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Research Article

Development and Validation of Stability Indicating RP-HPLC Method of Analysis for Determination Related Substances of Levothyroxine Sodium in Bulk and Formulation

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ABSTRACT

A new, simple, rapid, accurate and precise high-performance thin layer chromatography (HPLC) method has been developed and validated according to the guidelines of the International Conference on Harmonization (ICH Q2(R1) for the estimation of Levothyroxine Sodium related substances in bulk and formulation. The chromatographic analysis was performed by an Waters HPLC instrument using a YMC Pack Pro C18 RS (150 x 4.6) mm, 3µm and mobile phase A comprising Buffer pH 3.9 in water and mobile phase acetonitrile MeoH (90:10 v/v) and 1 ml orthophosphoric acid at flow rate of 0.8 ml/min. The eluent was monitored at 225 nm for determination of Levothyroxine Sodium and Impurity. Linearity was recorded at various concentrations ranges 1-10 µg /ml for Levothyroxine Sodium and 1-7 µg /ml for Levothyroxine Sodium related substances. Recovery RSD values for all the analytes were within the acceptable range. The developed method was found to be robust. A simple, precise, accurate, linear and rapid RP-HPLC method was developed and validated as per ICH guidelines. The results suggest that the developed method was found to be robust and it can be applicable in routine analysis and efficiently used for the estimation of Levothyroxine Sodium related substances in bulk as well as combined dosage form.

Keywords: Method development, Validation, HPLC, Levothyroxine Sodium.

INTRODUCTION

Levothyroxine Sodium (L-T4) is a synthetic hormone extensively utilized to manage hypothyroidism, producing effects that closely mimic those of the naturally produced thyroid hormone. The primary biological function of thyroid hormones is to elevate the basal metabolic rate, resulting in an increased utilization of substrates, heightened enzyme activity, and the secretion of various other hormones. Additionally, thyroid hormones play a vital role in the overall development of organs and tissues. For both fetal and postnatal stages, Thyroxine is crucial for the proper development of neurons and the growth of their extensions. The deficiency of thyroid hormones in both children and adults leads to a gradual reduction in the reactivity of the nervous system, affecting both motor functions and intellectual capabilities[1-2]. Hypothyroidism stands as one of the most prevalent endocrine disorders, with over 95% of cases originating in the thyroid (primary hypothyroidism). The recommended treatment involves administering an appropriate dosage of L-T4, enabling patients to improve their thyroid function clinically by restoring physiological levels of L-T4 and maintaining thyrotropin (TSH) concentrations within the lower half of the normal range. [3-6]

It's worth noting that Levothyroxine Sodium is a challenging drug to work with due to its poor solubility, as only 1-part dissolves in 700 parts of water and 300 parts of ethanol. This drug is virtually insoluble in acetone, chloroform, and ether, but it exhibits solubility in alkaline hydroxides; however, the resulting solutions are unstable. Additionally, L-T4 tablets need protection from light exposure. These unique characteristics make it difficult to establish a precise and reproducible analytical method for quantifying the drug content in tablets, especially considering that typical treatment doses are administered in microgram quantities. [7-8]

The primary objective of this study is to establish a Reverse Phase High-Performance Liquid Chromatography (HPLC) method that is straightforward, reliable, sensitive, and cost-effective. This method should be capable of effectively separating and analyzing impurities, even when they are present at extremely low levels. In our investigation, we aim to quantify impurities down to the 0.2% specification limit in the drug substance, and achieve this with a high degree of accuracy, all while employing a simplified elution system and mobile phase, rather than complex elution techniques that would extend the analysis time. The proposed method is subjected to validation according to the guidelines provided by the International Council for Harmonisation (ICH), specifically addressing parameters such as accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), linearity, robustness, and ruggedness.

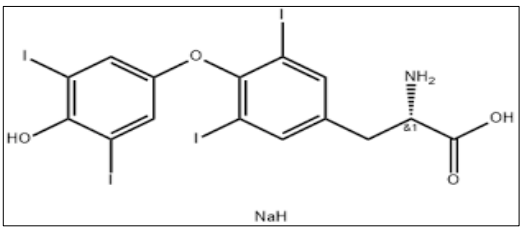

Figure 1: Chemical structure of Levothyroxine

MATERIALS AND METHODS

Chemicals and Reagents

Levothyroxine Sodium (Purity 99% by HPLC) was purchased from M/s Sigma Aldrich Solution (Merck), India. All the other chemicals of analytical grade were used for the proposed study.

Instruments

HPLC system of (Waters Alliance) was used for method development and analysis. The system was equipped with a PDA wavelength detector was used for the analysis. Chromatographic analysis was carried out at room temperature**.** Ultrasonicator (PCi Analyticals) is use for mobile phase degasing. Vibra HT (Essae) analytical balance was used for weighing of chemicals.

Preparation of 2N sodium hydroxide solution:

Dissolve 8 gm of sodium hydroxide pellets in 100 mL of water and mix well.

Preparation of 0.2N sodium hydroxide solution:

Dissolve 0.8 gm of sodium hydroxide pellets in 100 mL of water and mix well.

Preparation of mobile phase A:

Dissolve about 4.85 gm of sulfamic acid and about 0.75 gm of sodium hydroxide pellets in 1000 mL of water. Adjust the pH of solution to 3.9 ± 0.05 with 2N sodium hydroxide solution and 0.2N sodium hydroxide solution. Filter the solution through 0.45µm PVDF membrane filter and degas. (While adjusting the pH of mobile phase A, use 2N sodium hydroxide solution up to the pH of about 3.0 and then for fine adjustment of pH, use 0.2N sodium hydroxide solution up to the pH of 3.9 ± 0.05).

Preparation of mobile phase B:

Prepare a solvent mixture of acetonitrile and methanol, in the ratio of 900:100 (% v/v) in a suitable container. Add 1.0 mL of orthophosphoric acid into this solvent mixture. Mix well and degas the final mixture.

Preparation of diluent A:

Dissolve about 400 mg of sodium hydroxide pellets in 500 mL of water and cool. Add 500 mL of methanol and mix well.

Preparation of diluent B:

Prepare a mixture of buffer solution (0.1% Triethylamine) and methanol in the ratio of 50:50 (% v/v) and degas.

Preparation of standard stock solution:

Accurately weigh and transfer about 50 mg of Levothyroxine Sodium anhydrous into a 100 mL clean and dry volumetric flask. Add about 70 mL of diluent A and sonicate to dissolve completely the make up the volume with diluent A and mix well. (Stock-I).

Preparation of impurity stock solution:

Accurately weigh and transfer about 1 mg of Levothyroxine sodium Impurity-A, Levothyroxine-N-methylamide, T3-acetic acid (Impurity-C), T4-acetic acid (Impurity-D), Impurity F, Impurity G and T4-Benzoic acid (Impurity-H) into a 10 mL clean and dry volumetric flask. Add about 8 mL of methanol and sonicate to dissolve completely. Dilute to volume with methanol and mix well. (Stock-II).

Preparation of standard calibration curve:

Calibration curve was prepared by diluting the stock-I and stock-II solution to achieve the seven different calibration standards representing 1 to 10 µg /ml strength of Levothyroxine and Levothyroxine related substance concentration 1 to 7 µg/ml for all Impurity. All these solutions were injected into HPLC column and the peak area of each solution was measured. The standard calibration curves of peak area Vs concentration (ng) were plotted.

Method Validation:

The validation of pre-optimized chromatographic method was performed according to the Q2 (R1) guidelines of International Conference for Harmonization (ICH). Various analytical method validation parameters like system suitability, linearity, range, LOD, LOQ, accuracy, precision and stability were assessed [9-10].

System Suitability:

Before performing the main analysis, the system suitability test was carried out using freshly prepared standard working system suitability solutions. Standard working solution was repeated analyzed by using proposed HPLC conditions. During analysis, various parameters viz. retention time, peak area, and the number of theoretical plates were measured. Acceptable upper limit of % RSD for peak area and retention time was set at 2 whereas acceptable lower limit of number of theoretical plates was set at 2000. System was considered to be suitable only when obtained values were within the set limits.

Preparation of system suitability solution:

Transfer about 5.0 mL of standard stock solution into a 50 mL clean and dry volumetric flask. Add 1.0 mL of impurity stock solution, dilute to volume with diluent and mix well. Filter the solution through 0.45 µm Glass membrane filter with discarding first 3 mL of the filtrate.

Linearity & Range:

Linearity of the proposed method was calculated by using seven different calibration standards of Levothyroxine Sodium and Impurity. The calibration curves were constructed using the calibration standards representing 1 to 10 µg/ml and 1 to 7 µg/mL strength of Levothyroxine Sodium and Levothyroxine Sodium Impurity. Concentration vs. peak areas were plotted, subjected to linear regression analysis and linearity in terms of R-squared values and respective range were reported.

Accuracy (% Recovery):

Accuracy of pre-optimized HPLC method was assessed using recovery studies by standard addition method. To the solutions with predefined amount of Levothyroxine Sodium and Impurity (1, 5 & 10 µg/mL and 1, 3, 7 µg/mL Respectively), its 80, 100 and 120 % amount was added externally and the % recovery of the drugs was calculated.

Precision:

The precision of the developed method was evaluated by performing Intra-day and Inter-day studies. Intra-day precision study was carried out by analyzing five replicates of three different concentrations of Levothyroxine Sodium and Impurity (1, 5 & 10 µg/mL and 1, 5, 10 µg/mL Respectively), at morning, afternoon and evening time of the same day. Similarly, inter-day precision study was carried out by analyzing the samples on three consecutive days. Intra- and inter-day precision results were expressed in terms of % RSD.

Limit of detection (LOD) and Limit of quantification (LOQ):

Lowest detection and lowest quantification limits were established by adopting signal to noise ratio method.

Estimation of Levothyroxine Sodium content in pharmaceutical formulation

Preparation of placebo solution:

Accurately weigh and transfer powder placebo 5000 mg (equivalent to 1250 mcg) of Levothyroxine Sodium into a 100 mL clean and dry volumetric flask. Add 25.0 mL of diluent and sonicate for about 10 minutes with vigorous intermittent shaking. Centrifuge the solution in a stoppered centrifuge tube at 4000 RPM for about 10 minutes. Filter the supernatant solution through 0.45 µm Glass membrane filter with discarding first 3 mL of the filtrate.

Preparation of sample solution:

Determine the average weight of not less than 20 tablets. Crush sufficient quantity of tablets into a fine powder. Accurately weigh and transfer tablet powder 5000 mg (equivalent to 1250 mcg) of Levothyroxine Sodium into a 100 mL clean and dry volumetric flask. Add 25.0 mL of diluent and sonicate for about 10 minutes with vigorous intermittent shaking. Centrifuge the solution in a stoppered centrifuge tube at 4000 RPM for about 10 minutes. Filter the supernatant solution through 0.45 µm Glass membrane filter with discarding first 3 mL of the filtrate.

RESULTS AND DISCUSSION

Optimization of RP-HPLC Method:

While developing HPLC method for estimation of Levothyroxine Sodium, various mobile phase combinations and the stationary phases were tried. Selection of mobile phase composition and stationary phases was based on the solubility behavior, pKa values and the relative retention of Levothyroxine Sodium was optimally resolved (Fig. 2B) over YMC Pack Pro C18 RS (150 x 4.6) mm, 3µm column. The details of optimized chromatographic conditions are shown in Table 1.

Table 1: The optimized chromatographic conditions

Separation variable	Optimized conditions	
Chromatography	Waters Alliance	
Column	YMC Pack Pro C18 RS (150 x 4.6) mm, 3µm	
Flow rate	0.8 mL/minute	
Injection Volume	50 µL	
Total Run Time	85 Min	
Temperature	30 ^{0}C	
Detection wavelength	225 nm	
Mode	Gradient	
Gradient Program		
Time (minutes)	**Mobile phase-A (%)**	**Mobile phase-B (%)**
0	70	30
10	70	30
32	65	35
70	20	80
75	20	80
78	70	30
85	70	30

System suitability:

During system suitability test, RSD of all parameter were calculated to evaluate the suitability of the developed method. From the results, it was found that %RSD for retention time and peak area was less than 2 and the number of theoretical plates were more than 2000 (Table 2). On the basis of obtained results, it was found that system is suitable for the analysis. The details of system suitability results are summarized in Table 2.

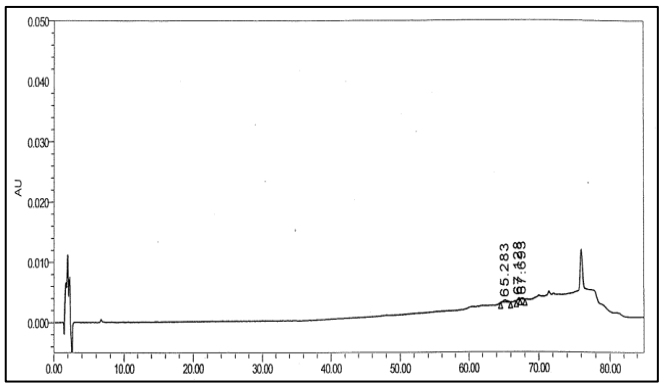

Figure 2: A typical RP-HPLC chromatogram of Blank

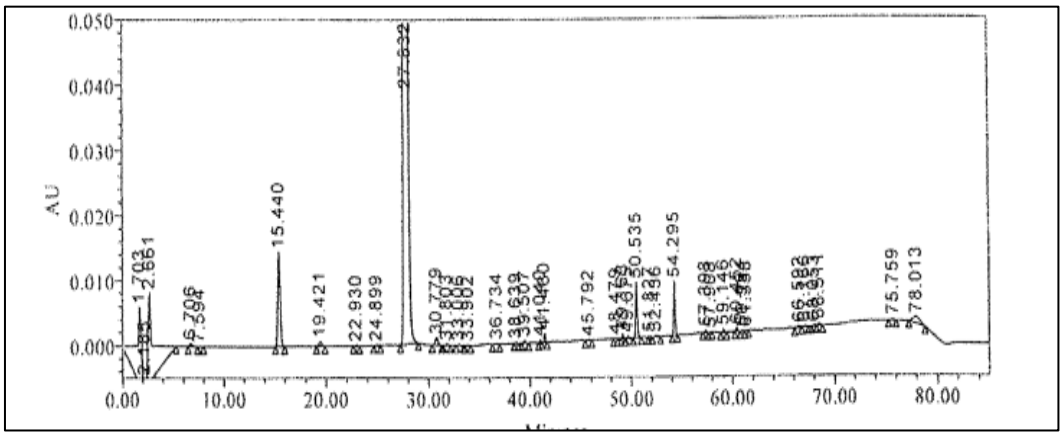

Figure 2: B typical RP-HPLC chromatogram Levothyroxine Sodium (RT-27.63), Impurity-A (RT-15.44), Levothyroxine-N-methylamide (RT-30.77), Impurity-C (RT-50.535) Impurity-D (RT-60.12) Impurity-F (RT- 54.29) Impurity-G (RT-57.88), Impurity H (RT-59.14)

Table 2: System suitability parameters

Sr.No.	Sample	Parameter		
		Retention Time (%RSD ≤ 2%)	Area (%RSD ≤ 2%)	Theoretical plates ≥ 2000
1	Levothyroxine Sodium	0.9854	0.9918	5148
2	Impurity-A	0.7849	0.4856	4571
3	Levothyroxine-N-methylamide	0.9248	0.6815	7813
4	Impurity-C	0.9124	0.2844	6612
5	Impurity-D	0.8216	0.3589	5584
6	Impurity F	0.4851	0.7183	8142
7	Impurity G	0.6218	0.4875	9148
8	Impurity-H	0.5491	0.6284	6281

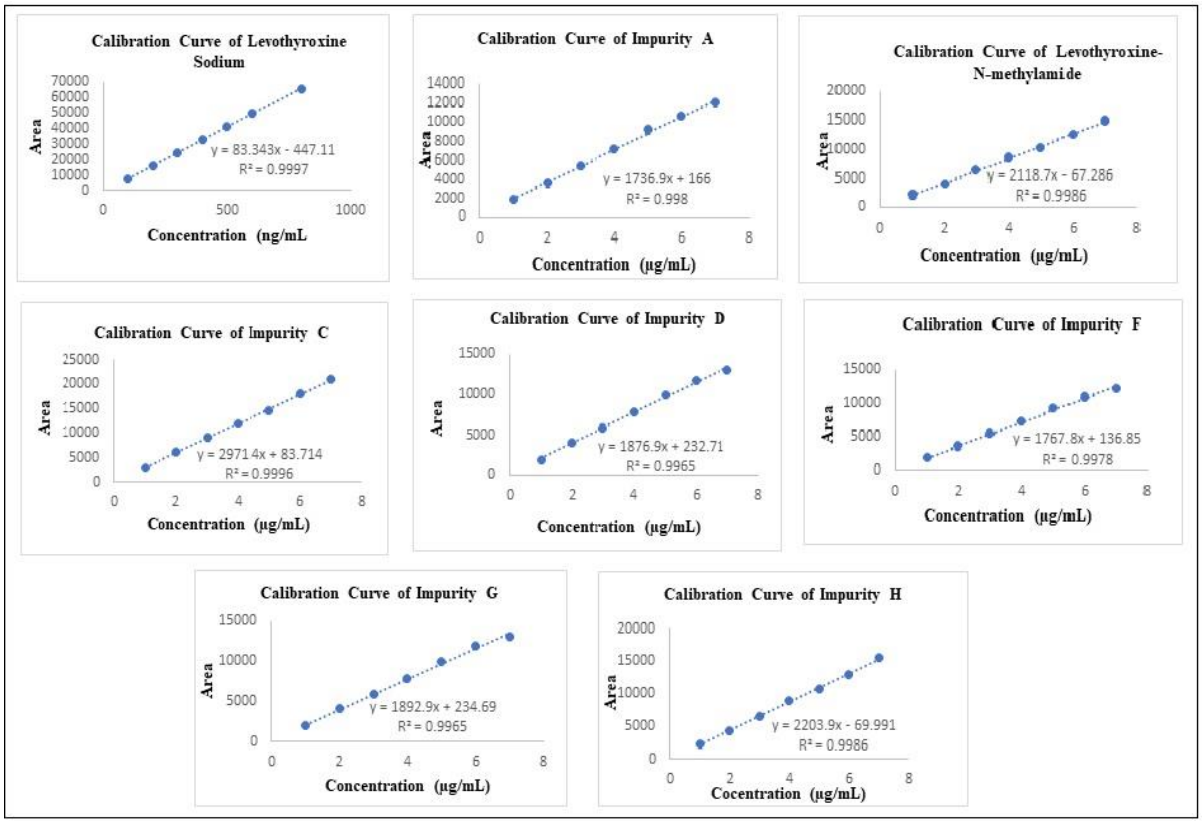

Figure 3: Calibration curve for Levothyroxine and Impurity

Table 3: Linearity Data

Sr. No.	Sample	Linearity Parameter			
		Conc. Range	Slope	y-intercept	R^2
1	Levothyroxine Sodium	1-10 µg/ml	83.343	447.11	0.9997
2	Impurity-A	1-7 µg/mL	1736.9	166	0.998
3	Levothyroxine-N-methylamide	1-7 µg/mL	2118.7	67.286	0.9986
4	Impurity-C	1-7 µg/mL	2971.4	83.714	0.9996
5	Impurity-D	1-7 µg/mL	1876.9	232.71	0.9965
6	Impurity F	1-7 µg/mL	1767.8	136.85	0.9978
7	Impurity G	1-7 µg/mL	1892.9	234.69	0.9965
8	Impurity-H	1-7 µg/mL	2203.9	69.991	0.9986

Table 4: Recovery studies

Sr. No.	Sample	Spiked level			Mean % Recovery	% RSD
		80%	100%	120%		
		% Recovery				
1	Levothyroxine Sodium	99.85	99.48	100.2	99.84	0.3994
2	Impurity-A	99.52	100.21	100.68	100.14	0.2003
3	Levothyroxine-N-methylamide	98.16	99.43	99.15	98.91	0.3957
4	Impurity-C	99.43	100.51	100.14	100.03	0.3001
5	Impurity-D	99.37	98.28	99.15	98.93	0.4947
6	Impurity F	99.91	98.16	98.25	98.77	0.7902
7	Impurity G	98.99	99.19	99.15	99.11	0.5947
8	Impurity-H	98.72	100.25	99.49	99.49	0.6964

Table 5: Intra-day precision data for Levothyroxine Sodium

Sample	Intra-day precision				Inter-day precision data			
	LQC	MQC	HQC	% RSD	LQC	MQC	HQC	% RSD
	% Recovery				% Recovery			
Levothyroxine Sodium	100.10	99.63	100.35	1.05	100.05	99.68	100.40	0.8451
Impurity-A	99.67	100.36	100.83	0.8967	99.72	100.41	100.88	0.8972
Levothyroxine-N-methylamide	98.31	99.58	99.30	0.9831	98.36	99.63	99.35	0.9836
Impurity-C	99.58	100.66	100.29	0.7958	99.63	100.71	100.34	0.7463
Impurity-D	99.52	98.43	99.30	0.9952	99.57	98.48	99.35	0.2957
Impurity F	100.06	98.31	98.40	1.161	100.11	98.36	98.45	0.9810
Impurity G	99.14	99.34	99.30	0.7414	99.19	99.39	99.35	0.4919
Impurity-H	98.87	100.40	99.64	0.3887	98.92	100.45	99.69	1.12

Method Validation:

Linearity and Range:

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven-point calibration curve of Levothyroxine Sodium and Impurity were constructed. Different concentrations and peak area values are depicted in Table 3. Calibration curve when subjected to least square regression analysis yielded an equation; shown in Fig. 3

from the linearity study, it was revealed that, there is a linear relationship between response and amount of drug and impurity within the range 1-10 µg/ml and 1 – 7µg/ml.

Accuracy (percentage Recovery) :

Accuracy is the closeness of test results to the true value obtained by proposed method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For levothyroxine sodium and levothyroxine sodium related substances, accuracy was determined using recovery studies. At 80, 100 and 120 % standard addition, mean recovery of Levothyroxine Sodium was found to be in between 98.77 to 100.14 %. The relative standard deviation (% RSD) was found to be less than 2 (Table 4). From the results of accuracy studies, it was concluded that the proposed method is accurate.

Precision:

Precision was studied by analysis LQC, MQC and HQC STDs of the Levothyroxine Sodium at concentrations covering the entire calibration range**.** The results expressed in terms of % RSD for the intra- and inter-day precision study (Table 5). Percent RSD values of intra-day precision study were found to be in between 0.3887 to 1.161 whereas inter-day precision was found to be in between 0.2957 to 1.12. It was concluded that the analytical technique showed good repeatability.

LOD and LOQ:

LOD and LOQ of proposed HPLC method was found to be 0.09 µg/ml and 0.41 µg/ml. (Table No.6) Lower LOQ value indicated that proposed method would be sensitive enough to quantify the Levothyroxine Sodium and Impurity content of samples at its lower level.

Table 6: LOD and LOQ

Sample	LOD (µg/mL)	LOQ (µg/mL)
Levothyroxine Sodium	0.10	0.30
Impurity-A	0.15	0.28
Levothyroxine-N-methylamide	0.11	0.33
Impurity-C	0.09	0.35
Impurity-D	0.15	0.41
Impurity F	0.12	0.37
Impurity G	0.11	0.39
Impurity-H	0.15	0.40

Estimation of in Levothyroxine Sodium Related Substances in pharmaceutical formulation

Proposed validated analytical method was successfully applied to the determination of Levothyroxine Sodium related substances in pharmaceutical formulation. By proposed HPLC method, Levothyroxine Sodium related substances in the tablet formulation was found within limits (Less than 2%) Further, it was found that proposed HPLC method is specific for the Levothyroxine Sodium related substances.

CONCLUSION

An accurate, precise, sensitive yet robust HPLC method was developed and validated for the determination of related substances of Levothyroxine Sodium in bulk and formulation. Proposed HPLC method was found to be specific for related substances of Levothyroxine Sodium and was free from any interference of formulation excipients. Proposed HPLC method can be used for routine analysis of Levothyroxine Sodium related substances in bulk as well as formulation.

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