ISSN 1061-9348, Journal of Analytical Chemistry, 2013, Vol. 68, No. 6, pp. 510–515. © Pleiades Publishing, Ltd., 2013.

ARTICLES

A Derivative UV Spectrophotometry Method for the Determination of Levothyroxine Sodium in Tablets[1]

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Received May 26, 2011; in final form July 05, 2012

Abstract—A derivative UV (**D-UV**) spectrophotometric method was developed for the determination of Levothyroxine Sodium (L-T_4) in tablets of different doses. Quantification was performed using the second derivative of the absorption spectrum at 253 nm ($^2D_{253}$) in methanol : water (50 : 50; v/v) (pH 11.2). The method was validated and compared with an HPLC procedure carried out using a RP-18 column (125 × 4 mm, 5 μm) and methanol : phosphoric acid (0.1%) (70 : 30, v/v) (pH 3) as mobile phase. Flow rate was set at 1.5 mL/min, and detection was performed at 225 nm. The proposed D-UV method was linear in the range 3.0–40.0 μg/mL with an appropriate precision and accuracy, and it was selective for the drug under study. On the other hand, results obtained by $^2D_{253}$ analysis were similar to those obtained by HPLC, with no statistically significant differences between them. Therefore, it was concluded that the developed method is suitable for the determination of L-T_4 in tablets at the tested doses.

Keywords: levothyroxine sodium, derivative UV spectrophotometry, high performance liquid chromatography

DOI: 10.1134/S1061934813060075

INTRODUCTION

Levothyroxine Sodium (L-T_4, Fig. 1) is a synthetic hormone widely used for the treatment of hypothyroidism, with effects identical to those of the natural hormone produced by the thyroid gland. The biological action of thyroid hormones is to increase the basal metabolic rate, and thus entailing an increase of substrates use, enzymes activity and other hormones secretion. In addition to this, it contributes to the general development of organs and tissues; fetal and postnatal thyroxine is essential for the correct development of neurons and growth of their extensions. Thyroid hormones deficiency in childhood and adults involves a progressive reduction in nervous system reactivity, affecting both the motor function and the intellectual aspects [1].

Hypothyroidism is one of the most frequent endocrine diseases, and more than 95% of the cases have a thyroid (primary) origin [2], requiring treatment with an appropriate dose of L-T_4. This substitutive treatment allows patients to clinically improve their thyroid hypofunction recovering physiological concentration levels of L-T_4 and keeping thyrotropin (TSH) concentrations in the lower half of the normal range [3].

Levothyroxine Sodium is an unstable drug with poor solubility: 1 part dissolves in 700 parts of water and 300 parts of ethanol. It is practically insoluble in acetone, chloroform and ether, and soluble in alkaline hydroxide, but the solutions are unstable [4]. Furthermore, L-T_4 tablets must be protected from light [5]. Due to these characteristics it is difficult to find an exact and reproducible analytical method for the drug determination in tablets, moreover considering that typical treatment doses are in the order of micrograms.

Most of the analytical literature methods are HPLC methods [6–9]. Other methods include electrospray ionization tandem mass spectrometry (**ESI-MS/MS**) [10] and inductively coupled plasma mass spectrometry [11]. Despite an extensive search, no derivative UV spectrophotometry (D-UV) method for the determination of L-T_4 could be found in the literature. Consequently, it would be useful to develop a new method allowing for the quantification of this drug in vitro in a simple, fast, and accessible way with reliable results.

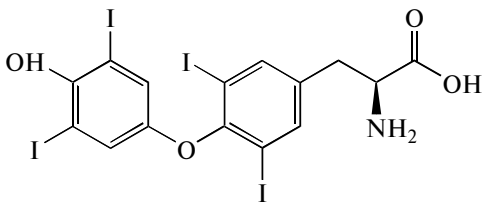

Fig. 1. Chemical structure of Levothyroxine.

[1] The article is published in the original.

The aim of the present work was to develop and validate a derivative UV spectrophotometry method that could be used in routine quality control for the determination of L-T_4 in tablets containing different amounts of the drug. In order to bring more confidence to the method, it was compared with an HPLC one, modified from the USP 31 [12] proposed method.

Since commercially available products of L-T_4 usually consist in scored tablets, it is a very common practice to break them in order to obtain the required treatment dose, but jeopardizing the uniformity of the administered units [13]. Thus, another goal of the present study was to assess the effect of the breaking process in the uniformity of dosage units. To accomplish the aforementioned, the percentage of drug loss due to the breaking was evaluated by quantifying the drug content of the divided tablets.

EXPERIMENTAL

Chemicals. Levothyroxine Sodium Reference Substance (L-T_4 RS) of 89.2% purity from Montpellier S.A. Quemistry was used. The L-T_4 products were purchased from the market and used as samples. These products were from two different laboratories (A and B) and consisted in scored tablets containing different amounts of the drug: 50 and 100 μg from Laboratory A; 50, 100 and 200 μg from Laboratory B. Chromatographic grade methanol and analytical grade phosphoric acid were used as solvent and for the mobile phase, respectively.

Apparatus and conditions. For the D-UV method, a methanol : water (50 : 50; v/v) (pH 11.2) mixture was used as solvent. The pH of the mixture was adjusted to 11.2 with 1 M NaOH. Quantification was performed using the second derivative from the absorption spectrum at 253 nm ($^2D_{253}$). A Thermo spectrophotometer, Heliosbeta model (Thermo Fisher Scientific, Waltham, MA, USA) was used. The chromatographic method used for comparation (modified from the USP 31 [12] one and validated in our laboratory) was carried out in an HPLC system consisting in a 322-H2 series pump, a 155/156 UV/Vis detector and a workstation equipped with the UniPoint LC 3.3 version software (Gilson SAS, Villiers-Le-Bel, France). A Rheodyne 7125 manual sample injector (Rheodyne, CA, USA) with a fixed volume of 20 μL was also used. Chromatographic conditions were: LiChrocart RP-18 (125 × 4 mm i.d., 5 μm particle size) column (Merck, Darmstadt, Germany) as stationary phase and methanol : phosphoric acid (0.1%) (70 : 30; v/v) (pH 3.0) as mobile phase. Flow rate was set at 1.5 mL/min, and detection was performed at 225 nm.

Standard preparations. The standard stock solution was prepared by dissolving about 10 mg of accurately weighed L-T_4 RS in 100 mL of the methanol : water (50 : 50; v/v) (pH 11.2) mixture, in order to obtain a concentration of 100 μg/mL. For complete dissolution of the drug, a 30 min sonication at room temperature was needed. Stock solution was stored at 4°C and protected from light. Standard solutions for each analytical method were prepared by appropriate dilution of the stock solution. For the $^2D_{253}$ method, dilutions were performed with the same medium to obtain a concentration of 10 μg/mL. For the HPLC method, filtered methanol was used to obtain a standard solution of 2 μg/mL.

Validation of analytical methods. The following validation parameters were determined: linearity and precision (for both $^2D_{253}$ and HPLC methods), specificity and accuracy (only for $^2D_{253}$ method), according to pharmacopoeia specifications [12, 14].

For the $^2D_{253}$ method, linearity was demonstrated through a standard calibration curve in the range of 3.0–40.0 μg/mL, with seven concentration levels.

The system precision was determined by repeated measuring ($n = 6$) of the response of the same standard solution (approximately 10 μg/mL) at 253 nm, and expressed as relative standard deviation (%RSD). The method precision (as %RSD) and accuracy (as %Recovered) were assessed by a recovery assay at three concentration levels: 6.0, 10.0 and 30.0 μg/mL. At each level, three independent samples were prepared by adding a mix of excipients to the corresponding amount of L-T_4 RS. The excipients:drug ratio was the corresponding to the lowest of the tested doses (and therefore, the maximum one). The excipients' mixture was prepared according to the formulation declared by one of the products tested (product A), since no excipients description was declared for the other product (B). The results were statistically evaluated using Student's *t*-test.

The specificity of the method was established through the analysis of excipients solutions responses. Placebo tablets (mixture of excipients) were prepared with lactose, microcrystalline cellulose, starch and magnesium stearate according to the proportions declared in the 50 μg L-T_4 product from Laboratory A. The mixture was then dissolved in methanol : water (50 : 50; v/v) (pH 11.2), sonicated for 30 min at room temperature and centrifuged at 3500 rpm for 25 min. The absorption spectrum of the clear centrifugate was measured and the response corresponding to the second derivative at 253 nm was recorded.

For the HPLC method, the linearity was studied in range of 0.8–10 μg/mL by a standard calibration curve at six concentration levels. System precision was assessed at the concentration of 2 μg/mL (100%) using the same procedure described above for the $^2D_{253}$ method, and expressed as %RSD. The method precision was determined as the %RSD obtained for three independent samples prepared at 2 μg/mL.

Method application: analysis of L-T_4 tablets. The method was applied to assay brand products of L-T_4 with different drug amounts: entire doses of 50 and 100 μg from two laboratories (A and B), and 100 μg

Table 1. Results of the recovery assay of L-T_4 from the excipient mixture samples spiked with different amounts of L-T_4 RS

Theoretical concentration of L-T_4 RS in the samples (μg/mL)	Recovered concentration of L-T_4 RS in the samples (μg/mL)	Recovery (%)
6.15	5.95	96.75
5.99	6.02	100.50
5.99	6.12	102.17
10.08	10.26	101.79
9.99	10.12	101.30
10.08	10.14	100.59
28.10	28.56	101.64
28.37	27.53	97.04
28.63	29.24	102.13
	Mean	*100.43*
	%RSD	*2.08*

dose generated by division of a 200 μg tablet (only from B). The results were statistically compared with those obtained with the HPLC method by an Analysis of Variance (ANOVA) followed by the comparison of the obtained means values according to the Fisher's LSD (*Least Significant Difference*) and the Tukey's HSD (*Honestly Significant Difference*) methods [15].

Sample solutions. Ten tablets were accurately weighed and then powdered. For triplicate, an amount of powder equivalent to 100 μg of L-T_4 was transferred to a 10 mL volumetric flask and dissolved with methanol : water (50 : 50; v/v) (pH 11.2). These solutions were sonicated for 30 min at room temperature, transferred to 15 mL centrifuge tubes and then centrifuged 20 min at 3500 rpm. In the D-UV methodology, the absorption spectrum of the clear centrifugate was recorded and the response at 253 nm of the second derivative of the spectrum was determined. The amount of L-T_4, expressed as percent of the labeled amount (%LA), was obtained by calculation using a standard solution of L-T_4 RS. For the HPLC method, a clear portion of the centrifuged sample was diluted with filter methanol to obtain a final concentration of 2 μg/mL. These solutions were centrifuged at 13000 rpm for 10 min at 4°C and injected in the HPLC system.

To assay the L-T_4 100 μg dose generated by division, ten 200 μg tablets from Laboratory B were divided into halves using a cutting element and both parts were separately weighed. Samples corresponding to each half were prepared and assayed following the same procedure described above for entire tablets (only by the $^2D_{253}$ method).

RESULTS AND DISCUSSION

Figure 2 shows the zero order spectrum from a 10.0 μg/mL L-T_4 RS solution (a), the second order derivative spectrum of the same solution (b, continuous line) and the second derivative spectra of the clear centrifugate of the excipient mixture (b, dot line).

D-UV method. A linear response was observed in the concentration range 3.0–40.0 μg/mL, with a coefficient of determination $r^2 = 0.9998$. The intercept (a) and the slope (b) with the respective 95% confidence interval were $a = -0.0009 \pm 0.0012$ and $b = 0.00397 \pm 0.00006$ respectively. Employing Student's t-test, a linear correlation was observed ($p < 0.05$), assuming that there was no correlation between X and Y as null hypothesis. The residuals' sum was 3.5×10^{-18}. The limit of quantification (LOQ) was 3.0 μg/mL according to the lowest standard concentration on the calibration curve, since a signal-to-noise ratio equal or greater than 10 : 1 was obtained when comparing measured signals from 3.0 μg/mL samples ($n = 3$) with those of blank samples (excipients mixture solutions) [16].

The system precision expressed as relative standard deviation (%RSD) was 1.2%. Results of the recovery assay are presented in Table 1. The accuracy and method precision at each concentration level, expressed as %Recovered (±%RSD), was 99.8 (±2.8), 101.2 (±0.6) and 100.3 (±2.8) for the 6.0, 10.0 and 30.0 μg/mL concentrations, respectively. As shown in Table 1, the average recovery for the three levels studied ($n = 9$) was 100.4%, with a %RSD of 2.1. According to Student's t-test, the method was accurate ($p > 0.05$).

As it can be seen in Fig. 2b, the excipients signal at 253 nm was practically equal to zero (the obtained values were around 0.001 in all cases) and did not inter-

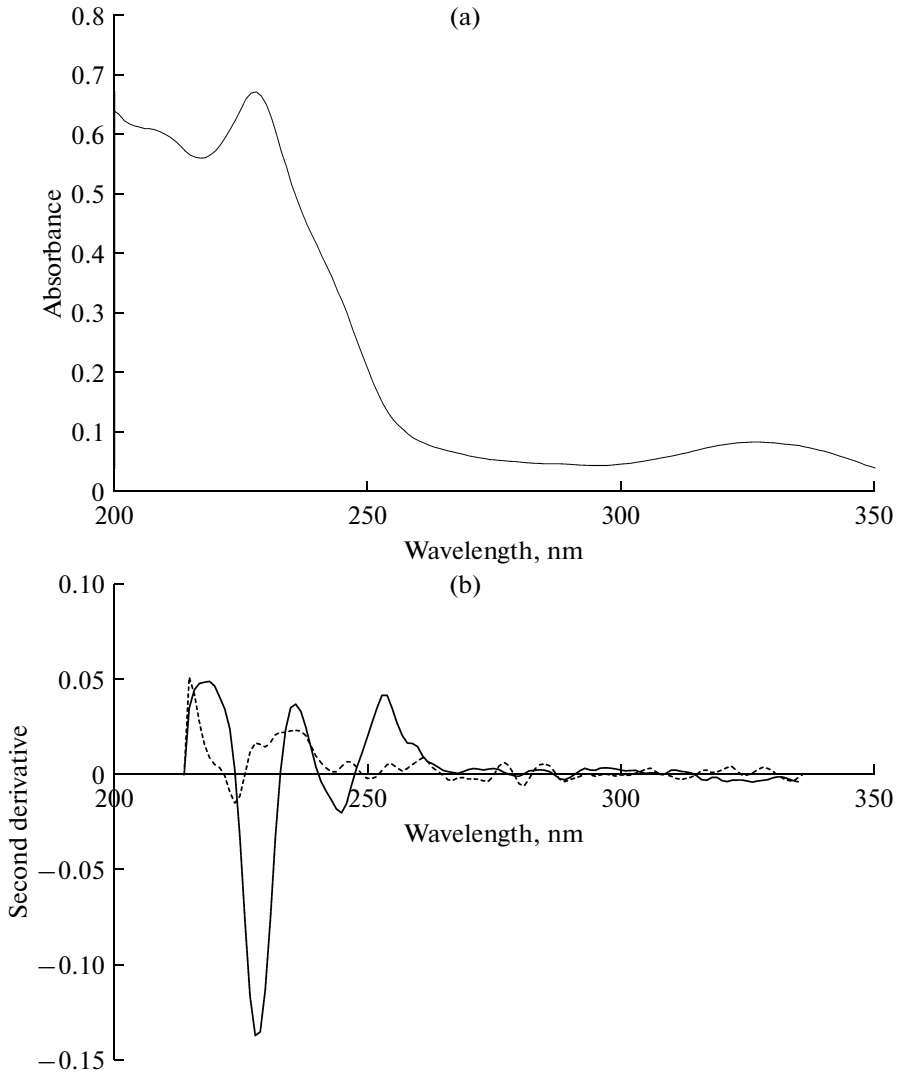

Fig. 2. UV spectrum of a L-T_4 RS solution (10 μg/mL) prepared in methanol : water (50 : 50; v/v) (pH 11.2). (a) Zero order spectrum. (b) Second derivative spectrum (continuous line) superimposed with the second derivative spectrum of the excipient mixture also prepared in methanol : water (50 : 50; v/v) (pH 11.2) (dotted line).

fere with the L-T_4 signal at any of the studied levels. Therefore, the method was specific to the drug.

HPLC method. The linear regression analysis performed in the concentration range 0.8–10 μg/mL resulted in a coefficient of determination $r^2 = 0.9992$, and the intercept (a) and the slope (b) with the 95% confidence interval were $a = (-3.1 \pm 4.1) \times 10^3$ and $b = (29.3 \pm 1.2) \times 10^3$, respectively. The residuals' sum was -1.4×10^{-10} and the linear correlation was confirmed ($p < 0.05$) according to the Student's t-test.

The system precision, expressed as relative standard deviation (%RSD), obtained for the concentration level of L-T_4 RS solution of 2.0 μg/mL was 2.2%, and the method precision determined at the same level with three independent samples was 2.8%. Figure 3 shows the chromatogram obtained for the 2.0 μg/mL L-T_4 SR solution.

Comparison between proposed $^2D_{253}$ and HPLC method. The results obtained from the assay of L-T_4 tablets by both $^2D_{253}$ and HPLC methods are presented in Table 2. According to the USP 31 [12], the tablet content may be within the range 90–110% of the labelled amount (%LA) of L-T_4. In all cases, this acceptance criteria was fulfilled.

To compare the results obtained by $^2D_{253}$ to those obtained with the HPLC method, an ANOVA was performed for each studied formulation, with the analytical method ($^2D_{253}$ or HPLC) as fixed effect (treatment). Once the null hypothesis of equal means was accepted ($p > 0.05$), treatment group means were compared by Fisher's LSD and Tukey's HSD statisti-

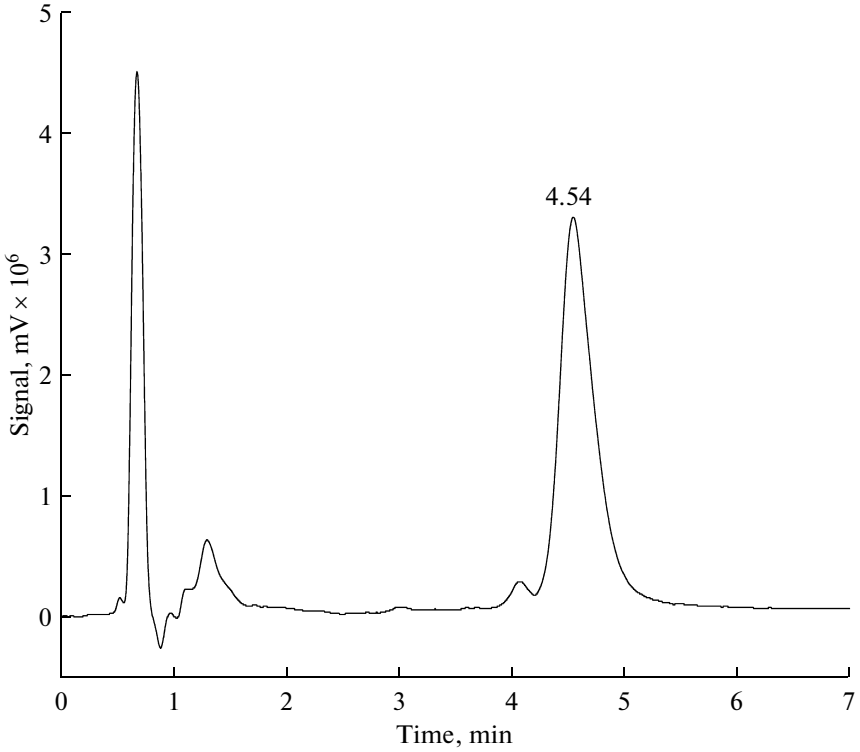

Fig. 3. HPLC chromatogram obtained for a L-T_4 RS solution (2.0 μg/mL) in methanol.

cal methods [15] to confirm that there was no statistically significant difference between them. Both statistics were calculated (ΔLSD and ΔHSD) and then compared with the means difference between both methods, $|X_{2D} - X_{HPLC}|$, being X_{2D} and X_{HPLC} the %LA obtained by $^2D_{253}$ and HPLC, respectively. The means difference was considered statistically significant when it was greater than the calculated statistics. As it can be seen in Table 2, there was no significant difference between both analytical methods for all the tested products and doses.

Table 3 presents the results of the assayed 100 μg L-T_4 doses generated by division of the 200 μg tablets from Laboratory B. The average %LA ± %RSD (half 1 plus half 2) was 88.0 ± 7.2 (n = 10), while the same value obtained for the entire 200 μg tablets assayed was 102.9 ± 0.9 (Table 2). These results seem to indicate that there is a loss of nearly 15% in the tablets' active drug amount during the breaking procedure.

So, a simple, rapid, reliable and specific second order derivate UV method was developed for the determination of L-T_4 in tablets. The validation protocol applied demonstrated the method to be accurate, reproducible, linear and sensitive for the drug quantification in a wide range of concentrations with no interference of the formulations' inactive ingredients.

Table 2. Results of the assay of different tablets doses (μg) of L-T_4 from both studied laboratories, A and B, by $^2D_{253}$ and HPLC methods. The content is expressed as mean percentage of the labeled amount (%LA) ± %RSD (n = 3). $|X_{2D}–X_{HPLC}|$ is the difference between the means obtained by each method. ΔLSD and ΔHSD are the statistics calculated according to Fisher's LSD and Tukey's HSD methods, respectively

	Laboratory A		Laboratory B		
	50 μg	100 μg	50 μg	100 μg	200 μg
$^2D_{253}$	99.2 ± 1.1	96.7 ± 2.0	107.4 ± 0.8	106.1 ± 1.1	103.0 ± 0.9
HPLC	97.3 ± 0.5	95.3 ± 0.6	107.5 ± 0.6	106.0 ± 0.3	
$\|X_{2\Delta}–X_{HPLC}\|$	*1.85*	*1.48*	*0.07*	*0.07*	
ΔLSD	1.94	1.94	1.94	1.94	
ΔHSD	3.26	3.26	3.26	3.26	

Table 3. Assay of LT_4 200 μg (LA) divided dose by $^2D_{253}$. %LA: percentage of the labeled amount

Tablet	Half 1		Half 2		Half 1 + 2
	Amount found (μg)	%LA	Amount found (μg)	%LA	%LA
1	80.60	40.30	79.35	39.67	79.97
2	77.59	38.79	88.39	44.19	82.99
3	84.62	42.31	89.89	44.94	87.25
4	75.66	37.83	83.93	41.96	79.79
5	79.31	39.65	90.50	45.25	84.90
6	81.50	40.75	100.23	50.11	90.86
7	83.62	41.81	91.21	45.60	87.41
8	88.27	44.14	97.58	48.79	92.92
9	92.43	46.22	97.09	48.54	94.76
10	99.54	49.77	97.83	48.91	98.68
				Range	*[79.79–98.68]*
				Mean	*87.96*
				%RSD	*7.19*

Furthermore, it was demonstrated that the proposed method provides comparable results to those obtained by a HPLC methology, but with less costs and operative steps, when applied to the assay of L-T_4 tables with different drug amounts. Therefore, it can be concluded that the described $^2D_{253}$ method is suitable for the intended purpose.

With regard to the tablets' breaking process, the obtained results advice against this practice, since the resulting loss of active drug was not negligible. In the patients' daily practice, this could cause a decrease in the systemic levels of the drug, thus jeopardizing the effectiveness of the therapeutic treatment.

ACKNOWLEDGMENTS

A. Gregorini is a fellow of CIC (Scientific Investigation Commission). The authors are grateful to the Pharmaceutical College of La Plata and Montpellier Chemistry.

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