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Spectrophotometric Determination of Sulfamethoxazole in Pure and in Pharmaceutical Preparations by Diazotization and Coupling Reaction

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ABSTRACT

A highly sensitive, simple and accurate spectrophotometric method has been developed for quantitative determination of sulfamethoxazole(SMX) in both pure form and pharmaceutical preparations. In this method SMX is diazotized with equimolar of sodium nitrite($NaNO_2$) in acid medium of hydrochloric acid to form diazonium ion , which is reacted with 2,4,6-trihydroxybenzoic acid in alkaline medium of NaOH to form a yellow water soluble azo dye that has absorption maximum at 416 nm versus reagent blank. Beer's law is obeyed over the concentration range 0.2-16 µg. ml^{-1} with an excellent determination coefficient (r^2= 0.9996) and molar absorptivity 1.84×10^4 $l.mol^{-1}.cm^{-1}$. The recoveries are obtained in the range of 97.8 - 99.8% and the relative standard deviation is better than ±0.23% . The stoichiometry of the resulting azo dye has been also worked out and it is found to be 1:1 SMX: 2,4,6-trihydroxybenzoic acid. This method has been applied successfully for the determination of SMX in pharmaceutical preparations (tablets and oral suspension).

Keywords: Sulfamethoxazole; Diazotization; 2,4,6-Trihydroxybenzoic acid; Spectrophotometry.

التقدير الطيفي للسلفاميثوكسازول بحالته النقية وفي المستحضرات الصيدلانية بوساطة الازوتة والاقتران

الملخص

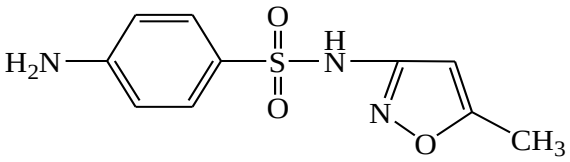
يتضمن البحث تطوير طريقة طيفية بسيطة و حساسة لتقدير كميات مايكروغرامية من السلفاميثوكسازول بحالته النقية وفي المستحضرات الصيدلانية. تعتمد الطريقة على ازوتة السلفاميثوكسازول في وسط حامضي من حامض الهيدروكلوريك مع كمية متكافئة من نتريت الصوديوم لتكوين ايون الديازونيوم الذي يتفاعل مع كاشف 6,4,2- ثلاثي هيدروكسيل حامض البنزويك في محلول قاعدي من هيدروكسيد الصوديوم لتكوين صبغة الازو ذات اللون الأصفر، المستقرة و الذائبة في الماء والتي أعطت أعلى امتصاص عند الطول ألموجي 416 نانوميتر وكان مدى الخطية الذي ينطبق على قانون بير يقع ضمن مدى التركيز من 0.2-16 مايكروغرام. مللتر$^{-1}$. وقيمة معامل التقدير(r^2= 0.9996) و قيمة الامتصاصية المولارية 1.84×10^4 لتر.مول$^{-1}$.سم$^{-1}$، بينما تراوحت قيم نسبة الاسترجاع بين 97.8 و 99.8 % بانحراف قياسي نسبي أفضل من ±0.23 وكانت النسبة التركيبية المولية بين السلفاميثوكسازول و الكاشف 6,4,2- ثلاثي هيدروكسيل حامض البنزويك 1:1، وقد طبقت الطريقة بنجاح لتقدير السلفاميثوكسازول على بعض المستحضرات الصيدلانية الحاوية على السلفاميثوكسازول في الأقراص الدوائية والمحلول المعلق.

الكلمات الدالة: سلفاميثوكسازول، ازوتة واقتران، 6,4,2- ثلاثي هيدروكسيل حامض البنزويك، تقدير مطيافي.

INTRODUCTION

Sulfamethoxazole (SMX) is a sulfonamide bacteriostatic antibiotic that is a highly effective chemotherapeutic agent, which competitively inhibits the bacterial enzyme dihydropteroate synthetase (Mandel and Petri, 2006). Sulfonamides are structural analogs and competitive antagonists of para-aminobenzoic acid (PABA). They inhibit normal bacterial utilization of PABA

for the synthesis of folic acid, an important metabolite in DNA synthesis (Mitscher, 2002). SMX is mostly marketed in combination with trimethoprim (TMP) as a co-trimazole dosage (Nazer *et al.*, 2001). A combination of TMP/SMX is an effective antimicrobial agent that is commonly used in dairy cattle for the treatment or prevention of respiratory infections and mastitis (Bedor *et al.*, 2008). SMX is short to medium acting agents used almost exclusively to treat urinary tract infections, eye infections and as a prophylaxis of rheumatic fever (Petri, 2001). It is commonly used to treat pneumoig, tuberculosis, mningitis and tonsillitis. SMX is white crystallized powder. It does not dissolve in ether and chloroform solvents. It has low solubility in water, but it dissolves in acetone (1 :30) and in ethanol (1: 50). On the other hand, it dissolves in alkaline hydroxide solutions (Barragry, 1994). SMX is chemically known as [4-amino -N-(5-methylisoxazole-3-yl) benzenesulfonamide] Fig. (1).



M.wt. = 253.28 g / mol

Fig. 1: The chemical structure of SMX

Several analytical techniques have been reported for determination of SMX in pharmaceutical formulations and biological fluids. Most of these techniques employed separation methods, such as solid phase extraction–liquid chromatography using an on line clean-up column coupled with amperometric detection employing a boron-doped diamond (BDD) electrode (Andrade *et al*., 2009), HPLC-tandem mass spectrometry (LC-MS/MS) (Mistri *et al.*, 2010) or capillary electrophoresis (Qing-Cui *et al.*, 2008). Other techniques such as differential pulse voltammetry (DPV) (Joseph and Kumar, 2010), square wave voltammetry (SWV) (Souza *et al.*, 2008), micellar electrokinetic capillary chromatography (Injac *et al.*, 2008), partial least square regression method (Givianrad *et al.*, 2013), flow injection system /HPLC (Sabriye *et al.*, 2011) and ratio derivative spectrophotometry (Hajian *et al.*, 2010) have been also used for determination of SMX. Most of these techniques are time consuming and expensive, as well as the most potentiometric methods which are used SMX as ion-selective electrodes are either not readily available in the market or expensive.

Many spectrophotometric methods have been also used for the determination of SMX in pharmaceutical preparations. Most of them included diazotization reaction of SMX and coupling with different coupling agents such as phloroglucinol (Upadhyay *et al.*, 2012), pyrogallol (Othman, 2005), 1-naphthol (Sinan and Al-Uzri, 2011), tropaeolin O (Boiko *et al*., 2011), γ-resorsolic acid (Mohammed and Zamel, 2017), 2-Naphthol (Shamsa and Amani, 2006) and diphenylamine (Khalaf *et al*., 2014). Other methods were either based on the charge transfer reaction with chloranilic acid to form complex (Adegoke *et al*., 2017), condensation reaction with 1,2-napthoquinone-4-sulphonic acid (Khalaf *et al*., 2017), Schiff's base reaction with p-dimethylaminobenzaldehyde (Siddappa *et al.*, 2011) or oxidation–reduction reaction with ferric ions and potassium ferricyanide by using resorcinol as reagent (Vijaya *et al.*, 2008). Some of these methods suffer from various limitations for example, low stability of the colored product formed and laborious (Adegoke *et al*., 2017). Others required heating, extraction (Upadhyay *et al.*, 2012), applicable to higher concentrations of the drug (Shamsa and Amani, 2006) or long time for the reaction to complete (Siddappa *et al.*, 2011), In the present study, a new coupling agent is employed to develop a simple, sensitive and inexpensive spectro-photometric method for the assay of SMX in both pure and in its dosage forms. The method is based on the diazotization reaction of SMX with equimolar of sodium nitrite in acid medium; the formed diazonium ion is then coupled with 2,4,6-trihydroxybenzoic acid in sodium hydroxide medium to form a yellow water soluble azo dye. This method does not need to get rid of

excess sodium nitrite (by addition sulfamic acid or ammonium sulfamate) because of the low concentration of sodium nitrite used by adding equimolar solution of SMX and sodium nitrite.

EXPERIMENTAL

Apparatus

All absorption spectra and absorbance measurements are performed using a CECILL CE 7200 recording spectrophotometer with 1-cm silica cells. The pH measurements are made with a professional TRANS BP 300.

Reagents

All experiments were performed with analytical – reagent grade chemicals.

SMX stock solution (500 µg / ml). Accurately weighed 0.05g of SMX (SDI-Iraq) was dissolved in 5 ml of ethanol and the volume was completed to 100 ml with distilled water. Working solution (200 µg / ml=$7.89x10^{-4}$ M) of SMX was prepared by diluting appropriate volume of the stock solution with distilled water.

2,4,6-Trihydroxybenzoic acid solution (0.1%w/v). It was prepared by dissolving 0.1g of 2,4,6-trihydroxybenzoic acid provided by (Fluka) in distilled water and completed to the mark in 100 ml calibrated flask. The solution was then transferred to a dark bottle . This solution was stable for at least one week.

Sodium nitrite solution ($2.89x10^{-3}$ M). This solution was prepared by dissolving 0.0200g of sodium nitrite in 100 ml distilled water. Working solution ($7.9x10^{-4}$ M) of sodium nitrite was then prepared by diluting 27.3 ml of the stock solution with distilled water in a 100 ml volumetric flask.

HCl (1M) and NaOH (1M)solutions. These solutions were also prepared.

Analytical Procedure for Calibration Curve

An aliquot 0.05-2.0 ml of a standard solution of SMX (200 µg/ml = 7.89×10^{-4} M) was transferred into a series of 25 ml calibrated flasks. To each flask an equimolar of sodium nitrite solution 7.89×10^{-4} M was added and followed by 2 ml of 1M hydrochloric acid solution and mixed thoroughly. After 3 minutes, 2 ml of 0.1% 2,4,6-trihydroxybenzoic acid and 2 ml of 1M sodium hydroxide solutions were added. The flasks were kept at room temperature (25 C° ±2) for 4 minutes and the contents were completed to the marks with distilled water and mixed well, then the absorbance of the product was measured at 416 nm against the corresponding reagent blank.

A linear relationship between absorption and SMX concentration in the range 10-400 µg of SMX /25ml was obtained. The apparent molar absorptivity has been found to be 1.84×10^{4} $l.mol^{-1}.cm^{-1}$. Fig. (2).

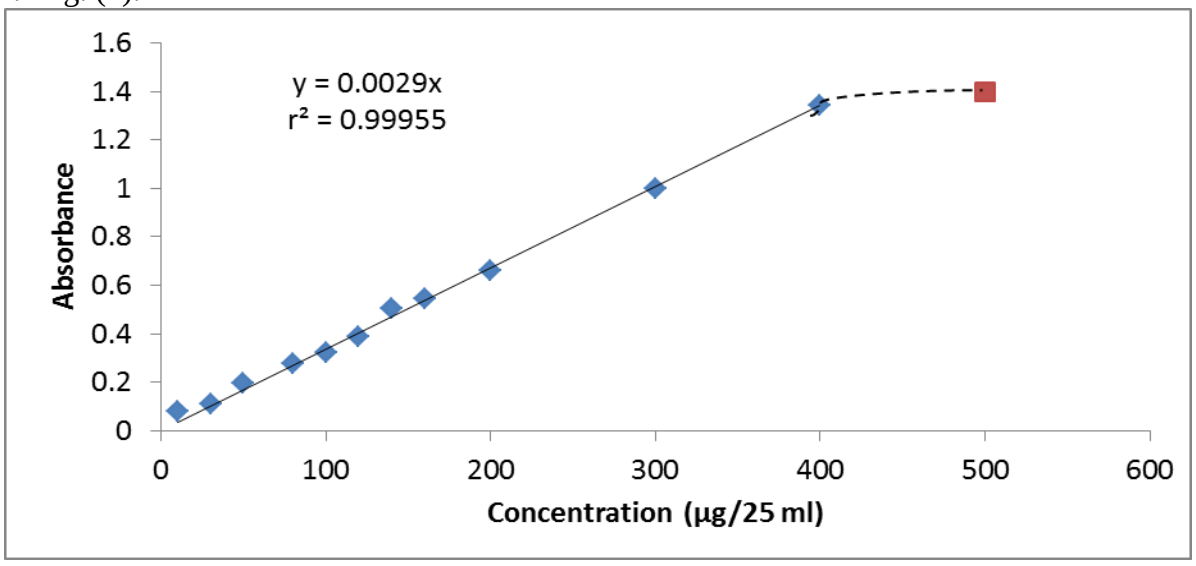


Fig. 2: The calibration curve for SMX determination

Procedure for the Assay of Pharmaceutical Preparations

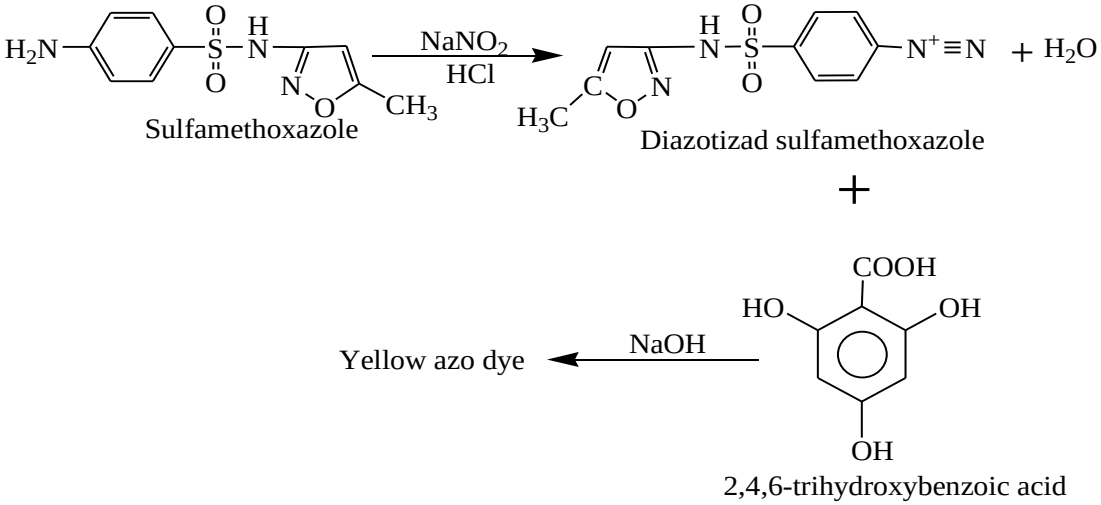
For tablets. Ten tablets (each containing 400 mg SMX and 80 mg TMP/ tablet) were weighed and crushed to powder. A portion of this powder, equivalent to 0.0500 g of SMX was weighed accurately and dissolved in 5ml of ethanol. The solution was mixed, warmed if necessary and filtered into a 100 ml volumetric flask. The residue was then washed with 5 ml of ethanol and the volume was diluted to the mark with distilled water. Each ml of this solution contains 500 µg SMX. Working solution (200 µg/ml =$7.9x10^{-4}$ M) of SMX is prepared by diluting 40 ml of the stock solution with distilled water into a 100 ml volumetric flask. An aliquot of the diluted drug solution was then treated as done in a recommended procedure.

For oral suspension, (200 mg SMX and 40 mg TMP /5 ml). A suitable volume 1. 25 ml of the oral suspension containing about 0.05 g of SMX was diluted with 5ml of ethanol and a portion of distilled water. The solution was filtered into 100 ml calibrated flask and the residue was washed with 5 ml of ethanol and finally the volume was diluted to the mark with distilled water to obtain a solution contains 500 µg/ml of SMX . Working solution (200 µg/ml =$7.89x10^{-4}$ M) of SMX was prepared by diluting 40 ml of the stock solution with distilled water into a 100 ml calibrated flask. An aliquot of the diluted drug solution was then treated as done in a recommended procedure.

RESULTS AND DISCUSSION

Principle of the colour reaction

Under the reaction conditions, SMX was diazotized with equimolar of sodium nitrite solution 7.89×10^{-4} M in the presence of acid solution of HCl to give the diazonium salt. The diazonium salt was then reacted with 2,4,6-trihydroxybenzoic acid as a coupling agent in alkaline solution of NaOH to form a coloured azo dye . A reaction sequence is shown in Scheme (1) .



Scheme (1) reaction sequence

Absorption maxima at 416 nm was exhibited due to formation of coloured azo dye. The formed coloured dye was exhibited maximum absorption at 416 nm against reagent blank solution. The intensity of the formed dye has been found to be proportional to the amount of SMX originally present in solution

Optimum Reaction Conditions

The effects of various parameters on the absorption intensity of the formed product were optimized. In the subsequent experiments, 1 ml of SMZ solution (200 µg/ml=7.89×10^{-4} M) with equimolar of sodium nitrite solution (1 ml of 7.89×10^{-4} M) was taken in 25 ml final volume and mixed with 1.5 ml of 1M hydrochloric acid, 1.5 ml of 2,4,6-trihydroxy- benzoic acid (0.1%) and 2 ml of 1M base and diluted to the mark with distilled water. The absorbance of solutions was measured at 416 nm versus reagent blank. This method does not need to get rid of excess sodium

nitrite (by addition of sulfamic acid or ammonium sulfamate) because of the low concentration of sodium nitrite used in equimolar solution of SMX and sodium nitrite.

Effect of Diazotization Acid

The effects of various acids solutions (conc.=1M) such as, HCl, CH_3COOH, HNO_3, H_2SO_4 and HCOOH have been investigated in diazotization of SMX in order to produce intense coloured azo dye and lower blank value. The experimental investigations showed that HCl was the most suitable acidic medium for obtaining maximum absorbance and it was used in all subsequent experiments. The effect of different volumes 0.5−3 ml of 1M HCl has been examined on the maximum absorbance of the formed product. Fig. (3) shows that 2 ml of 1MHCl were enough to obtain the maximum absorbance.

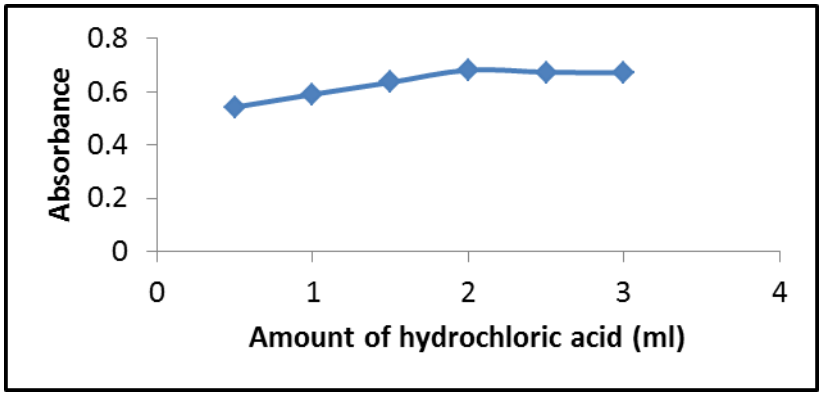


Fig. 3: Effect of the amount of 1M HCl on absorbance

The effect of temperature on diazotization was also studied. It was found that diazotization at 0-30 C° gave maximum colour intensity. The experimental results showed that the effect of time for 2 minutes or more gave the same results, so 3 minutes was selected for studies gives maximum colour intensity.

Effect of 2,4,6-Trihydroxybenzoic Acid Amount

The influence of various amounts of 2,4,6-trihydroxybenzoic acid as a coupling reagent on the formation of azo dye was investigated. The results in (Table 1) indicated that 2 ml of 0.1% 2,4,6-trihydroxybenzoic acid are the more suitable to give high absorbance value for the azo dye and can be considered optimum.

Table 1: Effect of 2,4,6-trihydroxybenzoic acid amount on absorbance

ml of 0.1% reagent	Absorbance / µg of SMX in 25 ml					r^2
	20	30	40	50	100	
0.5	0.065	0.103	0.119	0.166	0.239	0.97605
1.0	0.083	0.115	0.124	0.182	0.267	0.98142
1.5	0.097	0.129	0.144	0.195	0.281	0.98497
2.0	0.103	0.139	0.171	0.201	0.385	0.99949
2.5	0.098	0.111	0.145	0.176	0.289	0.99695
3.0	0.087	0.102	0.123	0.149	0.245	0.99896

The formation of azo dye was required 1minute for complete colour development after addition of 2,4,6-trihydroxybenzoic acid.

Effect of Base

The reaction of diazotized SMX with 2,4,6-trihydroxybenzoic acid was carried out in basic medium. Therefore, the effects of various alkaline solutions (conce=1M) were investigated such as, NaOH, $NaCO_3$, KOH and NH_4OH. The experimental investigations showed that the formation of the azo dye required a strong basic solutions of NaOH and KOH. While $NaCO_3$ and NH_4OH exhibited weak colour contrast which is apparently due to pH variation. The most suitable basic solution to give maximum absorbance is NaOH solution and it was employed in all subsequent experiments. The effect of different amounts 0.5−3 ml of 1M NaOH has been investigated on the absorbance of the formed product. Fig. (4) shows that 2 ml of 1M NaOH are enough to obtain high sensitivity.

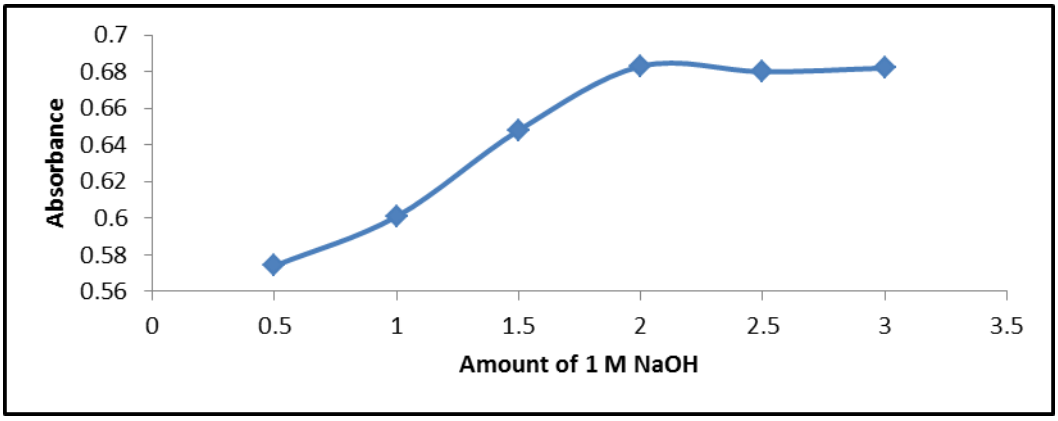


Fig. 4: Effect of the amount of 1M sodium hydroxide on absorbance.

Effect of Time on Colour Development

The effect of time on the stability of the coloured azo dye at 416 nm has been carried out by preparing two different amounts (50 and 200 µg) of SMX under the optimal experimental conditions, and the absorbance was measured at different time intervals up to 120 minutes. The results in Fig. (5) show that the absorbance reached maximum value after the reaction mixture solution was allowed to stand for 4 minutes and the absorbance remained maximum and constant for at least 120 minutes at room temperature.

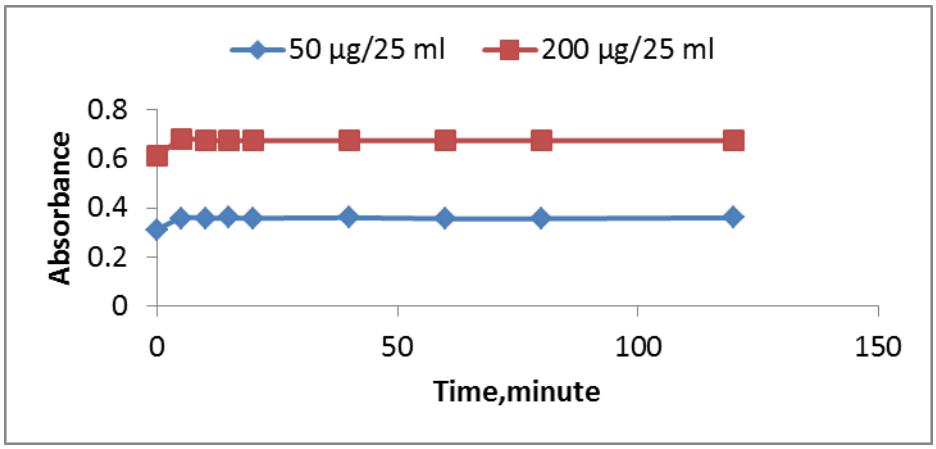


Fig. 5: Effect of time and amount of SMX on absorbance.

Final Absorption Spectra

Under the above optimized conditions, a yellow azo dye is formed by coupling of diazotized SMX with 2,4,6-trihydroxybenzoic acid in alkaline medium. This coloured dye exhibits maximum absorption at 416 nm against reagent blank as shown in Fig. (6). The corresponding reagent blank show a negligible absorbance at this wavelength.

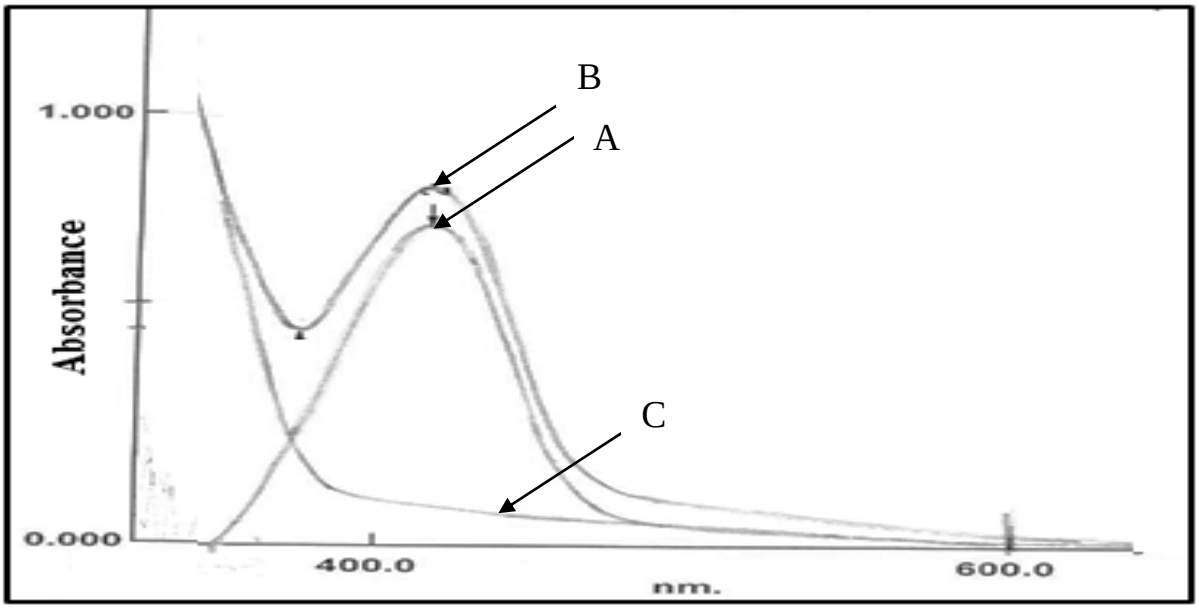


Fig. 6: Absorption spectra of (A) (8µg.ml^{-1}) of SMX (A) SMX verses reagent blank, (B) (8µg.ml^{-1}) of SMX verses distilled water, and (C) blank verses distilled water

Accuracy and Precision

The accuracy (recovery, %) and precision (R.S.D, %) of the proposed method were checked by analysis three different concentrations of SMX. The results in (Table 2) indicate that the method is satisfactory.

Table 2: Accuracy and precision of the method

Amount of SMX (µg/ml)		Error (%)	Recovery(%)*	R.S.D(%)*
Present	Found			
20	19.57	- 2.15	97.85	± 0.23
50	49.58	- 0.84	99.16	± 0.23
200	198.53	- 0.74	99.27	± 0.12
400	399.2	- 0. 20	99.80	± 0.06

* Average of five determinations.

Composition of azo dye

The stoichiometry of the product was investigated using the mole ratio and continuous variation methods. In mole ratio method, increased volumes 0.5 – 6 ml of 7.89 × 10^{-4} M 2,4,6-trihydroxybenzoic acid solution (V_R) were added to a 2 ml of 7.89 × 10^{-4} M of SMX (V_S) which was diazotized by using 2 ml of 7.89 × 10^{-4} M sodium nitrite in presence of 2ml of 1M HCl, 2 ml of 1M NaOH was added and the absorbances were measured at 416 nm after dilution to the mark with distilled water. In continuous variation method, volumes 0.5 – 4.5 ml of 7.89 × 10^{-4} M portions of SMX (V_S) were diazotized using equimolar of 7.89×10^{-4} M sodium nitrite and 2 ml of 1M HCl and coupled according to analytical procedure with the corresponding complementary volume of 7.89 ×10^{-4} M 2,4,6-trihydroxybenzoic acid solution (V_R) to give a total volume of 5 ml for V_S+ V_R in 2 ml of 1M NaOH and diluted to 25 ml with distilled water. The results obtained in Fig.7 and Fig.8 show that a 1:1 azo dye is formed between diazotized SMX (S) and 2,4,6-trihydroxybenzoic acid (R).

For the diazotization reaction, it would be expected that NH_2 group in SMX would be readily diazotized in HCl solution, and that diazonium ion would then react with a molecule of 2,4,6-

trihydroxybenzoic acid by electrophilic substitution at the 4-position of the coupling agent to produce an intense yellow azo dye in sodium hydroxide medium.

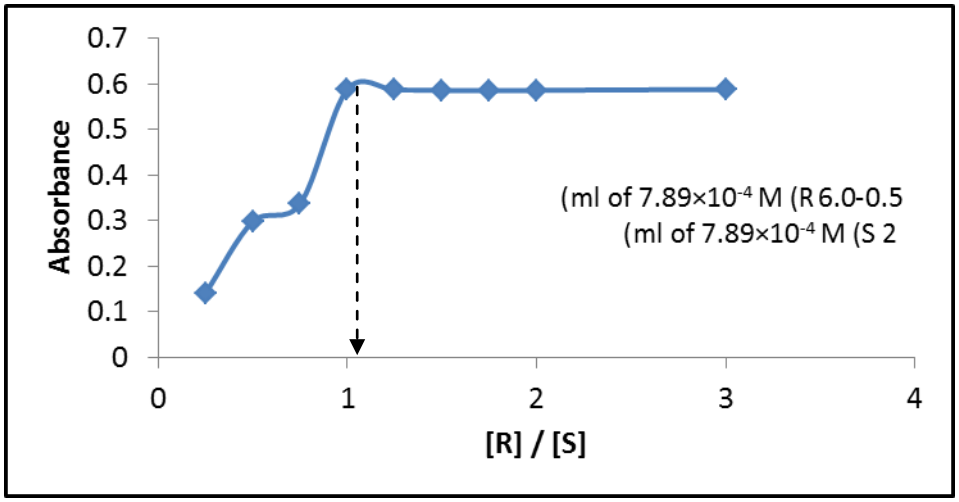


Fig. 7: Mole ratio plot

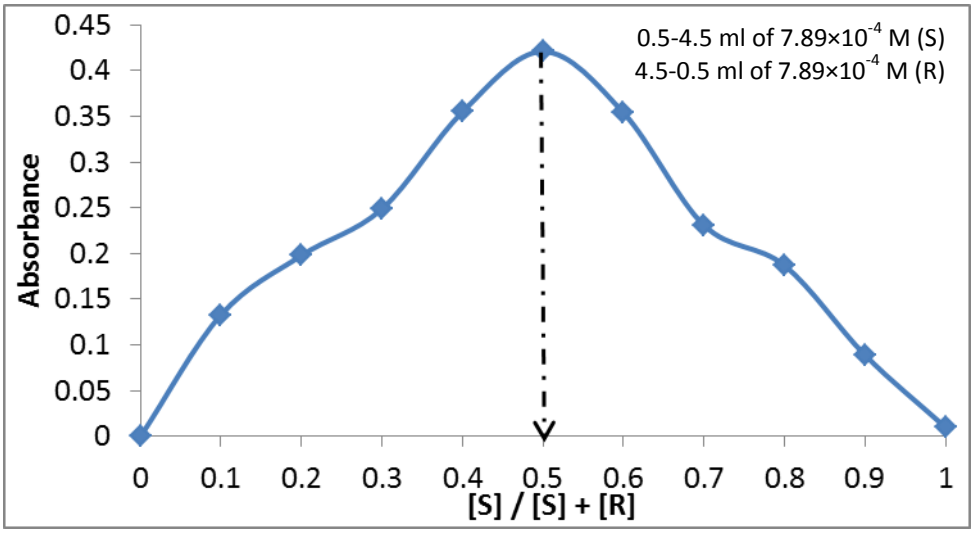
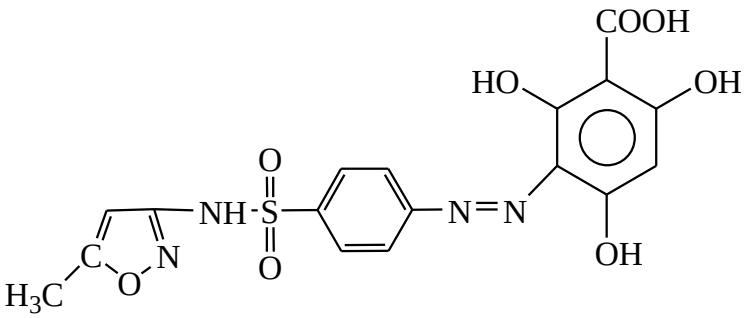


Fig. 8: Continuous variation plot

According to the results obtained in Fig. (7) and Fig. (8) The formation of the product azo dye can be written as follows Fig. (9):



Yellow azo dye

Fig. 9 : The composition of yellow azo dye

The effect of some common excipients frequently found with SMX in dosage forms such as: glucose, starch, lactose, sucrose and trimethoprim was investigated by adding different amounts of excipient to 200 μg of SMX. The results in (Table 3) indicate that there are no significant interferences produced by these excipients on the proposed procedure.

Table 3: Determination of SMX in the presence of excipients

Excipients	Recovery (%)* of 200 μg SMX / μg foreign compound added		
	100	250	500
Starch	99.4	100.3	100.6
Glucose	98.3	99.7	100.0
Lactose	99.0	98.8	100.2
Sucrose	101.9	101.6	102.2
Trimethoprim	100.7	102.8	114.2

* Average of five determinations

Pharmaceutical Applications

The proposed method was applied successfully to the analysis of SMX in various samples of dosage forms (tablets and suspensions) and the results were summarized in (Table 4). For all preparations examined, the assay results of proposed method are in good agreement with the declared content.

Table 4: Determination of SMX in pharmaceutical preparations

Pharmaceutical preparation	μg SMX present per 25 ml	μg SMX found per 25 ml	Relative error (%)	Recovery (%)*
Methoprim tablet (400 mg SMX and 80mg TMP/tablet) (S.D.I.- Iraq)	50	49.5	- 0.5	99.8
	200	198.8	-0.6	99.4
	400	399.7	-0.075	99.9
Ciplin tablet 400 mg /tablet Cipla Ltd. (India)	50	49.1	-1.80	98.2
	200	197.1	-1.45	98.5
	400	398.5	- 0.375	99.6
Balkatrin suspension 200 mg SMX/5ml (Jordan)	50	49.1	- 1.8	98.2
	200	197.9	-1.05	98.9
	400	398.7	- 0.325	99.6
Trimoks suspension 200 mg SMX/5ml (Turkia)	50	48.89	- 2.22	97.7
	200	198.20	-0.9	99.1
	400	400.4	0.56	100.1

* Average of three determinations.

Evaluation of the Proposed Method

According to the difficulties of using the standard method for determination of SMX in its pharmaceutical preparations, so that a standard addition method has been used for its simplicity which proves that the proposed method was applied successfully for the determination of SMX without interferences Fig. (10) and (Table 5).

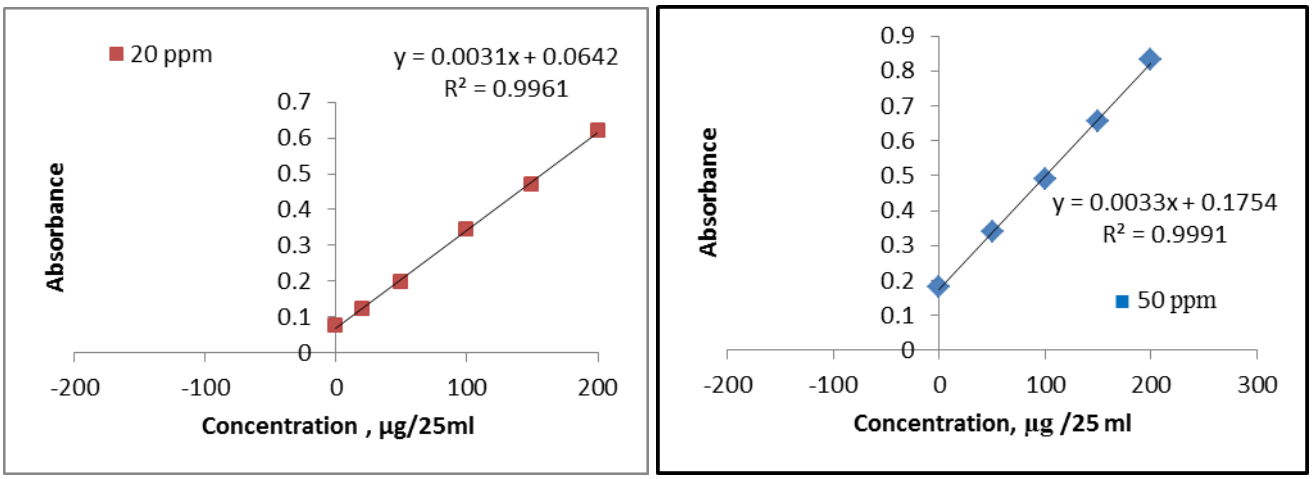


Fig. 10: Graphs of standard addition method of SMX in tablet, (Ciplin tablet)

Table 5: The results of standard addition method

Pharmaceutical preparation	SMX taken µg /20 ml	SMX measured µg /20 ml	Recovery, %
Ciplin (400 mg/tablet) Cipla Ltd. / India	20	20.71	103.6
	50	53.15	106.3

CONCLUSION

The proposed method offers clear advantages for the fast determination of SMX in the presence of the related compounds, such as TMP in pharmaceutical preparations. The method was found to be simple, economical, selective, sensitive, did not require the removal of excipients, temperature control, expensive reagents and organic solvents. It was also accurate, precise enough to be successfully adopted as an alternative to the existing spectrophotometric method and evaluation of SMX in both pure form and in its pharmaceutical preparations.

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