DEVELOPMENT OF SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF AMOXICILLIN BY DIAZOCOUPLING REACTION IN PHARMACEUTICAL FORMULATIONS

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TÓM TẮT

XÂY DỰNG PHƯƠNG PHÁP QUANG PHỔ XÁC ĐỊNH AMOXICILIN TRONG MỘT SỐ CHẾ PHẨM DƯỢC BẰNG CÁC PHẢN ỨNG GHÉP NỐI ĐIAZO HÓA

Hai phương pháp đơn giản, nhanh, nhạy và chính xác đã được xây dựng để xác định amoxicilin trong các chế phẩm dược bằng phương pháp quang phổ. Cơ sở của hai phương pháp dựa trên phản ứng ghép nối giữa amoxicilin với các muối diazo của anilin (phương pháp A) và axit sunfanilic (phương pháp B) trong môi trường kiềm tạo thành hợp chất azo màu vàng có cực đại hấp thụ tương ứng ở 398 và 435nm. Ảnh hưởng của các yếu tố nh nồng độ nitrit, thuốc thử, môi trường và thứ tự thêm thuốc thử đã được khảo sát. Khoảng nồng độ tuân theo định luật lambert-Beer là 0,5-12,0 µg/mL và 1,0-20,0 µg/mL với giới hạn phát hiện (LOD) là 0,098 µg. mL^{-1} và 0,166g. mL^{-1}, hệ số hấp thụ mol $2,99.10^4$ l. $mol^{-1}.cm^{-1}$ và $2,58.10^4$ $l.mol^{-1}.cm^{-1}$ tương ứng đối với phương pháp A và B. Các tá dược trong thành phần thuốc không gây cản đến phép xác định amoxicilin. Phương pháp đã được áp dụng thành công để xác định amoxicilin trong một số chế phẩm dược (viên nang và viên nén).

Keywords: *Amoxicillin, UV-Vis, phản ứng điazo hóa anilin, phản ứng điazo hóa sunfanilic*.

1. INTRODUCTION

Amoxicillin (AMX) *(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 is a β-lactam antibiotic used as an antibacterial drug in the treatment of infections caused by *gram-positive,gram-negative* bacteria (Fig. 1)[1].

A number of analytical methods have been reported for the determination of amoxicillin in pure drug, pharmaceutical dosage forms and biological samples using spectrophotometric[2-3], spectrofluorimetric [4], capillary electrophoresis [5], electrochemical analysis[6]. In the pharmacopoeia, liquid chromatography is used to determine AMX in various preparations such as tablets, capsules, sprays, oral and injectable [7]. Spectrophotometric methods are the most convenient techniques because of their inherent simplicity, high sensitivity, low cost and wide applicability in laboratories. Different spectrophotometric methods have been used in the determination of amoxicillin by using various reagents such as: diazotized reagents (o-nitroaniline, p-nitroaniline, p-amino benzoic acid, procaine and metoclopramide)[8-10], π-acceptor reagents (p-chloranil, tetracyanoethylene, 4-nitrophenol, 2,4-dinitrophenol, 3,5-dinitrobenzoic acid, 3,5-dinitrosalicylic acid) [11], redox reagents (Ce(IV), Fe(III), N-bromosuccinimide, N-chlorosuccinimide, Folin-Ciocalteu)[12].

However, the disadvantages of using these methods are that the reaction is often narrow linearity range, requiring heating or extraction, reaction time lasts, use of non-aqueous

systems, low stability of the colored product formed, many excipients and organic substances absorb light in the same spectrum range.

The present work is a development simple, sensitive and selective spectrophotometric method for determination of amoxicillin in dosage forms. Based on the diazotization of aniline and sulfanilic acid with nitrite ion in acidic medium to colorless diazonium ion, which subsequently coupled with amoxicillin to form a colored azo dye in the alkaline medium. The proposed methods were applied to the determination of amoxicillin in pharmaceutical preparation.

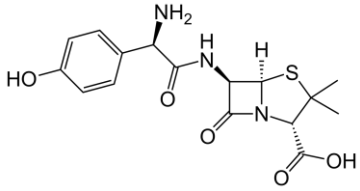

Figure 1. Chemical structure of amoxicillin

2. EXPERIMENTAL

2.1. Equipment and chemicals

A Biochrom Model SP-60 double beam, UV-Vis spectrophotometer (Biochrom Ltd., UK) with 1.0 cm matched quartz cells was used for absorbance measurements.

All chemicals used were of analytical grade and twice distilled water was used for dilution of reagents and samples. Amoxicillin trihydrate, aniline (Maya - R, China, certified to be 99%), sulfanilic acid (Merck, Germany, certified to be 99%) were used and other chemicals used were of analytical reagent grade.

The following available commercial preparations were analyzed: Amoxicillin capsules labeled to contain 500 mg of amoxicillin per capsule (Mekophar Chemical Pharmaceutical Joint Stock Company and Pharbaco, Viet Nam); Amoxicillin tablets labeled to contain 500 mg of amoxicillin and and 125 mg clavulanic acid per tablet (Agumentin^{TM}, Glaxo SmithKline, UK and Klamentin 625, DHG pharmaceutical joint – stock company, Viet Nam).

2.2. Preparation of solutions

A stock solution of amoxicillin (1mg/mL) in twice distilled water. The working standard solution of amoxicillin containing 100µg/mL was prepared by dilution with distilled water twice.

Sulfanilic acid solution (0.5%w/v) was prepared by dissolving 0.25 g of sulfanilic acid in 20 mL of a 1N hydrochloric acid solution. After dissolution, the volume was completed to 50 mL in a volumetric flask with twice distilled water.

Aniline solution (0.5%w/v) was prepared by dissolving 0.25 g aniline in 20 mL of a 1 N hydrochloric acid solution. After dissolution, the volume was completed to 50 mL in a volumetric flask with twice distilled water.

Hydrochloric acid solution, 1 N. This solution was prepared by diluting 8.5 mL of the concentrated acid to 100 mL with distilled water.

Sodium nitrite solution (0.5%w/v) was prepared by dissolving 0.25 g of sodium nitrite in twice distilled water and diluting to the mark in 50 mL volumetric flask.

Potassium hydroxide solution (0.5N) was prepared by dissoslving 2.81 g of potassium hydroxide in twice distilled water and diluting to the mark in 100 mL volumetric flask.

Sodium carbonate solution (l.0N) was prepared by dissoslv 5.30 g of sodium carbonate in twice distilled water and diluting to the mark in 100 mL volumetric flask.

2.3. Pharmaceutical preparations of amoxicillin

Capsules: Weigh and mix the contents of twenty capsules (each one contains 500 mg amoxicillin), an accurately weighed amount of powder equivalent 1g of amoxicillin transferred in to a 100 mL beaker. Using a magnetic stirrer, the powder was completely disintegrated in distilled water twice, filtered through a Whatman filter paper No 40 and diluted up to 100 mL with distilled water twice in a volumetric flask. Amoxicillin solution was preapared at the concentration of 100 ug/ml in distilled water twice The tablets were also prepared using the same procedure.

2.4. Procedure and calibration graph

Method A

Into a series of 10 mL volumetric flasks, to each flask 3.0 mL of sodium nitrit 0.5%), and 0.7 mL of anilin (0.5%) were added and a reaction time of 5 minutes at 0-5°C was given for completion of the reaction to colorless

diazonium ion. Next, an aliquot of a standard solution containing (0.5–12) µg of AMX was added to the diazo reagent followed by 0.7 mL of 5N potassium hydroxide solution and the mixture was left to stand for 5 min. Finally the volume in each flask was brought up to the 10 mL mark with twice distilled water. The absorbances of yellow-colored chromogen were measured at 398 nm against the reagent blank. The reagent blank is prepared in the same manner but without AMX. The colored chromogen was stable for 3 hours.

Method B

Into a series of 10 mL volumetric flasks, to each flask 2.5 mL of sodium nitrite (0.5%), and 0.7 mL of sulfanilic acid (0,5%w/v) were added and a reaction time of 5 minutes at 0-5°C was given for completion of the reaction to colorless diazonium ion. Next, 1.5mL of 1N Na_2CO_3 solution was added to the diazo reagent followed by standard solution containing (1.0-20) µg of AMX and the mixture was left to stand for 5 min. Finally the volume in each flask was brought up to the 10 mL mark with twice distilled water. The absorbances of yellow-colored chromogen were measured at 435 nm against the reagent blank. The reagent blank is prepared in the same manner but without AMX. The colored chromogen was stable for 3 hours.

2.5. Statistical analysis

Method was validated according to ICH Guidelines[13], in terms of linearity and range, accuracy and precision, limit of detection (LOD), limit of quantitation (LOQ). Calculation and processing of data were done using the programs Origin Pro 8.5.1(USA).

3. RESULTS AND DISCUSSION

3.1. Study of the optimum reaction conditions

The optimization of the methods was carefully studied to achieve complete reaction formation, highest sensitivity and maximum absorbance. The following parameters were optimized such as sodium nitrite concentration, reagent amount, alkaline medium and order of additions.

3.1.1. Effect of nitrite amount

The effect of adding various amounts of sodium nitrite solution on absorbance of 5 and 8 µg.mL^{-1} AMX for method A and B were examined. The amount of sodium nitrite was varied between 0.5-4.0 mL of 0.5% (w/v) sodium nitrite in water (Fig.2). Maximum absorbance was observed when the volume of 3.0 mL and 2.5 mL of 0.5% sodium nitrite for method A and B, respectively.

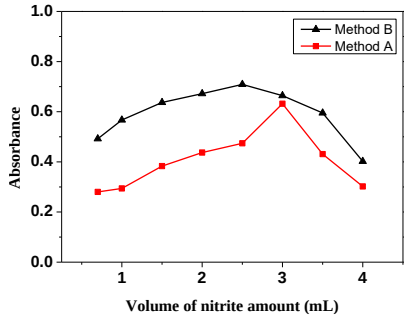

Figure 2. Effect of sodium nitrit amount on absorbance (The concentration of amoxicillin was 5 and 8µg.mL^{-1} for method A (λ_{max} = 398 nm) and B (λ_{max} = 435 nm), respectively)

3.1.2. Effect of reagent amount

The effect of aniline and sulfanilic acid amount on the colour intensity of the dye has been studied in the range of (0.1-2.0) mL (Fig.3). The greatest absorbance intensity was obtained with 0.7 mL for both reagents.

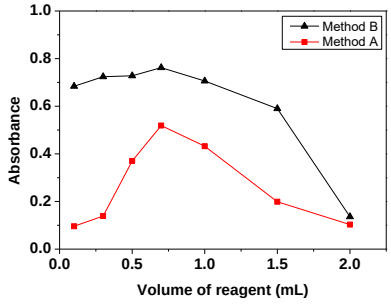

Figure 3. Effect of reagent amount on absorbance
(The concentration of amoxicillin was 5 and 8µg.mL^{-1} for method A (λ_{max} = 398 nm) and B (λ_{max} = 435 nm), respectively)

3.1.3. Effect of alkaline amount

The coupling reaction of diazotized reagents with AMX was formed in alkaline medium. Therefore, the effects of different alkaline solutions were studied such as sodium hydroxide, potassium hydroxide, ammonium hydroxide, sodium carbonate and sodium bicarbonate. It was found that potassium

hydroxide and sodium carbonate were the most suitable alkaline medium for a maximum absorbance for methods A and B respectively and were used in all subsequent experiments. The effect of different volumes of 0.5N KOH and 1N Na_2CO_3 were investigated by varying its volume between (0.3-2.5 mL) keeping other parameters constant. The results are depicted in Figure 4. The results were indicated that 0.7 mL of 0.5N KOH and 1.5 mL of 1N Na_2CO_3 for method A and B, respectively gave the highest absorbance for azo coupling reaction, so it has been selected in the subsequent experiments.

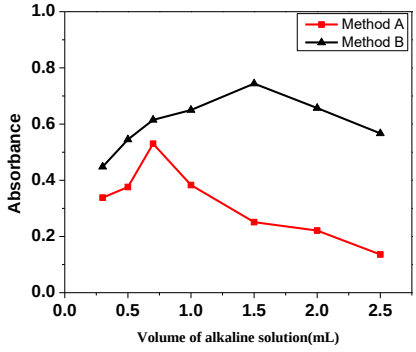

Figure 4. Effect of alkaline amount on absorbance
(The concentration of amoxicillin was 5 and 8µg.mL^{-} for method A (λ_{max} = 398 nm) and B (λ_{max} = 435 nm), respectively)

3.1.4. Order of addition of reagents

The effect of the order of addition on the absorbance of the product was studied under the optimum experimental conditions. Different orders of addition of reagents were examined and it was found that the order of addition of reagents by mixing sodium nitrite with aniline (method A) then AMX and potassium hydroxide or sodium nitrite with sulfanilic acid (method B) then sodium carbonate and AMX gave the highest absorbance and were used in all subsequent experiments. Any changes in the order of addition of reagents have an effect on the formation of azo-dye and the sensitivity of the system. The option was selected in order to optimize the results are:

Method A: $NaNO_2$ + Reagents + AMX + KOH
Method B: $NaNO_2$ + Reagents + Na_2CO_3 + AMX

3.1.5. Molecule ratio determination and mechanism of reaction

Job's method of continuous variation of equimolar solutions was employed: 2.74×10^{-3}M standard solution of AMX and 2.74×10^{-3}M solution of aniline, a 5.84×10^{-4}M standard solution of AMX and 5.84×10^{-4}M solution of sulfanilic acid for method A and B respectively, were used. The results obtained in (Fig.5) show that a 1:1 azo dye was formed between AMX and diazotized reagent for both method at 398 and 435nm, respectively.

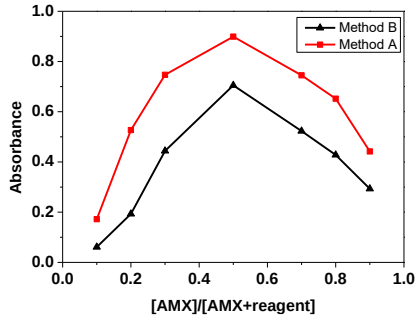

Figure 5. Job's method of continuous variation graph for the reaction of AMX with aniline (λ_{max} = 398 nm) and sulfanilic acid (λ_{max} = 435 nm))

The reactions of methods A and B occur in two steps:in the first step the reaction of aniline (method A) or sulfanilic acid (method B) with sodium nitrite occurs in an acid medium producing the diazo compound. In the second step, the diazo compound in alkaline medium coupled with the amoxicillin at the ortho position of the phenolic ring to form a yellow colored azo dye in an alkaline medium having λ_{max} at 398 nm (method A), 435 nm (method B). The yellow color azo dye is stable for about 3 h. The possible reaction mechanism had been shown in Figure 6.

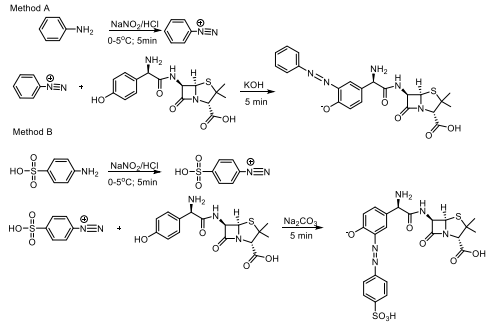

Figure 6. Proposed mechanism of the reaction between AMX and diazotized aniline or sulfanilic acid

3.1.6. Final absorption spectra

The absorbance spectra of the yellow products have a maximum absorption at 398 nm and 435nm for method A and B respectively are shown in Figure 7. The above mentioned blanks have practically negligible absorption in both systems

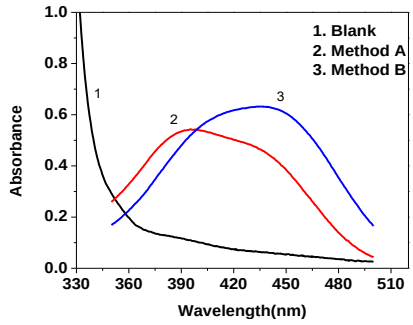

Figure 7. Absorbance spectra of method A and B (The concentration of amoxicillin was 5 and 8µg.mL^{-1} for A and B method, respectively)

3.2. Validation of the proposed method

The optical characteristics such as Beer's law limits, limit of detection (LOD) and quantification (LOQ), Sandell's sensitivity and molar absorptivity were calculated for the proposed methods and the results are summarized in Table 1. LOD and LOQ (LOQ) of the method are given by $3.3\times\frac{SD}{b}$ and $10\times\frac{SD}{b}$ respectively, where SD is the standard deviation of blank absorbance values, b is the slope of the calibration curve equation. The values of molar absorptivity, Sandell's sensitivity and LOD (Table1) indicate the sensitivity of the proposed methods and these values showed that method A more sensitive compared to method B.

The accuracy and precision of the methods were determined by preparing solutions of three different concentrations of amoxicillin and analyzing them in six replicates. The calculated standard deviation, percentage relative standard deviation (RSD%), percentage relative error (RE%) for the proposed methods and the results are presented in Table 2. The percentage relative error and percentage relative standard deviation calculated using the following equations:

RE(%)=[(founded–added)/added]x100;

RSD(%)= $\frac{SD}{\overline{X}}\times 100$; where SD is the standard deviation of the measurement values, $\overline{X}$ is the average value of the measurement.

The low values of the relative standard deviation percentage and relative error percentage specify the high precision and the good accuracy of the method.

Table 1. Analytical characteristics of the proposed methods

Parameters	Proposed methods	
	Method A	Method B
Reagent	Aniline	Sulfanilic acid
Colour	Yellow	Yellow
Wavelengths λ_{max} (nm)	398	435
Stability of colored products (h)	3	3
Stoichiometric ratio	1:1	1:1
Lambert-Beer law range (µg.mL^{-1})	0.5 – 12	1 – 20
Limit of detection (µg.mL^{-1})	0.098	0.166
Limit of quantitation (µg.mL^{-1})	0.294	0.554
Molar absorptivity (L.mol^{-1}.cm^{-1})	$2.99\text{x}10^{4}$	$2.58\text{x}10^{4}$
Sandell's sensitivity (µg/cm^2)	0.0157	0.0212
Regression equation (Y = bx +a)	Y=0.0818×x +0.042	Y=0.0707 ×x+0.0389
Slope (b)	0.0818	0.0707
Intercept (a)	0.0422	0.0389
Correlation coefficient (R^2)	0.998	0.997

Table 2. Evaluation of accuracy and precision of the proposed methods (n=6)

Method	Amount taken (µg.mL^{-1})	Amount found (µg.mL^{-1})	RSD (%)	RE (%)
Aniline	3.00	3.07	3.11	2.47
	6.00	5.97	3.65	-0.50
	10.00	9.92	3.08	-0.80
Sulfanilic acid	3.00	3.10	2.61	3.33
	6.00	6.02	2..23	0.33
	10.00	9.96	2.76	-0.40

3.3. Effects of interference

The effect of commonly utilized excipients in drug formulation was studied. The investigated amoxicillin was studied in the presence of various excipients e.g magnesium stearate, glucose, lactose, ascorbic acid The results indicated that there is no interference from the degradation, indicating a high selectivity for determining the studied AMX in its dosage forms.

3.4. Analysis of pharmaceutical preparations.

The two suggested methods were applied successfully to the determination of AMX in commercial capsule and tablet. Six replicate determinations were made. Table 3 shows that satisfactory recovery data were obtained and the recovery efficiency varies from 99.39 to 101.36% for Method A and 95.81 to 101.47% for method B indicating high accuracy of methods. The proposed method compares favorably with other reported methods. As shown in table 4 the proposed methods is more high sensitivity than other methods, needs no heating and are free from interference with common excipients.

Tablet 3. Results of analysis of capsule and tablet formulation containing AMX (n=6)

Pharmaceutical preparation	Labeled amount (mg/form)	Recovery (%) ± SD	
		Method A	Method B
Amoxicillin (Mekophar)	500 mg/capsule	99.39±0.17	96.64±0.20
Amoxicillin (Pharbaco)	500 mg/capsule	101.36±0.16	95.81±0.23
Amoxicillin (Agumentin)	500 mg/tablet	100.01±0.26	97.50±0.21
Amoxicillin (Klamentin)	500 mg/tablet	100.20±0.16	101.47±0.22

Table 4. Comparison of the proposed methods with other spectrophotometric methods

N_o	Reagent	λ_{max} (nm)	Range of determination ($\mu g.\ mL^{-1}$)	Molar absorbitivity ($L.mol^{-1}.cm^{-1}$)	Remarks	Ref.
1	O-nitroaniline	440	1-5	7.1×10^3	Narrow linear range	[8]
2	Metoclopramide	479	0.3-3.0	2.35×10^5	Narrow linear range and involved extraction steps with micelles of a non-ionic surfctant	[9]
3	4-nitrophenol	445	1-18	2.2×10^4	Involves contact time and heating	[11]
4	2,4-dinitrophenol	438	1-20	2.2×10^4		
5	3,5-dinitrobenzoic acid	336	1-24	2.8×10^4		
6	3,5-dinitrosalicylic acid	429	1-22	2.6×10^4		
7	Ce (IV)	397	5-30	6.3×10^3	Less sensitive	[12]
8	Fe (III)	397	5-30	5.8×10^3		
9	Sodium1,2-naphthoquinone-4-sulfonate	468	0.8-120	3.91×10^3	Involves contact time and less sensitive	[14]
10	Mo (V)–thiocyanate	467	7.5–85	5.28×10^3	Less sensitive and and involved extraction steps	[15]
11	Aniline	398	0.5-12	2.99×10^4	High sensitive	This paper
	Sulfanilic acid	435	1.0-20	2.58×10^4		

4. CONCLUSIONS

This article reports the use of aniline (method A) and sulfanilic acid (method B) as diazotized reagents for the spectrophotometric determination of amoxicillin. Method A was found to be more sensitive compared to method B for the assay of AMX. No interference from common excipients was encountered. The proposed methods are found to be simple, sensitive, selective, accurate, precise and can be used in the determination of amoxicillin in different pharmaceutical preparations (capsules and tablets).

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