidone (PVP) were studied by analyzing synthetic sample solutions containing  $4 \mu\text{g mL}^{-1}$  of AMX and excess

amounts (25-fold excess) of each excipient, none of these substances interfered seriously (**Table -1**)

**Table(1): Determination of  $4 \mu\text{g mL}^{-1}$  of AMX in the presence of excipients**

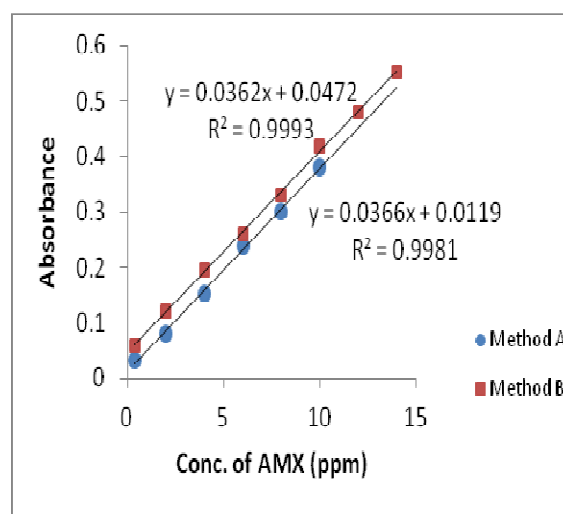
Method A				Method B		
Excipient  $100 \mu\text{g mL}^{-1}$	Conc. of AMX, $4 \mu\text{g mL}^{-1}$	$E_{\text{rel.}},^*$ %	Rec., %	Conc. of AMX, $4 \mu\text{g mL}^{-1}$	$E_{\text{rel.}}$ %	Rec., %
	Found			Found		
Starch	4.028	0.700	100.700	3.914	-2.150	97.850
Mg-stearate	3.945	-1.375	98.625	3.935	-1.625	98.375
Lactose	3.917	-2.075	97.925	3.892	-2.700	97.300
PVP	4.055	1.375	101.375	3.914	-2.150	97.850
Talc	3.945	-1.375	98.625	3.957	-1.075	98.925

$E_{\text{rel.}}$  = Relative error

### Analytical characteristics of spectrophotometric method

For the proposed methods, a calibration graph, were obtained by the procedures described previously and a series of standard solutions was analyzed in triplicate to test the linearity (**Figure-7**). The molar absorptivity ( $\epsilon$ ), the Sandell's sensitivity ( $S$ ), the slope ( $a$ ) and the intercept ( $b$ ) were determined and are included in (**Table -2**). The accuracy and precision of the proposed methods were tested by analyzing five replicate of AMX by proposed spectrophotometric methods for three different concentrations of AMX. The values of relative standard deviation RSD% and relative error  $E_{\text{rel.}}\%$  are summarized in the same table. These values indicated the high accuracy and precision of the proposed methods. The limit of detection (LOD) was determined by taking the ratio of the standard deviation (SD) of the blank

with respect to water and the slope of the calibration curve multiplied by the factor three<sup>(32)</sup>



(Figure-7): Calibration graph of AMX

### Pharmaceutical application

The proposed methods were applied successfully to the analysis of different pharmaceutical preparations (capsules and injections) containing AMX and the results are summarized in Table (3). For all preparations examined, the assay results of the proposed methods were in good agreement with the declared content. The results in (table-4)

are in accordance with those obtained by the official spectrophotometric method<sup>(31)</sup> using imidazole-mercury reagent<sup>(31)</sup> as being in pharmaceutical Samarra. Statistical analysis<sup>(32)</sup>, and by applying F-test and t- test, at 95% confidence level. The calculated values for F(4.668, 2.93) and t (0.419,0.57) for method A and B respectively did not exceed the critical values of  $F_{4,4} = 9.605$  and  $t = 2.306$  ( $n_1+n_2-2=8$ ). These confirming that there are no significant differences between the proposed methods with the official method with respect to precision and accuracy in the determination of AMX in pharmaceutical preparations.

### Conclusions

The proposed methods were found to be very simple, rapid, low cost, and fairly selective than some of the reported methods. They had an advantage of being accurate, did not require the removal of excipients, any chemical sample pretreatment, temperature control, pH control, solvent extraction step, and expensive reagents and solvents. The proposed method was applied to the analysis of AMX in capsules and injections, and can be used for the routine analysis of commercial formulations instead of the tedious official method.

(Table -2): Analytical parameters of spectrophotometric method

Parameters	Method A	Method B
$\lambda_{\max}$ (nm)	435	450
Linearity range, $\mu\text{g ml}^{-1}$	0.4-10	0.4-14
Molar absorptivity ( $\text{L mol}^{-1} \text{cm}^{-1}$ )	$1.914 \times 10^4$	$2.544 \times 10^4$
Sandell's sensitivity ( $\mu\text{g ml}^{-1}$ )	$21.912 \times 10^{-3}$	$16.486 \times 10^{-3}$
Regression equation	$Y = 0.0366X + 0.0119$	$Y = 0.0362X + 0.0472$
Linearity (r)	0.9990	0.9997
Limit of detection ( $\mu\text{g ml}^{-1}$ )	0.1877	0.1916
Relative standard deviation (RSD%)*	1.079	1.180
Average of recovery%	99.811	99.434
$E_{\text{rel}} \%$	-0.189	-0.566
Stability (hr.)	2	2
Molar ratio (D:R)	1:2	1:1
Color	Yellow	Yellow



**(Table-3): Application of the proposed methods for determination of AMX in pharmaceutical preparations**

Pharmaceutical preparation	Method A					Method B				
	Conc. of AMX, $\mu\text{g ml}^{-1}$		$E_{\text{rel}}$ , %	Rec., %	RSD %	Conc. of AMX, $\mu\text{g ml}^{-1}$		$E_{\text{rel}}$ , %	Rec., %	RSD %
	Present	Found*				Present	Found			
Amoline(injection 500mg) Oubari-pharma-Syria	4.000 6.000	4.046 6.044	1.150 0.733	101.150 100.733	0.873 0.779	4.000 6,000	3.979 5.977	-0.525 -0.383	99.475 99.617	0.884 0.314
Amoxicillin(injection 500mg) Pan pharma-France	4.000 6.000	3.963 5.975	-0.925 -0.417	99.075 99.583	1.225 0.417	4.000 6.000	4.041 6.068	1.025 1.133	101.025 101.133	0.711 0.692
Amoxicillin(capsule 500mg) Ajanta-Limited-India	4.000 6.000	3.981 5.950	-0.475 -0.833	99.525 99.167	1.080 0.341	4.000 6.000	3.959 5.908	-1.025 -1.533	98.975 98.467	0.513 0.389
Amoxicillin(capsule 500mg) SDI-Iraq	4.000 6.000	4.028 6.000	0.700 0.000	100.700 100.000	0.827 0.536	4.000 6.000	4.050 5.954	1.250 -0.766	101.250 99.233	0.647 0.546

\*Average of four determinations

**(Table-4): Comparison of the proposed methods with official method for determination of pharmaceutical preparations of AMX.**

Pharmaceutical preparation	Recovery, %		
	Proposed method (A)	Proposed method (B)	Official method <sup>(31)</sup>
Pure AMX	100.000	100.000	100.000
Amoline(injection)/Syria	100.942	99.546	102.500
Amoxicillin(injection)/France	99.329	101.079	101.000
Amoxicillin(capsule)-India	99.346	98.721	98.000
Amoxicillin(capsule)-Iraq	100.350	100.242	99.000

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