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Determination of Thyroxine by Spectrophotometric and High Performance Liquid Chromatographic Methods

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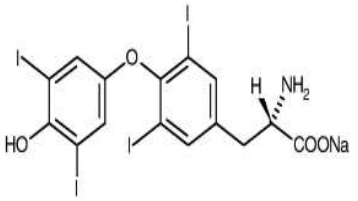
***Abstract*- A simple, and sensitive spectrophotometric and high performance liquid Chromatographic methods have been used for the determination of levo-thyroxine sodium (LTHS) in both pure and its pharmaceutical preparations. The spectrophotometric method is based on the oxidative coupling reaction of LTHS with 4-aminoantipyrine reagent in alkaline medium in the presence of potassium periodate (PIP) as oxidizing agent to produce an intense yellow to orange colored, water soluble and stable product, which shows maximum absorption at 444 nm. Beer's law is obeyed over the range 5-125µg.ml^{-1} of LTHS, limit of detection (LOD) of 0.197 µg.ml^{-1}, limit of quantization (LOQ) of 0.658 µg.ml^{-1}, a molar absorptive of 1.9176×10^{4} l.mol^{-1}.cm^{-1},and Sand ell's sensitivity index of 0.041 µg.cm^{-2}, a relative error of –2.96 to +2.14% and a relative standard deviation of±0.87 to ±3.34%, depending on the concentration level HPLC method has been developed for the measurement of LTHS, the method is based on using C_{18} column and the mobile phase consisted from acetonitrile: methanol: water (40:30:30, V:V:V) with flow rate 1 ml. min^{-1} . The detection is done by using UV detector at wavelength 230 nm and calibration curve shows good linearity in the concentration range from 10 to 200 ppm (0.2-4.0)µg injected with a relative error of - 0.582 to + 0.1% and a relative standard deviation is better than ±0.784% depending on the concentration level. The methods have been successfully applied to the determination of LTHS in pharmaceutical preparations**

***Keywords*-Thyroxin, spectrophotometer, oxidative coupling, HPLC, pharmaceutical preparations.**

I. 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) :



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

The thyroid gland mostly secrets 3-5-3`-5`-tetraiodothyronine or thyroxin (T4) which is mono de-iodinated to 3-3`-5-thiiodathyronine (T3), The level of free thyroxine (T4) in the blood is an important correlate of an individual's thyroid status. Evidencefor this concept has been recently reviewed (4,5) .Few spectrophotometric methods are available for quantification assay of LTHS in pharmaceutical preparation.(6-9). High performance liquid chromatography (HPLC) is one of the most powerful and versatile tool for the quantitative determination of LTHS (10-13), also hyphenated techn-iques(14) and GS as a famous types of chromatographic techniques have been used(15). Other analytical techniques have been reviewed in literature in determination included flow injection- chemiluminescence (16-19), pulls polargraphic (20), electrochemiluminescence (21-22) rad-ioimmunoassay (23) and electrochemical (24-26) .

II. EXPIMENTAL SETUP

Apparatus

A JASCOV - 630 UV / Vis spectro-photometer , with 1cm matched quartz cells were used for all measurement . pH measurements have been done BHANNA 211 pH-meter. BEL ENGNEERING balance has been used for weight. A Shimadzu LC-20AT Liquid Chromatographic Shimadzu (HPLC) with C18 stainless steel column (15 cm x 4.6 mm) has been used.

Reagents

All chemicals used were of analytical grade and LTHS standard material was provided from Sigma company. Chemicals for HPLC were of analytical HPLC grades,

Solutions

LTHS solution (500 $\mu g.ml^{-1}$) -

This solution was prepared by dissolving 0.0500 g of LTHS in 50 ml distilled water(with 5 drops of 1M NaOH) then diluted to 100 ml in a volumetric flask with distilled water.

4-Amindantipyrine(4-AAP) solution($4\times10^{-3}M$) -

This solution was prepared by dissolving 0.203 g of 4-AAP in 100 ml of warm distilled water in a volumetric flask.

Potassium periodate(PPI) solution (0.01M) -

This solution was prepared by dissolving 0.2296 g of PIP in 100 ml ofdistilled water(heating needed to increase solubility) in a volumetric flask.

Sodium hydroxide solution(2 M) -

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

Dosage solution (20 $\mu g.ml^{-1}$) -

Twenty tablets (each tablet contain 50 µg. of LTHS) were dissolved in 40 ml of alkaline (5 drops of 0.1M NaOH) distilled water with heating in water-bath for 10 minutes and then filtered through filter paper ,then the volume completed to the mark in 50 ml volumetric flask with distilled water. While the HPLC dosage solution, the twenty tablets dissolved in 40 ml of the mobile phase (acetonitrile: methanol: water 40:30:30 ,V:V:V) and then filtered through filter paper ,then the volume completed to the mark in 50 ml volumetric flask with the mobile phase.

Procedure and calibration graph Spectrophotometric method -

To a series of 10 ml calibrated flasks an increasing volumes 0.1-2.5 ml of LTHS (500 $\mu g.ml^{-1}$), transfer 2.5 ml of 4-AAP reagent then 2 ml of PIP added, (shaking the solutions), followed by 0.25 ml of 2M NaOH solution (low conc. of NaOH gives low intensity) and the volumes were completed to the mark with distilled water, the absorbances has been measured at 444 nm against reagent blank. The calibration graph was linear over the range 5-125 $\mu g.ml^{-1}$ (Fig. 1). The apparent molar absorptivity referred to LTHS, has been found to be 1.9172×10^{4} $L.mol^{-1}.cm^{-1}$.

HPLC method -

LTHS standard solutions prepared in the concentration between 10-200 µg/ml (0.2-4 µg LTHS injected) in mobile phase. The mobile phase consists of acetonitrile: methanol: water (40:30:30,V:V:V) using isocratically eluted with flow rate equal to 1ml/min. Twenty µl of each standard solution was injected to C18 column at 40 ° C , the area of the peaks was plotted against LTHS concentration and the detection was done by using UV detector at wavelength 230 nm. The peak of LTHS was followed at 5.530 min..A linear calibration graph was obtained between the area under the peaks andConcentration over the range 0.2- 4 µg LTHS injected (Fig. 2).

III.RESULTS AND DISCUSSION

Optimization of Spectrophotometric Selection of coupling reagent -

The effect of different organic agents on the absorption intensity and color contrast has been investigated for better analytical results. The reagents tested were: p-aminobenzoicacid, 4- aminoantipyrine, 4-aminophenol

and N-1-naphthylethylendiamine. Only 4-aminoantipyrine (4-AAP) gives the maximum absorption intensity, other reagent give low or no color constant, therefore it was selected for subsequent investigations.

Selection the amount of coupling reagent -

The results in table I indicated that 2.5 ml of 4-AAP was the optimum amount, it cover the rang of concentration from 125-750 µg of LTHS with determination coefficient =0.998, therefore it was selected for subsequent investigations

Selection of oxidizing agent -

Different types and amounts of oxidizing agents (0.01 M) were investigated in alkaline medium with different .The results indicated that using of potassium periodate as oxidizing agent give the more sensitive reaction (highest intensity),other oxidizing reagent such as N –chlorosuccinimide and N-bromosuccinimide give no reaction ,therefore it was recommended for subsequent experiments with optimum amount 2 ml.

Effect of pH -

The previous experiment shows that the reaction occurred in alkaline medium, so that the effect of different bases on the reaction has been investigated , the result in table II indicated that the reaction need strong alkaline medium , therefore different amounts of 2MNaOH (low conc. gives low intensity) has been tested ,the results shows that 0.25 ml was the optimum volume, therefore it was recommended in the subsequence experiments.

Effect of temperature on reaction -

The oxidative coupling reactions depended on temperature, therefore the suggested procedure has been tested in different temperature(from room temp. 25± 1 to 60 º C with 20 minutes heating) ,the results indicated that room temp. was the optimums temperature according to high intensity and the highest stability.

The stability of the colored product -

The effect of time on the development and stability period of the colored product was investigated under the optimum conditions of the reaction. The results indicate that the absorbance of the colored product remained constant for at least 110 minutes and this time was sufficient for several determination (Table III).

Final absorption spectra -

The absorption spectra of the colored product formed from the reaction between LTHS and 4-AAP reagent in presence of PPI in an alkaline medium shows maximum absorption at 444 nm in contrast to the reagent blank, which shows a weak absorption at the same wavelength (Fig.3)

HPLC method -

Selection of wavelength:

The absorption spectrum of 100 µg .ml^{-1} of LTHS prepared in various mixture of mobile phase (Fig. 4 and Table IV) .The results indicated that the mobil phase consist from acetonitrile – methanol – water gave high area and excepted value of capacity factor(K) at wavelength at 230 nm . Therefore, 230 nm has been used for UV-detection.

Selection of mobile phase:

The results in table IV indicated that the composition of mobile phase No. V gives the optimum results, therefore the effect of different compositions has been studied (Table V). The results indicated that using acetonitrile: methanole : water with ratio (40:30:30 V:V:V) as a mobile phase gives symmetrical peak and good value of capacity factor , therefore it followed in the next experiments .

Selection of flow rate:

The effect of the rate of flowing mobile phase has been studied, the flowing rate equal to1ml.$min.^{-1}$ which gives excepted capacity factor withclear chromatogram and good sharpness has been fixed.

Application of the methods:

The proposed methods were applied to determine LTHS in two pharmaceutical formulations. On

applying proposed procedures, good recoveries and precisions were obtained as shown in table VI.

IV.CONCLUSION

The suggested methods for the determination of LTHS are simple, sensitive, accurate and can be applicable in determination LTHS in dosage form without resorting to an extraction step.

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Table I
Effect of the amount in of reagent absorbance

No.	Acetonitrile: Methanol: Water	Retention time (min.)	Capacity factor (K)
I	20:40:40	4.462	0.904
II	30:40:30	4.856	1.435
III	40:30:30	5.530	2.767
IV	50:20:30	3.652	2.013
V	60:20:20	2.314	1.907
VI	70:10:20	3.842	0.182

Table II
The effect of pH on absorbance

Base used (1ml of 2 M)	$\lambda_{max.(nm)}$	Absorbance	
		Sample VS. blank	Blank VS. D.W.*
Without	No colour contrast		
NaOH	444	1.1508	0.0540
KOH	438	0.9488	0.0634
Na_2CO_3	432	0.7845	0.0524
$NaHCO_3$	425	0.6956	0.0511

Table III
Effect of LTHS amount and time on absorbance

Amount of 0.01 M 4-AAP (ml)	Absorbance//μg of LTHS				$-R^2$
	125	250	500	750	
1	0.2142	0.4250	0.8531	1.1560	0.993
2	0.2410	0.5988	0.9671	1.4512	0.994
2.5	0.3145	0.6470	1.1091	1.8732	0.998
3.0	0.2395	0.3840	0.8443	1.4268	0.988

Table IV

Selection optimum wavelength corresponding to the mobile phase used

µg of LTHS	Absorbance / minute standing time							
	10	20	30	40	50	60	90	120
250	0.5590	0.5588	0.5582	0.5578	0.5570	0.5562	0.5542	0.5252
500	1.1542	1.1538	1.1536	1.1528	1.1518	1.1508	1.1485	1.1245

Table V

Selection the composition of mobile phase

No	Mobile phase solution	λ_{max}, nm	Retention time (min.)	Area	Capacity factor (K)
I	Acetonitrile-water 80:20	319	4.783	2172610.5	1.504
II	Methanol- Water 80:20	316	2.250	3710358.4	1.343
		262	4.779	3271742.6	1.062
III	Ethanol-Water 80:20	282	4.742	3727220.8	1.390
IV	Acetonitrile-Ethanol-Water 50:20:30	278	4.782	3628618.4	0.328
		312	4.800	33127400.2	0.329
V	Acetonitrile-Methanol-Water 50:20:30	230	5.766	3848252.6	2.736

Table VI

Application of the methods

Pharmacetal preparations	Specter. Method			HPLC method		
	LTHS taken, µg	Relative error, %*	R.S.D,%*	µg LTHS injected	Relative error, %*	R.S.D,%*
Berlin tablet 50µg LTHS/ tablet	50	+0.90	±1.68	2	-0.100	± 0.784
	100	-2.96	±0.87	4	+0.100	± 0.234
Merck tablet 50µg LTHS/ tablet	50	+1.47	±2.15	4	-0.582	±0.061

* Average of five determinations

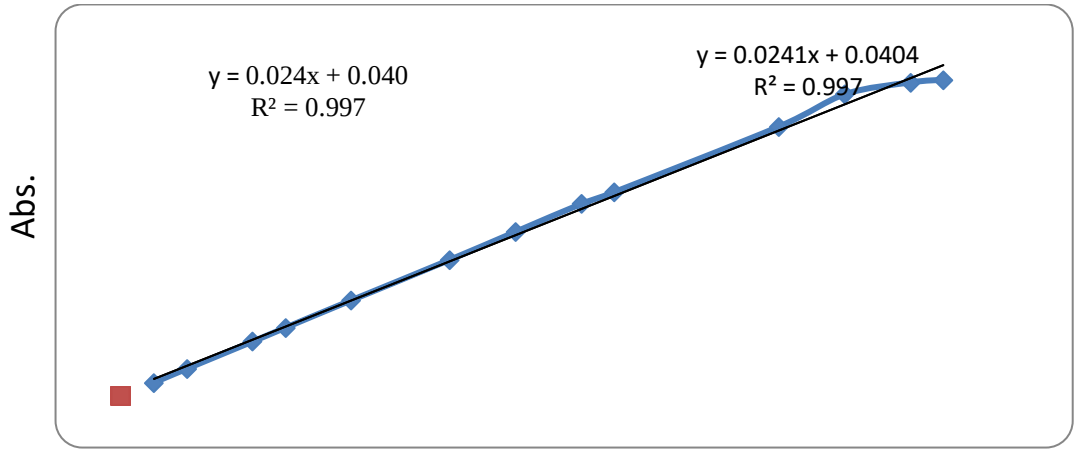

Fig. 1. Calibration graph of spectrophotometric method

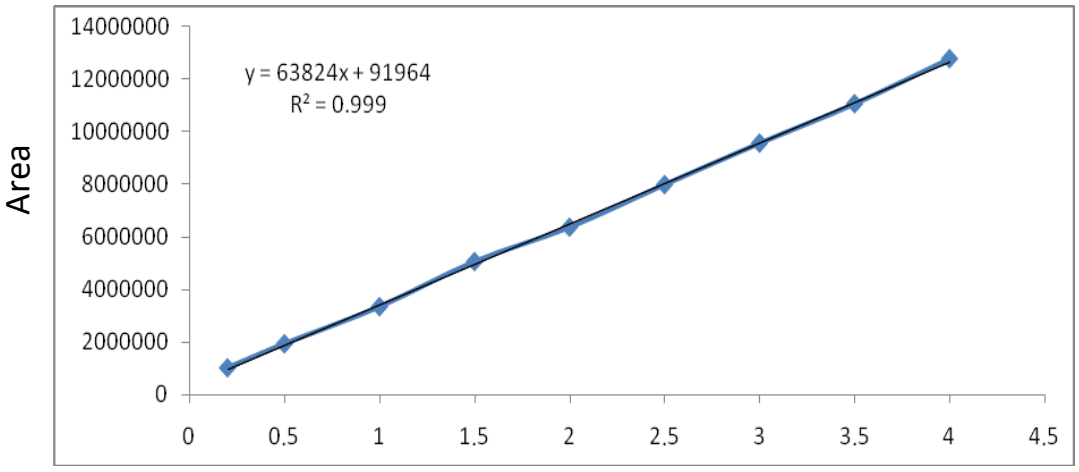

Fig. 2. Calibration graph of HPLC method

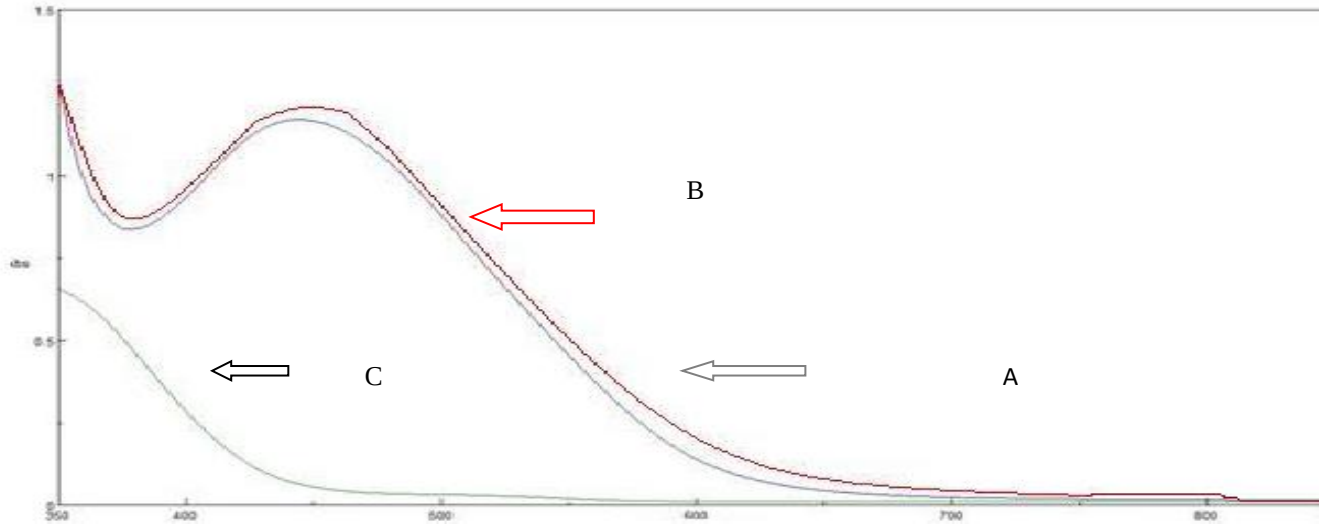

Fig. 3. Absorption spectra of 100 µg treated according to the recommended procedure and measured against (A) blank, (B) distilled water and (C) blank measured against distilled water

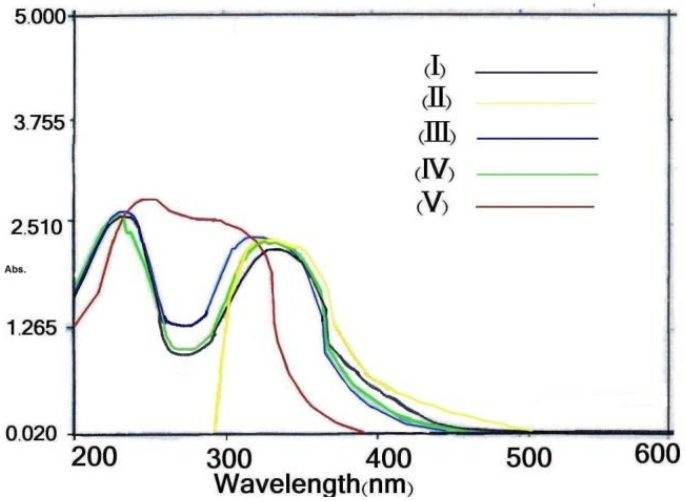

Fig. 4. Absorption spectra of dissolved in different mixture of mobile phase.