Spectrophotometric Determination of Thymol in Pharmaceutical Preparations Via Oxidative Coupling Reaction with 2,4-dinitrophenylhydrazine in the Presence of Potassium Periodate

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Abstract
A new, simple and sensitive spectrophotometric method for the determination of Thymol in pure and mouth wash preparations has been proposed in this study. The method was based on oxidation of 2,4-dinitrophenylhydrazine with potassium periodate and coupling with Thymol in alkaline medium to form an intense violet water-soluble dye that is stable and has a maximum absorption at 570 nm. A graph of absorbance versus concentration shows that Beer’s law was obeyed over the concentration range of 0.25-10 μg.mL⁻¹ of Thymol, with detection limits of 0.063 μg.mL⁻¹. All experimental parameters that affect the development and stability of the colored product were carefully studied and the proposed method was successfully applied to the determination of Thymol in mouth wash preparations.

Key words: Thymol, 2, 4-dinitrophenylhydrazine, potassium periodate, spectrophotometry.

Introduction:
Thymol is a 5-methyl-2-(methyl ethyl) phenol, C₁₀H₁₄O, whereas its chemical structure is shown in figure 1-[1]:

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Figure 1- Structure of Thymol

Thymol is a monoterpenoid phenol derivatives. It is found in natural substances, spice plants. It is found in thyme, oregano and wolf's bane. Thymol is widely used in the chemical industry to stabilize and to store solutions and serum samples [2].

Thymol resembles phenol in its action, but owing to its insolubility in the fluids of the body, it is absorbed much more slowly; it is also less irritant to wounds, while its germicidal action is greater than that of phenol, though less than that of naphthol. In alcoholic solution, it penetrates the skin and produces local anaesthesia. It is used as an antiseptic lotion and mouth wash (1 in 1000), or as Liquor Thymolis Composites; as a paint in ringworm (1 in 10 of alcohol, or alcohol and ether); and as an ointment (1 in 24 of soft paraffin, the Thymol being dissolved with the aid of heat) in eczema, psoriasis, broken chilblains, parasitic skin affections, and burns.

An ointment perfumed with oil of lavender, is used to keep off mosquitoes. Thymol in oily solution (1 or 2 per cent.) is applied to the respiratory passages by means of a spray in nasal catarrh and it's also used to medicate absorbent gauze and wool for use as surgical dressings.

A number of analytical methods have been reported for the determination of Thymol, these included high pressure liquid chromatography [3-8], liquid chromatography with electrochemical detection [9], gas chromatography [10-15], differential-pulse voltammetry [16], spectrometry [17-19], colorimetric analysis [20],TLC[21,22] and Flow injection spectrophotometry[23]. However, some of these methods are time consuming and/or require expensive equipment and conditions. In this work, rapid and sensitive method using spectrophotometric detection at 570 nm was proposed for the determination of Thymol in pharmaceutical preparations. The method is based on an oxidation of 2, 4-dinitrophenylhydrazin with potassium periodate and reaction with Thymol in alkaline medium. The analytical procedure is safe, simple, fast and accurate. It has been satisfactorily applied to the determination of Thymol in pure and mouth wash preparations.

Experimental Apparatus
All spectral and absorbance measurements were carried out on a Shimadzu UV-Visible-260 digital double-beam recording spectrophotometer (Tokyo-Japan) and using 1 cm quartz cells.

Preparation of solutions
Thymol stock solution (1000 μg.mL⁻¹): prepared by dissolving 0.100 gm amount of pure Thymol (BDH) in 5 mL of ethanol then complete to 100 mL in a volumetric flask with distilled water. Thymol working solution (100 μg.mL⁻¹), was prepared by dilution of 10 mL of the stock solution to 100 mL volumetric flask with distilled water.

2,4-dinitrophenylhydrazine (2,4-NPH) (1 × 10⁻³ M): was prepared by dissolving 0.019814 g of 2,4,DNPH (BDH) in 2mL of concentrated sulfuric acid then complete to 100mL in a volumetric flask with distilled water.

Potassium periodate (5×10⁻³M) : A 0.09544g of potassium periodate (BDH) was dissolved in distilled water and diluting to mark in 100 mL volumetric flask.

Sodium hydroxide (0.5M) : was prepared by dissolving 5 g of sodium hydroxide (BDH) in distilled water and diluting to mark in 250 mL volumetric flask.

Pharmaceutical preparations of Thymol
Pharmaceutical preparations were obtained from commercial sources. 1-Listerine antiseptic (USA): containing 0.063% Thymol. 2-Breath Rx (mouth rinse-anti bacterial-USA): containing 0.060% Thymol.

General procedure for calibration
An aliquot of sample containing 0.025-2.5 mL of pure Thymol(100μg.mL⁻¹) was transfered into a series of 25 mL standard flask. Add 1 mL of potassium periodate (5×10⁻³ M), and 1 mL of 2,4-DNPH(1×10⁻³ M), then 5mL of sodium hydroxide(0.5M), the contents of the flasks were diluted to the mark with distilled water, mixed well and left for 15 min at room temperature, the absorbance of the violet compound formed was measured at 570 nm against a reagent blank containing all materials.
except Thymol. A calibration graph was drawn and the regression equation was calculated. For the optimization of conditions a solution of 2mL of pure Thymol (100µg.ml⁻¹) was used in a final volume of 25mL.

**Procedure for Mouth wash:**

Two types of mouthwash were analyzed by the developed methods, these include:-

1-Breath Rx (mouth rinse-anti bacterial-USA), this type of mouthwash containing 0.060% Thymol. Transfer 20 mL of the mouthwash preparation to a 50 mL volumetric flask, add 5 mL of ethanol and dilute to the mark with distilled water. The concentration of this solution was (240 µg.ml⁻¹) stock solution. Working solution of 100 µg.ml⁻¹ was prepared by simple dilution of the stock solution with distilled water.

2-Listerine-antiseptic(USA): containing 0.063% Thymol. Transfer 20 mL of the mouthwash preparation to a 50 mL volumetric flask then add 5 mL of ethanol and dilute to the mark with distilled water. The concentration of this prepared solution was (252µg.mL⁻¹) stock solution, (100 µg.mL⁻¹) solution was prepared by simple dilution of the stock solution with distilled water.

**Results and discussion Absorption spectra**

When a diluted aqueous solution of Thymol was added to the oxidized 2,4-DNPH in alkaline medium, an intense violet product formed immediately, which became stable after 15 min. The violet product has a maximum absorption at 570nm. Figure 2 shows the spectra of the product formed and of the reagent blank, the maximum absorption at 570 nm produce from mixing [1mL of Thymol (100µg.ml⁻¹), 1mL of potassium periodate (5 × 10⁻³ M), 0.5 mL of 2,4-DNPH (1 × 10⁻³ M) and 5mL of sodium hydroxide(0.5M) and diluted to 25mL with distilled water] measured versus reagent blank [it contains all components except Thymol] which has negligible absorbance at this wavelength.

![Figure 2- Absorbance spectra of thymol (4µg mL⁻¹) treated as described under procedure above and measured against blank(A). The reagent blank contains all components except Thymol measured against distilled water(B).](image)

**Effect of order of addition**

The order of addition of the reagents is an essential part of the experiment, it was found that the order of addition of the reagent cited under general procedure gave a maximum color intensity and a minimum absorbance of the blank and was used in all subsequent experiments.

**Optimization of the experimental condition**

The effects of various parameters on the absorption intensity of the formed products were optimized. The effects of different alkaline solutions (0.5 M) were studied such as sodium hydroxide, sodium carbonate, potassium hydroxide and ammonium hydroxide. It was found that sodium hydroxide was the most suitable alkaline medium for a maximum absorbance and was used in all subsequent experiments. The effect of different volumes of sodium hydroxide (0.5M) were studied on the maximum absorbance by varying the volume of 2,4-DNPH between (0.1-1.5mL). It was found that 1mL of 2,4-DNPH (1 × 10⁻³ M) gave the highest absorbance figure 4. Effect of different volumes (0.1–2 mL) of potassium periodate (5 × 10⁻³ M) was examined on the maximum absorbance of the
formed product. Figure 5- Shows that 1 mL of potassium periodate solution was enough to obtain a maximum absorbance.

**Figure 3- Effect** of the volume of NaOH (0.5M) for determination of Thymol (8µg.mL⁻¹).

**Figure 4- Effect** of the volume of 2,4-DNPH (1×10⁻³M) for determination of Thymol (8µg.mL⁻¹).

**Figure 5- Effect** of the volume of sodium periodate (5×10⁻³M) for determination of Thymol (8µg.mL⁻¹).

**Effect of reaction time**
Experimental results revealed that the colour intensity reaches a maximum after 2,4-DNPH solution had been oxidized with potassium periodate and reacted with Thymol in alkaline medium for 15min, therefore, a 15 min development time was suggested as the optimum reaction time and remain stable for 120 min.

**Structures of the products**
The stoichiometry of the reaction between Thymol and 2,4-DNPH was investigated using both continuous variation and molar ratio methods respectively. The results obtained figure (6 and 7) show that a (1:1) was formed between Thymol and 2,4-DNPH.
A reaction subsequent based on the above results is shown in Scheme 1.[24]

The product formed was soluble in water. The apparent stability constant was calculated by comparing the absorbance of a solution containing stoichiometric amount of Thymol (6.65x10^-4 M) (Aₐₛ) with that of a solution containing a five-fold excess of 2,4-DNPH reagent (Aₘ) and according to analytical procedure. The average stability constant (K) = 5.4x10^5 L.mol^-1, where [K = (1-α)/α²C] and α = Am - As/Am [25].

Analytical characteristics of spectrophotometric method

For the proposed method, a calibration graph, were obtained by the procedure described previously and a series of standard solutions was analyzed in triplicate to test the linearity figure 8. The molar absorptivity (ε), the Sandell’s sensitivity (S), the slope (a) and the intercept (b) were determined and are included in Table 1. The accuracy and precision of the proposed methods were tested by analyzing five replicate of Thymol using the proposed spectrophotometric method for three different concentrations of thymol. The values of relative standard deviation RSD% and relative error E_rel% are summarized in the same table. These values indicated a high accuracy and precision of the proposed method. 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 a factor of three[26].
Table 1- Analytical parameters of spectrophotometric method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>570</td>
</tr>
<tr>
<td>Linearity range, $\mu g$ ml$^{-1}$</td>
<td>0.25-10</td>
</tr>
<tr>
<td>Molar absorptivity (L mol$^{-1}$ cm$^{-1}$)</td>
<td>2.2×10$^4$</td>
</tr>
<tr>
<td>Sandell’s sensitivity($\mu g$ ml$^{-1}$)</td>
<td>0.67×10$^{-2}$</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$Y=0.1471X + 0.009$</td>
</tr>
<tr>
<td>Linearity (r)</td>
<td>0.9969</td>
</tr>
<tr>
<td>Limit of detection( L.O.D*)  ($\mu g$ ml$^{-1}$)</td>
<td>0.063</td>
</tr>
<tr>
<td>Relative standard deviation (RSD%)*</td>
<td>1.55</td>
</tr>
<tr>
<td>Average of recovery%</td>
<td>101.12</td>
</tr>
<tr>
<td>$E_{\text{rel}}$ %</td>
<td>1.12</td>
</tr>
<tr>
<td>Molar ratio (D:R)</td>
<td>1:1</td>
</tr>
<tr>
<td>$\sigma$ =standard deviation of blank</td>
<td>$S_1= (1.01)$</td>
</tr>
<tr>
<td>$F$ calculated=S$2^2$/</td>
<td>$S_1^2=2.004$</td>
</tr>
<tr>
<td>$F$ theoretical =19.0</td>
<td>F theoretical &gt; F calculated</td>
</tr>
</tbody>
</table>

*L.O.D= (3$\sigma$/S),  $\sigma$ =standard deviation of blank S=slope

Pharmaceutical application

The suggested methods were applied to the quantitative determination of Thymol in mouth wash formulation. Two types of mouth wash preparations containing Thymol were analyzed and they gave a good accuracy and precision as shown in Table 2. The proposed method was compared successfully with the official method [27].

Table 2- Application of the proposed and official methods for the determination of mouth wash containing Thymol

<table>
<thead>
<tr>
<th>Mouth wash samples</th>
<th>Proposed method</th>
<th>Official method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery, %</td>
<td>RSD%</td>
</tr>
<tr>
<td></td>
<td>(Xi−Xi‾)</td>
<td></td>
</tr>
<tr>
<td>Breath Rx</td>
<td>99.6</td>
<td>1.569</td>
</tr>
<tr>
<td>Listerine</td>
<td>99.2</td>
<td>4.181</td>
</tr>
</tbody>
</table>

F-test and T-test showed that there was no significant difference between the proposed method and the official method using 4-aminoantipyrine (4-AAP) and potassium ferricyanide Table 3.

Table 3- The comparison of the proposed method with standard method using t- and F-statistical tests

<table>
<thead>
<tr>
<th>The pharmaceutical preparations for 2.0 $\mu g$.ml$^{-1}$</th>
<th>The proposed method</th>
<th>The official method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rec.%</td>
<td>(Xi−Xi‾)$^2$</td>
<td>Rec.%</td>
</tr>
<tr>
<td>Pure Thymol</td>
<td>101.12</td>
<td>98.00</td>
</tr>
<tr>
<td>Breath Rx</td>
<td>99.6</td>
<td>99.48</td>
</tr>
<tr>
<td>Listerine</td>
<td>99.2</td>
<td>100.86</td>
</tr>
</tbody>
</table>

at 95% confidence level

$$(\Xi_i)=99.97$$

$$(\Xi_i)^2=99.44$$

$$S_1= (1.01)$$

$$S_2= (1.43)$$

$$F_{\text{calculated}}=S_2^2/S_1^2=2.004$$

$$F_{\text{theoretical}}=19.0$$

$$F_{\text{theoretical}} > F_{\text{calculated}}$$

$$t_{\text{calculated}}= 1.239$$

$$t_{\text{theoretical}}= 2.776$$

$$t_{\text{theoretical}} > t_{\text{calculated}}$$

A standard additions method was used to avoid and correct the chemical interferences that present in mouth wash preparations. It involves adding increment volumes (0-1.5mL) of standard solution of...
100μg.mL$^{-1}$ to a fixed volume sample (0.2 mL of 100μg.mL$^{-1}$) and employing the conditions described under procedure. It gave a good accuracy and precision Table 4.

**Table 4-** Application of the standard additions method and official methods for the determination of mouth wash containing Thymol

<table>
<thead>
<tr>
<th>Mouth wash samples</th>
<th>[Thymol] depend on st. addition*</th>
<th>Standard additions method</th>
<th>Official method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breath Rx</td>
<td>0.80</td>
<td>100.0</td>
<td>99.48</td>
</tr>
<tr>
<td>Listerine</td>
<td>0.81</td>
<td>101.25</td>
<td>100.86</td>
</tr>
</tbody>
</table>

*Standard additions

**Conclusions**

The proposed method was 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 Thymol in mouth wash and can be used for the routine analysis.

**References:**

2. Szentandrassy, N. **2003.** Effects of thymol on cardiac and skeletal muscle. Ph.D. Thesis. Department of Physiology, Medical School, Medical and Health Science Center, University of Debrecen.


