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OPTIMIZATION AND AUTHENTICATION OF RP-HPLC PROCEDURE FOR DETERMINING SYNTHETIC THYROID HORMONE REPLACEMENT

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ABSTRACT

This study presents the development, optimization, and validation of a robust reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of levothyroxine and liothyronine in pharmaceutical formulations. Levothyroxine (T4) and liothyronine (T3) are synthetic thyroid hormones crucial for managing hypothyroidism and other endocrine disorders. Given the sensitivity and low concentration of these hormones in formulations, precise analytical techniques are imperative. The optimized RP-HPLC method employed a C18 column with a mobile phase of acetonitrile and water (25:75 v/v), adjusted to pH 6.2, with UV detection at 300 nm. The method demonstrated excellent linearity (10–100 µg/mL), precision, accuracy, specificity, and robustness in accordance with ICH guidelines. Forced degradation studies under acidic, basic, oxidative, thermal, and photolytic conditions confirmed the method's stability-indicating nature. Complementary UV spectrophotometric methods, including absorption correction and first-order derivative techniques, were also validated and applied. The results indicate high assay accuracy (within 99–101%) and minimal interference from excipients or degradation products. This validated RP-HPLC method ensures reliable quality control of thyroid hormone formulations, enhancing the safety and efficacy of endocrine pharmacotherapy.

Keyword: *Levothyroxine, Liothyronine, RP-HPLC, Stability-Indicating Method, Thyroid Hormone Analysis*

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1. INTRODUCTION

Thyroid hormone replacement therapy, primarily levothyroxine and liothyronine, is crucial for managing hypothyroidism and related endocrine disorders, with levothyroxine being the most prescribed medication globally **(Gharib et al., 2005; Garber et al., 2012)**. Reverse-phase high-performance liquid chromatography (RP-HPLC) is a gold standard for determining and controlling synthetic thyroid hormones due to its precision, sensitivity, and reproducibility. The efficacy of these hormones depends on their bioavailability and chemical stability **(Colucci et al., 2011)**. Moreover, the pharmacokinetic differences between T3 and T4, including half-life and onset of action, necessitate exact quantification to ensure therapeutic efficacy and to minimize adverse effects such as hyperthyroidism or hypothyroidism due to under- or overdosing **(Larsen et al., 2016)**. Therefore, analytical methods used for the determination of these hormones in bulk drugs and finished pharmaceutical products must meet stringent validation criteria, including accuracy, precision, specificity, linearity, and robustness. Reverse-phase HPLC, particularly with ultraviolet (UV) detection, has been extensively studied and applied in pharmaceutical analysis of levothyroxine and liothyronine **(Furlanetto et al., 2013; Sethi, 2008)**. Thyroid hormones face challenges due to degradation and low concentration in pharmaceutical formulations. Optimized chromatographic conditions, including mobile phases, column selection, detection wavelength, and flow rate, are needed for robust results. Method optimization in RP-HPLC involves adjusting parameters to improve resolution, peak symmetry, retention time, and maintain ionization state **(Katz et al., 2000)**. Similarly, the use of C18 columns in RP-HPLC offers high retention and separation efficiency for hydrophobic and moderately polar compounds such as T4 and T3 **(Dong, 2006)**. Authentication and validation of the developed RP-HPLC method are equally important to ensure compliance with regulatory standards set by the International Council for Harmonisation (ICH), the United States Pharmacopeia (USP), and other pharmacopeial guidelines. Key validation parameters include linearity, limit of detection (LOD), limit of quantitation (LOQ), precision (intra-day and inter-day), accuracy (recovery studies), specificity, and robustness **(ICH, 2005)**. Moreover, system suitability testing (SST) must be performed to confirm the reliability of the chromatographic system before sample analysis. Recent studies have also explored the use of diode-array detection (DAD), mass spectrometry (MS), and fluorescence detection in combination with RP-HPLC to enhance sensitivity and selectivity in thyroid hormone analysis **(Garg et al., 2017; Mahajan et al., 2020)**. UV detection is widely used for thyroid hormone authentication in pharmaceutical formulations due to its cost-effectiveness and simplicity, but concerns over inconsistent and counterfeit products have led to concerns about quality control **(Khandelwal et al., 2008)**. This study aims to develop and optimize a robust RP-HPLC method for the quantification of levothyroxine and liothyronine in commercial formulations,

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contributing to quality assurance in endocrine pharmacotherapy and addressing analytical challenges in thyroid hormone analysis, thereby enhancing the reliability of thyroid hormone analysis.

2. MATERIAL AND METHOD

2.1 Identification and characterization of drugs

2.1.1 Physical characterization of drug

At the beginning, the drugs Levothyroxine and Liothyronine were visually characterized by shape, colour and scent. All these parameters were reported in the literature and contrasted with the study.

2.2 Identification by IR:

The sample concentration of KBr should be in the 0.2 percent to 1 percent range. The pellet is much thicker than a liquid film, so a lower concentration (Beer's Law) is required in the sample. Roughly 80 mg of the mixture is needed for the die set to be used. Too high a concentration typically produces problems with receiving transparent pellets. This pellet is held in the sample cell and collected from 4000- 400 c.m-1 and IR spectra are scanned.

2.3 Solubility in different solvent:

It was carried out in different solvents like water, methanol, ethanol, acetonitrile, HCl and NaOH. Drug was dissolved up to the saturation of the solution and weight in mg per 10 ml of the solvent was recorded and solubility in each solvent was calculated.

2.4 Identification of drugs by Melting point determination:

Melting point of drugs was determined by using Digital melting point apparatus.

2.5 Estimation of levothyroxine and liothyronine from their Combination Drug Product by Spectrophotometric Methods

To select an appropriate solvent for the analysis, 10 µg/mL solutions of both levothyroxine (LVT) and liothyronine (LIO) were prepared separately in 0.1 N NaOH, 0.1 N HCl, and methanol. The UV overlay spectra of both drugs in the different solvents were recorded and compared. Among the tested solvents, methanol provided the most distinct and stable spectral characteristics for both compounds. Therefore, methanol was selected as the common solvent for further analysis **(Bodiwala et al., 2022)**.

2.5.1 Absorption Correction Method

Standard stock solutions of levothyroxine (LVT) and liothyronine (LIO) were prepared by dissolving 30 mg of LVT and 100 mg of LIO in methanol and making up the volume to 100 mL, resulting in concentrations of 300 µg/mL and 1000 µg/mL, respectively. For wavelength determination, separate

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solutions of 5 µg/mL LVT and 10 µg/mL LIO were scanned in the UV range of 200–400 nm using methanol as the solvent. LVT exhibited maximum absorbance at 242 nm, and LIO at 360 nm. Calibration curves were constructed by preparing serial dilutions of LVT (5–20 µg/mL) and LIO (10–50 µg/mL), measuring absorbance at their respective wavelengths. Method validation followed ICH guidelines, assessing linearity (LVT: 5–30 µg/mL, LIO: 10–60 µg/mL), with correlation coefficients and regression equations determined. Precision was evaluated via repeatability (%RSD of six replicate readings of 5 µg/mL LVT and 20 µg/mL LIO), intraday and interday precision using three concentrations for each drug. Accuracy was assessed through recovery studies using the standard addition method at 80%, 100%, and 120% levels. Robustness was examined by analyzing mixed LVT\:LIO ratios (1:2 and 2:1), and ruggedness by triplicate analysis of three mixed concentrations on the same day. LOD and LOQ were calculated using standard deviation and slope from calibration data, with formulas: LOD = 3.3 × (SD/slope) and LOQ = 10 × (SD/slope) **(Mahmood et al., 2020)**.

2.5.2 Estimation of LVT and LIO in Combined Dosage Form:

Twenty tablets (label claim: levothyroxine 30 mcg + liothyronine 5 mcg) were weighing and crushed to get a fine powder. An accurately weigh powder equivalent to 30 mcg of LVT and 5 mcg of LIO was transfer to 50 ml volumetric flask and the volume was made by methanol and sonicated for 25-30 minutes then filtered through Whatman Filter No. 42. Few ml of filtrate was removed then 4 ml solution was pipette out in 10 ml volumetric flask and diluted with methanol. The absorbance was measured at 242 nm for LVT and 372 nm for LIO. Finally, concentration of both drugs was calculated using straight line equation.

2.6 First Order Derivative Spectrophotometric Method

A first derivative UV spectrophotometric method was developed for the simultaneous estimation of Levofloxacin (LVT) and Lornoxicam (LIO). Spectra were recorded in spectrum mode with medium scan speed, using a wavelength range of 200–400 nm, scan pitch of 0.1 nm, derivative order 1, smoothing factor 1, and a digital differentiation Δλ (N) of 2 nm. Standard solutions were prepared in methanol: 10 µg/mL LVT by serial dilution from a 100 mg/100 mL stock, and 100 µg/mL LIO from a 10 mg/100 mL stock. First derivative spectra of 5 µg/mL LVT and 20 µg/mL LIO revealed zero crossing points at 242 nm and 372 nm, respectively **(Ferreira et al., 2020)**.

2.6.1 Calibration curves were constructed: Series A (5–20 µg/mL LVT) and Series B (5–30 µg/mL LIO) were measured at 242 nm and 372 nm, respectively. In Series C, mixtures containing 1.5–9 µg/mL LVT and 5–30 µg/mL LIO were analyzed for simultaneous determination. The method was validated as per ICH guidelines. Linearity was confirmed (n=6), with respective correlation coefficients calculated. Precision

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studies (repeatability, intraday, and interday) were performed using mixed concentrations, with %RSD calculated. Accuracy was assessed via standard addition method (80%, 100%, 120%) with satisfactory recoveries. Robustness was tested by varying drug ratios (1:2, 2:1) and ruggedness by using different instruments. LOD and LOQ were determined using the formula: LOD = 3.3(SD/Slope), LOQ = 10(SD/Slope), where SD was based on 10 measurements of the lowest concentration and slope from calibration curves.

2.6.2 Estimation of LVT and LIO in Combined Dosage Form

Twenty tablets were weighing and crushed to get a powder form. An accurately weigh powder equivalent to 1.5 mg of LVT and 5 mg of LIO was transfer to 50 ml volumetric flask and the volume was made up to the mark using methanol as solvent and resulted solution was sonicated for 25–30 minutes then filtered through Whatman Filter No. 40. Few ml of filtrate were discarded then 2 ml solution was pipette out in 10 ml volumetric flask and diluted with methanol. Zero order spectrum of this solution was taken and converted into first derivative spectrum. Then absorbance was measured at 242 nm for LVT and 372 nm for LIO. Finally concentration of both drugs was calculated using straight line equation of first derivative method.

2.7 Estimation of LVT and LIO from their Combination Drug Product by RP-HPLC Method

The HPLC analysis was performed using a Shimadzu LC-2010CHT system equipped with a UV detector and Class VP software (version 2.31). A Kromasil C18 column (250 mm × 4.6 mm i.d., 5 µm particle size) was used for separation. The mobile phase consisted of a mixture including 0.02 M disodium hydrogen phosphate buffer (pH adjusted to 7.0 with ortho-phosphoric acid), HPLC-grade acetonitrile, methanol, and water. The flow rate was maintained at 1.0 mL/min, with detection carried out at 240 nm and an injection volume of 20 µL. An electronic analytical balance (Sartorious CD 2150) and a pH meter (Eutech Instruments, Model 313927) were used during reagent preparation. Standard reagents included Indapamide, Amlodipine besylate, and solvents (methanol, acetonitrile, and water) of HPLC grade obtained from E. Merck Ltd.

2.8 Development of HPLC Method

Pharmaceutical-grade Levothyroxine and Liothyronine were obtained from Intas Pharm. Pvt. Ltd., India. HPLC-grade water and acetonitrile were sourced from Rankem (Ranbaxy Fine Chemicals, New Delhi), and other analytical-grade chemicals were procured locally. All dilutions were carried out using standard volumetric glassware. The analysis was performed on a Shimadzu HPLC system equipped with an LC-10AT vp solvent delivery module, SPD-10A UV-Visible detector, and Rheodyne 7725i injector with a 20 µL sample loop. Injections were made using a Hamilton Bonodaz AG microliter syringe. Chromatographic

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separation was achieved on a Phenomenex C18 column (250 × 4.6 mm i.d.) with a matching guard column, using a mobile phase of acetonitrile:water (25:75 v/v), adjusted to pH 6.2 with phosphate buffer. The flow rate was maintained at 1.0 mL/min, and detection was carried out at 300 nm. Stock solutions of each drug (600 µg/mL) were prepared by dissolving 6 mg of the drug in 10 mL of mobile phase. Mixed working standards (15–150 µg/mL) were prepared by appropriate dilution. For sample preparation, twenty tablets containing either Levothyroxine (100 mg) or Liothyronine (100 mg) were weighed, crushed, and an amount equivalent to 25 mg was dissolved in mobile phase in a 25 mL volumetric flask. After filtration, appropriate dilutions were made to yield concentrations of 70, 30, 80, and 95 µg/mL. A 100 µL volume of the prepared sample was injected into the HPLC system for analysis **(Kumbhar et al., 2020)**.

2.9 Quality Control Standards:

Quality control (QC) standards and stock solutions of Levothyroxine and Liothyronine were prepared by dissolving 6 mg and 10 mg of each drug in 10 mL and 100 mL of mobile phase, respectively, to obtain concentrations of 600 µg/mL and 100 µg/mL. Working standards were prepared in low (40 µg/mL), medium (80 µg/mL), and high (150 µg/mL) concentration ranges using mobile phase as the diluent. Chromatographic separation was performed on a Shimadzu HPLC system equipped with LC-10AT vp solvent delivery module, SPD-10A UV-Vis detector, and Rheodyne 7725i injector with a 20 µL loop. The C18 analytical column (Phenomenex, 250 × 4.6 mm i.d.) with a matching guard column was used. The mobile phase consisted of acetonitrile:water (25:75 v/v), adjusted to pH 6.2 with phosphate buffer, delivered at 1.0 mL/min in isocratic mode. Detection was carried out at 300 nm. Calibration curves were constructed over a concentration range of 10–100 µg/mL for both drugs, with regression coefficients (r^2) of 0.9974 for Levothyroxine and 0.9989 for Liothyronine. The method was validated for linearity, accuracy, precision, specificity, stability, LOD, and LOQ **(Sebaiy et al., 2022)**.

2.10 Forced degradation studies

Forced degradation studies for Levothyroxine and Liothyronine were conducted under various stress conditions including acid and base hydrolysis, oxidation, dry heat, and sunlight exposure. A sample stock solution (100 µg/mL) of each drug was prepared by dissolving powdered API equivalent to 50 mg of Levothyroxine in mobile phase, filtered through a 0.2 µm nylon membrane, and appropriately diluted. For alkaline degradation, 0.1 mL of stock solution was mixed with 0.1 mL of 0.1N NaOH and kept at room temperature for 1 hour. Acid degradation was carried out by adding 0.1 mL of 0.1N HCl to 0.1 mL of the stock and holding the mixture for 2 hours at room temperature. Oxidative degradation involved treating the stock solution with 0.1 mL of 3% hydrogen peroxide for 1 hour. Thermal degradation was done by placing solutions of 5 µg/mL Levothyroxine and 15 µg/mL Liothyronine in an oven at 75°C for 1 hour, while photodegradation was performed by exposing the same concentration solutions to direct

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sunlight for 1 hour. All degraded samples were diluted with mobile phase to obtain final concentrations of 5 µg/mL and 15 µg/mL for Levothyroxine and Liothyronine, respectively. Non-degraded control samples were prepared at the same concentrations. Each sample (20 µL) was injected into the HPLC system and analyzed under the previously described chromatographic conditions.

2.11 New Stability Indicating Analytical Method Development And Validation For Estimation of Levothyroxine Using RP-HPLC

Method II: Stability-Indicating RP-HPLC Method for Levothyroxine

A new RP-HPLC method was developed and validated for the estimation of Levothyroxine in pharmaceutical dosage forms. The optimal mobile phase was found to be 10mM KH_2PO_4: methanol (20:80 v/v), filtered and sonicated before use, with a flow rate of 1.0 ml/min and detection at 234 nm. A C18 column (250 mm × 4.6 mm, 5 µm) was used at ambient temperature. Methanol was selected as the diluent. The retention time of Levothyroxine was 5.658 ± 0.4 min. Calibration standards were prepared in the range of 5–25 µg/ml, and the method showed good linearity, precision, and accuracy. System suitability parameters met the required criteria, and the method was validated as per ICH Q2(R1) guidelines for specificity, accuracy, precision (repeatability, intermediate), robustness, LOD, and LOQ. The method proved to be simple, accurate, robust, and suitable for routine analysis **(Carmona et al., 2022)**.

2.12 Forced degradation studies

To evaluate the stability of Levothyroxine as per ICH guidelines, forced degradation studies were conducted under various stress conditions. Acid degradation involved treating 10 mg of drug with 0.1 N HCl at 40°C for 8 hours, while alkaline hydrolysis was performed using 0.1 N NaOH at room temperature for 2 hours. Oxidative degradation was induced using 0.2% hydrogen peroxide at 40°C for 24 hours. For thermal degradation, the drug was exposed to 75°C in an oven for 2 weeks. In each case, samples were diluted to 10 µg/ml and analyzed by HPLC using a UV detector (injection volume: 20 µl). The extent of degradation was calculated using the calibration curve.

2.13 New Stability Indicating Analytical Method Development and Validation for Estimation of liothyronine Using RP-HPLC

A validated RP-HPLC method was developed for estimating liothyronine using an Agilent 1200 system with VWD and ELSD detectors, processed through Chemstation software. The optimal mobile phase was 0.2M acetic acid and acetonitrile (60:40 v/v, pH 7 adjusted with TEA), filtered and sonicated before use. A PLRPs analytical column (250 × 4.6 mm, 5 µm) was used at ambient temperature, with a flow rate of 1.0 ml/min and UV detection at 210 nm. Acetonitrile served as the diluent. The retention time was 4.156 ± 0.4 min. Calibration standards (5–25 µg/ml) were prepared from a 1000 µg/ml stock solution and analyzed to establish linearity. System suitability was confirmed through six replicate injections. The method was

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applied successfully for the analysis of commercial liothyronine formulations **(Shewale and Chaturvedi, 2022)**.

2.14 Validation of developed Method

The developed RP-HPLC method for liothyronine was validated as per ICH Q2(R1) guidelines. Linearity was confirmed over the 5–25 µg/ml range with a strong correlation between concentration and peak area. Specificity demonstrated clear detection of liothyronine without interference from other components. Accuracy was assessed through recovery studies at 80%, 100%, and 120% levels. Precision was evaluated by repeatability and intermediate precision (day-to-day and analyst-to-analyst), showing consistent results. Robustness was confirmed by slight variations in the mobile phase composition (60:40 to 65:35 v/v), with no significant effect on method performance. LOD and LOQ were calculated from the standard deviation and slope of the calibration curve, confirming the method's sensitivity **(Sunder and Mittal, 2018)**.

2.15 Forced degradation studies

Forced degradation studies were performed to assess the stability of liothyronine in line with ICH guidelines. Samples (10 mg) were subjected to acid (0.1 N HCl, 40°C, 8 h), alkaline (0.1 N NaOH, RT, 2 h), oxidative (0.2% H_2O_2, 40°C, 24 h), and thermal (75°C, 2 weeks) stress conditions. Each degraded sample was diluted to 10 µg/ml and analyzed by HPLC using a UV detector (injection volume: 20 µl). Degradation was quantified using the drug's calibration curve.

3. RESULT

3.1 Identification and characterization of drugs

3.1.1 Physical characterization of drug

At the beginning, the drugs Levothyroxine and Liothyronine were visually characterized by shape, colour and scent.

3.1.2 Identification by IR:

The peak values that are properties of the drug are seen in the IR spectrum of Levothyroxine and Liothyronine and the graph is seen in Figure 6.1- 6.2.

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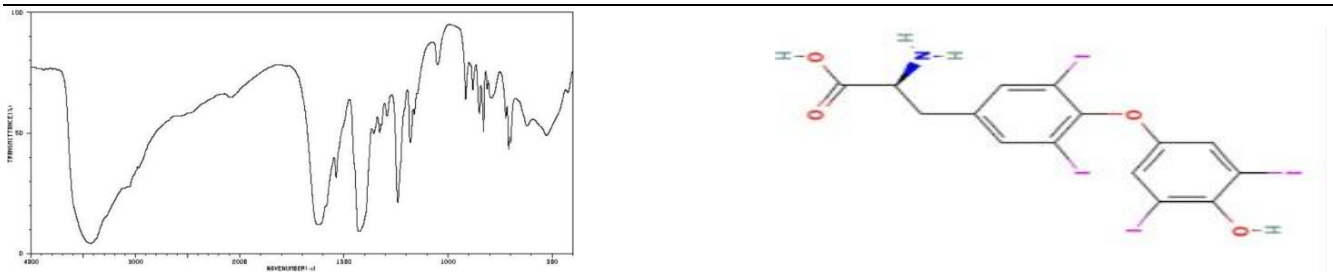

Figure 1 IR and Structure interpretation of drug Levothyroxine

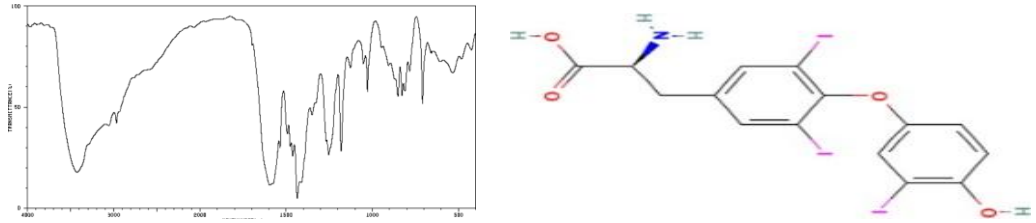

Figure 2 IR and Structure interpretation of drug Liothyronine

3.2 Solubility in different solvent:

Table 1 Solubility in Different Solvent

Solvent	Levothyroxine	Liothyronine
Water	Slightly soluble	Partially soluble
Methanol/ Ethanol	Freely soluble	Freely soluble
Acetonitrile	Freely soluble	Freely soluble
Ether	Slightly soluble	Very slightly soluble
0.1 N HCl	Freely soluble	Practically
0.1 N NaOH	Practically insoluble	soluble

3.3 Identification of drugs by Melting point determination:

Table 2 Melting Point of Drugs

Drug name	Melting point	Standard value
Levothyroxine	198–200 ˚C	199–201 ˚C
Liothyronine	167–169 ˚C	171–177 ˚C

3.4 Estimation of levothyroxine and liothyronine from their Combination Drug Product by Spectrophotometric Methods

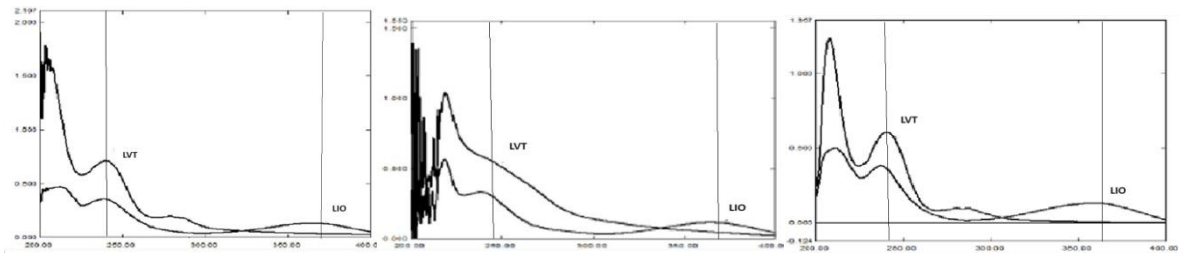

Figure 3 Overlain UV spectra of LVT and LIO in 0.1 N HCl, 0.1 N NaOH and Methanol

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3.5 Estimation of levothyroxine and liothyronine from their combination drug product by spectrophotometric methods

3.5.1 Absorption Correction method

Absorption correction method was successfully developed for simultaneous determination of LVT and LIO in combined dosage form. The results obtained are discussed below.

3.5.1.1 Wavelength Selection for LVT and LIO or Absorption Correction Method

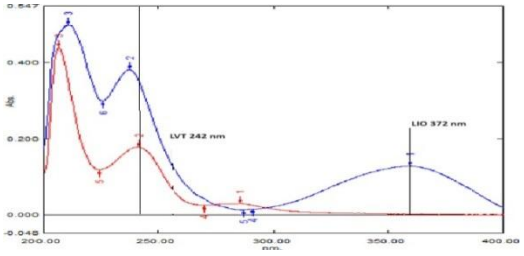

Figure 4 Overlain UV spectra of LVT and (2) LIO for Absorption Correction Method

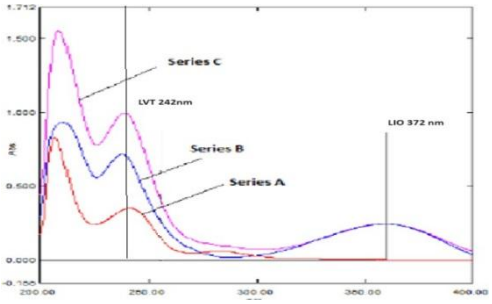

Figure 5 Overlain UV spectra of SERIES A, B and C for Absorption Correction Method

3.5.2 Calibration Curves

3 series of calibration curve were prepared **SERIES A:** Varying concentration of LVT alone **SERIES B:** Varying concentration of LIO alone **SERIES C:** Varying concentration of LVT and varying concentration of LIO

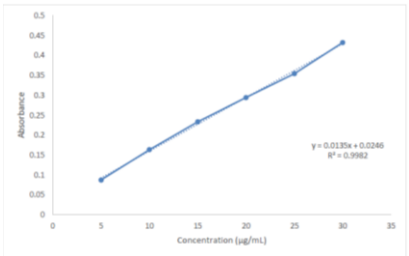

Figure 6 Calibration curve of standard LVT at 242 nm

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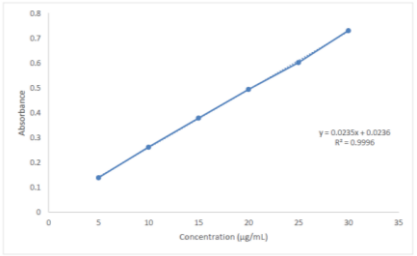

Figure 7 Calibration curve of standard LIO at 360 nm

3.6 Validation Parameters

3.6.1 Linearity and Range

Correlation coefficients for both the drugs were near to one, indicate linearity of method within the given range.

3.6.2 Precision

Table 3 Repeatability Data for LVT and LIO by Absorption Correction Method

Concentration (µg/mL)		Absorbance	
LVT	**LIO**	**LVT**	**LIO**
5	20	0.143	0.242
5	20	0.142	0.243
5	20	0.141	0.241
5	20	0.142	0.243
5	20	0.14	0.24
5	20	0.142	0.242
Average		0.14167	0.24183
SD		0.00103	0.00117
% RSD		0.729	0.4834

Table 4 Intraday Precision Data LVT and LIO by Absorption Correction Method

Concentration (µg/mL)		% RSD	
LVT	**LIO**	**LVT**	**LIO**
5	20	0.411	0.425
10	30	0.471	0.715
15	50	0.41	0.685

Table 5 Interday Precision data LVT and LIO by Absorption Correction Method

Concentration (µg/mL)		% RSD	
LVT	**LIO**	**LVT**	**LIO**
5	20	0.708	0.492
10	30	0.718	0.714
15	50	0.541	0.749

3.6.3 Accuracy

Table 6 Accuracy Data for LVT by Absorption Correction Method

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% Spiking	Total amount of LVT (µg/mL)	Amount of LVT found (µg/mL) Mean ± S.D (n = 3)	%Recovery	Mean % Recovery
80	10.8	10.75688± 0.065	99.6007	100.091
100	12	12.12498± 0.025	101.042	
120	13.2	13.15106± 0.065	99.6293	

Table 7 Accuracy Data for LIO by Absorption Correction Method

% Spiking	Total amount of LIO (µg/mL)	Amount of LIO found (µg/mL) Mean ± S.D (n = 3)	%Recovery	Mean % Recovery
80	36	36.0732 ± 0.098	100.203	99.787
100	40	39.2935 ± 0.098	98.2338	
120	44	44.4065 ± 0.129	100.924	

3.6.4 Robustness/ Ruggedness

Table 8 Robustness Data LVT and LIO by Absorption Correction Method

Concentration (µg/mL)		% RSD	
LVT	LIO	LVT	LIO
5	20	0.408	0.425
10	30	0.542	0.568
15	50	0.408	0.535

Table 9 Ruggedness Data LVT and LIO by Absorption Correction Method

Concentration (µg/mL)		% RSD	
LVT	LIO	LVT	LIO
5	20	0.703	0.423
10	30	0.717	0.712
15	50	0.541	0.639

3.6.5 LOD and LOQ

Table 10 LOD and LOQ Data for LVT and LIO by Absorption Correction Method

Parameter	LVT (µg/mL)	LIO (µg/mL)
SD	0.00097	0.0017
Mean slope	0.02339	0.0118
LOD (µg/mL)	0.136	0.475
LOQ (µg/mL)	0.413	1.44

3.7 Simultaneous Estimation of LVT and LIO in Pharmaceutical Dosage Form

Applicability of the proposed method was analyzed in available tablet formulation.

Table 11 Assay of Combined Marketed Dosage of LVT and LIO by Absorption Correction Method

Tablet	Label Claim (mcg)	Concentration prepared (µg/mL)		Concentration Found (µg/mL)		% Mean Assay (n = 6)	
	LVT:LIO	LVT	LIO	LVT	LIO	LVT	LIO
Shytomel		5	20	4.91	18.53	99.32	98.02

3.7.1 Chromatograms of mobile phase trial

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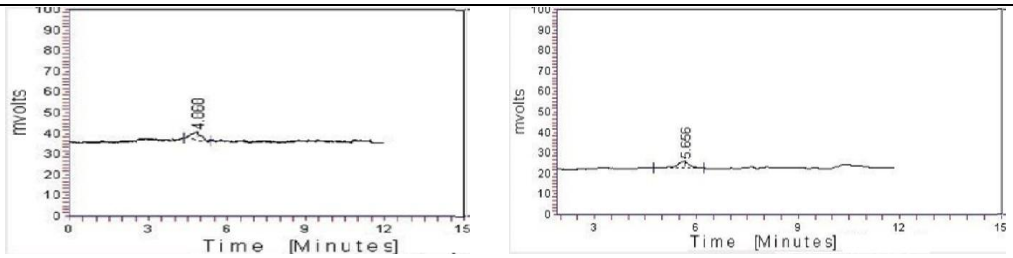


Figure 8 Trail graph of Water: Methanol (50: 50 v/v) & Water: Acetonitrile (50: 50 v/v)

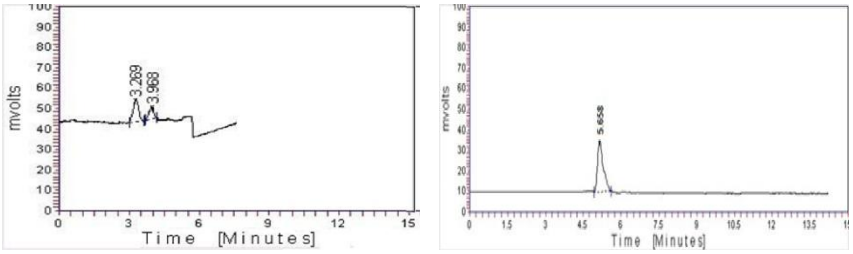


Figure 9 Trail graph of ACN: Methanol (50: 50 v/v) & Suitable graph of Enoxaparin sodium in 10mM KH2PO4: Methanol (20:80 v/v)

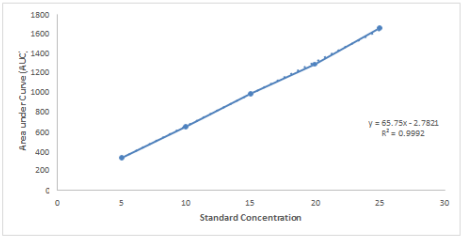
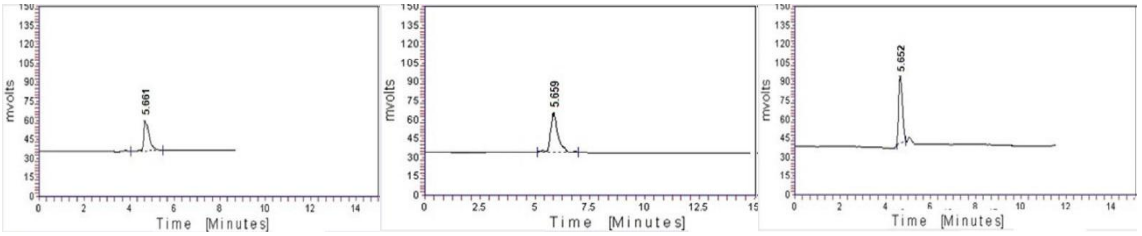


Figure 10 Calibration Curve of levothyroxine



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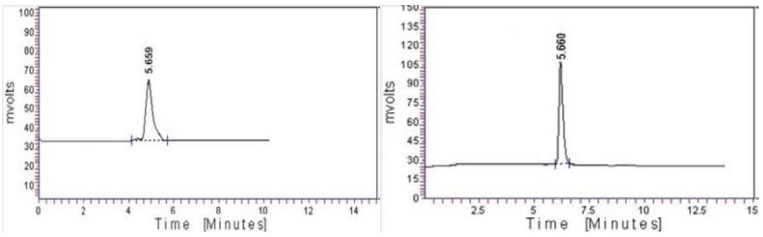

Figure 11 Chromatogram of levothyroxine 5 ppm, 10 ppm, 15 ppm, 20 ppm & 25ppm 3.8 Validation of developed Method

3.8.1 Linearity

The linearity of the analytical method is its ability to achieve a result (within a specified range) that is directly proportional to the analyte region in the sample. After analysis of five different (from 5 to 25 µg/ ml) concentrations, the calibration plot was contracted and areas were reported three times for each concentration, and the mean area was determined. The equation of regression equation and the correlation coefficient are given.

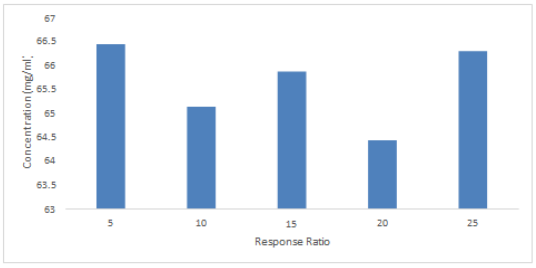

Figure 12 Response Ratio Curve of levothyroxine Specificity

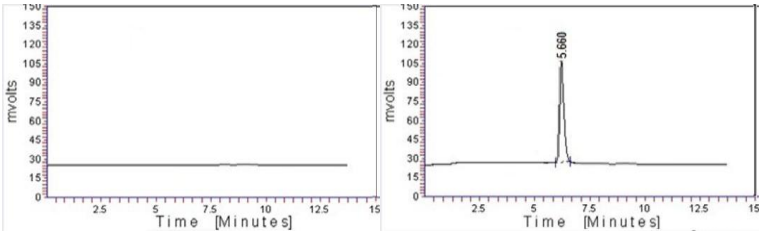

Figure 13 Chromatogram of the blank & standard drug

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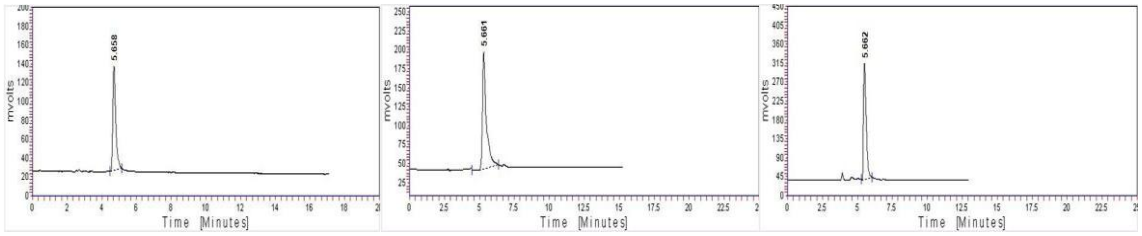

Figure 14 Chromatogram of Recovery Study of levothyroxine (80% Level), (100% Level) & (120% Level)

3.8.2 Repeatability

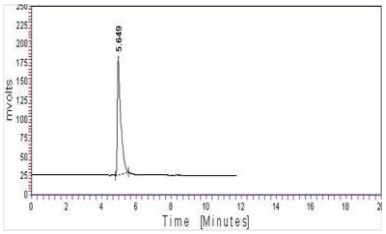

Figure 15 Chromatogram of Repeatability of levothyroxine

3.8.3 Intermediate Precision

A. Day To Day Precision

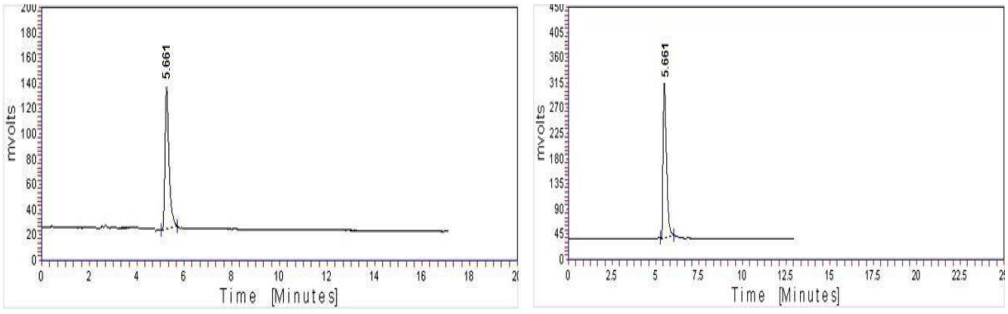

Figure 16 Chromatogram of Day-to-day variation of levothyroxine & Analyst to analyst of levothyroxine

3.8.4 Robustness

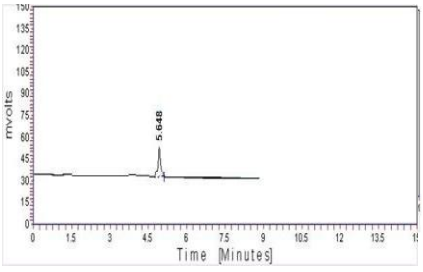

Figure 17 Chromatogram of Robustness of levothyroxine

Supriya Sethi[1]*, Raju Choksey[2], Dr. Neha Jain[3]

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3.9 Detection Limit and Quantitation Limit

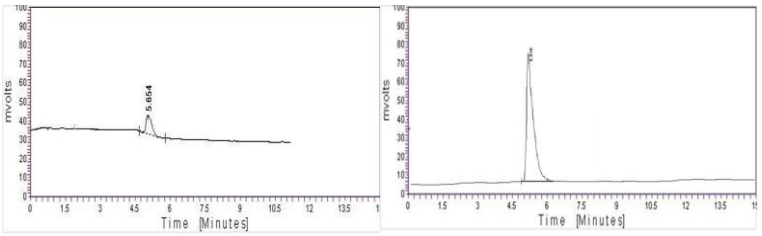

Figure 18 Chromatogram of Limit of detection of levothyroxine & Limit of quantification of levothyroxine

3.10 Results of Forced Degradation studies

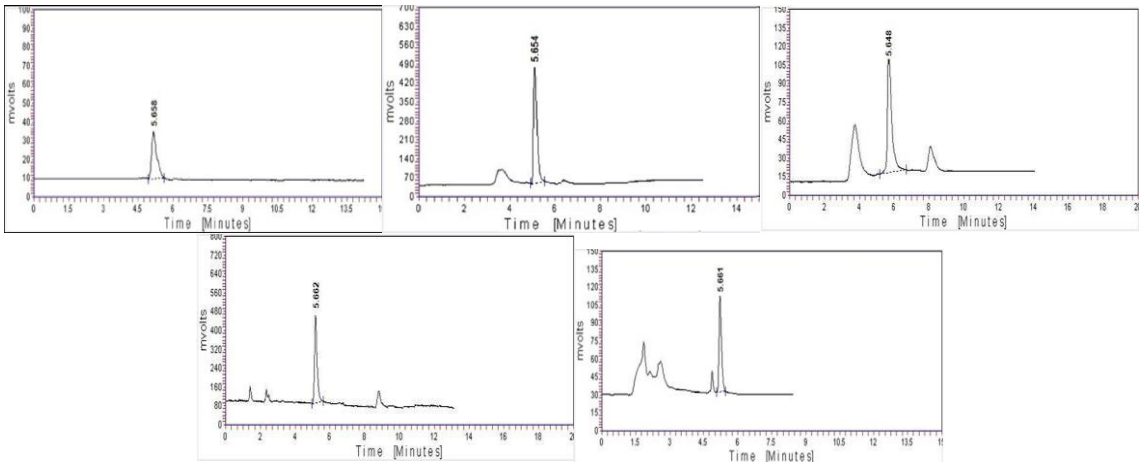

Figure 19 Graph of Chromatogram of standard Drugs, Acidic hydrolysis, Alkaline hydrolysis, Oxidative degradation & Photolytic degradation

3.11 Spectroscopic Method Development for The Estimation of Liothyronine

3.11.1 Selection of wavelength for liothyronine

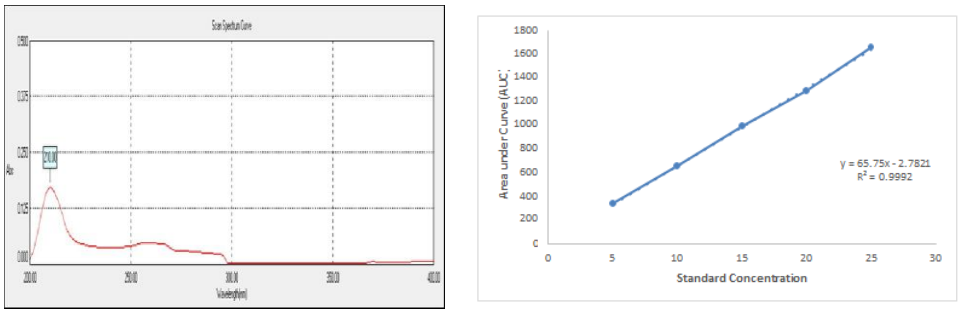

Figure 20 Determination of λmax of liothyronine & Calibration Curve of liothyronine

3.12 Validation of developed method

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3.12.1 Linearity

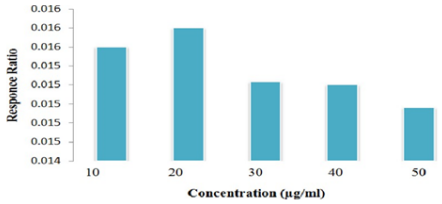

Figure 21 Response Ratio graph of liothyronine

3.12.2 Accuracy:

Table 12 Recovery study

Parameters	FNDP (80% level)	Liothyronine (100% level)	liothyronine (120% level)
MEAN SD	99.168	99.45	99.63
SD*	0.354	0.108	0.371
% RSD	0.357	0.108	0.372

* Mean of 3 replicate and 5 concentrations

3.12.3 Precision

Table 13 Precision studies

Parameters	Repeatability	Intermediate Precision	Analyst-to-Analyst Variation	Reproducibility
% Mean	99.196	99.332	99.257	98.979
S.D.	0.189	0.153	0.158	0.182
% R.S.D.	0.191	0.155	0.159	0.184

4. DISCUSSION

An RP-HPLC method for simultaneous estimation of Levothyroxine and Liothyronine were developed and validated. The amounts attained by the proposed method are between 99.13% and 101.07%, within the acceptance level of 90% to 110%. The results obtained specify that the proposed method is rapid, accurate, selective, and reproducible. Linearity was observed over a concentration range of 10 to 100 µg.mL-1 for all both drugs. It can be used for the routine analysis of formulations covering any one of the above drugs or their groupings without any alteration in the assay. The main advantage of the method is the common chromatographic conditions adopted for all formulations. Therefore, the proposed method decreases the time mandatory for switch over of chromatographic conditions, equilibration of column and post column blushing that are typically related when different formulations are analyzed.

A new, simple, rapid, selective, precise and accurate isocratic reverse phase high performance liquid chromatography assay has been developed for simultaneous estimation of various tablet formulations. The separation was achieved by using C-18 column (Phenomenax, 250 x 4.6mm i.d.) coupled with a guard column of same material, in mobile phase Acetonitrile: Water (25:75). The pH of mobile phase was adjusted to 6.0 ± 0.1 with 50% ortho phosphoric acid. The flow rate was 1.0 mL.min-1 and the separated drugs were detected using UV detector at the wavelength of 300 nm. The retention time

of various formulations was noted to be 2.08 and 5.05 min, respectively for Levothyroxine and Liothyronine, indicative of rather shorter analysis time. The method was validated as per ICH guidelines. The proposed method was found to be accurate, reproducible, and consistent. It was successfully applied for the analysis of these drugs in marketed formulations and could be effectively used for the routine analysis of formulations containing any one of the above drugs, or a combination, without any alteration in the chromatographic conditions.

A stability‑indicating reverse phase high performance liquid chromatography method was developed and validated for Levothyroxine and Liothyronine. The wavelength selected for quantitation was 285 nm. The method has been validated for linearity, accuracy, precision, robustness, limit of detection and limit of quantitation. Linearity was observed in the concentration range of 10-100 µg/ml for both drugs. For RP‑HPLC, the separation was achieved by C-18 column (Phenomenax, 250 x 4.6 mm i.d.) coupled using in mobile phase Acetonitrile: Water (25:75) as mobile phase with flow rate 1 ml/min. The

retention time of Levothyroxine and Liothyronine were found to be 2.08 min and 5.02 min, respectively. During force degradation, drug product was exposed to hydrolysis (acid and base hydrolysis), H_2O_2, thermal degradation and photo degradation. The percent degradation was found to be 10 to 20% for both Levothyroxine and Liothyronine in the given condition. The method specifically estimates both the drugs in presence of all the degradants generated during forced degradation study. The developed methods were simple, specific and economic, which can be used for simultaneous estimation of both drugs in any combination of dosage forms.

The mobile phase consisting of Acetonitrile: Water (25:75) as mobile phase, at 1 ml/min flow rate was optimized which gave two sharp, well‑resolved peaks with minimum tailing factor for Levothyroxine and Liothyronine. The retention times for Levothyroxine and Liothyronine were 2.08 min and 5.02 min, respectively. UV overlain spectra of both drugs showed that both drugs absorbed appreciably at 285 nm, so this wavelength was selected as the detection wavelength. The calibration curve for 2.08 min and 5.02 min, respectively was found to be linear over the range of 10-100 µg/ml respectively. The data of regression analysis of the calibration curves. The proposed method was successfully applied to the determination of both drugs in their combined dosage form. The degradation study indicated that the drug degrades as shown by the decreased areas in the peaks when compared with peak areas of the same concentration of the non-degraded drug, without giving any additional degradation peaks. Percent degradation was calculated by comparing the areas of the degraded peaks in each degradation condition with the corresponding areas of the peaks of both the drugs under non degradation condition.

For forced degradation with 0.1 N HCl at 2 h, 0.1 NaOH at 1 h, 3% v/v H2O2, 75° at 1 h and

photo degradation at 1h were done. The % degradation was found to be 10 to 20% for both drugs in their tablet dosage form in the given condition using developed HPLC method. In the proposed study, a stability‑indicating HPLC method was developed for the simultaneous estimation of both drugs and validated as per ICH guidelines. Statistical analysis proved that method was accurate, precise, and repeatable. The developed method was found to be simple, sensitive and selective for analysis of both drugs in combination without any interference from the excipients. The method specifically estimates both the drugs in presence of all the degradants generated during forced degradation study. Assay results for combined dosage form using proposed method showed 99.02±0.14% of Levothyroxine and 97.33±0.257% of Liothyronine. The results indicated

the suitability of the method to study stability of Levothyroxine and Liothyronine under various forced degradation conditions acid, base, dry heat, oxidation and photolytic degradation. It can be concluded that the method separates the drugs from their degradation products; it may be employed for analysis of stability for their tablet dosage form.

CONCLUSION

In conclusion, this study successfully developed and validated a robust, accurate, and stability-indicating RP-HPLC method for the simultaneous estimation of levothyroxine and liothyronine in pharmaceutical formulations. The method demonstrated excellent linearity, precision, specificity, and sensitivity, meeting ICH guidelines. It proved effective in distinguishing both drugs from their degradation products under various stress conditions, making it highly suitable for routine quality control and stability assessment. The simplicity, reproducibility, and reliability of the method ensure its practical applicability in pharmaceutical analysis and regulatory compliance for thyroid hormone therapies.

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