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Spectrophotometric Determination of L- Thyroxine via Diazo-Coupling with Diazotized p-Nitroaniline

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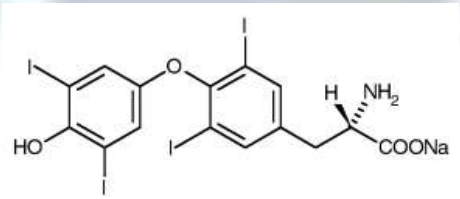
Abstract: A simple and sensitive spectrophotometric method was described for the determination of L-thyroxine sodium (LTHS) as pure and in its pharmaceutical formulation (tablet). The method was based on the coupling of LTHS with diazotized p-nitroaniline, in an alkaline medium (pH=12.2). The dye shows an absorption maximum at 540 nm and obeys Beer's law over a range of 1 to 25 µg.ml^{-1} of LTHS. The molar absorptivity and Sandell's sensitivity of the colored azo dye are 6.23 x 10^5 l mol^{-1}cm^{-1} and 0.00128 µg.cm^{-2} respectively. The relative standard deviation is not more than 2.47 % (n=5). The azo dye formed was stable for at least 90 minute. The colored azo dye was found to be 1:1 LTHS: diazotized p-nitroaniline. The stability constant under optimized conditions and at room temperature was 2.306 x 10^5 l.mole^{-1}. The method was applied successfully for the assay of LTHS in pharmaceutical preparation (tablet).

Keywords: L- Thyroxine, diazotized paranitroaniline, Determination, Spectrophotometry, Diazo-coupling.

INTRODUCTION

Thyroid hormones, thyroxine (T4) and thyronine (T3), are iodine-containing hormones secreted by the thyroid gland. They are responsible for the regulation of diverse biochemical processes essential for normal metabolic and neuronal activity. T3 is more biologically potent than T4 but T4 is normally present in human serum in approximately 50-fold excess of circulating T3 and constitute more than 90% of the circulating protein bound iodine. Thyroxine has L- and D-forms. The L-form is twice as active physiologically as the racemic product, and the D-form has very little activity(1,2), L-thyroxine sodium has the following chemical structure(3) :



M.wt = 799 g/mol. (anhydrous)

L-thyroxine sodium ($C_{15}H_{10}I_4NNaO_4$,xH_2O)

The reports found in the literature for L-thyroxine determination concentrate on HPLC (4-7),HPLC tandem – mass(8) GC – mass (9) differential pullspolargraphic(10) ,electrochemical (11-13) ,electrochemiluminescence(14-15), radioimmunoassay(16) , flow injection- chemiluminescence (17-20) .

To the best of our knowledge, few spectrophotometric method is available for quantification of LTHS in pharmaceutical preparation.(21-23) . This paper describes visible spectrophotometric method based on the diazo-coupling reaction of LTHS with diazotized p-nitroaniline.

EXPERIMENTAL

Apparatus:

A JASCOV - 630 UV / Vis spectrophotometer and with 1cm matched quartz cells were used for all measurement of absorbance and for the constructed absorption spectrums.

Reagents:

All chemicals used in this study are analytical grade reagents, and distilled water was used for preparing the reagent solutions.

L- thyroxine sodium solution 100 µg.ml^{-1}.

This solution was prepared by dissolving 0.0100 g of LTHS in distilled water in presence of 0.1M sodium hydroxide and the volume was completed to the mark in a 100 ml volumetric flask with distilled water.

Diazotized p-nitroaniline, 0.005 M.

This solution was prepared by dissolving 0.0690 g of p-nitroaniline in 20 ml of HCl (1M) followed by further dilution to 80 ml with distilled water. The mixture boiled until all p-nitroaniline was dissolved then the solution transferred to 100 volumetric flask and cooled at (0 - 5) ºC in an ice-bath, a 0.0345 g of sodium nitrite was added then stirred vigorously after 5 minutes the solution made up to 100 ml with cooled distilled water and stored in an amber bottle in a refrigerator.

Sodium hydroxide solution ,1M .

This solution was prepared by appropriate dilution of the concentrated volumetric (BDH) solution with distilled water and then transferred to a plastic bottle.

Recommended procedure and calibration graph

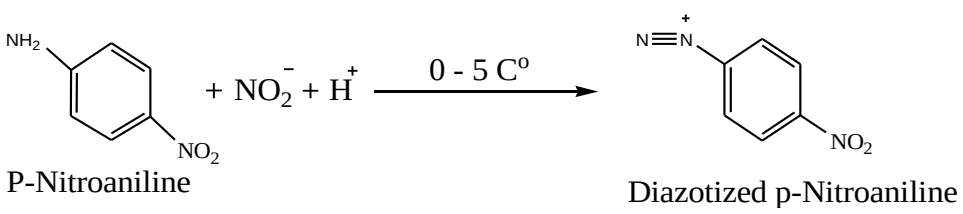
An aliquot of the sample solution containing 10-250 µg of LTHS was transferred into a series of 10 ml volumetric flask. Then 2.5 ml of diazotized p-nitroaniline (0.005M) and 1.5 ml of sodium hydroxide solutions (1M) were added, then the contents were diluted to the mark with distilled water and mixed well. The absorbance of the colored azo dye was measured at 540 nm against the reagent blank which prepared in a similar way but without the addition of LTHS. The calibration graph was linear over the range of 1-25 µg.ml^{-1}.

Determination of LTHS in tablet.

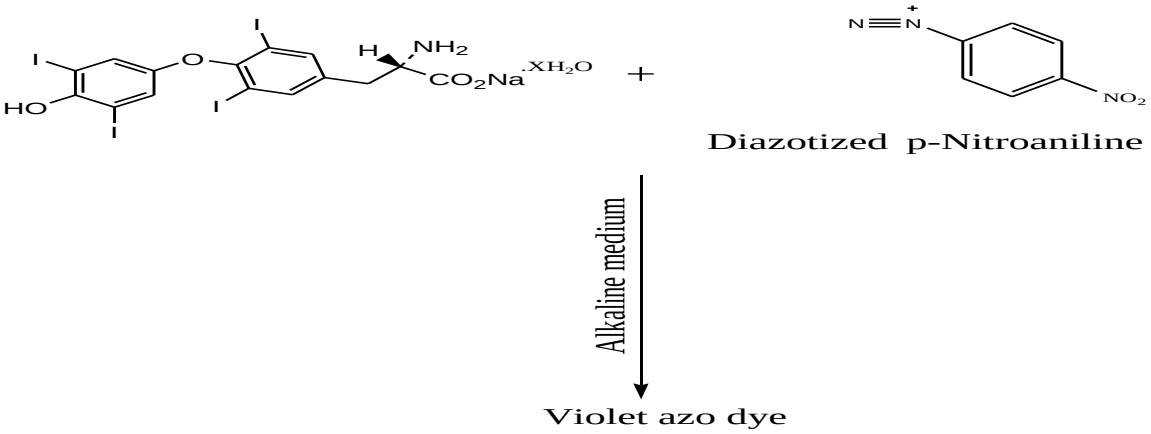
Twenty tablets (each tablet contain 50 µg LTHS) were finely powdered and dissolved in distilled water in presence of 5 ml of 0.1 M sodium hydroxide with shaking for 10 min, then filtrated and the volume of the filtrate was transferred to a 50 ml measuring flask and completed to the mark with distilled water to prepared 20 µg.ml^{-1} solution of LTHS.

RESULTS AND DISCUSSION

The method involves the diazo-coupling reaction between LTHS with diazotized p-nitroaniline in an alkaline medium to give a violet colored azo dye. Two steps are involved in the reaction that produces the colored dye. The first step included the preparation of diazotized as mentioned before, the second step, involved the coupling of the diazonium ion with LTHS in an alkaline medium to form a stable azo dye.
The two steps reaction is described in Scheme 1.



P-Nitroaniline Diazotized p-Nitroaniline



Scheme 1. Steps of the main reactions

The effect of various variables on the color development of the azo dye formed from the reaction of LTHS with diazotized p-nitroanline was tested and the optimum conditions have been selected.

Choice of diazotized

The effect of different diazotized reagent(0.005M) on the absorbance of the colored azo dye was studied .The results indicated that diazotised p – nitroaniline was the optimum reagent according to the highest intensity of the colored product(Table 1).

Table 1. The chosen of diazotized reagent.

Diazotized reagent,0.005M	**Absorbance**	**λmax(nm)**	**Δλ (nm)***
5-Amino-2-chlorobenzotriflouride	0.3342	530	140
p-Nitroaniline	0.5576	540	150
m-Nitroaniline	0.3209	517	140

*$\Delta\lambda\ (nm)_{=\text{Color contrast}}\ \lambda_{max}^{S}-\lambda_{max}^{B}$ when S =azo dye, B = Blank

Effect of reagent concentrations

It was observed from the results of Table 2 that 2.5 ml of (0.005M) solution of diazotized p-nitroaniline(DPNA) was required for a complete diazo-coupling according to the highest intensity of the formed azo dye and the value of determination`s coefficient(R2). This volume was recommended in the subsequent experiments .

Table 2. Effect of diazotized p-nitroanline on absorbance.

Amount of (0.005M)DPNA reagent solution(ml)	**Absorbance/µg of LTHS**						**R^2**
	10	25	50	80	100	150	
1.0	0.0449	0.1180	0.2210	0.4140	0.5560	0.7852	0.994
2	0.0544	0.1508	0.3218	0.4152	0.5158	0.7260	0.990
2.5	0.0639	0.1855	0.3728	0.5562	0.7460	1.1185	0.998
3	0.0355	0.1282	0.2653	0.4435	0.5460	0.7332	0.989

Effect of Base

The effect of different bases (1 ml of 1M of each base was added) on the absorbance of the azo dye was studied. The results indicate that the reaction needs a strong alkaline medium, and 1.5 ml of NaOH was recommended in the subsequent experiment according to the highest intensity and to the highest value of the color contrast of the formed azo dye.

The stability of the formed azo dye

The effect of time on the development and stability period of the colored azo dye was investigated under the optimum conditions of the reaction. The results were quite satisfying and the absorbance of the colored dye remained constant for at least 90 minutes .

Final absorption spectra

The absorption spectra of LTHS - p-nitroanline azo dye and its corresponding reagent blank are shown in Fig. 1. The azo dye shows maximum absorption at 540 nm in contrast to the reagent blank which shows a nil absorption at 540 nm.

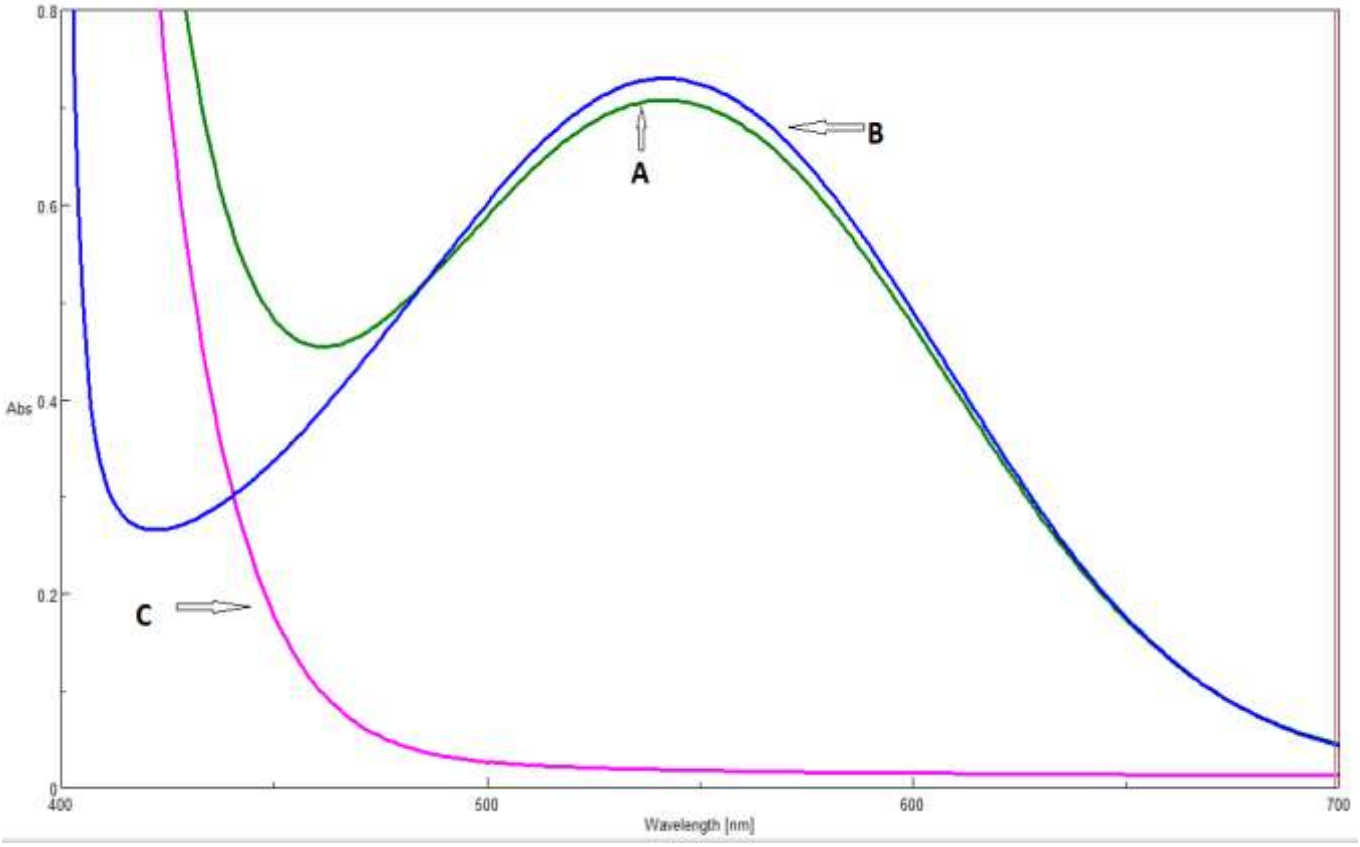
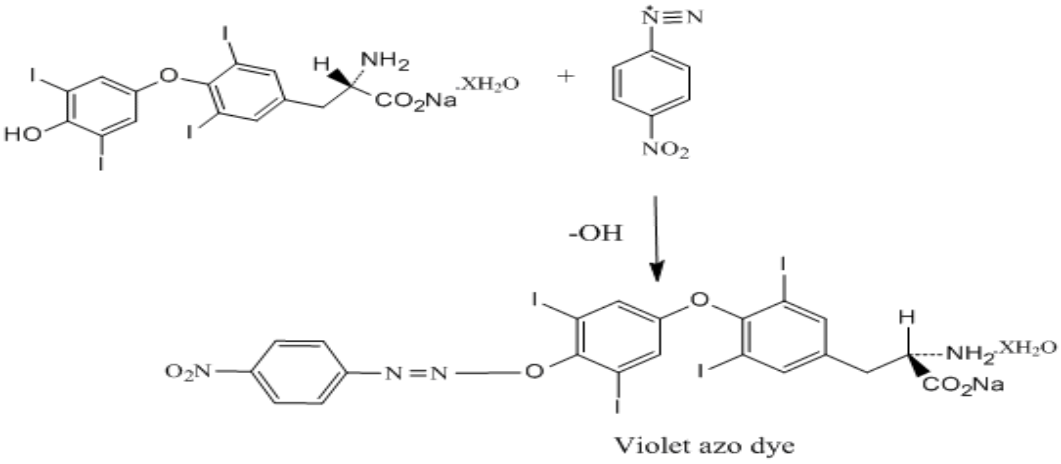


Fig. 1. Absorption spectra of 100 µg LTHS treated according to the recommended procedures and measured against (A) blank (B) distilled water and (C) blank against distilled water.

The nature of the reaction's product

Job's method and mole ratio methods (23) have been used in estimation of reaction ratio stoichiometric amount of LTHS with DPNA. The obtained result showed that a 1:1 LTHS to DPNA ratio is obtained. Therefore the suggested structure was as shown below (Scheme 2).



Scheme2 . Suggested structure of LTHS – DPNA azo dye

Method validation

Table 3 shows the optical characteristics such as Beer's law limits, molar absorptivity,Sandell's sensitivity, absorption maxima, and the regression analysis using the method of least square and the determination coefficient of the regression plots . The precision of the method was very good as shown by the corresponding RSD% value.

Table 3. Optical characteristics and precision data

Effect of interferences

Parameter	Proposed method
Beer's law limits($\mu g.ml^{-1}$)	1 -25
Molar Absorptivity($l.\ mol.^{-1}cm^{-1}$)	6.23×10^5
Sandell's Sensitivity($\mu g.cm^{-2}$)	0.00128
λ_{max} (nm)	540
Regression Equation (Y= sx +c) Slope= s Intercept = c	 0.078 0.006
Determination Coefficient (R^2)	0.999
Precision(% RSD)	Not more than 2.47%

In order to assess the possible analytical applications of the present proposed method, the interfering effect of foreign substances (glucose ,starch, Arabic gum and lactose) on the determination of 100 µg of LTHS is study. It is evident from the results that the proposed method has good selectivity.

Application of the method

The proposed method has been successfully applied in the determination of LTHS in tablet dosage form .The results in Table 4 indicate that the suggested method has a good precision, and the RSD % is not more than 2.47%.

Table 4. Appliction of the prposed method

Pharmaceutical preparation	Amount taken (µg)	Amount measured (µg)	Recovery,%*	RSD,%*
Tablet (LTHS) 50 µg / tablet (Merck)	50	51.33	102.66	2.17
Tablet (LTHS) 50 µg / tablet(Berlin)	50	50.86	101.72	2.47

The performance of the proposed method was assessed by calculation of t-test compared with the standard method (3) for 95% confidence level with eight degrees of freedom. The results showed that the t-value was less than the critical value, indicated that there were no significant differences between the proposed and standard method for estimation of LTHS in tablet dosage.

Conclusion

The proposed method was simple and has a good sensitivity and it is the first proposed visible method in quantitative determination of LTHS, it based on diazo-coupling of LTHS with diazotized p-nitroaniline.

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