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Analytical Development

High Performance Liquid Chromatography Method Development and Validation for Separation of Liothyronine Sodium Related Substances Using a Quality by Design Approach

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Abstract **Original Research Article**

The application of the Quality by Design (QbD) principles in developing a novel high performance liquid chromatography (HPLC) method for the analysis of liothyronine sodium related substances for finished product using Fusion QbD® software is explored. The effect of various chromatographic parameters including, column stationary phase, initial hold time and gradient time on separations were systematically investigated. Results show that optimal separations of these compounds in a standard solution can be achieved using a X Bridge C18 column (150 mm × 4.6 µm, 5µm), maintained at sample temperature 15°C by using pH 2.0 buffer solution as mobile phase-A and methanol as mobile phase-B with a flow rate of 1.0 ml/min at 225 nm of detection and a gradient time of 38 minutes. The injection volume is 200 µl. Pre-validation studies of the method were performed and all the parameters met the acceptance criteria. The results are demonstrated that optimized method is selective, linear, precise, repeatable and accurate.

Keywords: Fusion, liothyronine sodium, method development, quality by design, related substances.

1. INTRODUCTION

Liothyronine Sodium (L-triiodothyronine or LT_3Na) is typically used to treat patients with hypothyroidism, a condition wherein the thyroid gland does not produce enough thyroid hormone. It is also used to help decrease the size of enlarged thyroid glands (goiter) and treat thyroid cancer [1]. As we mentioned above, Liothyronine Sodium is a white or slightly coloured, hygroscopic powder, practically insoluble in water, slightly soluble in ethanol (96 %) and dissolves in dilute solutions of alkali hydroxides. Liothyronine Sodium has the chemical structure depicted below, and it is chemically described as O-(4-hydroxy-3-iodophenyl)-3, 5-diido-monosodium salt (1:1) (Figure 1). The molecular weight of Liothyronine Sodium is 672.96 g/mol and molecular formula is $C_{15}H_{11}I_3NNaO_4$ [2].

According to The Wickman Survey hypothyroidism, which is the most general endocrine disorders, observed an annual diagnosis of the disease at a rate of 4.1 per 1000 for women and 0.6 per 1000 for men [3]. Hypothyroidism has been handled with Levothyroxine (L-T4), the sodium salted form of thyroid hormone (TH) tetraiodothyronine (T4) since the 1930s [4]. L-T4 has a half-life of 6 days and its transformed to T3 for ensure stable and physiological amount of T3. Liothyronine arrives its maximum points 2 to 4 hours after oral ingestion and has a half-life of 1 day. Therefore, stable levels cannot be maintained with a once-daily dose of liothyronine [5].

T3 is the most potent thyroid hormone, and its affinity for the thyroid hormone receptor is 10 to 20 times that of thyroxine [6]. In addition to this, it shows that replacement therapy with L-T4 does not increase adequate T3 levels, but the combined form of L-T4 with liothyronine (L-T3, the synthetic pharmaceutical form of T3) does [4]. Clinical trials for the treatment of hypothyroidism were conducted in the early 1970s using 40 to 60 ug per day of liothyronine in combination with L-T4 as pharmaceutical doses of thyroid hormones [5].

On the other hand, combination therapy was observed to improve quality of life and depression [4]. The use of combined therapy has good benefits on depression and physical conditions [5].

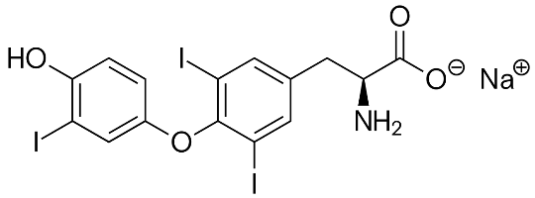


Figure 1: Chemical structure of Liothyronine Sodium

Scientists working on drug discovery have recently looked into a variety of highly effective low-dose molecule pharmaceutical candidates. The pharmacological development of such low-dose pharmaceuticals presents considerable difficulties in product design and optimization.

A low-dose pharmaceutical product may include as little as a few micrograms of substance in each dosage unit. The ratio of excipients to medicine might range from 1.000 to 10.000, which is very different from a typical pharmaceutical product. Major quality of the product features such; might be significantly impacted by the formulation composition's properties;

- Reliability of the powder blend's ingredients and the final product,
- Stability in the production process and the product's shelf life,
- Low potency as a consequence of lost manufacturing.

Variation in potency (high RSD) in test for blending samples of final product is a frequent issue with blending in a low-dose pharmaceuticals.

Because of the large ratio of excipients to active pharmaceutical ingredients (APIs) in a low-dose drug product, stability is another major task. Excipients used in pharmaceuticals influence both the stability and processability of the final product. Excipients, like APIs, may include manufacturing-related process leftovers and degradation products. One of the most often utilized excipients in solid oral pharmaceutical forms is lactose. While using lactose, liothyronine may be decomposed by Maillard reaction. Liothyronine and other drug compounds that include primary, secondary, or tertiary amines are sensitive to reacting with residual aldehydes or reducing sugars that contain aldehydic groups. Some excipients like lactose and microcrystalline cellulose will adsorb water, which affects the pharmaceutical's micro- environment. Degradation may occur fast if the API is moisture-sensitive, like Liothyronine sodium.

Absence of content uniformity may be caused by the physical characteristics of API (particle size, shape, and density) and by poorly blending quantities of API with the excipients.

For analytical chemists, developing analytical methods, validating methods, and ensuring product quality face huge challenges. The development of reliable methods for analyzing low-dose of drug substances and impurities in pharmaceutical forms, resolving testing interferences caused by excipient impurities, and determining the drug substance residue on production equipment after cleaning may be highly challenging. For instance, the following techniques can result in improve the sensitivity for impurity screening by high-pressure liquid chromatography (HPLC);

- Sample preparation,
- Better detection methods (mass spectrometry, electrochemical/fluorescent detector),
- Injecting a large volume [7].

The term Quality by Design (QbD), implied by the International Conference on Harmonisation (ICH), has gained much popularity in pharmaceutical development since 2009. In the beginning, the QbD methodology centered on the development of pharmaceutical manufacturing. However, it has lately been performed the development and optimization of analytical methods and is called Analytical Quality by Design (AQbD). The AQbD methodology is quite similar to the QbD process as described in ICH Q8 [8]. Fusion QbD is a design software system that can be connected with liquid chromatography (LC) equipment and enables researchers to design and set up automated experiments with sufficient data points for method screening and optimization [9-10]. Up to this day there are no RP-HPLC methods for liothyronine sodium tablets and its related compounds in the literature.

Therefore, the current study focuses on developing, easy, rapid and more sensitive analytical method through popular QbD approach for determination of Liothyronine sodium related substances in tablet form. The pre-validation of the developed method was performed based on ICH Q2 (R1) guidelines [11].

2. MATERIALS AND METHODS

2.1. Chemicals and Eluents

Analytical reference standards of Liothyronine sodium (assigned purity, 101.10%) and Impurity A (assigned purity; 97.70%) were purchased from Peptido GmbH, Germany. Impurity B (assigned purity, 94.60%), Impurity C (assigned purity; 96.81%), Impurity D (assigned purity; 95.64%) were purchased from Hemarsh Technologies, India. Impurity E (assigned purity; 98.12%) was purchased from SimSon Pharma Ltd., India. Liothyronine Sodium Tablets, containing 20 mcg of Liothyronine Sodium were developed and produced in Abdi Ibrahim Research & Development laboratory.

All reagents used in the experimental work were of HPLC grade. Methanol and sodium hydroxide were purchased from Merck (Darmstadt, Germany).

Water used was purified by a Sartorius Arium® Pro water purification system (Sartorius Lab Instruments GmbH, Goettingen, Germany).

All solutions were protected from light by use of amber colored volumetric flask.

2.2. Preparation of Standard and Sample Solution

Stock Impurities Solutions

0.01 mg/ml of impurity A, impurity B, impurity C, impurity D, or impurity E in diluent (30:70 v/v pH 2.0 Buffer:Methanol).

Standard Solution

0.0002 mg/ml of Liothyronine Sodium in diluent.

System Suitability Solution

0.0002 mg/ml of Liothyronine Sodium and Impurity A in diluent.

Sample Solution

0.02 mg/ml of Liothyronine Sodium in diluent.

2.3. H-Class Equipment and Chromatographic Conditions

The method development study was performed on an Acquity H-Class chromatography system comprised of a quaternary solvent manager (QSM), an autosampler, a photodiode-array (PDA) detector and a Column Manager equipped with two Auxiliary Column Managers to accommodate two columns (Waters, Milford, MA, USA). The QSM is a high pressure pump that can simultaneously pump four degassed solvents (A, B, C and D) through the system. Injection volume was set to 10 µl and the flow rate was 0.3 ml/min with UV detection at 225 nm.

Fusion QbD software (version 9.9.0, S-Matrix, Eureka, California, USA) was used to create a generalized full factorial design to generate a representative subset of all possible combination factors, including gradient profile, initial hold time, mobile phase and column type. The gradient profile was applied with an initial hold of 0.1-3.0 min at 20% strong solvent followed by a linear ramp to 80% strong solvent and a final hold of 3.0 min hold at 80% strong solvent before re-equilibration to initial conditions. The columns used in the screening experiments were all (100 mm × 2.1 mm) in dimensions and included: a BEH C18 (1.7 µm) and a BEH PHENYL (1.7 µm). The Fusion QbD software interacts directly with the chromatography data software Empower 3 (Waters, Milford, MA, USA) such that no further manual involvement was needed.

2.4. Design of Experiments

Our method development strategy involved column screening to identify the stationary phase in combination with parameters such as mobile phase, gradient time, initial hold time and column type with the best resolution (Rs > 2.0) for all compounds (Liothyronine Sodium and five of its impurities). Table 1 presents the experimental design used in the column and mobile phase, gradient time, initial hold time combination scouting phase.

Since the strongest acidic pKa value of liothyronine sodium is 0.3, it was aimed to select pH in the range of pKa ± 1, which is the usable range in the mobile phase buffer [12]. For this purpose, weak acid TFAA and pH 2.0 buffers were preferred. Accordingly, weak solvents were determined as pH 2.0 buffer and 0.1% TFAA. Methanol and Acetonitrile were preferred as strong solvents. BEH Phenyl (100 mm × 2.1 mm, 1.7 µm) and BEH C18 (100 mm × 2.1 mm, 1.7 µm) column were used. Our aim in using UPLC is to separate the peaks in a short injection time and to minimize the trial time with QbD. The wavelength was chosen as 225 nm. The pump flow rate was selected 0.3 ml/min. Initial hold time 0.5 min to 4.0 min range was tried. In the gradient program, 5.0% and 20% were chosen as the initial organic %. By this method, it was aimed to develop a new method ability of separating five defined impurities (Impurity A, Impurity B, Impurity C, Impurity D and Impurity E) of Liothyronine sodium.

Reservoir Assignments

Reservoir A	Level
pH 2.0	---
Reservoir B	**Level**
0.1 % TFAA	---
Reservoir C	**Level**
MeOH	---
Reservoir D	**Level**
ACN	---

Column Assignments

Column Valve Position	Column Level
ValvePosition_1	BEH C18
ValvePosition_2	BEH PHENYL

Experiment Constants

Constant Name	Constant Value
Pump Flow Rate	0.300
Injection Volume	10.0
Oven Temperature	35.0
Wavelength	225
Equilibration Time	3.0
Initial Hold % Organic	20.0
Final Hold Time	3.0
Final Hold % Organic	80.0
Ramp Up to Wash Time	0.5
Column Wash Time	2.0
Column Wash % Organic	100.0

Figure 2: Variation and range testing by Quality by Design Approach

Table 1: Design of Experiments for the Scouting

Run No	Strong Solvent Type	Weak Solvent Type	Initial Hold Time (min.)	Gradient Time (min.)	Column Type
1.a.1.a	MeOH	pH 2.0	3.0	6.0	BEH C18
2.a.1.a	MeOH	pH 2.0	1.6	9.0	BEH C18
3.a.1.a	MeOH	pH 2.0	1.6	9.0	BEH PHENYL
4.a.1.a	MeOH	pH 2.0	3.0	12.0	BEH PHENYL
5.a.1.a	MeOH	pH 2.0	0.1	12.0	BEH C18
6.a.1.a	MeOH	pH 2.0	0.1	6.0	BEH C18
7.a.1.a	MeOH	pH 2.0	1.6	9.0	BEH PHENYL
8.a.1.a	MeOH	pH 2.0	1.6	9.0	BEH C18
9.a.1.a	MeOH	pH 2.0	0.1	6.0	BEH C18
10.a.1.a	ACN	pH 2.0	1.6	9.0	BEH C18
11.a.1.a	ACN	pH 2.0	2.3	7.5	BEH PHENYL
12.a.1.a	ACN	pH 2.0	0.8	10.5	BEH PHENYL
13.a.1.a	ACN	pH 2.0	3.0	12.0	BEH C18
14.a.1.a	ACN	pH 2.0	3.0	9.0	BEH C18
15.a.1.a	ACN	pH 2.0	1.6	6.0	BEH PHENYL
16.a.1.a	ACN	pH 2.0	1.6	9.0	BEH C18
17.a.1.a	ACN	pH 2.0	1.6	9.0	BEH C18
18.a.1.a	ACN	pH 2.0	0.1	12.0	BEH PHENYL
19.a.1.a	ACN	pH 2.0	1.6	9.0	BEH C18
20.a.1.a	ACN	pH 2.0	0.1	12.0	BEH PHENYL
21.a.1.a	ACN	pH 2.0	0.1	6.0	BEH PHENYL
22.a.1.a	ACN	pH 2.0	3.0	12.0	BEH C18
23.a.1.a	MeOH	0.1 % TFAA	3.0	6.0	BEH PHENYL
24.a.1.a	MeOH	0.1 % TFAA	3.0	9.0	BEH C18
25.a.1.a	MeOH	0.1 % TFAA	1.6	9.0	BEH C18
26.a.1.a	MeOH	0.1 % TFAA	1.6	9.0	BEH PHENYL
27.a.1.a	MeOH	0.1 % TFAA	1.6	9.0	BEH PHENYL
28.a.1.a	MeOH	0.1 % TFAA	2.3	10.5	BEH PHENYL
29.a.1.a	MeOH	0.1 % TFAA	0.8	7.5	BEH PHENYL
30.a.1.a	MeOH	0.1 % TFAA	1.6	9.0	BEH C18
31.a.1.a	MeOH	0.1 % TFAA	3.0	12.0	BEH C18
32.a.1.a	MeOH	0.1 % TFAA	0.1	12.0	BEH PHENYL
33.a.1.a	MeOH	0.1 % TFAA	1.6	6.0	BEH PHENYL
34.a.1.a	ACN	0.1 % TFAA	1.6	9.0	BEH PHENYL
35.a.1.a	ACN	0.1 % TFAA	1.6	9.0	BEH C18
36.a.1.a	ACN	0.1 % TFAA	1.6	9.0	BEH PHENYL
37.a.1.a	ACN	0.1 % TFAA	0.1	12.0	BEH C18
38.a.1.a	ACN	0.1 % TFAA	1.6	9.0	BEH C18
39.a.1.a	ACN	0.1 % TFAA	3.0	6.0	BEH C18
40.a.1.a	ACN	0.1 % TFAA	0.1	6.0	BEH C18
41.a.1.a	ACN	0.1 % TFAA	3.0	12.0	BEH PHENYL
42.a.1.a	ACN	0.1 % TFAA	0.1	6.0	BEH C18

2.5. Pre-validation Procedure

Pre-validation of method is carried out in accordance with ICH Q2 (R1) guideline [11] for the related substances of finished product. The method was pre-validated for parameters like specificity, linearity and range, response factor, limit of detection (LOD) & limit of quantitative (LOQ), system suitability, repeatability, accuracy.

2.5.1. Specificity

Specificity test is ability of the method to measure the analyte response in the presence of other substances or expected to be present. For determination of specificity, injection of blank, placebo, individual impurities, standard, sample and spiked sample solutions were prepared and injected into HPLC system. There should be no interfering peak at the retention time of liothyronine sodium and its known impurities with blank and placebo in standard sample and spiked sample solutions chromatograms. Spectrum of liothyronine sodium and its known impurities peaks in the chromatogram of obtained from standard sample and spiked sample solution should have no interference from blank and placebo solutions. For liothyronine

sodium peak in standard solution chromatogram and known impurities peaks in sample and spiked sample solutions chromatograms, there should be purity angle < purity threshold.

2.5.2. Linearity

The linearity of an analytical method is its ability to elicit test results that proportional to the concentration of analytes in the samples within a given range. The test solutions are prepared for related substance method from liothyronine sodium and its known impurities stock solution at various concentration levels. The linearity plot is constructed at different concentration levels between LOQ level and 120% of specification. Using the peak area versus the concentration of test solutions, the calibration curve is plotted and the regression equations are measured. The system of least squares is used to measure the slope, coefficient and intercept of correlation. The correlation coefficient (R) should be less than 0.99.

2.5.3. Response Factor

Response factors (RF) of the known impurities are used for the establising of exact amount of impurities present in pharmaceutical dosage form. The response factor is the ratio between a signal manufactured by active pharmaceutical ingredient (API) and impurity below the same condition. Response factors are figure out using the following equation (Eq. 1);

$$RF = \frac{\text{Response of API}}{\text{Response of impurity}} \quad \text{(Eq. 1)}$$

2.5.4. LOD and LOQ

The study is performed to find the lowest acceptable value where the analyte is quantified (LOQ) and detected (LOD) with an acceptable accuracy and precision. LOD and LOQ values are calculated with the determination curve containing the LOD level. The RSD% of liothyronine sodium and its impurities standards peaks in the chromatograms at LOQ level after determination should not be more than 10.0%. The peaks at LOD level should be detectable. Calculate using below formula (Eq. 2 and Eq. 3).

$$\text{Detection Limit (DL)} = \frac{3.3 \times \sigma}{S} \quad \text{(Eq. 2)}$$

$$\text{Quantification Limit (QL)} = \frac{10 \times \sigma}{S} \quad \text{(Eq. 3)}$$

σ = Standard deviation (The residual standard deviation of a regression line or the standard deviation of y-intercept of regression line)
S = The slope of the regression line

2.5.5. System Suitability

System suitability parameters are calculated to check the performance of the system as symmetry factor, theoretical plate count, resolution and retention time for analyte peak.

2.5.6. Repeatability

In repeatability, a homogeneous sample of a single batch should be analyzed. This indicates whether a method is giving consistent results of a single batch. Injecting spiked sample preparation method precision is performed. The precision of method is achieved by calculating RSD% of spiked sample results at specification limits for six measurements. The RSD% should be less than 4.0%.

2.5.7. Accuracy

The accuracy of method is the degree of closeness between the true value of analytes in the sample and the value determined by the method. Analysis was performed by using standard addition procedure. At each level, three determinations were performed. From this individual recovery% and RSD% were calculated (RSD < 5.0%). The recovery% should be between 90.0% to 110.0%.

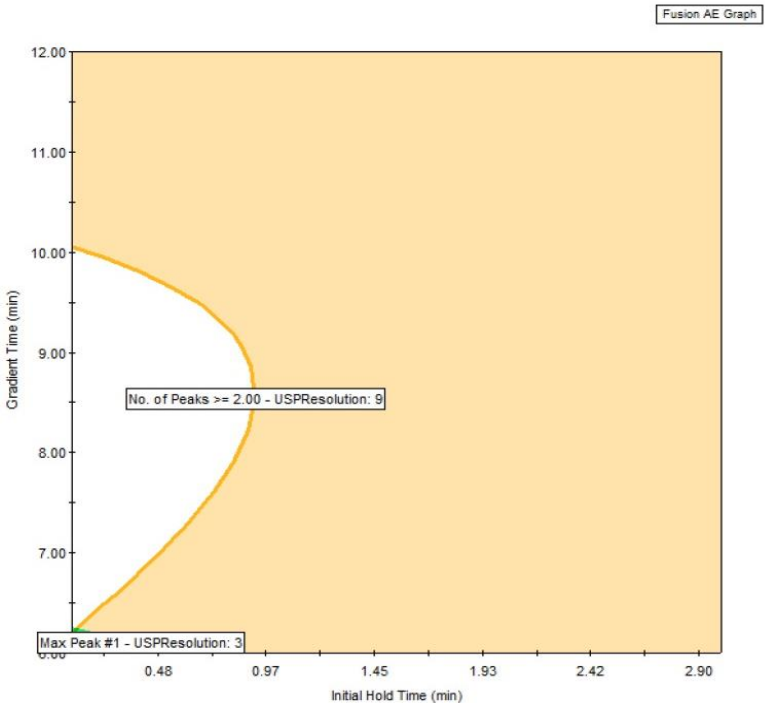
3. RESULTS AND DISCUSSION

3.1. Developed Method

The performance targets (multiple peak responses, USP resolution) for the screening phase were determined after the experiments were completed and the chromatogram was integrated. These are;

1) Number of integrated peaks in each chromatogram,
2) Number of peaks which are baseline separated (USP resolution Rs > 2.0),
3) USP resolution of liothyronine.

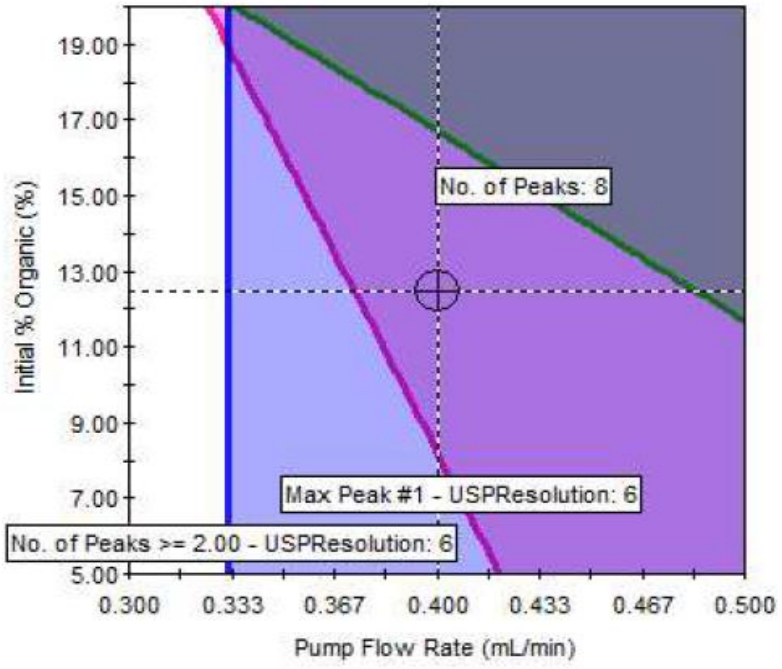
Gradient time, initial hold time, mobile phase as well as column type were all variables to be screened. This resulted in a method development design requiring only 42 experiments to obtain optimal method performance. The design was subsequently exported, with ready to use methods, to the chromatographic data system (CDS). After chromatographic runs were completed, the results were imported back to Fusion QbD for data analysis. The best overall answer search was applied based on the desired performance goals. The software used these goals along with a regression model that is fitted to the results to predict the conditions that would offer optimal method performance.



Response Variable Goals

Name	Units	Goal	Lower Bound	Upper Bound
No. of Peaks	( )	Maximize	8	---
No. of Peaks >= 2.00 - USPResolution	( )	Maximize	9	---
Max Peak #1 - USPResolution	( )	Maximize	3	---

Figure 3: **The design of the method optimization study with Fusion QbD and the APR region, comparing the gradient time (min.) versus the initial hold time (min.). The user-specified performance targets that this design was successful in achieving are displayed in the table under the graph**



Response Variable Goals

Name	Units	Goal	Color	Lower Bound	Upper Bound	Crosshair Prediction
No. of Peaks		Maximize	Green	8		12
No. of Peaks >= 2.00 - USPResolution		Maximize	Blue	6		8
Max Peak #1 - USPResolution		Maximize	Fuchsia	6.000000000		15.240482329

Figure 4: The design of the method optimization study with Fusion QbD and the APR region, comparing the initial % organic (%) versus the pump flow rate (ml/min). The user-specified performance targets that this design was successful in achieving are displayed in the table under the graph

The spiked sample was chosen as the "worst-case" situation for data analysis and additional technique development. For data analysis, integrated chromatograms for the spiked sample injections were uploaded into Fusion QbD. To create pattern response data, a "peak count" performance target was chosen. The technique variable combinations (column, gradient duration) that would provide the "Best Overall Answer (BOA)" or maximal peak count were predicted by Fusion QbD. After the fast-screening investigation was finished, these factors were taken over into technique optimization [13].

The acceptable performance region (APR) was established by achieving chromatographic system suitable performance targets, including peak count and USP resolution, after design execution, chromatogram integration, and data transfer into Fusion QbD. According to the APR, a gradient period of 6 to 10 minutes and an initial hold time of 0 to 1 minute would produce a reliable chromatographic separation (Figure 3.). The APR also projected that an initial % organic (%) of 5 to 20 and a pump flow rate (ml/min.) of 0.300 to 0.333 would produce a reliable chromatographic separation (Figure 4). Within the APR region, all system suitability requirements for peak count and USP resolution were met.

By examining spiked sample preparations under the variables specified at the APR region, the method verification was achieved. The Waters Acquity BEH C18 Column was used as a result, with pH 2.0 buffer as mobile phase-A and methanol as mobile phase-B. The column temperature was operated at 25°C with a 0.1 minute hold at 20% B. After then, the gradient was boosted to 80% over 12.1 minutes. The trial was carried out with a constant flow rate, injection volume, and UV detection wavelength of 0.3 ml/min, 10 µL, and 225 nm, respectively.

Since the HPLC method was chosen as the assay method in these studies, the UPLC method was adapted to HPLC method with Column Calculator, considering the Mass Balance compatibility.

Variable Settings

Variable	Level Setting
Strong Solvent Type	MeOH
Weak Solvent Type	pH 2.0
Pump Flow Rate	0.300
Initial Hold Time	0.5
Initial % Organic	5.0
Column Type	BEH C18

Figure 5: BOA results predicted by Fusion QbD

Table 2: Method parameters for UPLC selected by Fusion QbD and HPLC calculated by Column Calculator

Equipment	**UPLC**			**HPLC**		
Column	Waters Acquity BEH C18 100 mm × 2.1 mm, 1.7 µm			Waters XBridge C18 150 mm × 4.6 mm, 5 µm		
Wavelength	225 nm			225 nm		
Column Flow	0.3 ml/min.			1.0 ml/min.		
Column Temperature	35°C			25°C		
Autosampler Temperature	25°C			15°C		
Gradient Program	**Time (min.)**	**Mobile Phase A (%)**	**Mobile Phase B (%)**	**Time (min.)**	**Mobile Phase A (%)**	**Mobile Phase B (%)**
	0	80	20	**0**	80	20
	0.1	80	20	**1.0**	80	20
	12.1	20	80	**26.0**	20	80
	15.1	20	80	**32.6**	20	80
	15.6	80	20	**33.6**	80	20
	17.6	80	20	**38.0**	80	20

3.2. Method Validation

3.2.1. Specificity

Liothyronine sodium and its known impurities were well separated from blank and their excipient. There was no interference of blank and placebo with the main peak and its impurities. For liothyronine sodium peak in standard, sample and spiked sample solutions chromatograms, purity angle < purity threshold criteria was fulfilled. Therefore, the method was specific. The specificity results were tabulated in Table 3.

Table 3: Peak purity of components in spiked sample solution

Name of Solution	Peak Purity		Purity Criteria	Retention Time (min)
	Purity Angle	Purity Threshold		
Impurity B	23.291	46.554	Pass	9.941
Impurity E	9.789	16.786	Pass	12.285
Liothyronine Sodium	0.108	0.368	Pass	20.732
Impurity A	3.341	6.293	Pass	22.845
Impurity C	3.087	5.831	Pass	25.422
Impurity D	4.004	6.243	Pass	27.276

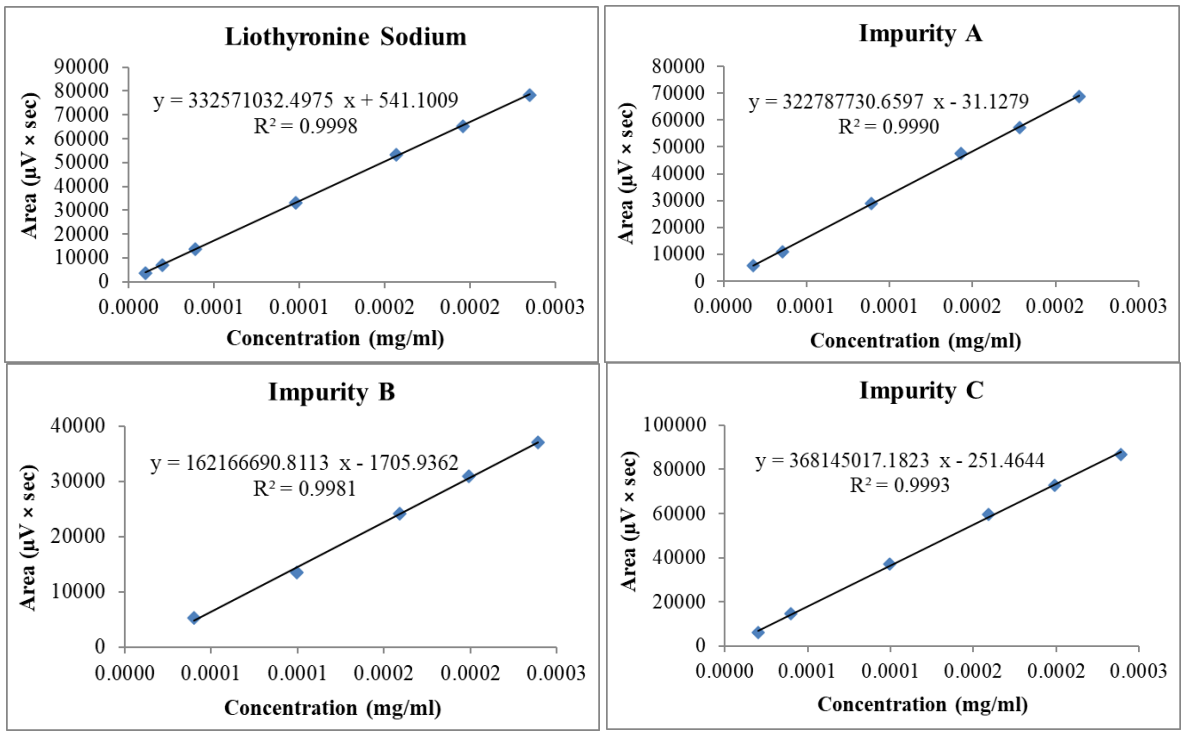
3.2.2. Linearity

The area of the linearity peak versus different concentrations was statistically evaluated for liothyronine sodium and its impurities. Correlation coefficient determination (R), slope and y-intercept values were calculated. The linear equation was shown in Table 4 and Figure 6. Following these results shows that the proposed method was linear.

Table 4: Linearity results of Liothyronine Sodium and its known impurities

Level%	Liothyronine Sodium	
	Concentration (mg/ml)	Area (µV × sec)
LOQ (5.6)	0.000010	3711
10	0.000020	6897
20	0.000039	13696
50	0.000098	33049
80	0.000156	53329
100	0.000196	65142
120	0.000235	78477
Corr. Coefficient	**0.9998**	
Slope	**332571032.4975**	
Intercept	**541.1009**	
Level%	**Liothyronine Sodium Impurity A**	
	Concentration (mg/ml)	**Area (µV × sec)**
LOQ (11.9)	0.000018	5724
20	0.000036	11036
50	0.000089	28827
80	0.000143	47626
100	0.000179	57045
120	0.000214	68670
Corr. Coefficient	**0.9990**	
Slope	**322787730.6597**	
Intercept	**-31.1279**	
Level%	**Liothyronine Sodium Impurity B**	
	Concentration (mg/ml)	**Area (µV × sec)**
LOQ (19.9)	0.000040	5356
50	0.000099	13531
80	0.000159	24085
100	0.000199	30884
120	0.000239	37046
Corr. Coefficient	**0.9981**	
Slope	**162166690.8113**	
Intercept	**-1705.9362**	

Level%	**Liothyronine Sodium Impurity C**	
	Concentration (mg/ml)	**Area (µV × sec)**
LOQ (9.4)	0.000020	6143
20	0.000040	14503
50	0.000100	37233
80	0.000159	59494
100	0.000199	72941
120	0.000239	86869
Corr. Coefficient	**0.9993**	
Slope	**368145017.1823**	
Intercept	**-251.4644**	
Level%	**Liothyronine Sodium Impurity D**	
	Concentration (mg/ml)	**Area (µV × sec)**
LOQ (16.0)	0.000028	10863
20	0.000037	14792
50	0.000093	33612
80	0.000148	53486
100	0.000186	62861
120	0.000223	75634
Corr. Coefficient	**0.9983**	
Slope	**330797578.9670**	
Intercept	**2470.0591**	
Level%	**Liothyronine Sodium Impurity E**	
	Concentration (mg/ml)	**Area (µV × sec)**
LOQ (12.9)	0.000024	9046
20	0.000039	13935
	0.000097	34508
80	0.000155	54933
100	0.000194	68322
120	0.000233	85089
Corr. Coefficient	**0.9989**	
Slope	**359664274.5379**	
Intercept	**-125.5063**	



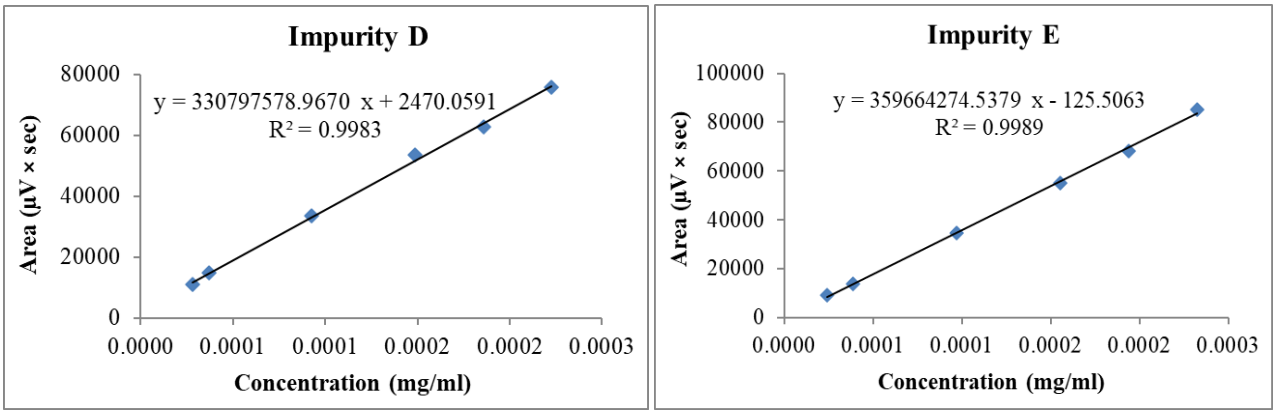


Figure 6: Linearity Curve of Liothyronine Sodium and its known impurities

3.2.3. Response Factor

Response factor (RF) was obtained from the slope ratios of the curves acquired as a result of the linear regression analysis performed in the determination of linearity parameter of active substance and its known impurity.

Table 5: RF and RRT results of Liothyronine Sodium and its known impurities

Sample Name	Slope	Relative Retention Time (RRT)	Response Factor (RF)
Liothyronine Sodium	332571032.4975	1.00	-
Liothyronine Sodium Impurity A	322787730.6597	1.10	1.03
Liothyronine Sodium Impurity B	162166690.8113	0.48	2.05
Liothyronine Sodium Impurity C	368145017.1823	1.23	0.90
Liothyronine Sodium Impurity D	330797578.9670	1.32	1.01
Liothyronine Sodium Impurity E	359664274.5379	0.83	0.92

3.2.4. LOD and LOQ

The study was performed to find the lowest acceptable value where the analyte is quantified (LOQ) and detected (LOD) with an acceptable accuracy and precision. The relative standard deviation (RSD%) of liothyronine sodium and its known impurities at the LOQ level is under the limit (Table 6 and Table 7).

Table 6: LOD & LOQ concentrations of Liothyronine Sodium and its known impurities

Sample Name	LOD (mg/ml)	LOQ (mg/ml)
Liothyronine Sodium	0.000004	0.000011
Liothyronine Sodium Impurity A	0.000007	0.000021
Liothyronine Sodium Impurity B	0.000013	0.000040
Liothyronine Sodium Impurity C	0.000006	0.000019
Liothyronine Sodium Impurity D	0.000010	0.000030
Liothyronine Sodium Impurity E	0.000008	0.000025

Table 7: LOQ areas (µV × sec) of Liothyronine Sodium and its known impurities

Sample No	Liothyronine Sodium	Impurity A	Impurity B	Impurity C	Impurity D	Impurity E
1	3703	5767	5427	6228	10860	9199
2	3719	5680	5285	6057	10866	8892
3	3841	5879	5512	6244	10950	8915
4	3755	5728	5266	6119	11115	8909
5	3869	5801	5293	6114	10955	8918
6	3775	5859	5347	6090	10838	9257
Mean	**3777**	**5786**	**5355**	**6142**	**10931**	**9015**
RSD%	**1.75**	**1.32**	**1.80**	**1.24**	**0.94**	**1.84**

3.2.5. System Suitability

Device suitability parameters have been assessed. The resolution between Liothyronine Sodium and Liothyronine Sodium Impurity A was calculated. The mean, SD and RSD% for peak area of liothyronine sodium was calculated. Also monitored symmetry factor, theorical plate count and retention time for analyte peak.

Table 8: System Suitability results of System Suitability Solution

Peak Name	Resolution
Liothyronine Sodium	-
Liothyronine Sodium Impurity A	8.12

Table 9: System Suitability results of Liothyronine Sodium Standard Solution

Injection No	Symmetry Factor	Theoretical Plate Count	Retention Time (min.)	Area (µV × sec)
1	0.7	78315	20.634	67403
2	0.8	74430	20.632	66763
3	0.8	100245	20.652	66458
4	0.7	100433	20.648	66576
5	0.8	98242	20.643	66891
6	0.8	98476	20.641	67776
Mean	0.8	91690	20.642	66978
SD	**0.052**	**11962**	**0.008**	**510.5**
RSD%	**6.74**	**13.1**	**0.04**	**0.76**

3.2.6. Repeatability

The precision of the method was tested by preparing 1.0% level spiked sample tested under the same conditions. The RSD% (< 4.0%) value indicates that the repeatability of the method has been proven (Table 10).

Table 10: Repeatability results of Liothyronine Sodium and its known impurities

Sample No	% of Related Substances				
	Impurity A	Impurity B	Impurity C	Impurity D	Impurity E
1	0.937	0.974	0.992	0.984	0.946
2	0.934	0.995	0.994	0.994	0.941
3	0.944	0.981	0.985	0.997	0.950
Mean	**0.938**	**0.983**	**0.991**	**0.992**	**0.945**
SD	**0.004**	**0.011**	**0.005**	**0.007**	**0.004**
RSD%	**0.53**	**1.07**	**0.46**	**0.68**	**0.46**

3.2.7. Accuracy

Accuracy for known impurities was conducted by adding impurities to placebo solution for LOQ level and sample solution for 100% intended. For unknown impurities was conducted adding liothyronine sodium to placebo solution for LOQ and 100% level intended. Liothyronine sodium and its known impurities recoveries% were measured and found to be within the limit (Table 11).

Table 11: Accuracy results of Liothyronine Sodium and its known impurities

Level%	Sample No	Recovery%				
		Impurity A	Impurity B	Impurity C	Impurity D	Impurity E
LOQ	1	99.6	97.7	96.2	101.2	98.7
	2	95.2	97.0	90.3	101.5	100.3
	3	97.7	98.7	96.1	104.8	96.7
100	1	99.9	99.2	99.5	102.1	102.7
	2	103.4	101.2	100.6	104.9	104.1
	3	100.5	102.1	99.4	102.9	101.8
Mean		**99.4**	**99.3**	**97.0**	**102.9**	**100.7**
RSD%		**2.80**	**1.99**	**3.87**	**1.57**	**2.69**

CONCLUSION

In this case study, we used the statistic Fusion QbD® software to develop a state-of-the-art related substances method for Liothyronine Sodium and its known impurities for finished product. Therefore, the proposed method can be used for routine analysis of the finished product and to verify the quality of the finished product during stability studies.

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