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RESEARCH ARTICLE

Development and Validation of HPLC-UV Method for the Determination of Levothyroxine in Orodispersible Minitablets for Pediatric Application

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Abstract: ***Background*:** Thyroid hormones play an important role in cognition and brain development. The determination of the content of levothyroxine, as well as related substances and dissolution test analysis, should be carried out by methods that are selective and highly sensitive due to the low concentration used in low dose orodispersible minitablets.

***Objectives*:** This study aims to develop and validate an analytical method by HPLC-UV for the quantification of levothyroxine and its related substances in pharmaceutical formulations; looking forward to being this method suitable for a future dissolution test analysis using tandem mass spectrometry detector.

***Methods*:** A Hypersil GOLD C18 (100 x 2.1 mm, 3µm) column was used with 25°C column temperature, 5 µL injection volume, 0.3 mL/min flow rate and detection at 225 nm. The mobile phase consisted of methanol: 0.05% formic acid (55:45). The developed method was validated for specificity, linearity, precision, accuracy and robustness.

***Results*:** The method is linear within the range of 2-20 µg mL-1 (R2=0.9982), which makes the method suitable for the evaluation of levothyroxine in pharmaceuticals formulations. LOQ was 0.17 µg/mL (0.85 ng on column) and LOD 0.05 µg/mL (0.25 ng on column) of LT4. Therefore, in terms of efficiency (1671), retention factor, k (6.79), Tailing factor, T (1.09) and resolution, Rs (5.11) the chromatographic method was found to be suitable according to USP 43.

***Conclusion*:** The HPLC UV method was found to be linear, specific, precise, accurate and robust, therefore it is suitable for the quality control of levothyroxine in pharmaceutical ODMTs.

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1. INTRODUCTION

Thyroid gland disorders is considered one of the most common endemic diseases that leads to insufficient production of thyroid hormone, which is principally involved in gene expression related to thermogenesis, regulation of cellular respiration and cell growth and differentiation [1, 2]. It is also involved in protein, carbohydrate and lipid metabolism. These processes take place when nuclear translocation of the hormone-receptor complex occurs. Triiodothyronine (T3) is responsible for this binding and is obtained by de-iodination of thyroxine in peripheral tissues [3, 4]. Thyroid hormones play an important role in cognition and brain development. During the first stage of life, irreversible alterations may occur if the thyroid gland is affected, especially at the level of the central nervous system. In order to carry out the appropriate treatment, a specific diagnosis and analysis is required [5, 6]. In the presence of a suspicious malignant thyroid nodule, fine needle aspiration (FNA) has been the recommended triage tool for more than two decades. It is still a matter of debate whether ROSE is worth using or not, but there is indeed an impact on the reduction of the nondiagnostic (NDR) [7].

The synthetic form of thyroid hormone (T4) is sodium;(2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]propanoate, and acts as a replacement therapy in this disease [8].

Numerous oral formulations of LT4, such as tablets, soft gel capsules, and liquid formulations, are, at the moment, commercially available [9,10]. However, given the large pharmaco-kinetic and dynamic variability that exists in paediatric patients and their constant growth, a dose range of 10-100 µg/kg/day is established [11]. No specific and appropriate formulation has been developed for paediatric patients so far.

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There is a global interest that has grown in recent years in individualised therapy in order to increase efficacy and decrease adverse effects. The development of orodispersible tablets as an alternative to conventional ones combines the advantages of the latter but also combines the advantages of liquid oral forms. Due to their small size and rapid disintegration once placed in the oral cavity, they become a specific and appropriate dosage form to be applied in paediatric population. These patients will have no problems in swallowing the orodispersible tablet, and it would also allow the correct dose adjustment to meet the patient's needs and treatment efficacy. In 12 recent years, interest has grown in the development of rapidly disintegrating tablets in the oral cavity as an alternative to conventional tablets. These formulations combine the advantages of liquid forms and oral solid forms. Hence orodispersible minitablets (ODMTs) stand out for their application in pediatrics, not only because of their small size, which would facilitate their administration and circumvent the swallowing problem but also allow the correct dose adjustment to meet the patient needs and treatment efficacy [12]. Due to the small concentrations used in this type of tablets, the development of selective and highly sensitive methodologies that are appropriate for content determination, related substances and dissolution tests applied to levothyroxine quality control are of great importance [13-15].

Although there are many reported analytical methods to test levothyroxine, these methods either involve a cumbersome sample treatment, such as derivatization and extraction processes [16-18] or do not have the appropriate sensibility for the determination of such low concentrations [19].

Moreover, the use of a small particle size and length column shortens the analysis time and reduces solvent consumption, providing high resolution, sensitivity, and robustness [20].

In addition, the methods included in international pharmacopeias (USP) [21] and most of the published methods use a mobile phase consisting of acetonitrile and phosphate-based buffers at a high flow [22]. The replacement of acetonitrile with methanol was possible due to the use of a column with the above- described characteristics, even at a very low flow rate (0.3 mL/min). On the other hand, the use of a non-volatile buffer in the mobile phase makes the method compatible to be applied in HPLC-ms/ms. This is a great advantage, given the possibility of applying this methodology to the MCODS dissolution test.

In the present study, we developed a simple, rapid and highly sensitive HPLC-UV method for the determination of levothyroxine and its related substances in ODMTs with lower solvent and sample consumption and with the appropriate sensitivity to be applied as a routine method for the quality control of levothyroxine ODMTs.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Sodium levothyroxine (sodium;(2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]propanoic acid), liothyronine ((2S)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]propanoic acid) were purchased from Sigma (St. Louise, MO, USA). Methanol (HPLC grade) and sodium hydroxide were supplied by E. Merck (Darmstadt, Germany). Formic acid was acquired from J.T Baker (Madrid, Spain). Ultrapure water obtained with an EASY pureTM RF (Barnstead , Dubuque, IA, USA) was used. All solutions were then degasified and filtered through a 0.45 µm nylon membrane (Micron Separations Inc., Westboro, MA, USA).

2.2. Equipment

HPLC analysis was performed using a Thermo Scientific HPLC (Waltham, Massachusetts) equipped with a quaternary pump (P4000), a temperature control, a vacuum degasser (SCM 1000), a dual UV detector (UV2000), an automatic injector (AS3000) and ChromQuest 5.0 software, which was used to control the instrumental parameters.

3. EXPERIMENTAL

3.1. Chromatographic Conditions

The chromatographic column was Hypersil GOLD C18 (100 x 2.1 mm, 3µm particle size) purchased from Thermo Scientific. An isocratic method was employed with a mobile phase consisting of methanol: 0.05% formic acid (55:45). The column temperature was maintained at 25°C, and the detection was monitored at 225 nm. The injection volume was 5 µL, and the mobile phase flow was set at 0.3 mL/min.

3.2. Preparations of Standard Solutions

A stock solution (1mg/mL) was prepared by dissolving levothyroxine in a mixture consisting of MeOH: 0.01M NaOH (50:50) (diluent solution). Standards solutions (for different sections) were prepared by diluting the stock solution with MeOH.

3.3. Sample Preparation

Ten minitablets of levothyroxine were introduced into a 25mL volumetric flask. An aliquot of 2.5 mL of the diluent solution was added and then sonicated for 10 minutes. The mixture was diluted to the final volume with MeOH to obtain a concentration of 10 µg/mL.

3.4. Validation

The validation of the developed HPLC method was accomplished following the International Conference on Harmonization (ICH) guidelines with respect to specificity, linearity, LOD, LOQ, precision, accuracy, and robustness.

3.4.1. Specificity

The specificity of the method was determined by comparing chromatograms of the excipient blank of the pharmaceutical formulation with a standard solution containing levothyroxine and liothyronine standard (STDx2), both at the same concentration.

3.4.2. Linearity, LOD and LOQ.

Linearity was carried out by means of five individual concentrations of levothyroxine prepared over a range of 2-20µg/mL (2, 5, 10, 15, 20 µg/mL), and each solution level was injected by triplicate. The LOD and LOQ were deter-

mined based on signal-to-noise ratio of 3:1 and 10:1 ratio, respectively.

3.4.3. Precision

Precision was evaluated for intra-day (n=6) and inter-day assays (n=18). For each sample solution, two replicate injections were made. Precision was expressed as RSD for peak areas and levothyroxine content.

3.4.4. Accuracy

Accuracy was calculated from recovery studies. Placebo samples prepared with all excipients contained in the orodispersible minitablet formulation were supplemented with LT4 at 80, 100, and 120% concentration levels of the nominal values. Preparations of each level were tested by triplicate, and two replicate injections were made.

3.4.5. Robustness

Different parameters were tested, such as column temperature, wavelength, injection volume, percentage of organic solvent and flow rate, with a ± 2% variation. For each case, the quantification of levothyroxine, tailing and capacity factor, and the number of theoretical plates (N) were evaluated to determine the influence of the parameter´s variation in the given chromatograms. In all cases, three injections of levothyroxine standard solution and three injections of the pharmaceutical formulation were made.

4. RESULTS AND DISCUSSION

4.1. HPLC-UV Method Development

Most of the reported HPLC methods for the analysis of levothyroxine in different matrices use a reversed-phase column and, as mobile phase a solution at an acidic pH value and organic solvent (methanol or acetonitrile). Currently, the USP-codified method for the determination of levothyroxine in tablets involves the use of an HPLC UV method where the liquid chromatograph is equipped with a 225-nm detector and a 4.6-mm × 25-cm column that contains packing L10. The flow rate is about 1.5 mL per minute and the mobile phase consist in a degassed and filtered mixture of water and acetonitrile (60:40) that contains 0.5 mL of phosphoric acid in each 1000 mL. Several conventional methods have been reported, as shown in Table **1**. The developed method proposes the use of a Hypersil GOLD C18 column (100 x 2.1 mm, 3µm particle size) with a reduced internal diameter and particle size. The use of this type of column allows an increase in sensitivity and selectivity with respect to conventional methods, achieving a reduction in solvent and sample consumption and also detection limits (mass on column) comparable to those of an HPLC method with a fluorescence detector. This makes the developed method suitable for the analysis of minitablets, which have doses 10 times lower than commercial pharmaceutical forms. In addition, the mobile phase was Methanol: 0.05% Formic Acid (55:45). The use of volatile solvents, such as formic acid instead of phosphoric acid, allows a future transfer of the chromatographic method to an HPLC MS/MS system for the determination of lower concentrations of levothyroxine in samples like biological fluids or dissolution test and prolongs the life of HPLC components by eliminating the damage for salts deposition.

Table 1. Comparison of reported chromatographic methods applies to Levothyroxine analysis in pharmaceuticals.

PARAMETERS			
Linear range (µg/mL)	2.0-20.0		
R2	0.9982		
LOD (µg/mL)	0.05 (0.25ng on column)		
LOQ (µg/mL)	0.17 (0.85 on column)		
Precision (RSD)			
Intra-day (n=6)			
Peak area	1.71		
Levothyroxine content	1.32		
Inter-day (n=18)			
Peak area	1.74		
Levothyroxine content	1.85		
Accuracy			
Spiked levels*	80%	100%	120%
%Recovery	99.8	99.7	99.3
RSD	0.52	0.47	0.2

*respect to the label content.

The column temperature was conserved at 25°C, and the UV detector was set at 225 nm, which allowed an adequate sensitivity with a suitable LOQ, LOD, and good resolution.

The chromatographic parameters were adjusted in order to obtain a good resolution and peak shape. The method was found to be adequate in terms of efficiency (1671), retention factor, k (6.79), Tailing factor, T (1.09) and resolution, Rs (5.11) according to USP 43 [21].

4.2. Specificity

Levothyroxine and its related compound liothyronine were resolved according to USP requirements. The resolution between these two was 5.11, which is in concordance with the USP specification of not less than 5. 21 No excipient interference was observed. Thus, the method proved to be specific (Fig. **1**).

4.3. Linearity

According to international guidelines, a good correlation was obtained, showing that the method is linear in a range from 2-20 µg/mL with a regression coefficient (R2) of 0.9982, which makes the method suitable for the evaluation of levothyroxine in pharmaceuticals formulations. LOQ (S/R=10) was 0.17 µg/mL, and LOD (S/R=3) was 0.05 µg/mL of LT4 (Table **2**).

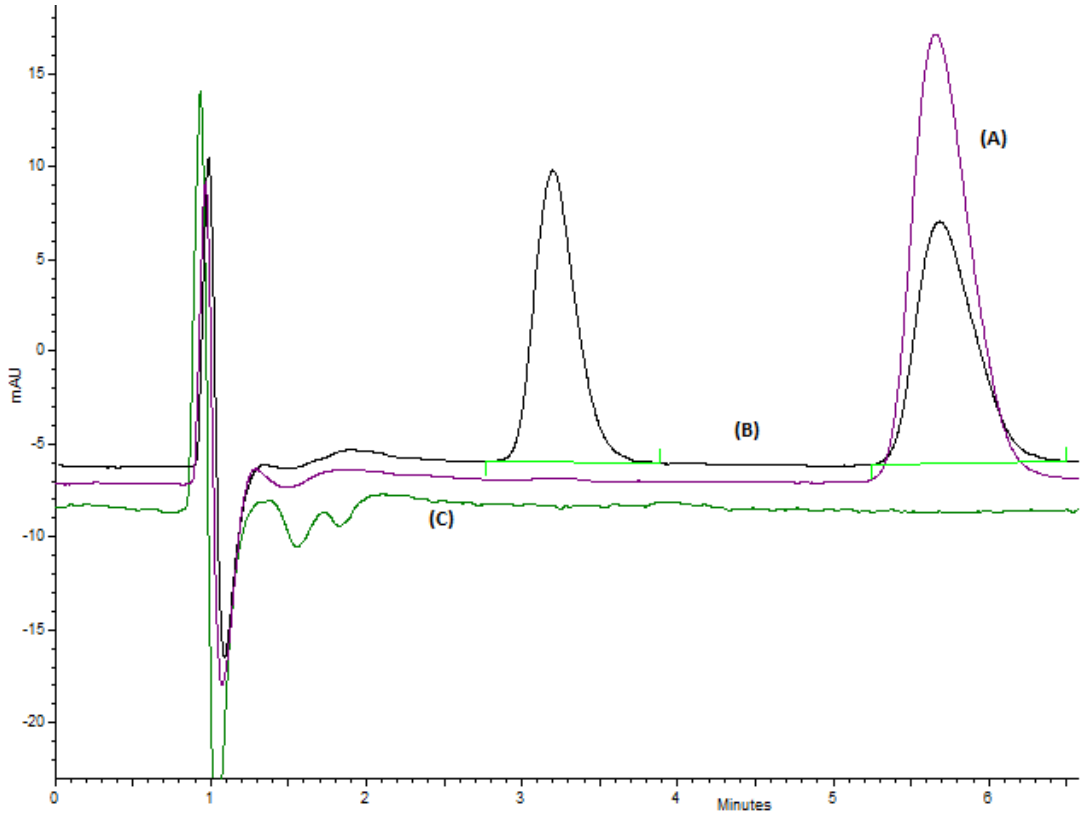

Fig. (1). (**A**) Chromatogram of the standard solution of levothyroxine. (**B**) Chromatogram of the standard solution of levothyroxine and liothyronine. (**C**) The experimental conditions used are described in the text. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Linearity, LOD and LOQ, precision and accuracy for Levothyroxine.

Ref	Accuracy (Recovery%)	Precision (%RSD)	LOQ	LOD	Inj. Vol	Detection	Mobile Phase	Sample Pre-treatment	Matrix
[16]	97.0 (3.0)	2.3	-	0.01 µg/mL (0.2ng)*	20 µL	Fluorescence; excitation, 257 and emisión, 425 nm	acetonitrile–0.02 M sodium dodecyl sulfate (SDS), adjusted to pH 3.5 with phosphoric acid (56:44, v/v).	fluorogenic derivatization	Tablets
[21]	90.0 – 110.0	<2.0	<0.08µg/mL (64 ng)*	-	800 µL	UV; 225 nm	0.01 M phosphate buffer (pH 3.0)–methanol (55:45, v/v)	The sample diluent was MeOH	Tablets dissolution samples
[22]	95.0 – 105.0	<2.0	2 µg/ml (100ng)*	1 µg/ml (50ng)*	50 µL	UV; 223 nm	trifluoroacetic acid (0.1%, v/v, pH 3)–acetonitrile	The sample diluent was 0.01 M methanolic NaOH	Pharmaceu-tical excipients

*ng per injection. - Not reported.

4.4. Precision

A good precision was observed for the developed method. The results for intra-day and inter-day precision are presented in Table **2**, showing low variability in peak areas and levothyroxine content (RSD lower than 2%).

4.5. Accuracy

Results from recovery studies agreed with the requirements of ICH guidelines. Percentages of recovery values were in the range between 99.7 -99.8 % (Table **2**).

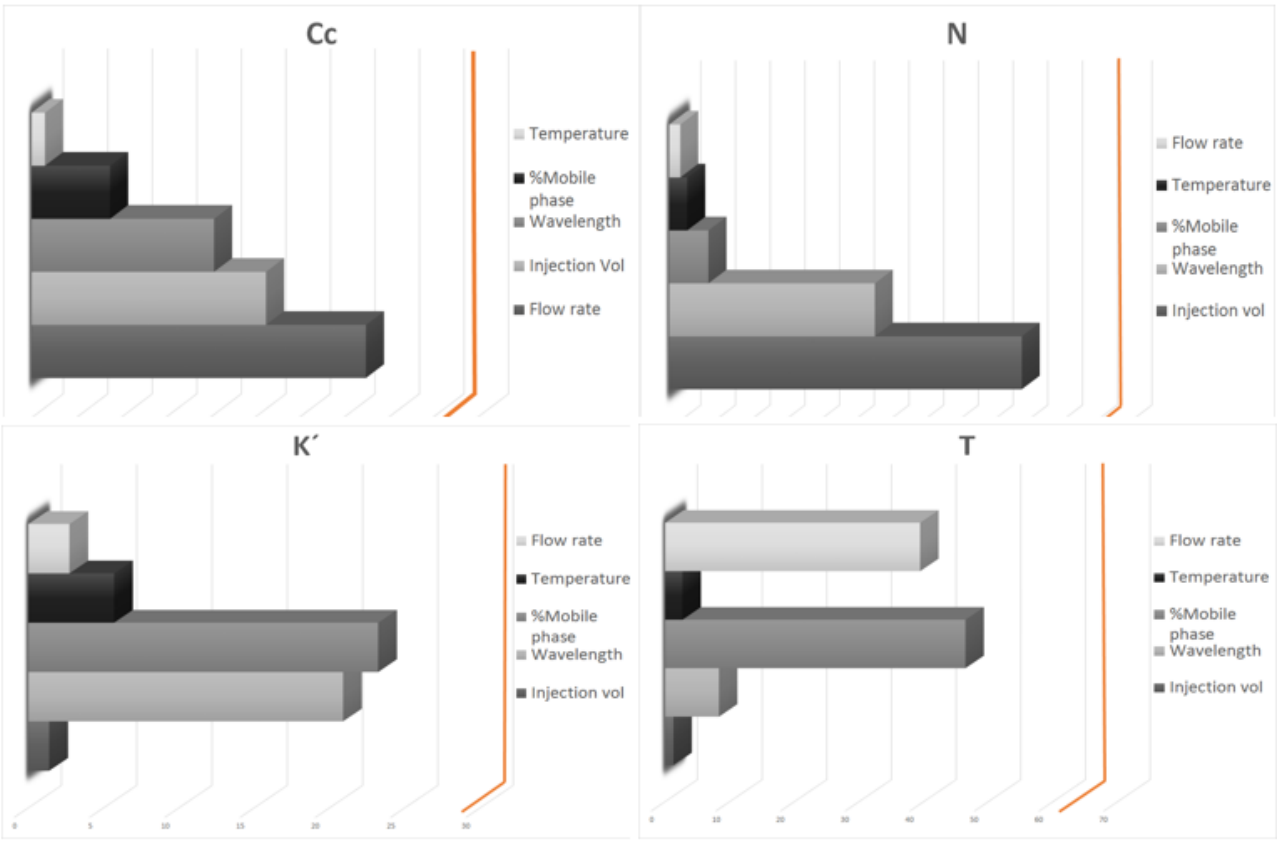

Fig. (2). Variation of operational parameters and their significance on chromatographic performance. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4.6. Robustness

Different modifications in the operational chromatographic parameters were analysed. By constructing standardised Pareto diagrams, it was possible to observe, as shown in Fig. (**2**), that none of the variables has a significant effect on the quantification of levothyroxine, number of theoretical plates, tail and capacity factor. Through the data obtained and statistical analyses performed, the robustness of the analytical method is confirmed.

CONCLUSION

A simple and highly sensitive HPLC method for the analysis of Levothyroxine in ODMTs was developed and constitutes the first one specifically developed for the determination of levothyroxine in ODMTs. One of the advantages of the proposed method in comparison with previous published methods is the possibility of a direct transfer to HPLC MS/MS since phosphates are replaced by formic acid. Moreover, it should be noted the high sensitivity of the method, even using a UV detector, which makes it suitable for routine laboratory use. In this sense, the developed method can be used for routine quality control of levothyroxine in pharmaceutical formulations. In addition, a full validation was performed according to international guidelines.

LIST OF ABBREVIATIONS

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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