

Spectrophotometric Determination of Thymol in Pharmaceutical Preparation via Diazotization Reaction with 4-aminoacetophenone

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ABSTRACT

A simple and sensitive spectrophotometric method is proposed for the determination of thymol. The method is based on the coupling reaction of the drug with diazotised 4-aminoacetophenone reagent in alkaline medium to produce an intense red coloured water - soluble and stable azo dye which exhibits a maximum absorption at 502 nm. Beer's law is obeyed over the concentration range 0.2 – 16 µg/ml with a molar absorptivity of 2.89×10^4 L.mol⁻¹.cm⁻¹. The limit of detection is 0.0216 µg/ml and the limit of quantitation is 0.072 µg/ml, average recovery is 101.6%, and RSD is less than 2.4%. The proposed method is applied successfully to the determination of thymol in pharmaceutical preparation.

Keywords: Thymol, 4-aminoacetophenone, assay, spectrophotometry, diazotization-coupling, pharmaceutical preparations.

-4

-4

16

502

$10^4 \times 2.89$

/

– 0.2

%101.6

/

0.072

/

0.0216

.%2.4

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-4

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INTRODUCTION

Thymol (2-isopropyl-5-methylphenol) is a white crystalline substance of pleasant aromatic odor and strong antiseptic properties. This molecule has a broad range of activities including antioxidant, anti-microbial preservative, antiseptic, antitussive, expectorant and antispasmodic properties (Singh *et al.*, 2004; Aeschbach *et al.*, 1994; Wadhwa *et al.*, 2010; British Pharm, 2007).

Different methods have been reported for the determination of thymol including gas chromatography (Nazal *et al.*, 2002; Ajay, 2006; Kohert *et al.*, 2002) liquid chromatographic (Vinas *et al.*, 2006; Gao *et al.*, 2010) HPLC (Ji *et al.*, 2004; Hajimehdipoor *et al.*, 2010) RV-HPLC (Alekseeva, 2009) HPLC-ED (Zima *et al.*, 2007) and differential-pulse voltammetry (Lau *et al.*, 1988).

Thymol in pharmaceutical preparation has been assayed by visible spectrophotometric procedures based on such varied reactions as redox (Bakheet, 1998), oxidative coupling (Al-Esawati, 2002; Mohammed, 2005; Al-Hafith, 2005; Al-Neaimy, 2009), diazotization and coupling (Al-Ramadani, 2007; Bashir *et al.*, 2007) (Romero *et al.*, 1994) and flow injection (Rodriguez *et al.*, 1999; Al-Abachi *et al.*, 2012).

The objective of investigation reported in this paper is to evaluate a sensitive and an accurate method for the assay of thymol in pharmaceutical preparations. This method is based on coupling of the drug with diazotized 4-aminoacetophenone to form a stable azo dye product.

EXPERIMENTAL

Apparatus

A Shimadzu model 1650 computerized spectrophotometer provided with 1.0-cm matched quartz cells was used for all absorbance measurements.

Waterbath BS-11 (koria) was used in the study.

Reagents

All chemicals used were analytical grade and obtained from Fluka and BDH companies.

Thymol standard solution (100 µg/ml). This solution is prepared by dissolving 0.01 g of pure thymol in 5 ml of ethanol and the volume completed to the mark with distilled water in a 100-ml volumetric flask.

4-aminoacetophenone reagent solution (0.1%). This solution is prepared by dissolving 0.1 g of the compound in distilled water and the volume is completed to the mark in a 100-ml volumetric flask with distilled water.

Hydrochloric acid solution (1N). This solution is prepared by diluting 8.4 ml of 11.8 N concentrated acid to the mark in a 100-ml volumetric flask with distilled water.

Sodium nitrite solution (1%). This solution is prepared by dissolving 1g of sodium nitrite in distilled water and diluted to the mark in a 100-ml volumetric flask with distilled water.

Sulphamic acid solution (2%). This solution is prepared by dissolving 2 g of sulphamic acid in distilled water and the volume is completed to the mark in a 100-ml volumetric flask.

Sodium hydroxide solution (1N). This solution is prepared by dissolving 4 g of sodium hydroxide in distilled water and the volume is completed to the mark in a 100 ml volumetric flask.

Interference solution (1000 µg/ml). An amount of 0.1 g of each foreign compound is dissolved and completed to 100 ml with distilled water.

cetyl trimethyl ammonium bromide(CTAB) surfactant solution (0.1%). This solution is prepared by dissolving 0.1 g of cetyl trimethyl ammonium bromide in distilled water and diluted to the mark in a 100-ml volumetric flask with distilled water.

Recommended procedure and calibration graph

Employing the established optimum condition, the calibration graph was constructed as follows:

To a series of 25-ml volumetric flask, 0.5 ml of 0.1% of 4-aminoacetophenone, 0.2 ml of 1N HCl and 0.5 ml of 1 % of NaNO_2 are transferred. The reaction mixture is shaken and left for 5 minutes then 0.5ml of 2 % sulphamic acid is added with shaking and left for 5 minutes. After that an aliquot of aqueous solution containing (5-700) µg of thymol is added, 3 ml of 0.1% CTAB solution and 1.0 ml of 1N of NaOH are also added, and the flasks are diluted with distilled water and after leaving for 10 minutes as a reaction time, the absorbance of coloured azo dye was measured versus reagent blank at 502 nm.

The calibration graph shown in Fig. 1 was linear over the range of 0.2-16 µg/ml of thymol. The higher concentration shows a negative deviation from Beer's law. The apparent molar absorptivity has been found to be $2.89 \times 10^4 \text{ L.mol}^{-1}.\text{cm}^{-1}$. The limit of detection is 0.0216 µg/ml and limit of quantitation is 0.072 µg/ml.

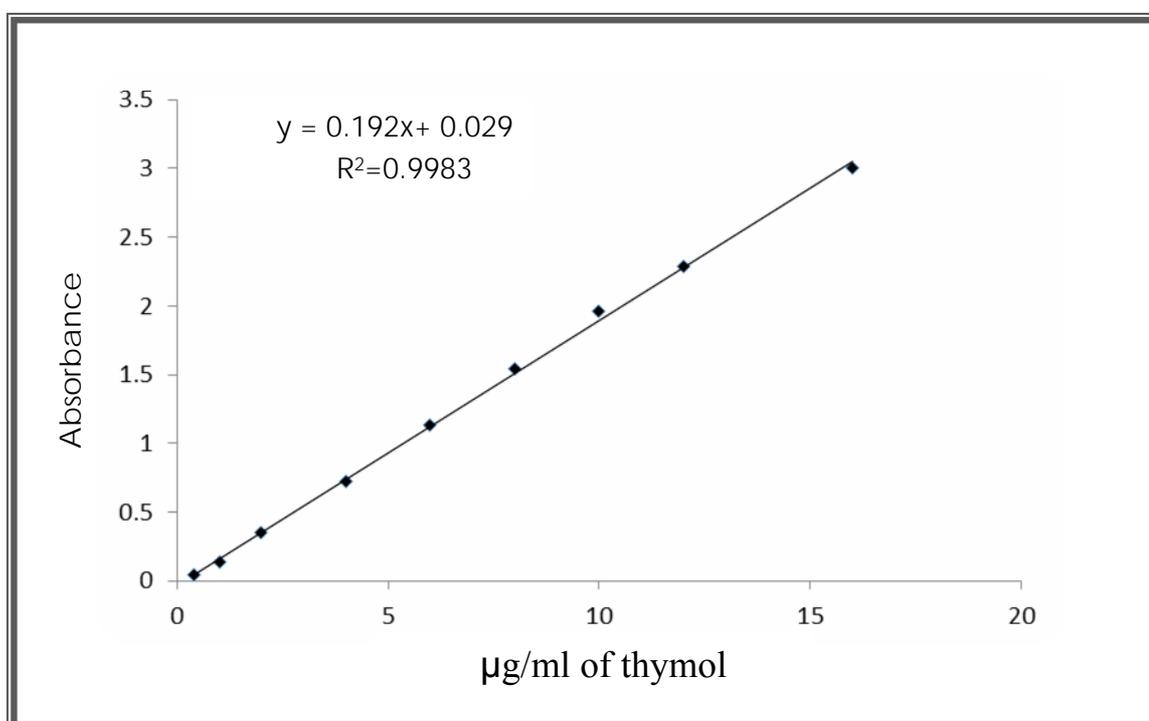


Fig. 1: Calibration graph for determination of thymol

Study of the optimum reaction condition

The various parameters affecting the colour intensity of the dye have been studied and optimum conditions are selected.

Effect of acid on diazotization formation

The effect of quality and quantity of acid on the intensity of the coloured dye is examined. Different volumes (0.1 – 1.0 ml) of 1 N of different acid solutions are transferred to volumetric flasks containing 1ml of 0.1% of reagent then 0.25 ml of (0.1%) NaNO₂ are added and shaken for 5 min, then 0.25 ml of (0.2 %) sulphamic acid was added and shaken for 5 min, after that the 1 ml of (100 ppm) thymol and 1 ml of (1N)NaOH are also added. The intensities of absorption are read against the reagent blank at 484 nm after diluting by distilled water to the mark. The results are shown in Table (1).

Table 1 : The effect of different acids on azo dye colour

Acid solution 1N	Absorbance / Volume of acid used (ml)					
	0.1	0.2	0.3	0.4	0.5	1.0
HCl	0.405	0.410	0.408	0.396	0.401	0.390
H ₂ SO ₄	0.409	0.405	0.401	0.408	0.392	0.389
HNO ₃	0.395	0.397	0.402	0.397	0.388	0.385
CH ₃ COOH	0.397	0.392	0.387	0.388	0.383	0.381

The results in Table 1 indicate that 0.2 ml of 1N HCl is considered as an optimum value therefore it is recommended for subsequent experiments.

Effect of nitrite amount and developing time

Different amounts of the 1% NaNO₂ solution are added and the time needed to complete the diazotization of 4-aminoacetophenone is studied by standing the solution after adding sodium nitrite solution for different time, the colored dye reached its maximum intensity when using 0.5 ml of 1% of NaNO₂ solution after 5 minutes as reaction standing time [Table (2)].

Table 2: Effect of 1% nitrite solution and developing time on azo dye coloured product

Developing time (min)	Absorbance / Volume of 1% nitrite solution (ml)				
	0.1	0.25	0.5	0.75	1.0
3	0.385	0.405	0.393	0.398	0.399
5	0.402	0.411	0.415	0.412	0.394
10	0.401	0.408	0.406	0.402	0.393
15	0.382	0.404	0.398	0.391	0.383

Effect of sulphamic acid amount and time

The amount effect of 2% sulphamic acid solution for removing the excess sodium nitrite with occasional shaking time are investigated. The results in Table (3) indicated that

0.5 ml of 2% sulphamic acid solution with 5 min shaking time are considered to be the most suitable, and therefore are selected subsequently.

Table 3 : Effect of sulphamic acid solution and time on the produced azo dye

Shaking time (min)	Absorbance / Volume of 2% sulphamic acid solution (ml)				
	0.1	0.25	0.5	0.75	1.0
3	0.371	0.388	0.390	0.392	0.386
5	0.398	0.410	0.412	0.406	0.409
10	0.391	0.402	0.407	0.409	0.408

*Absorbance without sulphamic acid =0.346

Effect of the reagent amount

The effect of 4-aminoacetophenone reagent amount on the maximum formation of the coloured product was investigated. The results shown in Table 4 indicated that 0.5 ml of 0.1% 4-aminoacetophenone solution is the optimum amount due to the higher colour intensity.

Table 4: The effect of 4-aminoacetophenone amount in azo dye colour intensity

Volume of 0.1% of reagent (ml)	0.25	0.5	1.0	1.5	2.0	2.5	3.0
Absorbance	0.432	0.440	0.421	0.416	0.408	0.429	0.403

Effect of base type and its amount

Preliminary experiments have shown that coupling reaction of diazotized 4-aminoacetophenone with thymol occurs in basic medium; therefore several bases (strong and weak) have been examined at different amounts. The results in Table (5) indicate that 1.0 ml of sodium hydroxide gives maximum absorbance so it was chosen in this method.

Table 5: The effect of different bases on azo dye colour

Base solution used (1N)	Absorbance / Volume of base used (ml)						
	0.25	0.5	1.0	1.5	2.0	2.5	3.0
NaOH	0.038	0.450	0.476	0.472	0.474	0.470	0.398
KOH	0.035	0.459	0.473	0.475	0.468	0.465	0.471
Na ₂ CO ₃	0.031	0.034	0.095	0.089	0.156	0.223	0.230
NaHCO ₃	0.029	0.030	0.045	0.066	0.097	0.173	0.211

Effect of surfactants

The effect of surfactants was studied by the addition of 1 ml of various types of surfactants (cationic, anionic and neutral) to the medium of reaction with two different orders.

The results in Table (6) indicate that cationic cetyl trimethyl ammonium bromide (CTAB) causes a red bathochromic shift of the absorption maxima wavelength of the produce dye from 484 nm to 502 nm, and also causes an increase in the colour intensity therefore this surfactant has been used for the subsequent experiments.

Table 6: The effect of different surfactants on azo dye colour

Surfactant solution (0.1% , 1ml)	Absorbance / Order of addition		
	I*	II**	λ_{\max}
Without	0.474	0.474	484
CTAB	0.478	0.481	502
SDS	0.450	0.443	486
Tween-20	0.423	0.439	482
Triton-80	0.427	0.448	483

* Reagent (R) +Nitrite (N)+ Sulphamic acid(A) + Thymol(D) +surfactant (S) +Base (B)

** R+N+A+D+B+S

In order to determine the optimum amount of CTAB, volumes from 0.5 – 5 ml of 0.1% of CTAB were examined, the results in Table (7) indicate that 3 ml of (0.1%) CTAB is the best for colour intensity so it was chosen for further studies.

Table 7: Effect of CTAB amount on colour intensity of the azo dye

CTAB (0.1% , ml)	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0
Absorbance	0.477	0.480	0.482	0.483	0.485	0.492	0.490	0.491

A summary of the optimum reaction conditions for this method is shown in Table (8).

Table 8: Summary of optimum reaction conditions for the proposed method

Material solution	Concentration	Optimum volume (ml)	Note
4-aminoacetophenone	0.1%	0.5	
Hydrochloric acid	1 N	0.2	
Sodium nitrite	1%	0.5	5 min standing time
Sulphamic acid	2%	0.5	5 min standing time
Sodium hydroxide	1 N	1.0	
CTAB solution	0.1%	3	
λ_{\max}			502nm

Effect of time and temperature

The effect of time with different temperatures in the range (0-60°C) has been investigated for maximum intensity of dye and the stability period. The results in Table (9) indicate that maximum absorbance reading is given after 10 min from mixing the components of the reaction and remains constant for at least 60 min.

Table 9: Effect of time and temperature on absorbance

Standing time (min)	Absorbance / Temperature (°C)		
	0.0	Room temp.*	60
5	0.475	0.477	0.472
10	0.476	0.482	0.471
15	0.473	0.481	0.472
20	0.468	0.483	0.466
25	0.469	0.479	0.465
30	0.469	0.478	0.468
35	0.471	0.481	0.465
40	0.472	0.479	0.463
56	0.470	0.480	0.463
60	0.468	0.480	0.465
70	0.468	0.481	0.462

* Room temperature = 11°C

Absorption spectra

Absorption spectra of the coloured dye formed by coupling of diazotized 4-aminoacetophenone with thymol in basic medium in presence of CTAB surfactant under the established optimum amounts show a maximum absorption at 502 nm in contrast to the reagent blank which shows no absorption in the visible region (Fig. 2)

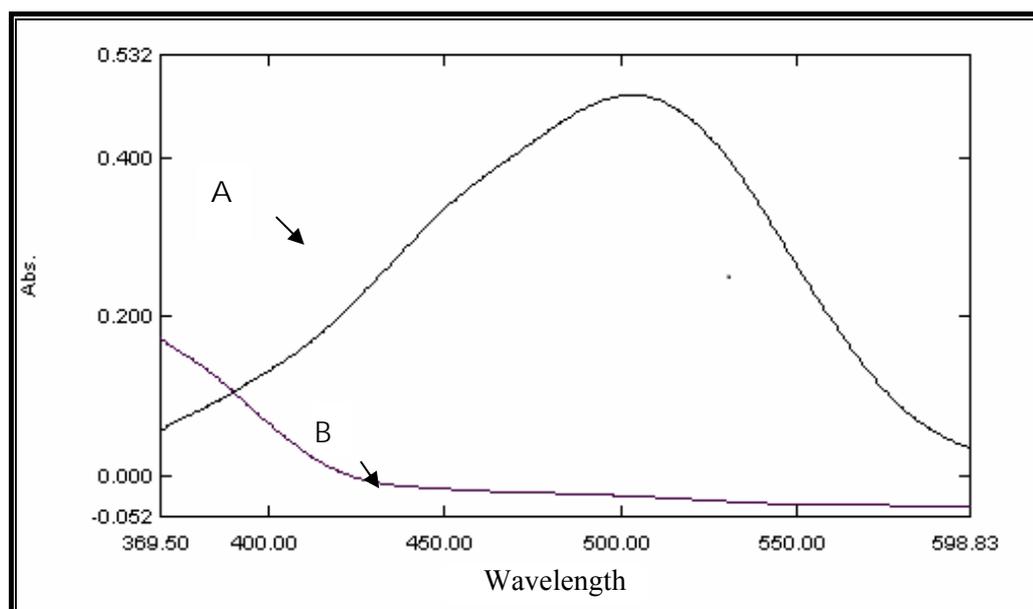


Fig. 2: Absorption spectra of (A) 50 µg thymol against reagent blank, (B) reagent blank against distilled water

Accuracy and Precision

To evaluate the accuracy (recovery %) and precision (RSD) of the calibration graph, a pure thymol was analyzed at three different concentrations in four replicates. The results shown in Table 10 indicated that a satisfactory accuracy and precision could be attended by using the proposed method.

Table 10: Accuracy and Precision

Concentration of thymol ($\mu\text{g/ml}$)	Recovery (%)	Average recovery* (%)	RSD* (%)
4	98.8	101.6	2.252
8	102.7		1.137
14	103.5		2.433

*Average for four determinations

Nature of the dye

Applying Job's method of continuous variations and mole-ratio method (Harvey, 2000) showed that the formed dye has the composition of 1:1, thymol: diazotized 4-aminoacetophenone as shown in (Fig. 3), therefore, the formed dye may be written as follows:

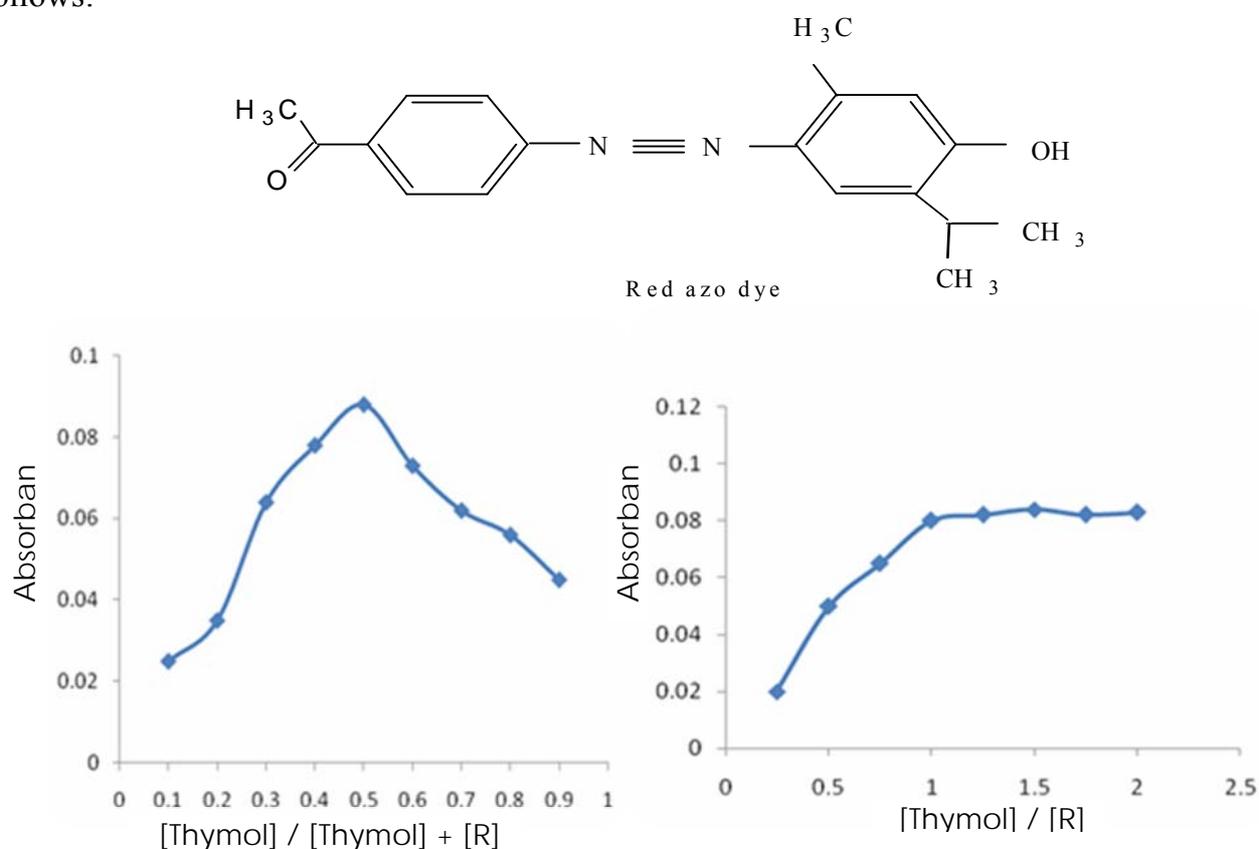


Fig. 3: (a) Job's plot and (b) mole-ratio plot for diazotized 4-aminoacetophenone coupled with thymol.

The apparent conditional stability constant of the produced azo dye described under the recommended procedure has been estimated to be $3.0 \times 10^5 \text{ L} \cdot \text{mol}^{-1}$.

Interferences

The effect of common excipients used in the pharmaceutical preparations was studied by analyzing a synthetic sample solution containing the quantity of drugs as mentioned in Table (11), in presence of different amounts of excipients. It was found that excipients show no significant interference.

Table 11: Effect of excipients for assay of thymol

Foreign compound	Recovery (%) of 100 µg thymol per µg foreign compound added			
	200	500	1000	2000
Starch	101.6	102.7	97.5	102.8
Arabic gum	100.3	103.7	102.6	101.8
Sodium chloride	97.9	99.7	100.1	99.2
Sodium bicarbonate	101.7	101.5	100.3	100.8
Glucose	98.3	101.5	101.4	99.7
Fructose	101.2	99.4	97.5	97.9
Lactose	100.8	100.2	98.4	102.5

Analytical applications

The proposed method was successfully applied to the determination of thymol in its pharmaceutical preparations as in Table (12). The obtained results were compared statistically by a student's t-test for accuracy and variance ratio F-test for precision with standard method (American P.H.A., 1975).

At the 95% confidence level with six degrees of freedom as cited in Table (13). The results showed that the t-test and F-test were less than the theoretical value ($t=2.306$, $F= 6.39$), indicating that there was no significant difference between the proposed method and the standard method.

Table 12: Determination of thymol in pharmaceutical preparations

Pharmaceutical Preparations	Certified Value	Amount Present (µg/ml)	Recovery* (%)	Drug content Found*
Lasstarime antiseptic Hams-syria	0.06%	4	103.33	0.0620
		8	99.20	0.0595
		12	102.50	0.0615
Listerix plus Mouthwash SIGMA – Egypt	0.064%	4	100.32	0.0642
		8	103.75	0.0664
		12	102.34	0.0655

*Average for four determinations

Table 13: The results of t-test and F-test analysis

Pharmaceutical Preparation	Recovery (%)*		t-test	F-test
	Present Method	Standard Method		
Lasstarime antiseptic	101.65	100.82	1.832	2.46
Listerix plus Mouthwash	102.14	103.31	1.953	3.62

*Average for five determinations

Comparison of methods

(Table 14) shows a comparison between the present method and another spectrophotometric method.

Table 14: Comparison of Methods

Analytical parameter	Present method	Literature method (Mohammad, 2005)
λ max (nm)	502	550
Temp. °C	Room temperature	Room temperature
Reaction medium	Aqueous	Aqueous
Type of reaction	Diazo-coupling	Oxidative coupling
Reagent	Diazotized 4-aminoacetophenone	<i>p</i> -phenylenediamine + sodium metaperiodate
Beer's law range ($\mu\text{g}\cdot\text{ml}^{-1}$)	0.2 - 16	0.4 - 24
Molar absorptivity ($\text{l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$)	2.89×10^4	7.45×10^3
Color of the dye	red	violet
Composition of the dye	1:1	1:1
Analytical application	Pharmaceutical preparations	Pharmaceutical preparations

The present method is more sensitive than the recently-published method for thymol (Mohammad, 2005).

CONCLUSION

A spectrophotometric method for the determination of thymol in an aqueous solution is described. The method is based on the azo-coupling reaction of thymol with diazotized 4-aminoacetophenone reagent in an alkaline medium. It is considered to be simple (it does not need heating and extraction), rapid (10 minutes development time), accurate (average recovery 101.6 %) and precise (RSD < 2 %), and the results suggested that there is no interference from excipients which are present in commercial dosage forms.

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