# Spectrophotometric determination of trimethoprim using 2,4-dinitro-1-fluorobenzene reagent

**Theia'a N. Al-Sabha**
Department of Chemistry
College of Education
University of Mosul

**Intesar A. Hamody**
Department of Chemistry
College of Education for Girls
University of Mosul

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**الملخص**

تم وصف طريقة طيفية سهلة لتقدير كميات مايكروغرامية من عقار التراي ميثوبريم . تعتمد الطريقة على التفاعل بين التراي ميثوبريم والكاشف ٤،٢–ثنائي نيترو– ١– فلوروبنزين في وسط الأسيتون . لقد وجد أن الناتج ذو لون أحمر وردي يمتلك طيفا امتصاصيا له أقصى امتصاص عند ٥٣٨ نانوميتر ويتكون بنسبة مولية ١:١. أمكن تطبيق قانون بير ضمن مدى التراكيز ١٠–٧٥ مايكروغرام/مللتر وقد بلغت الامتصاصية المولارية $1.917 \times 10^3$ لتر.مول$^{-1}$سم$^{-1}$ ودلالة ساندل 0.1514 مايكروغرام.سم$^{-2}$. تم حساب توافقية الطريقة (الانحراف القياسي النسبي) وكانت أفضل من ١.٧ % ودقة الطريقة (معدل نسبة الاسترجاع ) وكانت ١٠٠.٧٦. طبقت الطريقة بنجاح في تقدير تراي ميثوبريم في مستحضره الصيدلاني على شكل حبوب وتم مقارنة الطريقة المقترحة مع الطريقة المعتمدة في الدستور البريطاني . تم حساب قيمة اختبار t ووجد أنها تساوي ١.٨٢٨ عند مستوى ثقة ٩٥%، كما لوحظ أن الطريقة لا تعاني من تداخل المواد المضافة.

## Abstract

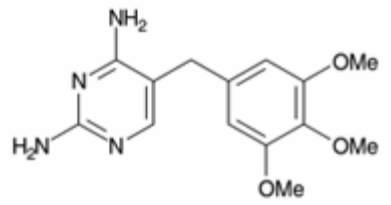
A simple spectrophotometric method for the determination of trimethoprim (TMP) in pure form and in its pharmaceutical formulation has been described. The method is based on the reaction of TMP with 2,4-dinitro-1-fluorobenzene reagent (DNFB) in acetone medium to give a highly rosy red colored product with maximum absorption at 538 nm with a molar ratio of 1:1. Beer's law is obeyed in the range 10-75 µg/ml with molar absorptivity of $1.917 \times 10^3$ l.mol$^{-1}$.cm$^{-1}$ and Sandell's sensitivity

of 0.1514 µg/cm$^2$. Precision (RSD) better than 1.7 % and accuracy (average recovery %) is 100.76 %. The suggested method has been applied to dosage forms as tablets and compared with the pharmacopoeial method, t-test is evaluated and found 1.828 at 95% confidence limit. The results show that there is no interference are present in commercial dosage forms.

Keywords: Spectrophotometry; 2,4-dinitro-1-fluorobenzene; TMP; spectrophotometry.

## Introduction

Trimethoprim[2,4-diamine-5-(3,4,5-trimethoxybenzyl) pyrimidine; (TMP)] (scheme 1), is known as a folic acid antagonist and is commonly used in combination with sulfonamides to treat gastrointestinal and respiratory tract infections and as a powerful bacteriostatic agent. TMP-sulfonamide formulations are also widely used as growth promoters. Residues of TMP may therefore occur even if the elimination time is short (elimination half-life is 5.5h for TMP in plasma) [1,2].




M.Wt = 290.3

Scheme 1. Structure formula of trimethoprim

Many analytical techniques have been employed for the determination of TMP. The generally used analytical techniques are electrochemical methods [3], selective membrane electrode [4], differential pulsed polarography and cyclic voltammetry [5], TLC [6,7], a TLC-densitometry [8], HPTLC [9], ion pair chromatography [10] and spectrodensitomety [11], a biomimetic bulk acoustic wave sensor was fabricated and applied for the determination of TMP in organic phase based on a molecular imprinting polymer [12]. In USP 26, HPLC is used for determination of TMP [13]. However; the determination of TMP and other drugs in biological systems, either individual or combined have been described by HPLC method [14-16].

Few spectrophotometric methods have been described for determination of TMP in the literature. These methods are based on using bromothymol blue, bromocresol green and alizarin red S as π-acceptors [17], oxidation of TMP by persulfate in alkaline medium [18] and ion-pair formation with bromophenol blue [19]. Other spectrophotometric methods are used for determination of TMP in a mixture with

sulphametoxazole depending upon derivative [20-22] extractive[23] and bivariate calibration [24] spectrophotometric methods.
The present work describes the spectrophotometric determination of TMP based on the reaction of the amine group with DNFB reagent in acetone medium.

## 2. Experimental

### 2.1. Apparatus

Shimadzu (UV-210) Double Beam Spectrophotometer with 1.0 cm silica cells was used to measure the absorbance. Heating of solutions is carried out on a water bath of frost instruments, LTD. The reading of pHs made on a PW 9420 philips pH meter supplied with an electrode type CE 10-12 pH. Weighing is carried out on a balance type of Mettler H 54 AR.

### 2.2. Reagents

Analytical grade chemicals, acetone was used. Standard solution of TMP (250µg/ml) in acetone was prepared. $1\times10^{-2}$M of DNFB (Sigma Co.) was prepared in acetone.

## 3. General procedure

Increasing volumes containing 0.2-1.5 ml of 250 µg/ml (i.e. 10-75 µg/ml) of TMP in pure form and 2 ml of DNFB were added in 5-ml volumetric flask and left for 40 min at 60° C. Then the solutions are cooled to room temperature and diluted to the mark with acetone solvent The absorbance was measured at 538 nm against the reagent blank.

## 4. Analysis of tablets

Ten tablets (each tablet containing 80 mg TMP) were accurately weighed and pulverized. A portion of the fine and homogenized powder equivalent to 80 mg TMP was accurately weighed and dissolved in about 15 ml of acetone. The solution was shaken thoroughly for about 10 min. and the residue was filtered through Whatmann no. 42 filter paper into 25 ml volumetric flask. The filtrate was diluted to the mark by repeated washing with acetone. The filtrate was diluted to get a 250µg/ml solution of TMP. An aliquot containing 10 to 75µg/ml was taken and the procedure as described above was followed. The absorbance was measured at 538 nm. The quantity per tablet was calculated from the standard calibration curve.

## 5. Results and discussion

In the preliminary investigation work, it was found that DNFB reagent reacted with TMP in acetone medium forming a rosy red colored product having two bands, the first band is a doublet width band with

maximum absorption at 380 nm and the reagent blank gave high absorption at this wavelength, the second band is a symmetrical band and more sharper than the doublet with maximum absorption at 538 nm which is the reagent blank almost gave no absorption at this wavelength, (Fig.1).Therefore the second band at 538 nm has been considered in subsequent experiments.

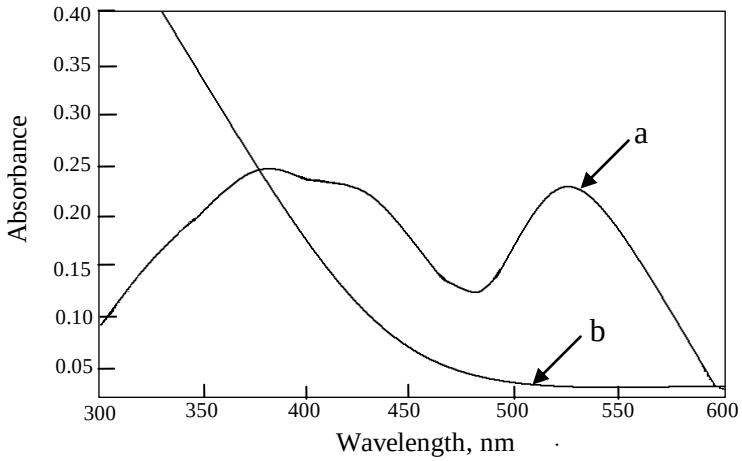



**Fig.1. Absorption spectra of (a) 60 µg $ml^{-1}$ TMP product with 2 ml of 0.01 M DNFB reagent aganist reagent blank and (b) reagent blank against acetone.**

## 5.1. Study of the optimum reaction conditions

The effect of various parameters on the absorption intensity of the colored product was studied and the reaction conditions are optimized.

### 5.1.1 Effect of solvent

Effect of various solvents such as ethanol, chloroform, acetonitrile, acetone and water for the sensitivity of the produced colored product were examined. It was found that acetone gave a rosy red colored product with maximum absorption at 538 nm, whereas other solvents gave low response with maximum absorption less at 400 nm, (Fig.2). Therefore acetone was chosen as organic medium in subsequent experiments.

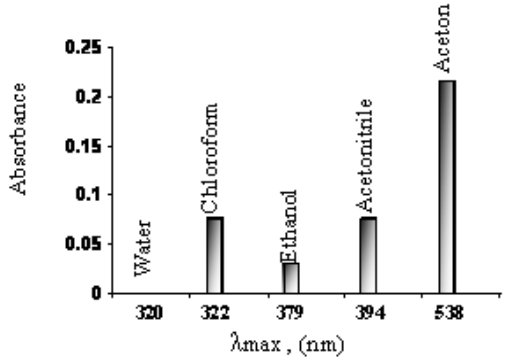



Fig.2. Effect of solvent on the absorption of 50 µg/ml trimethoprime

### 5.1.2. Effect of pH and buffer solutions

The effect of pH on the absorption of the product was studied using different pHs of HCl or NaOH ranged from 2 to 11.6. As seen in (Fig.3), it was found that the product formed with maximum absorption in the absence of acid or base (pH 7.4). Therefore different buffers such as borate, bicarbonate, phosphate and barbiton of the pH 7.4 were prepared and their effects on the sensitivity were examined. It was found that all of them decrease the absorbance.

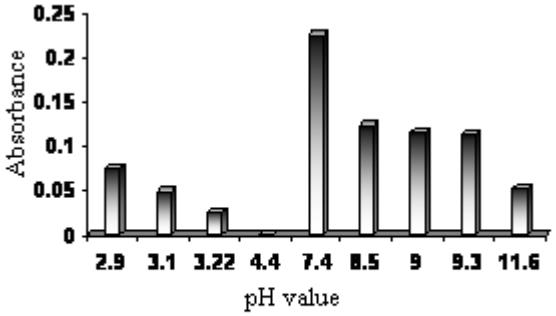



Fig.3 Effect of pH on the absorption of 50 μg/ml trimethoprime

### 5.1.3. Effect of temperature and reaction time

The reaction time was determined by following the color development at room temperature and in thermostatically controlled water-bath at different temperatures. The absorbance was measured against reagent blank treated similarly. It was observed that the sensitivity reached maximum after 40 min at 60°C and stable for 40 min after which it begun slowly fade, (Fig.4). This temperature and reaction time was chosen for the color development in subsequent experiments.

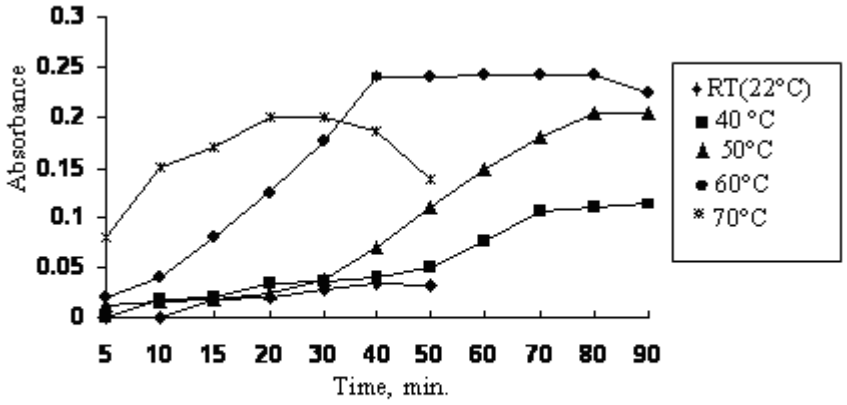



Fig.4. Effect of temperature and developing time on the absorbance of 50 µg/ml trimethoprime

### 5.1.4. Effect of DNFB reagent amount

The influence of DNFB concentration on the color intensity was studied by measuring the absorbance at the specified wavelength in the standard procedure for solutions containing the same drug amount but varying amount of DNFB. A volume of 2 ml of $1\times10^{-2}$ M in a total volume of 5 ml was found to be sufficient for full color development (Fig.5).

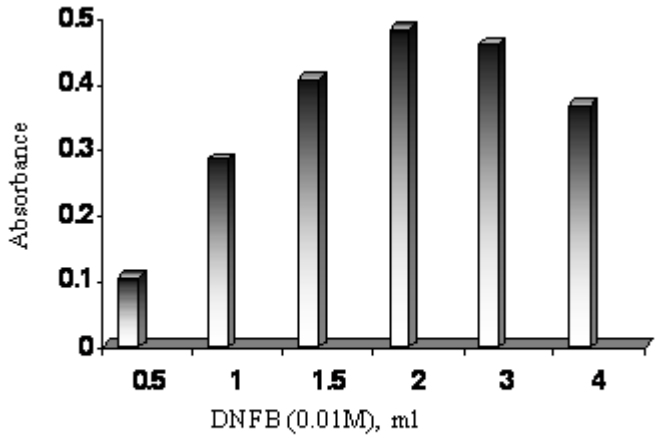



Fig.5 Effect of $1\times10^{-2}$M amount on the absorption intensity of 50 µg/ml trimethoprime

### 5.2. Precision and accuracy

The accuracy and precision of the proposed method was established by measuring the content of trimethoprim in pure form at three different concentration levels for six replicates at 25, 50 and 75µg/ml, (Table 1). The values of relative standard deviation and mean percent recovery obtained by the proposed method can be considered to be very satisfactory.

**Table 1 : Precision and accuracy of the proposed method**

| Amount added µg/ml | Recovery* (%) | Average recovery (%) | RSD* |
|---|---|---|---|
| 25 | 100.3 | 100.76 | 1.642 |
| 50 | 100.7 | | 0.912 |
| 75 | 101.3 | | 0.649 |

* Average of six determinations.

## 5.3. Quantification and Analytical Data

The absorbance of the formed product conform with Beer's law in the concentration range 10-75 µg/ml (Fig.6). The molar absorptivity is $1.917x10^3$ $l.mol^{-1}cm^{-1}$. The linearity was represented by the regression equation and the corresponding correlation coefficient for TMP determined by the proposed method were shown in Table 2.

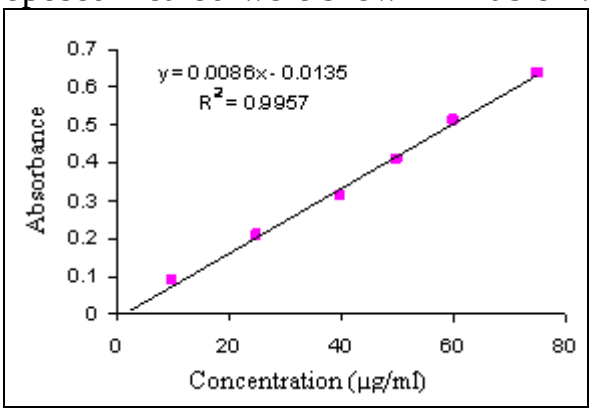



**Fig.6. Calibration graph of TMP drug**

**Table 2. Summary of optical characteristics and statistical data for the proposed method**

| **Parameters** | **Values** |
|---|---|
| $\lambda_{max}$ (nm) | 538 |
| Beer's law (µg/ml) | 10-75 |
| Molar absorptivity ($l.mol^{-1}cm^{-1}$) | $1.917x10^3$ |
| Sandell index ($\mu g/cm^2$) | 0.1514 |
| Regression equation (Y) * | |
| Slope (b) | 0.0086 |
| Intercept (a) | -0.0135 |
| Correlation coefficient ($R^2$) | 0.9957 |
| Average recovery % | 100.43 |
| Average relative standard deviation (RSD%)** | 1.067 |
| Temperature(°C) | 40 |
| Development time(min.) | 60 |
| Stability period (min.) | 30 |
| Final pH | 7.4 |

* Y= ac- b where C is the concentration of analyte (µg/ml) and Y is the absorbance unit.

** Calculated fro six determinations.

### 5.4. Interference

The extent of interference by some excipients which often accompany pharmaceutical preparations were determined by measuring the absorbance of solutions containing 40 µg/ml of TMP and various amounts(in mg) of excipients in final volume of 5 ml. It was found that the studied excipients do not interfere in the present method, even when present in large excess. An error of 5.0% in the absorbance readings was considered tolerable. Typical results are given in Table 3.

**Table 3: Effect of excipients for assay of TMP**

| excipients | Amount added (mg) | Relative error (E%) |
|---|---|---|
| Acacia | 0.1 | -0.55 |
| | 0.2 | -2.50 |
| | 0.4 | -1.39 |
| Glucose | 0.1 | -3.23 |
| | 0.2 | -2.11 |
| | 0.4 | 0.10 |
| Sodium chloride | 0.1 | -2.76 |
| | 0.2 | -0.69 |
| | 0.4 | -0.57 |
| Starch | 0.1 | -1.59 |
| | 0.2 | 1.38 |
| | 0.4 | 2.14 |

## 6. Application

The proposed method was successfully applied to determine TMP in pharmaceutical preparations as tablets. The obtained results were compared statistically by a Student's *t*-test for accuracy with the official method [25] (depending on potentiometric titration of pure drug dissolved in anhydrous acetic acid with perchloric acid) at the 95% confidence level with five degrees of freedom, as cited in Table 4. The results showed that the experimental *t*-test (1.828) less than the theoretical value (2.776), indicating that there was no significant difference between the proposed method and official method.

**Table 4: Assay of TMP tablet (80 mg) in pharmaceutical formulation by the proposed and official methods**

| Procedure applied | Pharmaceutical preparation | Drug amount taken | Recovery * (%) | Drug content found per tablet |
|---|---|---|---|---|

| | | (μg/ml) | | (mg) |
|---|---|---|---|---|
| Proposed method | Tablet (80 mg) | 10 | 97.62 | 78.09 |
| | | 20 | 98.88 | 79.10 |
| | | 25 | 98.56 | 78.84 |
| | | 50 | 96.32 | 77.05 |
| | | 75 | 97.24 | 77.79 |
| Official method [25] | Tablet (80 mg) | 25 | 102.08 | 81.66 |

*Every reading is an average of three determinations.

## 7. Stoichiometric Relationship

The molar ratio of the product formed between the studied drug and the reagent used was investigated applying the continuous variation (Job's) method using equimolar solutions of the drug and reagent ($1x10^{-3}$M). The results indicated that the product was formed in the ratio of 1:1 (Fig. 6). This finding supports that the interaction of the studied drug and the reagent used takes place at only one site which was the more sterically free terminal basic primary amino group.

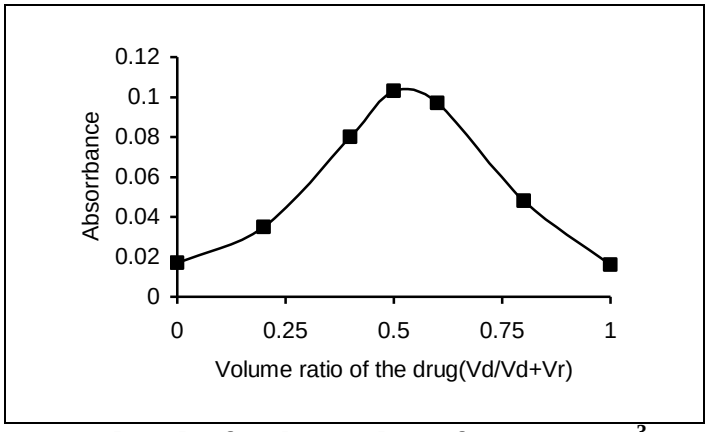
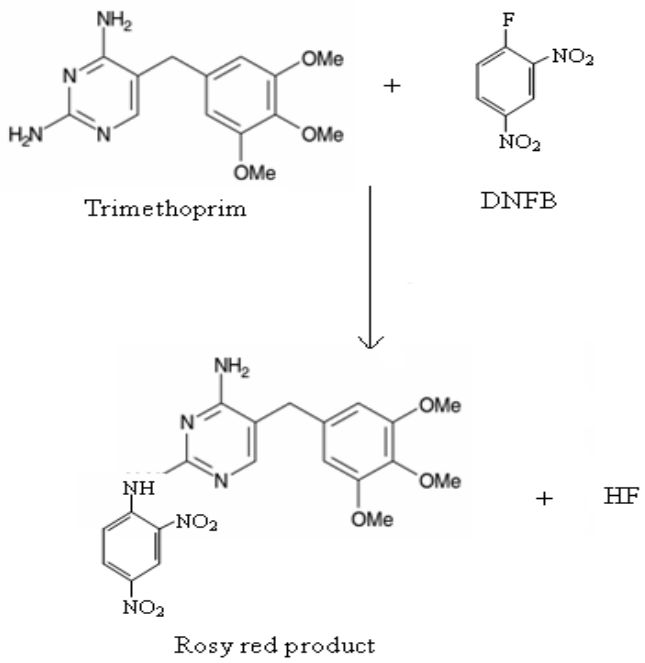



**Fig.7. Job's plot for the product of TMP ($1\times10^{-3}$M) with DNFB under the optimum conditions .**

## 8. Reaction mechanism:

The reaction of DNFB with drugs that own a free primary amine group results in the formation of coloured products[28]. This reaction was first introduced by Sanger[30] as means for determination of the DNA sequence. Based on the Job's method of continuous variation, it was found that TMP interacted with the DNFB in ratio of 1:1. This result indicates that the reaction between the drug and the reagent used takes place at only one site which was the more sterically free terminal amino group. The reaction is typical nucleophilic substitution and proceeds through an intermediate product as shown in scheme 2.




**Scheme 2. Reaction mechanism for TMP with DNFB reagent**

## 9. Conclusions

A spectrophotometric method has been developed for the determination of trace amounts of TMP based on its reaction with DNFB reagent in acetone medium. The proposed method is simple and precise and does not require solvent extraction step. However; the method required heating step to increase the reaction speed and sensitivity in addition to use of organic medium. The method was applied successfully for determination of TMP in its pharmaceutical tablets and compared favorably with the official method.

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