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QUALITY BY DESIGN DRIVEN RP-HPLC METHOD OPTIMIZATION FOR ANALYSIS OF LEVOTHYROXINE AND LIOTHYRONINE IN BULK AND TABLET DOSAGE FORM

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ABSTRACT
Background: Levothyroxine and Liothyronine are widely used in thyroid hormone replacement therapies. Simultaneously quantifying Levothyroxine and Liothyronine is important for managing thyroid hormone deficiency. **Aim:** This study aims to develop and validate an accurate and robust RP-HPLC method for simultaneously quantifying Levothyroxine and Liothyronine by utilizing Quality by Design (QbD). **Methodology:** Reversed phase chromatography was performed using a High Performance Liquid Chromatographic System (Agilent Technologies Ltd, 1100 series) equipped with a UV detector. The column used was Agilent C 18 (100 mm x 4.6 mm; 5µm) HPLC Column. The chromatographic separation was carried out using a mobile phase composed of Methanol and Formic acid (0.1%) (50:50 %v/v) with a flow rate of 1.2 ml/min, and a UV detector recorded the response at 254 nm. Design expert was used as software to evaluate experimental design studies (Stat-Ease Inc., Minneapolis, USA, Version 13.0). **Result and Discussion:** The RP-HPLC method was established to quantify Levothyroxine and Liothyronine simultaneously. The established method was linear, and correlation coefficients (R^2) were 0.9993 and 0.9994 for Levothyroxine and Liothyronine, respectively. Retention times of Levothyroxine and Liothyronine were 2.587 minutes and 3.035 minutes. Results of accuracy, precision studies, LOD, and LOQ were found within acceptable limits. **Conclusion:** A robust RP-HPLC method was developed for the simultaneous quantification of levothyroxine and liothyronine by utilizing a QbD. The QbD technique provided a systematic methodology for identifying and optimizing the critical parameters influencing the method's performance.

INTRODUCTION

Levothyroxine sodium (LT4) is O^4-(4-hydroxy-3,5-di-iodophenyl)-3,5-diiodo-L-tyrosinate, monosodium salt (Figure 1a), which is a slightly brownish yellow powder or white crystalline powder. Liothyronine sodium (LT3) is Monosodium L-3- [4- (4-hydroxy-3-iodophenoxy)-3, 5- di-iodophenyl] alanine sodium salt (Figure 1b), which is a white to light tan, odourless, crystalline powder [1-2].

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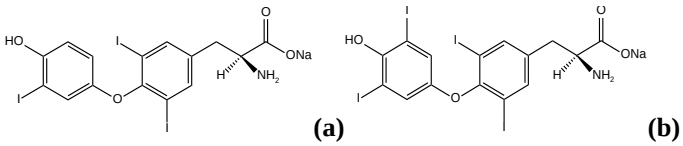

(a) **(b)**

Figure 1: Structure of (a) Levothyroxine sodium; (b) Liothyronine sodium

Thyroid disorders encompass a range of conditions affecting the thyroid gland, a butterfly-shaped gland in the neck region responsible for releasing hormones that regulate metabolism, growth, and development [3]. Graves' disease, another autoimmune disorder, is the primary cause of hyperthyroidism, where antibodies stimulate the thyroid to produce too much hormone [4]. According to the World Health Organization (WHO), it is estimated that approximately 750 million people globally suffer from thyroid-related conditions [5]. Synthetic thyroid hormones such as Levothyroxine and Liothyronine in treatment regimens underscore the importance of reliable analytical methods to ensure proper dosing and therapeutic efficacy [6]. Thyroid hormones are critical for cardiovascular, reproductive, and mental health, and crucial for normal growth and development of children [7,8]. Levothyroxine, a synthetic form of thyroxine (T4), is the primary treatment, as it normalizes hormone levels by being converted into the active hormone triiodothyronine (T3) in the body [9]. Accurate estimating these hormones in pharmaceutical formulations is critical to ensure therapeutic efficacy and patient safety. Though helpful, traditional analytical techniques, such as spectrophotometry and immunoassays, often lack the sensitivity and specificity required to quantify both Levothyroxine and Liothyronine simultaneously. HPLC has become a preferred analytical method due to its superior resolution, sensitivity, and reproducibility. Specifically, RP-HPLC is highly effective for separating small molecules, making it suitable for the simultaneous analysis of T4 and T3. Traditional HPLC method development utilizes a trial-and-error approach, lacks optimization, results in the wastage of organic solvents, and is time-consuming. Meanwhile, analytical QbD includes all important variables in method development and builds robustness into the method during development instead of in the final stage. Applying QbD technique saves time, reduces the use of valuable organic solvents, and enables the identification of critical factors for defining robustness [10]. According to a literature review, few RP-HPLC methods were reported to estimate either Levothyroxine or Liothyronine as a single drug. Still, no method was reported for the quantification of Levothyroxine and Liothyronine in combination by using HPLC, nor was there any QbD-based HPLC method reported in the literature [11-12]. Hence, developing an HPLC method to analyze levothyroxine or lithium is worthwhile by employing the quality by design (QbD) technique. This research focuses on developing and optimizing an HPLC method by employing Box Behnken Design to analyze Levothyroxine and Liothyronine.

MATERIALS AND METHODS

Chemicals and Materials

Merck Life Sciences Private Limited, Mumbai, provided methanol, water, and formic acid. A nylon membrane filter (0.45 µm size) was procured from Merck Life Sciences Private Limited, Bangalore. Levothyroxine and Liothyronine API were purchased from Swapnroop Drugs and Pharmaceuticals, Aurangabad. Chemicals required for research work were analytical grade, and HPLC-grade solvents were utilized.

Instruments and software

Chromatographic analysis was performed by utilizing HPLC (Agilent Technologies,1100 series) with Chemstation software equipped with a UV detector and Agilent C 18 (100 mm x 4.6 mm; 5 µm) column. Additional instruments include an Ultra-Violet visible Spectropotometer (Analytical Technologies Ltd. 2012), a sonicator (Labman Scientific Instruments LMUC Series), an analytical weighing balance (Wensar, DAB220), and a pH meter (Hanna Instruments, HI2211).

Chromatographic Conditions

Initially, the mobile phase comprised methanol and water in different ratios to estimate both drugs. During optimization of the method, Agilent C18 (ID: 250mm x 4.6mm, Particle size: 5 µm), Sapphirus C18 HP Classic (ID: 250mm x 4.6mm, Particle size: 5 µm) and Agilent C18 (ID: 100mm x 4.6mm, Particle size: 5 µm) column were used. Subsequently, several mobile phase (eluent) compositions were tried by using methanol and formic acid (0.1%) at varying pH levels (2.6, 2.8, 3.0) with different columns and flow rates to optimize the resolution and peak shape of Levothyroxine and liothyronine. After multiple experimental trials, eluent consisting of methanol and formic acid (0.1%) (38:62 %v/v) with pH 2.8, flow rate: 1 ml/min gave better peak shapes with satisfactory resolution quickly. An Agilent C18 (100 mm x 4.6 mm; 5 µm) column was employed for the chromatographic analysis, and the detection wavelength was 254 nm. The injection volume used for the trial was 20 µl, with

concentrations of 38 µg/ml and 9 µg/ml of Levothyroxine and Liothyronine, respectively.

Quality by Design Approach for HPLC Method Development

Selection of Analytical Target Profile (ATP)

An ATP consists of a description of the intended purpose, appropriate details on the product attributes to be measured, and relevant performance characteristics with associated performance criteria. The ATP includes the performance requirement for a single attribute or a set of quality attributes. The retention time, theoretical plates, and peak asymmetry were recognized as ATP for the proposed method [13].

Determine critical quality attributes (CQA)

The CQAs are the method parameters that directly affect ATP. The mobile composition, flow rate, and wavelength were identified as CQAs, which must be controlled to achieve the desired response range of ATP.

Selection of Design (DoE)

Box-Behnken Design (BBD) requires fewer experimental runs than Central Composite Designs (CCD) for the same number of factors, especially when studying quadratic relationships. BBD designs are less expensive. Hence, Box Behnken Design (BBD) was employed to optimize the HPLC method. Five center points were considered; 13 runs were generated by the design expert. The various interactions and quadratic effects of the mobile composition, flow rate, and wavelength on parameters such as retention time, theoretical plates, peak asymmetry, peak area, and resolution were evaluated using the ANOVA statistical tool. The ratio of solvents in the mobile phase affects the retention and separation of analytes. Optimizing this ratio is crucial for achieving desired retention times and resolution. The flow rate of the mobile phase influences the speed of analysis and peak shape. Adjusting the flow rate can optimize the analysis time and peak resolution. The wavelength used for detection is crucial for the sensitivity and specificity of the method. Optimizing the wavelength ensures that the analyte is detected accurately and effectively. Therefore, mobile phase ratio, flow rate, and wavelength were selected as independent variables. These were tested at three levels; the design was created with Design Expert® (Version 13.0, Stat-Ease Inc., USA) software. The dependent variables were the proposed independent variables' retention time, theoretical plates, peak asymmetry, peak area, and resolution.

Risk assessment

ICH Q8 and ICH Q9 guidelines were adopted for evaluating the method to study its robustness. Risk assessment was carried out based on prior knowledge and experience. The method parameters or its performance under various conditions, such as changes in mobile phase composition, flow rate, wavelength, etc., were evaluated [14,15].

Selection of Solvent (Diluent)

The mobile phase mixture, comprising Methanol and Formic acid (0.1%) in the ratio 38:62% v/v, was prepared and mixed well. Based on the solubility study, this mixture was selected as a solvent (diluent).

Selection of Detection wavelength

The separately prepared standard solutions of Levothyroxine and Liothyronine were scanned in the UV region using diluent as a blank. The recorded spectra revealed that the maximum absorbance (λmax) for levothyroxine and liothyronine occurred at 226 and 294 nm, respectively. The isobestic point was observed at 254 nm, so wavelength 254 nm was used for the quantification of Levothyroxine and Liothyronine.

Preparation of standard stock solution

Levothyroxine standard stock solution

Accurately weighed 19 mg of Levothyroxine and dissolved it in methanol in a 10 ml volumetric flask, then the volume was made up to the mark (10 ml) to achieve a concentration of 1900 µg/ml.

Liothyronine standard stock solution

Accurately weighed 4.5 mg of Liothyronine and dissolved it in methanol in a 10 ml volumetric flask, then the final volume was made up to the mark (10 ml) to obtain a concentration of 450 µg/ml.

Preparation of sample stock solution

To determine the content of levothyroxine and liothyronine in marketed tablets (the label claims 38 mcg of levothyroxine and 9 mcg of liothyronine), 20 tablets of powder were weighed 1.50 g, and the average powder weight was calculated as 0.075 g/tab. Tablets were triturated, and a powder equivalent to 75 mg of the drug was extracted and sonicated for 30 min. 0.2 ml of

supernatant was then diluted up to 10 ml with mobile phase, with 10 ml of Methanol. The resulting solution was injected into the HPLC, and the drug peak area was noted.

Method Validation

The optimized RP HPLC method was validated by ICH guidelines for various parameters including linearity, precision, accuracy, robustness, limit of detection and limit of quantification [16].

RESULTS

The chromatographic analysis revealed retention times of 2.587 min and 3.035 min for the two peaks of Levothyroxine and Liothyronine. The corresponding peak areas were 3582 and 336. The resolution between the peaks was determined to be 3.10, indicating good separation. Additionally, the theoretical plates were calculated to be 5795 and 6367, suggesting efficient column performance for the given conditions (Figure 2).

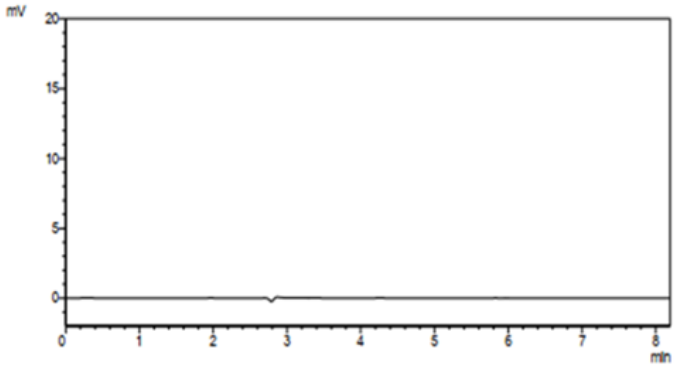

Figure 2: Chromatogram of blank

Experiments were carried out using HPLC, and the resolution of the peaks was assessed. All the three variables selected found critical for peak separation. The proposed model was evaluated by ANOVA. The analysis provides 2D (contour plot), 3 dimensional representations (3D response surface) by plotting the response against other two factors, and the third one kept constant at a desired level, and the 3D representation of as a response as shown in Figure 4–7, the probability values and F-values were noted for each factor, demonstrating robustness of developed method conditions at extended variations. The Design Expert software was utilized to evaluate the experimental design study. (Stat-Ease Inc. Minneapolis, USA, Version 13.0). Due to high competence with a limited number of runs, Box Behnken Design (BBD) and response surface methodology a model used for study. Three factors, three levels and five center points are selected for BBD, leads to 13 experimental runs, which were carried out. Standard and sample prepared and injected into chromatographic system. Retention time, theoretical plates, and peak asymmetry, peak area, resolution were measured as responses. For coefficients and nature of the robustness was evaluated by ANOVA with a linear approach. The significance and contribution of the factors were estimated by statistical ANOVA. The significance of the model was evaluated by the P values and F-value.

Optimized Chromatographic Conditions: Methanol: 0.1 % Formic acid, (50:50%v/v) was utilized as mobile phase. Flow rate was kept as 1.2 ml/min**,** with ambient column temperature and detection wavelength at 254 nm**.**

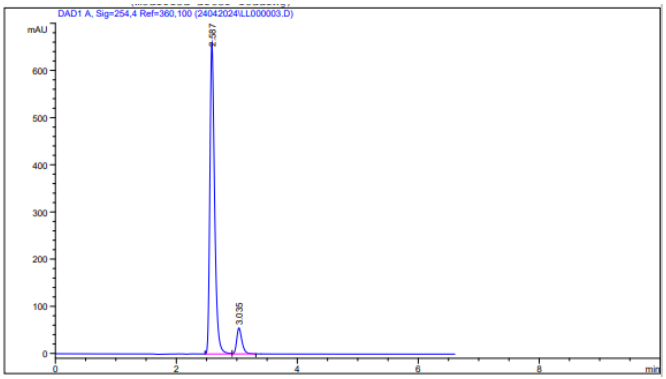

Figure 3: Optimized chromatogram for LT4 and LT3 by BBD

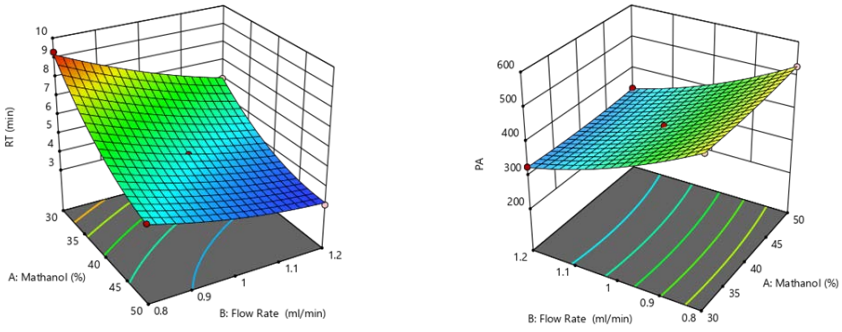

Figure 4: 3-D response surface plots depicting the effect of CQA specifically mobile phase composition, flow rate and wavelength on 1. Retention Time and 2. Peak Area of LT4

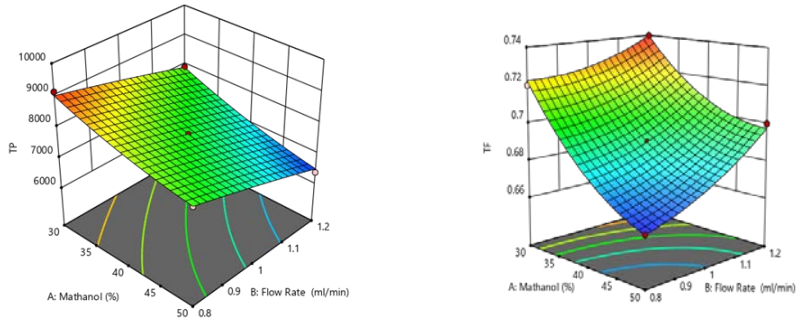

Figure 5: 3-D response surface plots depicting the effect of CQA namely mobile phase composition, flow rate and wavelength on 1. Theoretical Plate and 2. Asymmetric Factor of LT4.

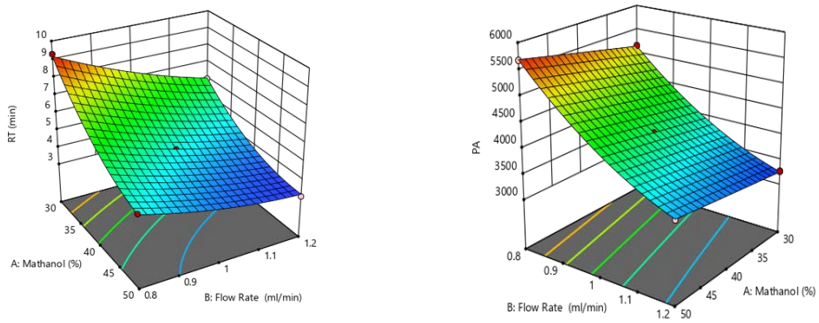

Figure 6: 3-D response surface plots depicting the effect of CQA namely mobile phase composition, flow rate and wavelength on 1. RT of Liothyronine and 2. Peak area of LT3.

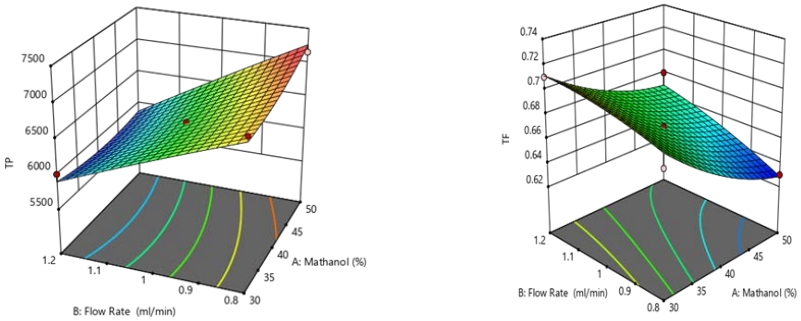

Figure 7: 3-D response surface plots depicting the influence of CQA namely mobile phase composition, flow rate and wavelength on 1. Theoretical Plate and 2. Tailing Factor of LT3.

Table 1: Optimisation of parameters by Box-Behnken Design for Levothyroxine.

	Factor A	Factor B	Factor C	Response 1	Response 2	Response 3	Response 4
Run	Methanol (%)	Flow rate (ml/min)	Wavelength (nm)	Retention Time (min)	Peak Area	Theoretical Plate	Tailing Factor
1	50	1.2	254	2.587	3582	5795	0.68
2	40	1.2	256	2.968	3143	5768	0.68
3	30	1.2	254	3.678	3263	6010	0.71
4	40	1	254	3.631	4177	6421	0.67
5	40	1.2	252	2.968	3632	5809	0.68
6	30	0.8	254	5.760	5155	6984	0.68
7	30	1	252	4.487	4282	6275	0.70
8	40	0.8	252	4.659	5763	6937	0.66
9	40	0.8	256	4.654	4997	7101	0.66
10	30	1	256	4.471	3546	6388	0.73
11	50	1	256	3.125	4081	6391	0.65
12	50	1	252	3.164	4678	6951	0.65
13	50	0.8	254	4.074	5674	7158	0.63

Table 2: Optimisation by Box-Behnken Design for Liothyronine

	Factor A	Factor B	Factor C	Response 1	Response 2	Response 3	Response 4
Run	Methanol (%)	Flow rate (ml/ min)	Wavelength (nm)	Retention Time (min)	Peak Area	Theoretical Plate	Tailing Factor
1	50	1.2	254	3.035	336	6367	0.70
2	40	1.2	256	3.925	376	6969	0.71
3	30	1.2	254	5.987	327	7920	0.74
4	40	1	254	4.792	399	7726	0.69
5	40	1.2	252	3.922	283	7128	0.70
6	30	0.8	254	9.295	517	9135	0.72
7	30	1	252	7.269	349	8402	0.73
8	40	0.8	252	6.123	449	8113	0.68
9	40	0.8	256	6.115	598	8263	0.68
10	30	1	256	7.221	466	8290	0.72
11	50	1	256	3.668	474	6981	0.67
12	50	1	252	3.707	359	7446	0.67
13	50	0.8	254	4.773	528	7868	0.66

Table 3: ANOVA responses of BBD Design for Levothyroxine and Liothyronine

ANOVA for quadratic model				
Variable	For Levothyroxine		For Liothyronine	
	P- value	F- value	P- value	F- value
Retention Time	0.0006	190	0.0001	522
Peak Area	0.012	4.39	0.0001	16.51
Theoretical Plate	0.0094	28.46	0.0437	9.71
Asymmetric factor	0.0014	101	0.0014	101

Method Validation

System suitability

The method was found to be suitable, meeting all the system suitability criteria. The system suitability results are summarized in Table 4.

Table 4: System suitability results

Parameters	Results		Acceptance criteria as per IP
	LT4	LT3	
Retention time (min)	4.07	4.77	NA
Tailing factor	0.63	0.66	NMT 2
Theoretical plate counts	7178	7868	NLT 2000
Resolution between RMO and VLD peak	–	3.43	NLT 2
% RSD for peak area of five replicate injections	0.87	0.39	NMT 2%

Linearity

The method demonstrated linearity in the concentration range of 19-95 µg/ml for Levothyroxine with an R^2 value of 0.9993 and 4.5-22.5 µg/ml for Liothyronine with an R^2 value of 0.9994. Figure 8 illustrates the method's suitability for analysis within the studied concentration range. Linearity results for Levothyroxine and Liothyronine are shown in Table 5.

Accuracy

Recovery studies were conducted to assess the accuracy of the developed method. Recovery pre-analyzed tablet solution was spiked with specific concentration of standard drug (80%, 100%, and 120%) and the recovery was subsequently analyzed. The statistical validation of recovery studies is provided in Table 6 and Table 7.

Table 5: Linearity results

Linearity level	Concentration (µg/ml)		Peak area response	
	LT4	LT3	LT4	LT3
1	19	4.5	1276	100
2	38	9	2449	206
3	57	13.5	3675	316
4	76	18	4963	424
5	95	22.5	6067	522

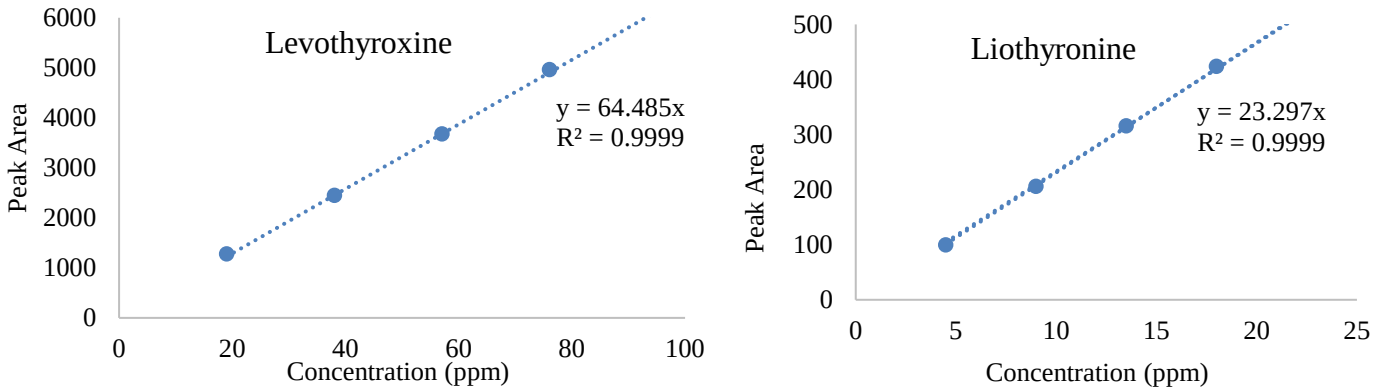

Figure 8: Calibration curve for the Linearity of LT4 and LT3

Precision

The method was established by analyzing various replicate standards of Levothyroxine and Liothyronine. All the solutions were analyzed five times to assess any intra-day and inter-day variation in the results. The findings for intra-day and inter-day variations are given in Tables 8 and 9.

Robustness

Small but deliberate changes in mobile phase composition, flow rate, and wavelength were made, and their effect on ATP was examined. Robustness parameters were found satisfactory.

Limit of detection (LOD) and Limit of Quantitation (LOQ)

The DL was 0.1149 µg/ml for Levothyroxine and 1.6252 µg/ml for Liothyronine, respectively, indicating that even small quantities of Levothyroxine and Liothyronine can be detected. The QL was 0.3483 µg/ml for Levothyroxine and 4.9249 µg/ml for Liothyronine, respectively, indicating that even small quantities of Levothyroxine and Liothyronine can be quantified.

Assay of Formulation

The percentage assay of the tablet was 100.16% for Levothyroxine and 99.61 % for Liothyronine.

Greenness of method

The greenness score of the developed analytical method was assessed and found to be 0.62 (Figure 9). The analytical greenness score was determined using the software tool AGREE: Analytical Greenness Calculator [17].

Figure 9: Result of the greenness of the method

Table 6: Results of Recovery Studies of Levothyroxine

Sr. No.	Conc. µg/ml	Amt. Added	Area	Amt. Found	Amt. Received	% Recovery
80 % Accuracy						
1	19	15.2	2239	34.24	15.24	100.28
2	19	15.2	2237	34.21	15.21	100.06
3	19	15.2	2237	34.20	34.20	100.05
Mean				34.21	15.23	100.17
SD				0.02	0.024	0.16
% RSD				0.06	0.155	0.16
100 % Accuracy						
1	19	19	2480	38.05	19.059	100.31
2	19	19	2475	38.03	19.038	100.22
3	19	19	2477	38.00	19.008	100.05
Mean				38.03	19.03	100.18
SD				0.02	0.036	0.19

% RSD				0.06	0.187	0.19
120 % Accuracy						
1	19	22.8	2711	41.71	22.714	99.63
2	19	22.8	2721	41.87	22.875	100.33
3	19	22.8	2715	41.65	22.596	99.74
Mean				41.74	22.800	99.98
SD				0.11	0.114	0.5
% RSD				0.27	0.5	0.5

Table 7: Result of Recovery Studies of Liothyronine

Sr. No.	Conc. µg/ml	Amt. Added	Area	Amt. Found	Amt. Received	% Recovery
80 % Accuracy						
1	4.5	3.6	185	8.08	3.58	99.48
2	4.5	3.6	186	8.13	3.63	100.72
3	4.5	3.6	186	8.1	3.61	100.16
Mean				8.10	3.6	100.1
SD				0.02	0.032	0.88
% RSD				0.31	0.875	0.87
100 % Accuracy						
1	4.5	4.5	209	9.082	4.58	101.83
2	4.5	4.5	208	9.029	4.52	100.65
3	4.5	4.5	208	9.108	4.56	101.56
Mean				9.073	4.56	101.24
SD				0.040	0.038	0.84
% RSD				0.443	0.83	0.83
120 % Accuracy						
1	4.5	5.4	228	9.914	5.414	100.26
2	4.5	5.4	229	9.924	5.424	100.60
3	4.5	5.4	229	9.919	5.421	100.38
Mean				9.919	5.42	100.36
SD				0.005	0.008	0.14
% RSD				0.050	0.139	0.14

Table 8: Result of Intra-Day and Inter-day Precision for Liothyronine

	Conc.	% Amt. Found	SD	% RSD
Intraday	4.5	100.58	0.88	0.87
	13.5	101.81	1.45	0.46
	22.5	99.49	0.37	0.07
Interday	4.5	100.55	0.22	0.22
	13.5	100.96	4.04	1.28
	22.5	99.53	2.95	0.56

Table 9: Result of Intra-Day and Inter-day Precision for Levothyroxine

	Conc.	% Amt. Found	SD	% RSD
Intraday	19	99.99	20.61	1.61
	57	99.06	18.78	0.52
	95	99.98	3.33	0.05
Interday	19	99.05	0.06	0.00
	57	99.23	0.68	0.02
	95	99.74	0.76	0.01

DISCUSSION

The developed method was optimized by employing Box Behnken Design, which generated 17 experimental runs with four repetitions of central points to evaluate the effect and interactions of critical method parameters on response and develop a model with adequate statistical parameters. The optimized RP-HPLC method employed a C18 column, methanol and 0.1 % formic acid (50:50, v/v) utilized as mobile phase, and the flow rate was 1.2 ml/min, with ambient column temperature and detection wavelength of 254 nm, achieving excellent separation and resolution of LT4 and LT3. 3-D response surface graphs shown in Figures 4 to 7 depict the effect of independent variables on the response. Experimental conditions in which the methanol percentage is between 45% and 50% in the mobile phase, the flow rate is between 1.1 mL/min and 1.2 mL/min, and the factor combination results in a minimum analysis time, optimum peak area, maximum theoretical plates, and low tailing factor. As shown in Table 3, the ANOVA results of Levothyroxine for a quadratic model for retention time suggested a P-value of 0.0006 and an F-value of 190, for peak area, a P-value of 0.012 and an F-value of 4.39, for theoretical plate, a P-value of 0.0094 and an F-value of 28.46, and tailing factor, a P-value of 0.0014 and an F-value of 102. The ANOVA results of Liothyronine for a quadratic model for retention time suggested a P-value of 0.0001 and an F-value of 523, for peak area, the P-value was 0.0001 and the F-value was 1651, for theoretical plate, the P-value was 0.0437 and the F-value was 9.71. For the tailing factor, the P-value was 0.0014, and the F-value was 101. The ANOVA analysis results for selected responses had a P-value below 0.05 and an F-value more than 2.5, indicating the developed and optimized HPLC method was statistically significant. The method was validated by ICH guidelines, showing acceptable results for different validation parameters such as linearity, precision, accuracy, robustness, limit of detection, and limit of quantification. Linearity was confirmed over a suitable concentration range for both analytes, with correlation coefficients (R^2) of 0.9993 and 0.9994 for Levothyroxine and Liothyronine, respectively, as shown in Figure 8. The method's accuracy was validated through recovery studies, showing recovery rates within acceptable limits, indicating that the method can accurately quantify the target analytes in pharmaceutical formulations, as shown in Tables 6 and 7. Precision studies, including both repeatability and intermediate precision, yielded low relative standard deviation (RSD) values, underscoring the method's reliability, as shown in Tables 8 and 9. Robustness was assessed by making deliberate variations in method parameters, which showed no significant impact on the method's performance, affirming its robustness.

CONCLUSION

The optimized HPLC method by BBD design for analysis of LT3 and LT4 is scientifically valid, efficient, accurate, and robust. Regulatory agencies like the US FDA encourage the implementation of QbD principles in product and analytical development and for submissions of dossiers, emphasizing that quality should be built into the product rather than relying on testing. This QbD-based method can be utilized for routine quality control analysis in pharmaceutical industries, contributing significantly to the assurance of the safety and efficacy of thyroid hormone therapies.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

FINANCIAL ASSISTANCE

NIL

AUTHOR CONTRIBUTION

Ramdas B. Darade and Sanjay S. Pekamwar designed the concept. Ramdas B. Darade performed the experimental work and contributed to preparing the manuscript. Sanjay S. Pekamwar supervised this research and contributed to interpreting statistical data.

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