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Research Article

Validated ion exchange HPLC method for the quantification of levothyroxine – a narrow therapeutic index drug – used for the treatment of hypothyroidism

Marwa Al Jamal[1], Malak Al Bathish[1], Azza Gazy[1]

1 *Department of Pharmaceutical Technology, Faculty of Pharmacy, Beirut Arab University, Beirut, Lebanon*

Corresponding author: Marwa Al Jamal (Marwa.jamal@bau.edu.lb)

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Abstract

Drugs with narrow therapeutic index (NTI-drugs) have been defined by the FDA as drugs with small differences between therapeutic and toxic doses that might lead to serious therapeutic failures or life-threatening adverse drug reactions. Levothyroxine sodium pentahydrate (LT4), a synthetic T4 hormone used for the treatment of hypothyroidism (a condition where there is a hormonal imbalance in the thyroid gland that is responsible for the regulation of several physiological, metabolic, cardiovascular, and neurological processes). LT4 is designated by the FDA as a narrow therapeutic index drug and is available in the market in the form of very low dose pharmaceutical formulations ranging from 25 mcg to 150 mcg. This requires that the pharmaceutical dosage form should contain the exact labeled amount of the active ingredient, LT4, such that safety and efficacy are maintained. Therefore, it is necessary to develop an a precise, accurate and sensitive analytical method for LT4 quantification considering the treatment doses being in micrograms. In the present work, an ion exchange HPLC method has been developed and validated for the determination of LT4 as per ICH guidelines. The developed method was found to be simple, sepcific, precise and accurate. The low LOD and LOQ values allowed the quantitfication of the active ingredient in different pharmaceutical products qualifying the method to be applied in quality control assays.

Keywords

Ion exchange HPLC, Quantification, Hypothyroidism, Narrow therapeutic index drug

Introduction

The thyroid gland, one of the most important glands in the human endocrine system, secretes triiodothyronine and thyroxine, two hormones that are essential for the regulation of several physiological, metabolic, cardiovascular, and neurological processes (Arthur and John 2006; Mondal et al. 2016). Accordingly, any imbalance in the level of such hormones would impact the entire body. Hypothyroidism is a condition where the thyroid gland doesn't produce and release enough hormones into the bloodstream. This condition could be treated by hormonal replacement therapy. Levothyroxine sodium pentahydrate (LT4) (Fig. 1), known as 3,5,3′,5′-tetraiodothyronine sodium salt, is a synthetic T4 hormone administered orally for the treatment of hypothyroidism as it depicts the natural hormone (Jonklaas et al. 2014).

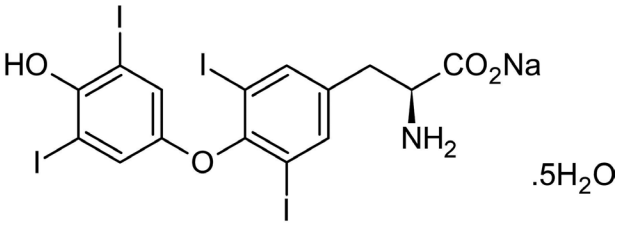

Figure 1. Chemical structure of LT4 sodium pentahydrate.

The FDA believes that NTI drug products require tighter product quality standards. Thus, NTI drugs must be carefully dosed and monitored. Moreover, Health care professionals, pharmaceutical scientists and regulatory agencies, stressed about the importance of similarity in terms of quality and quantity between a generic and its reference product for NTI drugs to be considered therapeutically equivalent. LT4 is designated by the FDA as a narrow therapeutic index (NTI) drug since it contains low doses of the active ingredient ranging from 25 to 150 mcg per tablet. Thereby it is of high importance that the marketed tablets contain the exact labeled amount of LT4 in order to maintain the highest efficacy and safety (Jonklaas et al. 2014; CDER 2000). In USA, UK and France, several pharmaceutical formulations containing LT4 have been recalled from the market due to therapeutic failure. This raised the concern that the marketed tablets do not meet the requirements of content uniformity and potency specification which was due to chemical instability of LT4 as a molecule and as a drug product. Accordingly, the national regulatory bodies issued a stricter potency specification where the content per tablet for LT4 should be between 95 and 105% (FDA 2007; MHRA 2013). Therefore, it is necessary to develop a sensitive, precise and accurate analytical method for LT4 determination that is able to quantify the low doses in pharmaceutical tablets.

Several analytical methods have been reported for the determination of LT4 in its pure, pharmaceutical dosage forms and in serum mostly using HPLC coupled to different detector types (Rapaka et al. 1981; Garnick et al. 1984; Kazemifard et al. 2001; Kannamkumarath et al. 2004; Gika et al 2005; Shah et al. 2008; Collier et al. 2014; Singare et al. 2016; Islam et al. 2018; Dutt et al. 2020; Thapa at al. 2020; Yunhee et al. 2021; Jisun et al. 2022; Naoyuki at al. 2022). Other analytical methods included derivative UV-spectrophotometry (Gregorini at al. 2013), electrospray ionization tandem mass spectrometry (ESI-MS/MS) (Lee et at. 2008), inductively coupled plasma mass spectrometry (Pabla et al. 2009) and immunoassays (Kunst et al. 1998; Stevenson et al. 1998; Frank et al. 2004; Dhatt et al. 2006).

In the present work, an ion-exchange HPLC method has been developed and validated for the determination of LT4 in its pure form and in pharmaceutical preparations. The proposed method was validated as per ICH guidelines and was found to be simple, sepcific, precise and accurate (ICH 2005). The low level of LOD and LOQ values allowed the quantitation of LT4 in different pharmaceutical brands (Euthyrox, Eltroxin) and thus the developed analytical method can be used in quality control assays.

Methodology

Experimental preparation of the mobile phase

The acetate buffer solution was prepared by weighing 1.64 g of anhydrous sodium acetate CH_3COONa and dissolving it in 1L HPLC grade water, then adjusting its pH to a value of 4.25 using acetic acid.

The mobile phase was filtered by passing it through 0.45 µm pore size membrane filter (Agilent technologies) at a pumping speed of 30 L/min (Glassco diaphragm Vacuum pump) and degassed for 30 min using SONOMATIC^R LANGFORD ultrasonic sonicator.

Preparation of 0.05% methanolic Sodium Hydroxide

A 0.1 N sodium hydroxide solution was prepared by weighing 4 g of NaOH and dissolving it in 1 L of HPLC grade water. To the above prepared solution an equal amount of methanol was added.

Preparation of LT4 standard solution (200 µg/mL)

The solution was prepared by accurately transferring 2 mg of LT4 standard powder into a 10-mL volumetric flask. The powder was dissolved and diluted to volume with 50% methanolic NaOH.

Chromatographic conditions

The chromatographic analysis was performed at ambient temperature with isocratic elution using HiQ sil NH_2 column at a flow rate of 1.2 mL/min and at a wavelength of 252 nm.

The mobile phase used for the chromatographic separation was prepared by adding HPLC grade acetonitrile to the acetate buffer solution of pH 4.25 in the ratio of (55:45, %V/V), respectively.

Construction of calibration graph

Aliquots of LT4 standard solution were transferred into a series of 10 mL volumetric flasks and completed to volume with 50% methanolic NaOH to give the final concentration ranges stated in Table 2.

The above solutions were filtered using 0.2 µm disposable filters and 20 µL portion of these solutions were injected in triplicates and chromatographed under the conditions mentioned above. The standard calibration graph was prepared by plotting the peak area values against the corresponding concentrations. Later, the concentration of LT4 was computed from this calibration graph.

Pharmaceutical tablets

Ten tablets of Euthyrox (100 µg) and Eltroxin (100 µg) were accurately weighed and finely powdered. Powder

amount equivalent to 250 µg was accurately weighed and transferred to a 25-mL volumetric flask, dissolved in around 10 mL 50% methanolic NaOH, shaken for 15 minutes, filtered using Whatmann No. 41 filter paper and completed to volume with the same solvent.

The above solutions were filtered using 0.2 µm disposable filters and 20 µL portion were injected in five replicates and chromatographed under the chromatographic conditions mentioned above. The peak area values were measured and the corresponding concentrations in the tablets were derived by referring to the calibration graph.

Results and discussion

Optimization of chromatographic conditions

Various physiochemical properties like pKa value, log P, solubility and absorption maximum (λ_{max}) of the drug must be known, as such parameters are important for HPLC method development. Log P and solubility helps in the selection of the mobile phase and sample solvent while pKa value helps in the determination of the pH of the mobile phase (Bakshi et al. 2002).

The HPLC method was developed to provide simple rapid and reliable quality control analysis of LT4. So, the most important aspect is to achieve elution with acceptable retention time, peak symmetry, and high sensitivity.

To attain this goal, the preliminary investigations were directed towards studying the effect of different variables on the system suitability in order to maximize the sensitivity of the method. The parameters assessed and optimized included column type, detection wavelength, type and quantity of organic modifier, aqueous phase concentration and pH, mobile phase additives and flow rate.

Detection wavelength

The absorption spectrum of the standard solution LT4 in 50% methanolic NaOH was performed over the wavelength range of 200–400 nm as shown in Fig. 2. Different wavelength values were tested; 252 nm was found to be the most effective λ for LT4 detection as it provided better detector responses together with the best sensitivity. Hence, 252 nm was set as the UV-detector wavelength for the determination of LT4.

Column type

Trials were performed on three different column types: two reversed-phase (C_8 and C_{18}) and the third one was an ion exchange column NH2. Upon using the RP columns, several problems were faced of which peak splitting and tailing, in addition to baseline noise. However, upon the use of anion exchange column (HiQ sil NH2), all these problems were resolved.

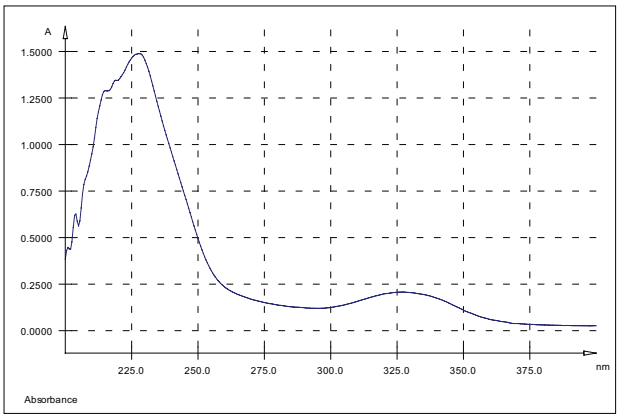

Figure 2. Absorption spectrum of 30 µg.mL^{-1} of standard LT4 in methanolic NaOH.

Mobile phase (composition, ratio, aqueous phase)

Mobile phase composition has a major effect on peak spacing, shape and retention time.

Methanol and acetonitrile have been tried as organic modifiers. Acetonitrile, due to its high eluting power, has been chosen, introduced into the mobile phase and allowed the elution of LT4 at reasonable retention time.

As LT4 contains acidic and basic functional groups, the retention time can vary with both, ionic strength and pH of the aqueous phase. Thus, the use of buffer- acetonitrile as mobile phase allows better elution of LT4. Phosphate buffer and acetate buffer were tried. The use of acetate buffer having the concentration of 0.02 M provided better ionization of LT4 and hence resulted in better chromatograms as an outcome.

The pH value of the acetate buffer was adjusted to 4.25 using acetic acid. At this pH value LT4 carboxylic group was completely ionized which provided optimum separation with the most symmetric, well-defined peaks, eluted after 3 min (Fig. 3).

The ratio of constituents of the mobile phase was also studied with respect to change in the ratio of acetonitrile: acetate buffer. Different ratios were tried including: 40:60, 45:55, 50:50, 55:45, 60:40, The optimal mobile phase composition was found to be acetonitrile: acetate buffer in the ratio

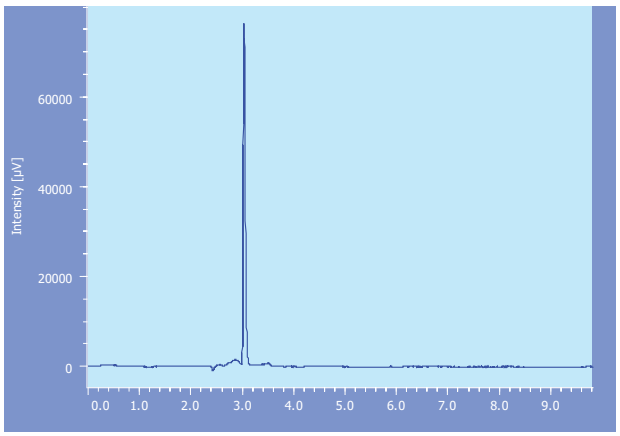

Figure 3. HPLC chromatogram of a 20µL injection of 20 µg.mL^{-1} of standard LT4 using the optimized chromatographic conditions.

of (55:45, %v/v). This mobile phase composition resulted in well-defined peaks with good shape and symmetry.

Flow rate

Various flow rate values in the range of 0.5–1.5 mL/min were tried. The best separation was achieved by adjusting the flow rate at 1.2 mL/min in an isocratic mode. Injection volume and run time were 20 µL and 10 minutes, respectively.

Method validation and statistical analysis

Under the optimized experimental conditions, the developed method was validated for system suitability, along with linearity, limits of detection (LOD) and quantification (LOQ), specificity, accuracy, precision (repeatability and intermediate precision) and robustness according to the procedures described in ICH guidelines (ICH 2005).

System suitability

According to FDA guidance 1994, system suitability tests are an integral part of any liquid chromatographic method (FDA). Under the optimized chromatographic conditions, system suitability parameters including capacity factor (k'), column efficiency expressed by the number of theoretical plates (N), tailing factor (T), and repeatability (RSD of peak response and retention time) were performed and listed in Table 1. The calculated parameters were satisfactory and indicative of good efficiency of the method for the determination of LT4.

Table 1. HPLC system suitability parameters for the determination of LT4 using the proposed method.

Retention time (t_R)	Capacity factor (k′)	N° of theoretical plates (N)	Tailing factor (T)
3.03 ± 0.07	4.41	23155	1.097

Method validation

Linearity and concentration range

Under the optimized experimental conditions, the standard calibration curve was constructed. The linear regression equation was obtained by the method of least squares by plotting the peak area against the concentrations of LT4. Linearity of the method was studied by injecting five concentrations prepared in methanolic NaOH keeping the injection volume constant.

Linearity parameters including correlation coefficient, intercept, slope, and the standard deviation of the residuals for the calibration data and concentration range are summarized in Table 2.

The correlation coefficient (r) obtained was higher than 0.999 with high values of F (low significant F) which confirmed the linearity of the calibration curves. An important statistical parameter for indicating the random error

Table 2. Assay parameters for the determination of LT4 using the proposed HPLC method.

Parameter	
Concentration range (µg/mL)	3.5–200
Regression equation	
Intercept (a)	-3317
Slope (b)	11934
Correlation coefficient (r)	0.9999
S_a	3792
S_b	46
$S_{y/x}$	7827
$(S_b)^2$	2125
% S_b	4611
F	66998
Significance F	2.2439×10^{-13}
LOD (µg/mL)	0.953
LOQ (µg/mL)	3.17

in the estimated values of y is the standard deviation of the residuals $S_{y/x}$. Also, the importance of $S_{y/x}$ originates from being used to calculate S_a and S_b, the standard deviation of the intercept (a) and the slope (b). These values showed the good linearity of the calibration graphs and the compliance to Beer's law. The variance test for the regression lines revealed that, for equal degrees of freedom, the increase in the variance ratio (F-values) means an increase in the mean squares due to regression and a decrease in the mean squares due to residuals, (i.e., the less is the scatter of experimental points around the regression line). Consequently, regression lines with high F-values (low significance F) are much better than those with lower ones. In conclusion, good regression lines show high values for both r and F statistical parameters (Mileer 2005).

Limit of detection and Limit of quantification

The LOD and LOQ were derived by calculating the signal-to-noise ratio. LOD and LOQ represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ. To determine the LOD and LOQ, serial dilutions of LT4 were prepared from the standard stock solution. The samples were injected in LC system and the measured signal from the samples were compared with those of blank samples. LOD and LOQ were calculated according to the ICH guidelines (ICH 2005). The sensitivity of the proposed method can be confirmed by the low LOD and LOQ values obtained in Table 2.

Accuracy

The accuracy of the proposed method was determined through a recovery study. Five different LT4 samples of known concentrations ranging from 10–100 µg/mL were prepared and chromatographed in triplicates under the optimized conditions. The amount of LT4 was estimated by measuring the peak area and fitting these values to the calibration curve equation. Good accuracy expressed as % recovery was obtained. The results, summarized in

Table 3. Accuracy data for the determination of LT4 using the proposed HPLC method.

LT4 concentration (µg/mL)	Mean Recovery ± SD[a]
	RSD%[b]
	Er%[c]
10	98.97 ± 2.03
	2.05
	1.03
20	99.57 ± 1.52
	1.53
	0.43
40	100.83 ± 1.47
	1.46
	- 0.83
60	99.97 ± 1.14
	1.14
	0.03
100	100.68 ± 1.36
	1.35
	-0.68

[a] Mean ± SD for the three determinations;
[b] % Relative standard deviation;
[c] % Relative error.

Table 3, show that the % recovery values do not exceed the accepted limits (98–102%), which demonstrate the accuracy of the developed method.

Precision

In order to evaluate the precision of the proposed method, repeatability (intra-day precision) and intermediate precision studies (inter-day precision) were performed.

Repeatability studies were performed by triplicate injections of standard LT4 solutions performed for three different concentrations within the calibration range (intra-day precision). The intermediate precision of the method was checked by repeating studies on three different days (inter-day analysis).

High precision, expressed as percentage RSD, were obtained proving the high precision of the method The results are summarized in Table 4.

Table 4. Intra-day and inter-day precision for the determination of LT4 using the proposed HPLC method.

LT4 concentration ($\mu g.mL^{-1}$)	Intra-day precision	Inter-day precision
	Mean Recovery ± SD[a]	Mean Recovery ± SD[a]
	RSD%[b]	RSD%[b]
	Er%[c]	Er%[c]
25	101.72 ± 1.64	99.95 ± 2.51
	1.61	2.51
	-1.72	0.05
100	98.68 ± 0.63	100.28 ± 1.48
	0.64	1.48
	1.32	-0.28
150	98.76 ± 0.30	99.71 ± 1.69
	0.3	1.69
	1.24	0.29

[a] Mean ± SD for the three determinations;
[b] % Relative standard deviation;
[c] % Relative error.

Specificity

The specificity of the method was determined by comparing test results obtained from the analysis of sample solutions containing excipients with that of the test results obtained from standard LT4 solutions. The peaks were well separated under the described chromatographic conditions where no interference from the excipients of the pharmaceutical tablets were shown.

Robustness

Robustness of the method was determined by small deliberate changes in flow rate, mobile phase pH and mobile phase ratio. The content of the drug was not adversely affected by these changes as evident from the low value of %RSD indicating that the method was robust. This study demonstrated that slight intended variations in the previously mentioned parameters have no significant effect in the determination of LT4 (Table 5).

Table 5. Robustness study of the chromatographic system for the determination of LT4 by the proposed HPLC method.

Parameter	Condition	Mean %Recovery ± SD[a]
		%RSD
		%Error
Flow rate (mL/min)	1.1	98.36 ± 1.34
		1.36
		1.64
	1.3	99.01 ± 0.76
		0.77
		0.99
Mobile phase composition (v/v)	54:46	101.72 ± 1.21
		1.19
		-1.72
	56:44	99.54 ± 0.89
		0.89
		0.46
pH	4.2	100.34 ± 0.94
		0.94
		-0.34
	4.3	99.34 ± 0.87
		0.88
		0.66

[a] Mean ± SD for the three determinations;
[b] % Relative standard deviation;
[c] % Relative error.

Pharmaceutical application

The applicability of the proposed method was tested in two different pharmaceutical formulations; Euthyrox tablet and Eltroxin tablet. LT4 peak was eluted at its specific retention time. The results for LT4 were comparable to the labelled amount expressed by high percentage recoveries with low % RSD which indicate high accuracy and precision in the determination of LT4. The absence of additional peaks indicates no interference of the excipients used in the tablets. The results obtained are summarized in Table 6. The proposed method, by the

Table 6. Determination of LT4 in pharmaceutical preparations using the proposed HPLC method.

	[a] Mean % Recovery ± SD	[b] % RSD	[c] %Error
Euthyrox 100mcg	98.59 ± 4.14	4.20	1.41
Eltroxin 100mcg	99.48 ± 3.26	3.28	0.52

[a] Mean ±SD for five determinations;
[b] Relative standard deviation;
[c] Relative error.

use of NH2 column, can be considered superior to the compendial method described in the European pharmacopeia. It was found to be more cost time effective due to a shorter run time of analysis in comparison to Ph. Eur. Method (CEEP 2019), where officially it is stated to be around 11 min. Based on literature review, statistical comparison wasn't possible as the two pharmaceutically available tested drugs were not analyzed previously using the Eur. Ph. method, pointing to the innovative work. Further investigations should be targeted to investigate bioequivalence and interchangeability between the two LT4 brands.

Conclusion

A novel, simple anion exchange HPLC method has been developed and validated for the determination of LT4. The analyte was eluted at relatively short retention time of 3.03 minutes on a NH_2 column at 252 nm. Consequently, the proposed analytical method can be considered cost and time effective. The method used for the assay of LT4 was validated and was found to be accurate and precise; where the low LOD and LOQ values allowed the quantitation of the studied drug in different pharmaceutical products qualifying the use of this HPLC method in quality control assays.

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