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(RESEARCH ARTICLE)

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Development and validation of RP-HPLC method for estimation of anti-hypothyroidism drug in capsule dosage form

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Abstract

A simple, accurate, sensitive and precise Reverse Phase-High Performance Liquid Chromatographic assay method for estimation of Levothyroxine sodium in dosage form was successfully developed. The chromatographic separation was performed on *Welch Ultisil XB-CN*, 150 x 4.6 mm, 5µm. column. The mobile phase consists of 0.05% OPA Buffer solution, and Acetonitrile in the ratio of 72:28, v/v. Mobile phase was delivered at a flow rate of 1.0 ml/min. Samples were injected 50 µL the column temperature was kept at 40 ^{0}C and sample temperature 15^{0}C. The wavelength 221 nm were selected for the evaluation of the chromatogram. The retention time of the drug was found to be 3.7 min. The developed method was found to be linear in a concentration range of 5-15 µg/ml of the drug (r^2= 0.999). The low value of % RSD indicates reproducibility of the method. Thus this method can be used for routine analysis of pomalidomide in formulation and to check the stability of bulk samples.

Keywords: Levothyroxine sodium; RP-HPLC; Method validation; Stability indicating assay method

1. Introduction

Livothyroxin is chemically (2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3diiodophenyl]propanoic acid. Levothyroxine is a synthetically prepared levo-isomer of the thyroid hormone thyroxine (T_4, a tetra-iodinated tyrosine derivative) that acts as a replacement in deficiency syndromes such as hypothyroidism. T_4 is the major hormone secreted from the thyroid gland and is chemically identical to the naturally secreted T_4: it increases metabolic rate, decreases thyroid-stimulating hormone (TSH) production from the anterior lobe of the pituitary gland, and, in peripheral tissues, is converted to T_3. Thyroxine is released from its precursor protein thyroglobulin through proteolysis and secreted into the blood where is it then peripherally deiodinated to form triiodothyronine (T_3) which exerts a broad spectrum of stimulatory effects on cell metabolism. T_4 and T_3 have a relative potency of ~1:4.

1.1. Structure

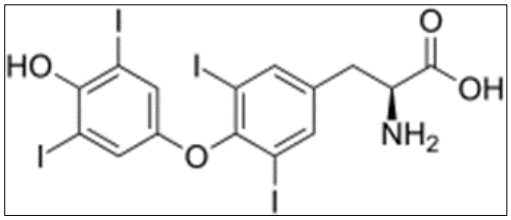

Figure 1 Structure of Levothyroxine

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Table 1 General profile of Levothyroxine

Category	Anti- hypothyroidism Agent
Chemical Name	(2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]propanoic acid
Molecular Formula	C15H11I4NO4
Molecular Weight	776.87 g/mol
Description	White to off white powder
Solubility	Slightly soluble in alcohol. Insoluble in acetone, chloroform, ether.
pKa	9.43
Melting point	235.5 °C

Instrumental techniques like HPLC play in a vital role in analysis. The brief outlines of some of the commonly used instrumental techniques are described below. Chromatographic techniques provide the means of separating the components of mixtures and simultaneous qualitative and quantitative analysis, as required. The linking of chromatographic and spectrometric techniques called hyphenation provides a powerful means of separating and identifying unknown compounds. Electrophoresis is another separation technique with similarities to chromatography that is useful for the separation technique of charged species. The principal separation techniques and their application are listed in Table 1.1[1]

2. Material and methods

2.1. Instruments

2.1.1. HPLC

Table 2 HPLC Instruments

Make	Waters e2695
Pump	Reciprocating Water-510
Detector	Waters 2695 PDA
Software	Empower PRO

2.1.2. SPECTROPHOTOMETER

Double beam UV-visible spectrophotometer with 10mm Matched quartz cells

Table 3 Spectrophotometer

Model	UV 1900i
Make	Shimadzu

2.1.3. ANALYTICAL BALANCE

Digital Analytical balance

Table 4 Digital Analytical balance

Model	XS205D0
Make	Mettler Toledo

2.1.4. PH METER

Digital Analytical balance

Table 5 Digital Analytical balance

Make	Thermo Scientific
Model	Orian Star A211

2.1.5. REAGENTS

Table 6 List of Reagent

Sr. No	Chemical	Make
1	Water	Rankem
2	Acetonitrile	Merck life science
3	Methanol	Merck life science
4	Phosphoric acid 88%	Merck life science
5	0.45µ PVDF Syringe Filter	Mdi

2.2. Preparation of Standard solution

2.2.1. Preparation of levothyroxine sodium Standard stock solution

Weigh and transfer Accurately Weighed 25 mg of levothyroxine sodium standard was take to 50 ml of volumetric flask. to it about 30 ml of diluent was added and sonicated to dissolve cool and diluted up to the mark with diluent and mixed. then Further 2 ml of this solution was diluted to 10 0ml with diluent. Mixed well. Filtered the sample solution through 0.45µ PVDF membrane syringe filter.

2.2.2. Preparation of levothyroxine sodium Sample solution for 0.2 mg

Were determined the average filled weighed of the 20 capsules. Transfer 10 capsule in 200 ml of volumetric flask. add 60 ml of diluent was added and sonicated for 30 minute with intermittent shaking, add 100 ml methanol, sonicated for 30 min with intermittent shaking. Make up volume with methanol. Stirrer on magnetic stirrer for 30 min at 500 RPM. Filtered the sample solution through 0.45µ PVDF membrane syringe filter.

2.3. Selection of Stationary phase

On the basis of reversed phase HPLC mode and number of carbon present in molecule (analyte) stationary phase with C18 bonded phase i.e *Welch, Ultisil XB-CN*, 150 x 4.6 mm, 5µm was selected.

2.4. Selection of Mobile Phase

The selection of mobile phase was done after assessing the solubility of drug in different solvent as well on the basis of literature survey and finally mobile phase was selected for is the mixture of Buffer solution and acetonitrile in the ratio of 72:28 v/v.

2.5. Optimization of Chromatographic Parameters

Optimization in HPLC was the process of finding a set of conditions that adequately separate and enable the quantification of the analyte from the endogenous material with acceptable accuracy, precision, sensitivity, specificity, cost, ease and speed.

2.6. Preparation of Levothyroxine sodium standard stock solution

Accurately weighed and transferred 20.00 mg of levothyroxine sodium and transferred in 100 ml of volumetric flask to it 70 ml of diluent was added and sonicated to dissolved then Further from this stock solution as per following table in to 100 ml of volumetric flask and the volume was made up with diluents up to the mark and mixed.

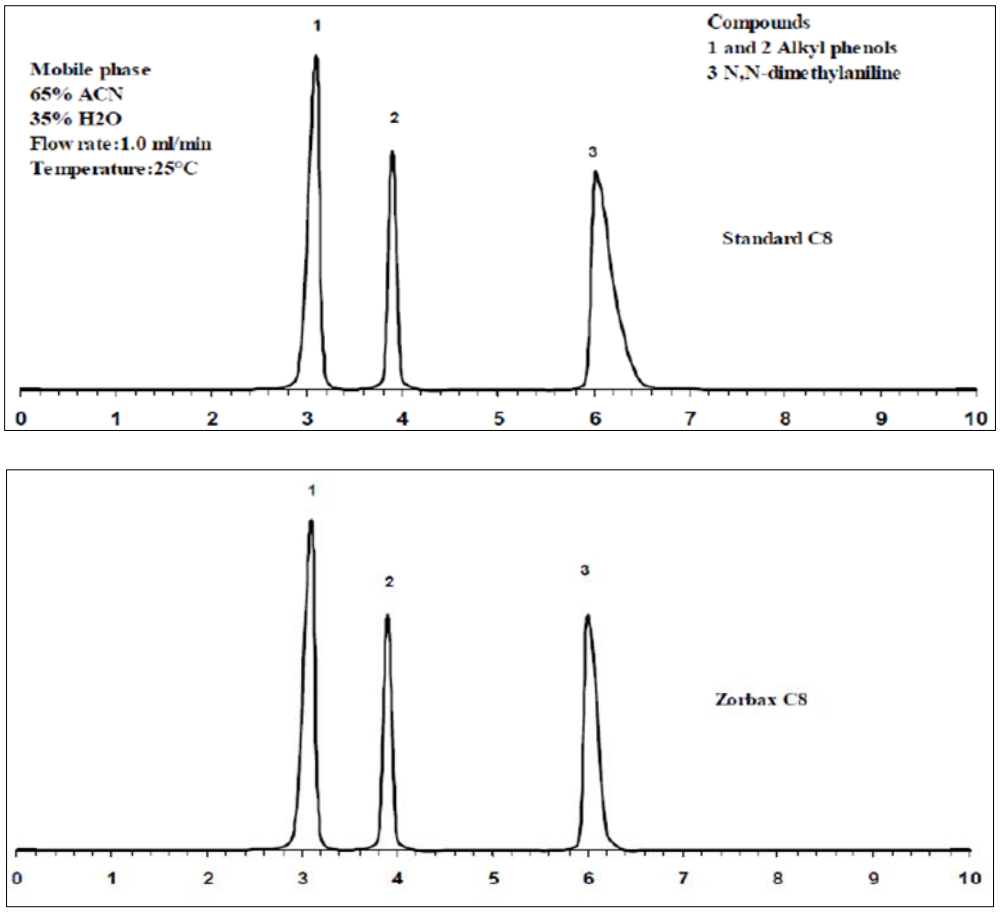

Figure 2 Asymmetric chromatograms (Concentration of Levothyroxine sodium: 200 ppm)

2.7. Method validation of RP-HPLC method

Once an analytical method is developed for its intended use, it must be validated. The extent of validation evolves with the drug development phase. Usually, a limited validation is carried out to support an Investigational New Drug (IND) application and a more extensive validation for New Drug Application (NDA) and Marketing Authorization Application (MAA). Typical parameters recommended by FDA, USP, and ICH are as follow.

2.7.1. Specificity

Specificity is the ability of the method to measure the analyte in the presence of other relevant components those are expected to be present in a sample. The relevant components might include impurities, degradants, matrix, etc. Lack of specificity of an individual procedure may be compensated by other supporting analytical procedure(s). Specificity can also be demonstrated by verification of the result with an independent analytical procedure. In the case of chromatographic separation, resolution factors should be obtained for critical separation. Tests for peak homogeneity, for example, by diode array detection (DAD) or mass spectrometry (MS) are recommended. The peak purity of analyte peak was evaluated in each degraded sample with respect to total peak purity and three point peak purity.

2.7.2. Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It is demonstrated directly on the drug substance by dilution of a standard stock solution of the drug product components, using the proposed procedure. For the establishment of linearity, minimum

of five concentrations are recommended by ICH guideline. The value of correlation co-efficient (r2) should fall around 0.99.

2.7.3. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision may be considered at two levels: repeatability and intermediate precision. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability

Repeatability study is performed by preparing a minimum of 6 determinations at 100% of the test concentration and analyzed as per the respective methodology.

Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The analyst should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. Here, intermediate precision of the method is checked by carrying out six independent assays of test sample preparation on the different day by another person under the same experimental condition and calculated the % RSD of assays.

2.7.4. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The evaluation of accuracy has got very prime importance as it deliberately force the method to extract the drug and impurities at higher and lower level.

2.7.5. Solution stability

Drug stability in pharmaceutical formulations/active pharmaceutical ingredients is a function of storage conditions and chemical properties of the drug, preservative and its impurities. Condition used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability data is required to show that the concentration and purity of analyte in the sample at the time of analysis corresponds to the concentration and purity of analyte at the time of sampling. Stability of sample solution was established by storage of sample solution at ambient temperature (25°C) for 24h.

2.7.6. Limit of detection

The limit of detection (LOD) for an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. The limit of detection is evaluated by serial dilutions of analyte stock solution in order to obtain signal to noise ratios of 3:1.

2.7.7. Limit of quantitation:

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The limit of quantitation (LOQ) is a parameter of quantitative assays for low levels of compounds in sample matrices. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1. The limit of quantification was evaluated by serial dilutions of analyte stock solution in order to obtain signal to noise ratios of 10:1.

2.7.8. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate

The factors chosen for all the drugs under investigation were the flow rate, mobile phase composition, pH of a mobile phase and using different lot of LC column. The observation shall be summarized and critical parameters shall be listed out in the validation report. System suitability parameter must be within the limit of acceptance criteria as mentioned in the method.

3. Results and discussion

A simple, precise and economic UV and RP-HPLC method was developed and validated for estimation of Levothyroxine sodium in bulk and capsule. The method was validated as per ICH guidelines by using various validation parameters such as Linearity, accuracy, precision, specificity and robustness.

3.1. Selection of Wavelength

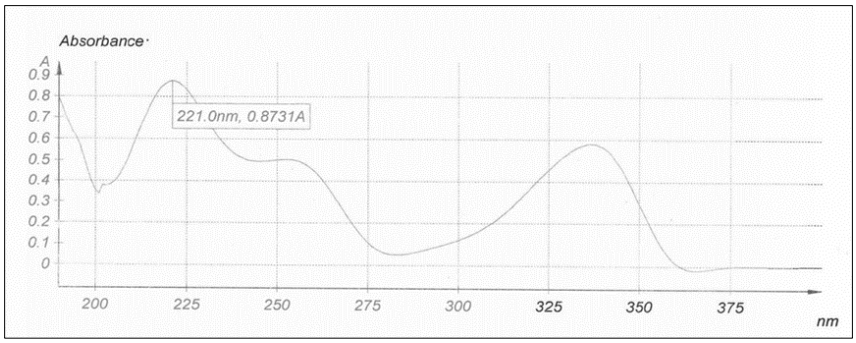

Figure 3 Spectra showing λ max of Levothyroxine sodium

3.2. Reverse Phase High Performance Liquid Chromatography Method Development Different trials taken were as follows

3.2.1. TRIAL: 1

Table 7 Trial 1 Chromatographic Conditions

Column	:	Inertsil ODS 2 50 x 4.6 mm, 3 µm.
Mobile Phase		water and methanol (60:40 v/v)
Flow Rate	:	1.5 ml/min
Injection Volume	:	10 µl
Wavelength	:	221 nm
Column oven Temp	:	25ºc
Auto Sampler Temp	:	15ºc
Run time	:	7 minutes
Retention time	:	About 4.1 minutes for levothyroxine sodium
Seal wash	:	Water : Methanol (90:10)
Needle wash	:	Water : Methanol (10:90)

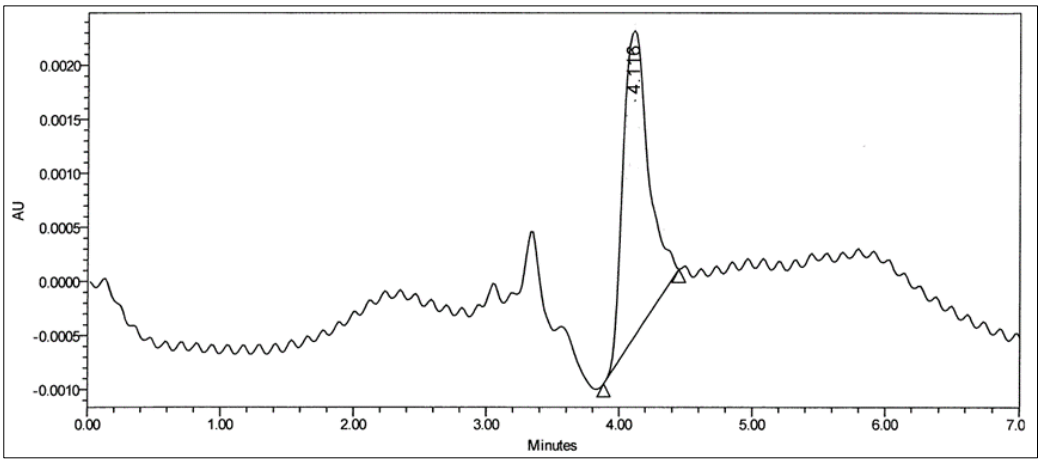

Figure 4 Typical chromatogram for Trial- 1

3.2.2. TRIAL: 2

Table 8. Trial 2 Chromatographic Condition

Column	**:**	**Inertsil ODS 2 50 x 4.6 mm, 3 µm.**
Mobile Phase		water and Acetonitrile (60:40 v/v)
Flow Rate	:	1.5 ml/min
Injection Volume	:	10 µl
Wavelength	:	221 nm
Column oven Temp	:	25ºc
Auto Sampler Temp	:	15ºc
Run time	:	10 minutes
Retention time	:	About 3.7 minutes for levothyroxine sodium
Seal wash	:	Water : Methanol (90:10)
Needle wash	:	Water : Methanol (10:90)

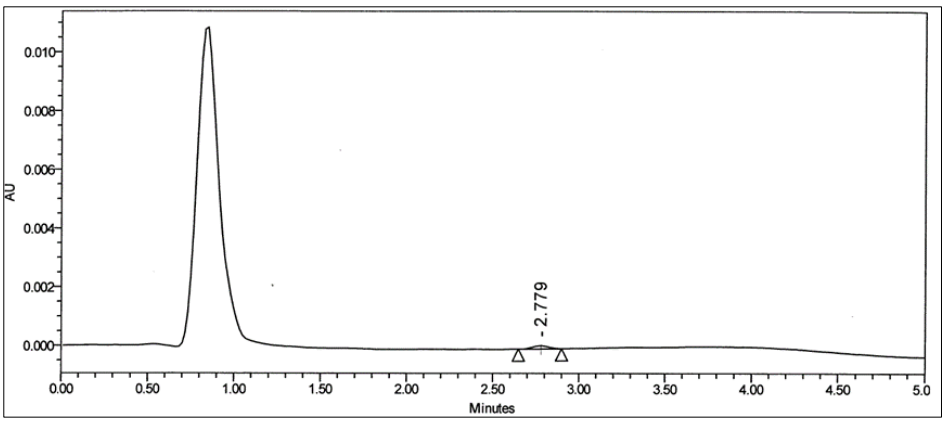

Figure 5 Typical chromatogram for Trial- 2

3.2.3. TRIAL: 3

Table 9 Trial 3 Chromatographic Condition

Column	:	Welch Ultisil XB-CN, 150 x 4.6 mm, 5µm.
Mobile Phase		Buffer solution and acetonitrile (60:40 v/v)
Flow Rate	:	1.5 ml/min
Injection Volume	:	50 µl
Wavelength	:	221 nm
Column oven Temp	:	40ºc
Auto Sampler Temp	:	15ºc
Run time	:	10 minutes
Retention time	:	About 3.7 minutes for levothyroxine sodium
Seal wash	:	Water : Methanol (90:10)
Needle wash	:	Water : Methanol (10:90)

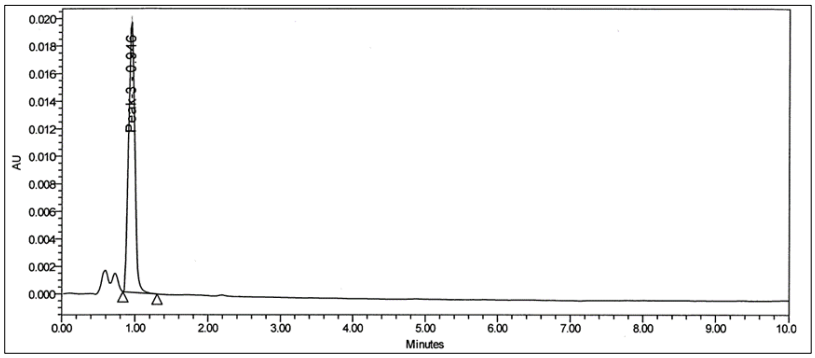

Figure 6 Typical chromatogram for Trial- 3

3.2.4. TRIAL: 4

Table 10 Trial 4 Chromatographic Condition

Column	:	Welch Ultisil XB-CN, 150 x 4.6 mm, 5µm.
Mobile Phase		Buffer solution and acetonitrile (72:28 v/v)
Flow Rate	:	1.0 ml/min
Injection Volume	:	50 µl
Wavelength	:	221 nm
Column oven Temp	:	40ºc
Auto Sampler Temp	:	15ºc
Run time	:	10 minutes
Retention time	:	About 3.7 minutes for levothyroxine sodium
Seal wash	:	Water : Methanol (90:10)
Needle wash	:	Water : Methanol (10:90)

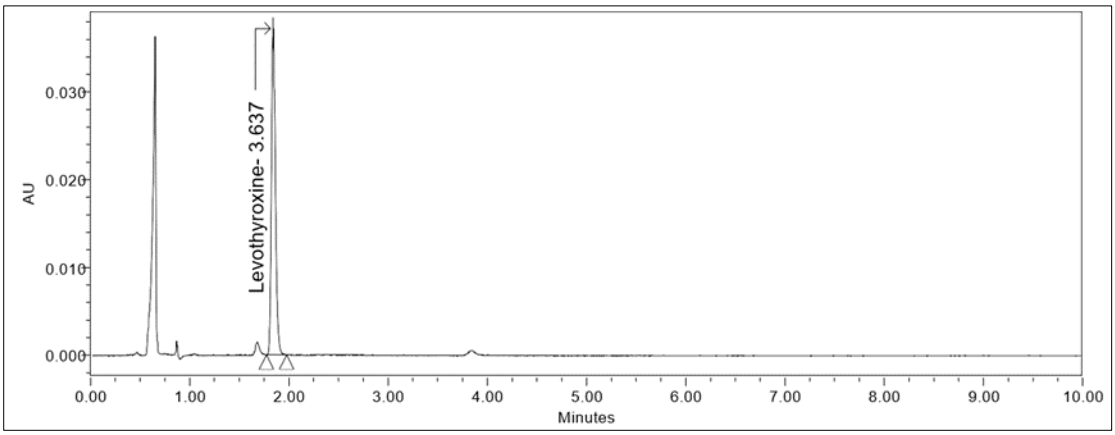

Figure 7 Typical chromatogram for Trial- 4

Table 11 System suitability test of Levothyroxine sodium

The Tailing factor for peak	**0.9**
The theoretical plates for peak	4578
Sr. No.	Area
1	32658
2	32525
3	32485
4	32565
5	32528
6	32452
Mean	32536
% RSD	0.2

Table 12 Specificity of Levothyroxine sodium (Identification and Interference)

Component	**Retention time (min)**	**Tailing factor**	**Theoretical plates**	**Purity angle**	**Purity threshold**
Blank	-	-	-	-	-
Placebo solution	-	-	-	-	-
Standard solution	3.520	1.0	4524	2.36	5.24
Sample solution	3.74	0.9	4235	2.15	5.38

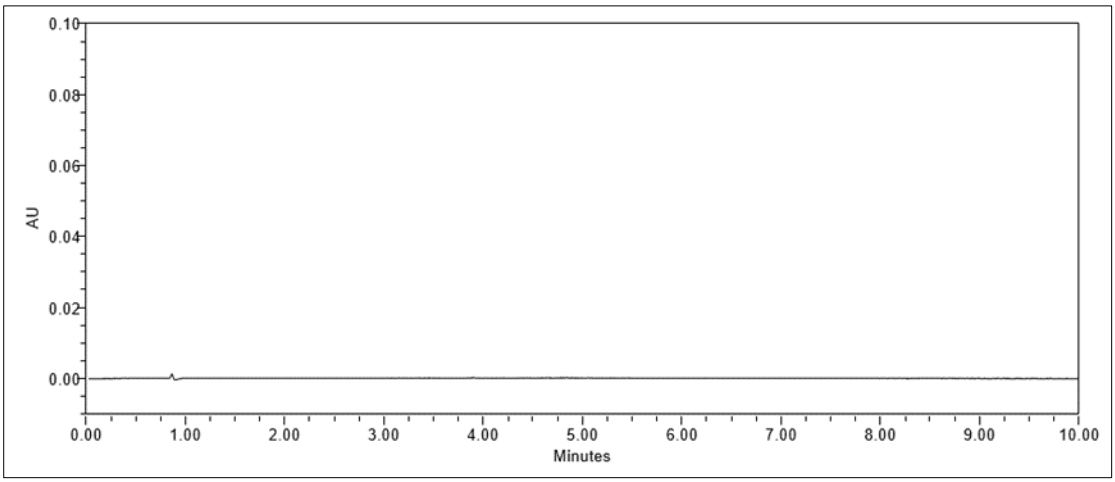

Figure 8 Chromatogram of Blank

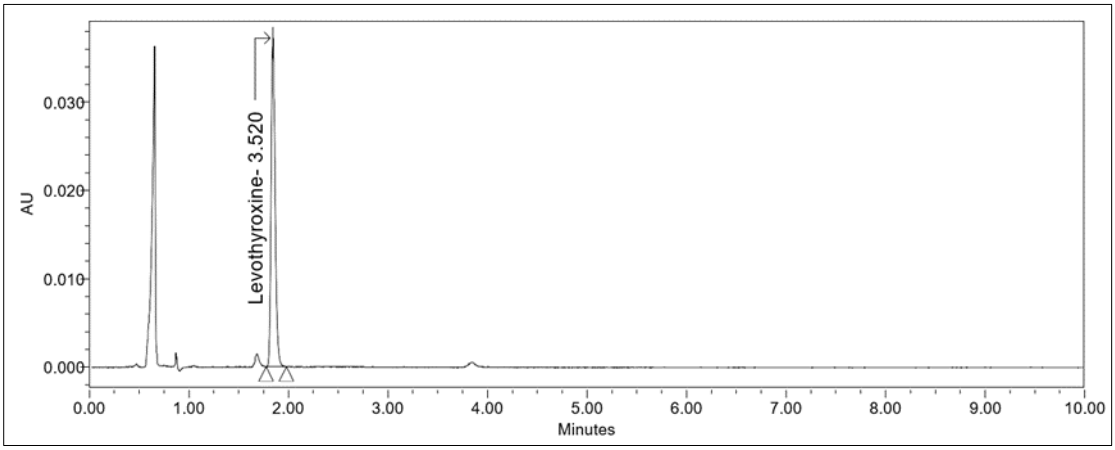

Figure 9 Chromatogram of Standard

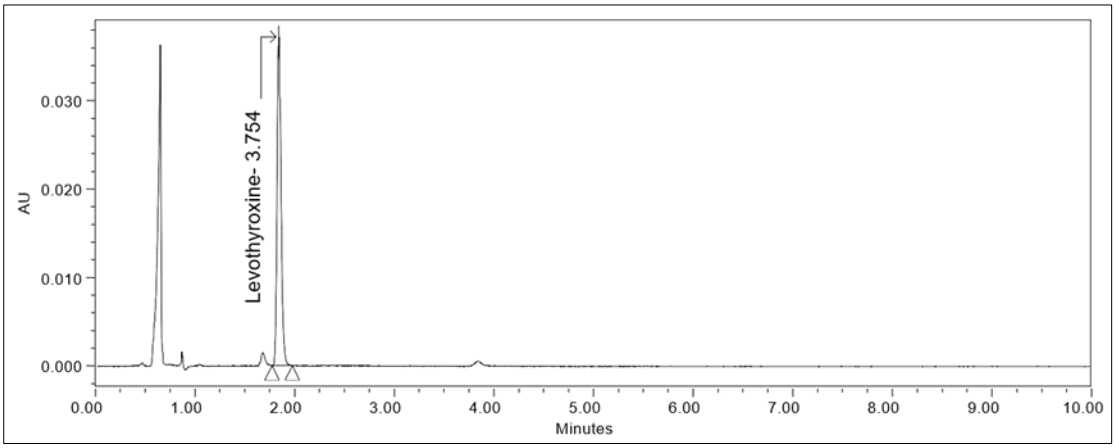

Figure 10 Chromatogram of Sample

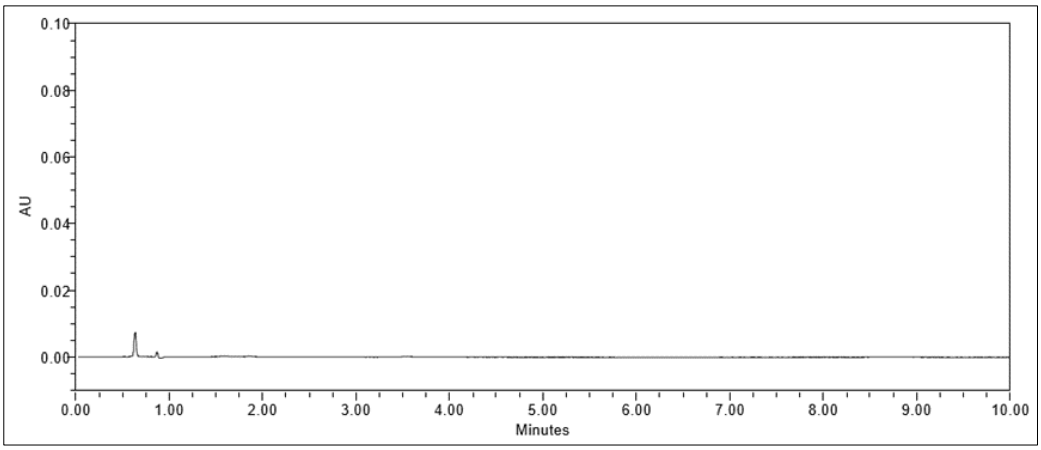

Figure 11 Chromatogram of Placebo

Table 13 Linearity of Levothyroxine sodium

Levothyroxine sodium				
Level (%)	**Concentration (ppm)**	**Response**		
		1	**2**	**Mean**
50	5	16534	16495	16515
80	8	26254	26301	26278
100	10	32264	32054	32159
120	12	39256	39125	39191
150	15	49038	49201	49120
Co-relation coefficient				0.9995
SLOPE				3256.1
Y-INTERCEPT				92.083

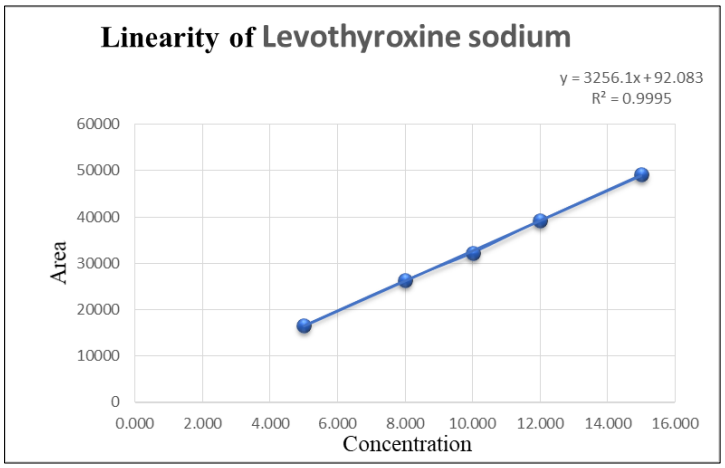

Figure 12 Linearity plot of Levothyroxine sodium

Table 14 % Recovery for Levothyroxine sodium

Level (%)	Theoretical concentration (mcg/mL)	% Recovery	Mean recovery%
50	5	100.3	100.3
	5	100.1	
	5	99.3	
100	10	99.4	99.4
	10	100.9	
	10	99.5	
150	15	100.5	100.4
	15	99.8	
	15	99.6	
Mean recovery			100.0

Table 15 Method precision Levothyroxine sodium

Sample No.	Area	% Assay
1	32268	99.6
2	32541	100.4
3	32365	99.9
4	32451	100.1
5	32314	99.7
6	32364	99.9
Mean		99.9
% RSD		0.3

Table 16 Intermediate Precision for Levothyroxine sodium

Parameter	Precision (Analyst-I)	Intermediate Precision (Analyst-II)
HPLC Instrument No.	AD/I-022	AD/I-024
Date of analysis	Day -1	Day -2
HPLC column No.	C18-130	C18-356
Sample No.	% Assay	
1	99.6	99.6
2	100.4	99.4
3	99.9	99.9
4	100.1	100.1
5	99.7	99.7

6	99.9	100.3
Mean	99.9	99.8
Absolute mean difference for % Assay from method precision and intermediate precision		0.3

Table 17 Robustness for Levothyroxine sodium

Changes in parameters	Values	%Assay	Absolute mean difference
Control	As per method	99.5	NA
Flow rate (±0.1 mL/min)	0.9 mL/min	100.4	0.9
	1.1 mL/min	99.1	-0.4
Column temperature (± 5°C)	30°C	99.8	0.3
	40°C	99.1	-0.4
Change in Wavelength (± 2 nm)	219	100.1	0.2
	223	99.3	0.9

4. Conclusion

HPLC has gained the valuable position in the field of analysis due to ease of performance, specificity, sensitivity and the analysis of sample of complex nature. This technique was employed in the present investigation for estimation of Levothyroxine sodium capsule formulation. HPLC Water2469 with *Welch Ultisil XB-CN*, 150 x 4.6 mm, 5µm. and UV/PDA detector with empower pro Software was used for the study. The standard and sample solution of Levothyroxine sodium were prepared in diluent. Different pure solvents of varying polarity in different proportions were tried as mobile phase for development of the chromatogram.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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