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Analytical Method Development and Validation of RP-HPLC Method for Estimation of Levothyroxine in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

Objective: To develop and validate a simple, accurate, and robust RP-HPLC method for the estimation of levothyroxine in bulk drug and pharmaceutical dosage forms, ensuring compliance with regulatory quality standards.

Method: A reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed using a C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase of phosphate buffer (pH 3.0) and methanol (55:45 v/v). The flow rate was set at 1.0 mL/min, detection wavelength at 225 nm, and injection volume was 10 µL. The method was validated as per ICH Q2(R1)[1]guidelines.

Result: The method exhibited excellent linearity in the range of 0.08–0.8 µg/mL with a correlation coefficient (r^2) of 0.999. Accuracy was within 95–105%, precision (%RSD) was less than 2%, LOD was 0.03 µg/mL, and LOQ was 0.09 µg/mL. The method also demonstrated strong specificity and robustness.

Conclusion: The validated RP-HPLC method is simple, sensitive, precise, and suitable for the routine quality control and stability analysis of levothyroxine sodium in pharmaceutical formulations, meeting all regulatory validation criteria.

Keywords: *Levothyroxine sodium, RP-HPLC, Method Validation, ICH Q2(R1), Assay, LOD, LOQ.*

1. INTRODUCTION

Levothyroxine is chemically(2S)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl] propionic acid

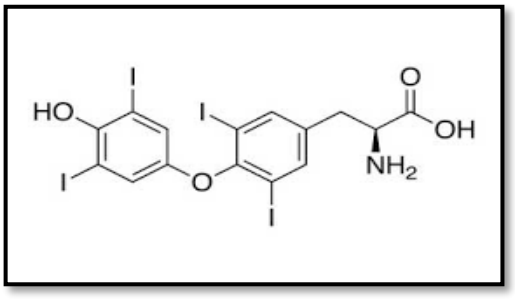

Fig. 1: Structure of Levothyroxine

The thyroid gland secretes thyroxine, a key endogenous hormone, in the synthesized form known as levothyroxine.Levothyroxine is also referred to as L-thyroxine or the brand-name product Synthroid. It is mainly used to treat hypothyroidism, a condition in which the thyroid gland is unable to produce enough of the thyroid hormones T4 (tetraiodothyronine or thyroxine) and T3 (triiodothyronine or Liothyronine), which results in decreased down-stream effects of these hormones. The symptoms of hypothyroidism, which include fatigue, elevated heart rate, depression[2], dry skin and hair, cramping in the muscles, constipation, weight gain, memory loss, and poor resistance to cold temperatures, start to appear when there are insufficient amounts of thyroid hormones going through the body.[3,4] Levothyroxinemore soluble in ethanol and very soluble in chloroform and ether. It has poor water solubility.The elimination half-life of Levothyroxine is 7.5 days.

When the pituitary gland releases Thyroid Stimulating Hormone (TSH), a healthy thyroid gland will create and release T4, which is then deiodinated (by type I or type II 5′-deiodinases)[5] to form its active metabolite T3. The majority of the physiological effects of thyroid hormones are exerted by T3, even though T4 is the main substance generated by the thyroid gland; the relative potencies of T4 and T3 are around 1:4 (T4:T3).15. Although T4 and T3 affect almost all of the body's cells, they have a particularly potent influence on the heart.[6].Thyroid health is therefore intimately related to a number of cardiac functions, such as heart rate, cardiac output, and systemic vascular resistance.[7]

➢ **Mechanism of action:**

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

Absorption ranges from 40% to 80%, primarily from the jejunum and upper ileum for orally administered T4 in the gastrointestinal tract. Increased by fasting, decreased in malabsorption syndromes, and certain foods. Absorption may decrease with age. Drugs like bile acid sequestrants, sucralfate, proton pump inhibitors, and minerals affect T4 absorption. To prevent insoluble chelates, levothyroxine should be taken on an empty stomach 2 hours before a meal and separated by 4 hours from interacting agents.T4 and T3 Metabolic Pathways are 70% of secreted T4 is deiodinated to equal amounts of T3 and reverse triiodothyronine (rT3). T4 is eliminated through sequential deiodination to T3, with 80% derived from peripheral T4. Hepatic conjugation to glucuronic and sulfuric acids is the major site of T4 and T3 elimination. Conjugated compounds reach the colon, are hydrolyzed, and eliminated as free compounds in feces. Other minor T4 metabolites have been identified.Kidneys primarily eliminate Thyroid hormones. Some reach colon, eliminated in feces. 20% of T4 eliminated in stool. Urinary T4 excretion decreases with age.

2. MATERIALS AND METHODS

Levothyroxine and its marketed formulation, Thyrox 150 tablets (Intas Pharmaceuticals), were used for the study. Analytical-grade reagents such as methanol, acetonitrile, and phosphate buffer were used, all of HPLC grade. Filtration was performed using 0.45 µm nylon and PVDF membrane filters. The chromatographic analysis was carried out using an Agilent 1260 Infinity II HPLC system equipped with a UV detector, and data was processed using OpenLabEZChrom software. Additional equipment included a Jasco UV-550 spectrophotometer, Aczet CY224C analytical balance, Thermo Scientific pH meter, and Bio-technic ultrasonicator. A reverse-phase HPLC method was developed using a C18 column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of phosphate buffer (pH 3.0) and methanol in a 55:45 v/v ratio, with a flow rate of 1.0 mL/min. Detection was performed at 225 nm with an injection volume of 10 µL. The method was validated according to ICH Q2(R1) guidelines for parameters including specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness.

- **Chromatographic condition**

The chromatographic analysis was performed using a **reverse-phase HPLC** system equipped with a **C18 column** (250 mm × 4.6 mm, 5 µm particle size). The mobile phase consisted of a mixture of **phosphate buffer (pH 3.0) and methanol in a 55:45 v/v ratio**. The flow rate was maintained at **1.0 mL/min**, with an **injection volume of 10 µL**. Detection was carried out at a **wavelength of 225 nm**, and the column temperature was kept at ambient conditions. The total run time was optimized to ensure good resolution, peak symmetry, and retention for levothyroxine.

3. CHARACTERISATION OF DRUG SUBSTANCE

- **Selection of solvent**

Table 1: Drug Solubility Summary

Sr. No.	Name of Solvent	Observation	Conclusion
1	Water	Drug Particles seen after sonication	Drug was slightly soluble in water.
2	Methanol	No Drug Particles seen after sonication	Drug was found soluble in methanol.
3	Water:Methanol (25:75 v/v)	No Drug Particles seen after sonication	Drug was found soluble in methanol.

Final Conclusion: Water:Methanol(25:75%v/v) will be used as a diluent for preparing stock solution.

- **Preparation of mobile phase** [8]

✓ **Preparation of 0.5% OPA Buffer solution:** An Accurately 0.5 mL of orthophosphoric acid was transferred in 1000mL of water, mixed well. Filtered through 0.45µ nylon membrane filter and degassed it.

✓ **Preparation of Mobile phase:** A mixture of 0.5 %OPA Buffer solution and acetonitrile was mixed in the ratio of 68:32; v/v. Mix well, sonicated to degassed it.

✓ **Preparation of Diluent:** Prepared amixture of0.1 N HCL and Methanolwas prepared inthe ratio of 30:70 v/v. mixed well.

✓ **Preparation of Blank:** Use diluents as blank.

- **Preparation of Standard Solution:**

✓ **Preparation of Levothyroxine Standard stock solution:** Weighed 20 mg Levothyroxine and dissolved in 50 mL of Diluent. (400 PPM of Levothyroxine)

Final Levothyroxine Sodium solution: Pipette out 2 mL of Levothyroxine stock solution and diluted up to 50 mL with Diluent. (16 PPM of Levothyroxine)

The API standard solutions were scanned separately between 400nm to 200nm. From the spectrum show high absorbance that select as a wavelength of drug. Selected wavelength was used for estimation of drugs. Diluent used as a Blank.

- **Preparation of Sample solution:**

Weighed and transferred 10 Levothyroxine tablets into 50 mL clean and dry volumetric flask. Added about 30 mL of diluent, sonicate for 30 minutes with intermittent shaking, at control room temperature and make volume upto mark with diluent and mix. Filter the sample solution through 0.45µ membrane nylon filter. Discard first 5.0 mL of filtrate and then collected the sample. **(Concentration of Sample Solution: 30 ppm)**

4. METHOD VALIDATION

Analytical Method Validation:

The RP-HPLC method for Levothyroxine was validated following ICH Q2(R1) guidelines. Key parameters included specificity, linearity, accuracy, precision (method and intermediate), robustness, LOD, and LOQ. The method ensures reliability and suitability for routine analysis.

1. System Suitability Test:

System suitability was assessed by evaluating chromatographic parameters like retention time, resolution, tailing factor, and theoretical plates. These parameters confirmed that the system performance was within acceptable limits before sample analysis.

2. Specificity Test (Identification, Interference, and Peak Purity):

Specificity was established by analyzing blank, placebo, and standard solutions. No interference from excipients or

degradation products was observed. **Peak purity** was confirmed using a diode array detector (DAD), ensuring the analyte peak was homogenous and pure under all conditions.

3. Linearity:

The method exhibited linearity over a suitable concentration range, typically using five concentration levels. The correlation coefficient (R^2) was found to be greater than 0.99, confirming a direct relationship between analyte concentration and response.

4. Accuracy (Recovery):

Accuracy was evaluated by recovery studies at different levels (typically 80%, 100%, and 120%). The percentage recovery was within the acceptable range, demonstrating that the method can accurately measure the drug content in formulations.

5. Precision:

- **Method Precision (Repeatability):** Six replicate injections of the same concentration showed consistent results with %RSD within acceptable limits.
- **Intermediate Precision:** Conducted by different analysts on different days using different instruments, and results confirmed method reproducibility under varied conditions.

6. Robustness:

The robustness of the method was evaluated by making small, deliberate changes in chromatographic conditions such as mobile phase composition, flow rate, pH, and column lot. The method remained unaffected, confirming its reliability under varied analytical conditions.

5. RESULTS AND DISCUSSION

- **Reverse Phase High Performance Liquid Chromatography Method Development and Optimization**

➢ **Chromatographic Condition:**

After several trials with the different combination and ratio of solvents and sharp peak.

Column	ZorbaxSB-CN,5µ,4.6 x150mm
Mobile Phase	0.5%OPABuffer solution and acetonitrile(68:32v/v)
Flow Rate	1.0mL/min
Injection Volume	10µL
Wavelength	210nm
Column Temp	40ºC
Sample Temp	10ºC
Run Time	8.0minutes

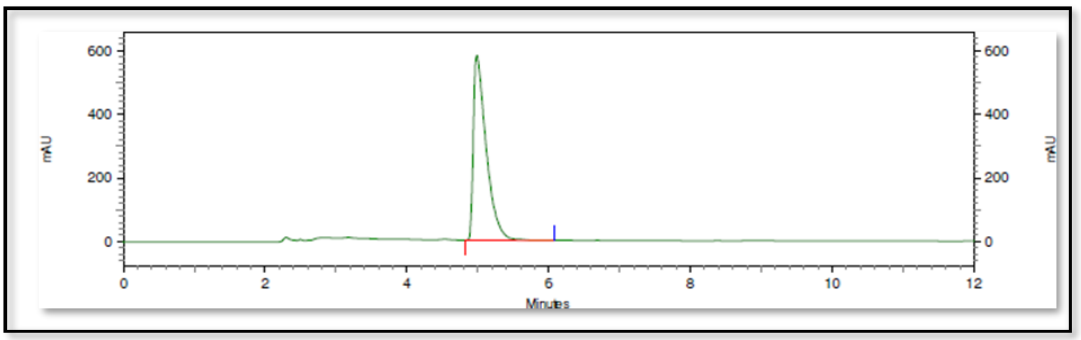

Fig. 2: Typical chromatogram for Levothyroxine

Observation: Levothyroxine eluted at 4.95 minutes with acceptable chromatography (Asymmetry: 1.23 and Theoretical plates 11265)

Conclusion:

- Method can be used for further analysis and will be subjected for validation.

➢ **Analytical Method Validation of RP-HPLC**

➢ **System Suitability:**

Table 2: System suitability test of Levothyroxine

Tailing Factor	**1.23**
Theoretical plates	**11246**
Injection No.	**Area**
1	8556204
2	8553106
3	8551426
4	8549567
5	8552664
Mean	**8552593**
%RSD	**0.05**

➢ **Conclusion:**

- The data demonstrates that the system suitability is within the acceptance criteria, thus the system is suitable.

➢ **Specificity: (Identification, Interference & Peak purity)**

Table 3: Specificity

Solution	**Specificity data**		
	Retention time (min)	**Purity Match**	
Blank solution	NA	NA	
Placebo solution	NA	NA	
Standard solution	4.94	Purity angle	Purity threshold
		2.55	3.74`
Sample solution	4.95	2.37	3.62

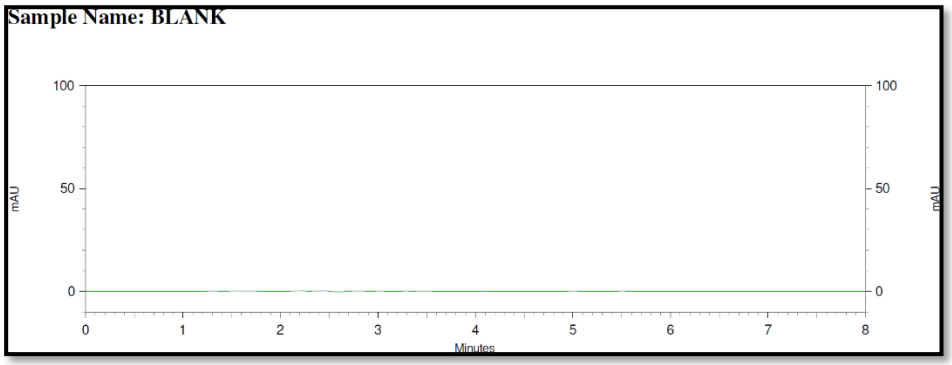

Fig 3: Chromatogram of blank

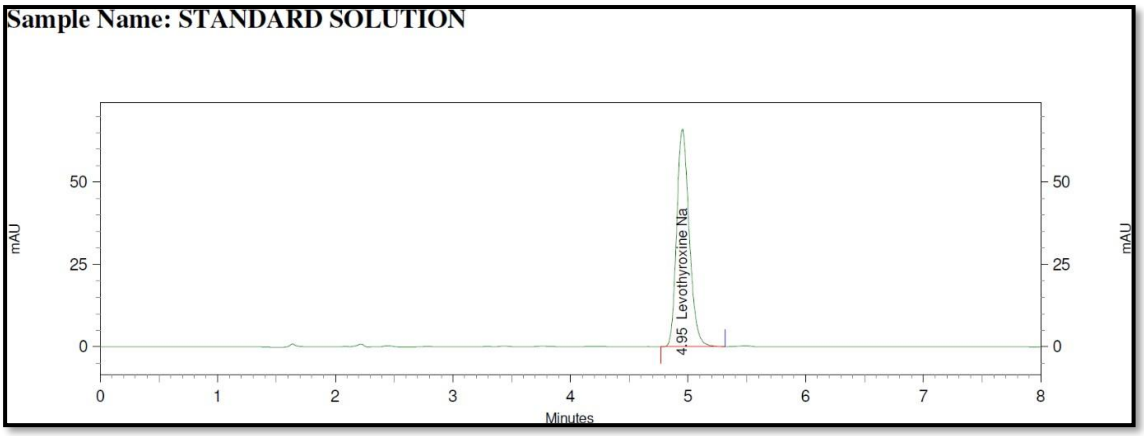

Fig 4: Chromatogram of Standard

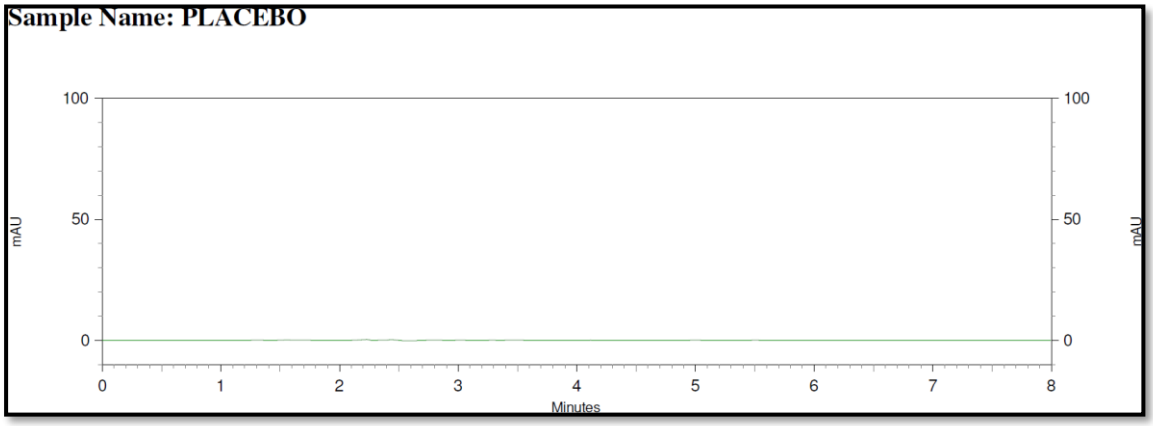

Fig 5 : Chromatogram of Sample

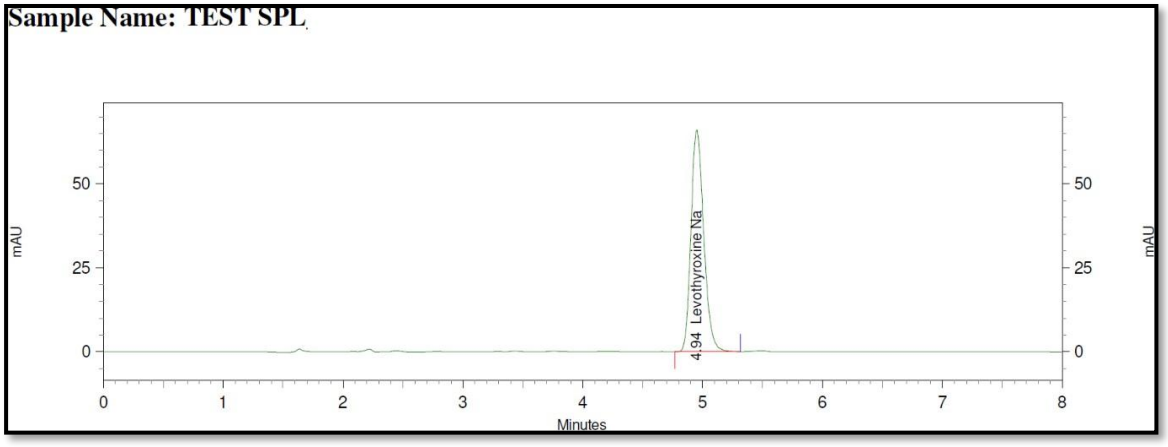

Fig 6: Chromatogram of Placebo

- **Conclusion:**
  - The data demonstrates that retention time in standard and sample is same for Levothyroxine Sodium peak.
  - The data demonstrates that here is no interference in blank and placebo at the retention time of Levothyroxine Sodium peak. Peak Purity match in both chromatograms obtained from Standard and Sample solution.

6. LINEARITY

Table 4: Linearity of Levothyroxine

Level	Conc(µg/mL)	Area	Mean
50%	15	4236521	4233206
		4229567	
		4233529	
75%	22.5	6475962	6470736
		6472651	
		6463596	
100%	30	8549521	8552680
		8553659	
		8554859	
125%	37.5	10656854	10652197
		10649853	
		10649883	
150%	45	12836950	12834633
		12835496	
		12831452	
Corr. Coeff			0.9999
Intercept			12039
Slope			284365
%Y-intercept			0.14

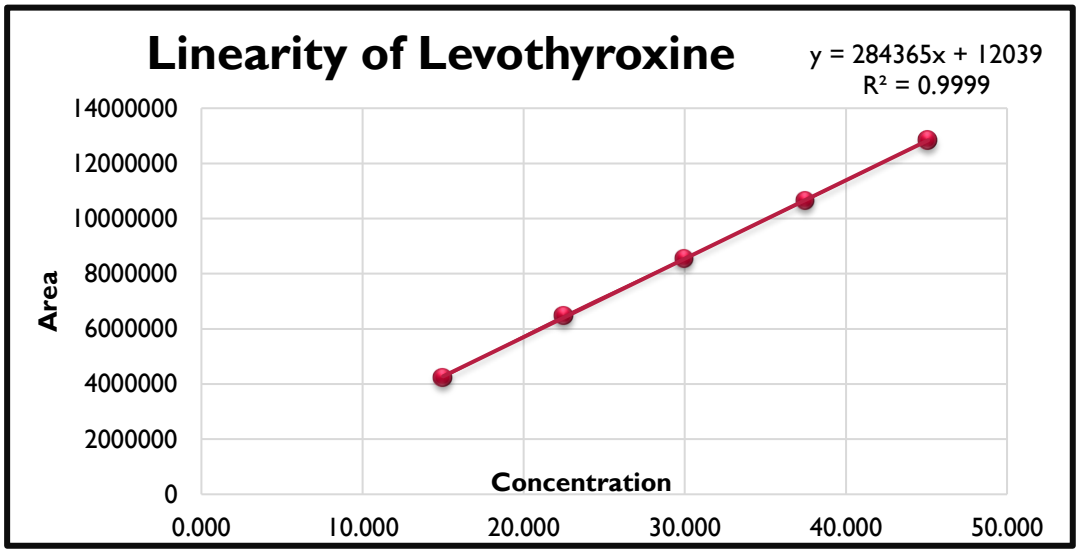

Fig 7: Linearity plot of Levothyroxine

- **Conclusion:**
  - The data shows that system suitability is fulfilled.
  - The data shows that the response is found to be linear.
  - Co-relation coefficient (r^2) was found 0.9999.

- **Accuracy (Recovery):**

Table 5:%Recovery for Levothyroxine

Level (%)	Levothyroxine Sodium Added Conc (µg/mL)	Levothyroxine Sodium Recovered conc	Area	% Recovery	Mean% Recovery
50	15.00	14.98	4259632	99.61	100.22
	15.00	15.03	4302568	100.61	
	15.00	15.02	4295416	100.45	
100	30.00	30.10	8639584	101.02	100.17
	30.00	30.05	8596521	100.51	
	30.00	29.90	8465967	98.99	
150	45.00	45.01	12836024	100.06	99.99
	45.00	45.04	12859856	100.24	
	45.00	44.95	12786520	99.67	

Conclusion:

- The data shows that the Mean recovery for 50% to 150% is in the range of 98.0%-102.0% and individual recovery for 50% to 150% is in the range of 95.0% - 105.0%.

- **Precision:**
  - **Method Precision:**

Table 6: Method precision

Sample	Area	%Assay
Sample1	8326521	97.31
Sample2	8396562	98.15
Sample3	8406372	98.25
Sample4	8369406	97.77
Sample5	8426991	98.53
Sample6	8410356	98.37
Mean		**98.07**
STDDEV		**0.4502**
%RSD		**0.459**

Conclusion:

- The data shows that system suitability is fulfilled.
- The data shows that %RSD for % Assay is within the acceptance criteria and hence the method is precise

➢ **Intermediate Precision:**

Table 7 : Intermediate Precision

Sample	**Area**	**%Assay**
Sample1	8369429	97.82
Sample2	8406522	98.34
Sample3	8396952	98.24
Sample4	8312093	97.15
Sample5	8356934	97.63
Sample6	8425668	98.50
Mean		**97.95**
STD DEV		**0.5102**
%RSD		**0.521**

Table 8 : Intermediate Precision pool Data

Parameter	**Method Precision (Analyst-I)**	**Intermediate Precision (Analyst-II)**
HPLCNO.	**AD/HPLC-02**	**AD/HPLC-04**
Column No.	**HPLC-041**	**HPLC-037**
Sample No.	**%Assay**	
1	97.31	97.82
2	98.15	98.34
3	98.25	98.24
4	97.77	97.15
5	98.53	97.63
6	98.37	98.50
Mean	98.07	97.95
Mean of Precision % Assay	**98.01**	
Absolute Mean Difference % assay	**0.5**	

➢ **Conclusion:**

- The data shows that system suitability is fulfilled.
- The data shows that %Assay is of six samples is not more than 2.0
- The data shows that %Assay is within the acceptance criteria and hence the method is rugged.

➢ **Robustness:**

Table 9: Robustness for Levothyroxine

Change in parameter	Condition	Area	Absolute difference Of %Assay
Control	As per method	8326521	NA
Changeinflowrate1.0 ml/min(±0.1ml/min)	1.1ml/min	8423530	1.2
	0.9ml/min	8193024	-1.6
Change in wavelength (±2 nm)	212nm	8202561	-1.5
	208nm	8435219	1.3

➢ **Conclusion:**

- System suitability criteria were fulfilled.

The difference of Area value in each modified condition is within acceptance criteria.

7. CONCLUSION:

The Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method proved to be effective for the estimation of Levothyroxine in tablet formulation. Its simplicity, sensitivity, and ability to handle complex samples make it highly suitable for pharmaceutical analysis. The use of HPLC Agilent 1260 Infinity II with Zorbax SB-CN column and UV/PDA detector ensured accurate detection. The mobile phase comprising 0.5% orthophosphoric acid (OPA) and acetonitrile provided optimal chromatographic results. A detection wavelength of 210 nm was selected based on the λmax obtained from UV scanning. The developed method showed good resolution, appropriate retention time, and a tailing factor of less than 2. Standard and sample solutions were prepared and analyzed using validated conditions. The results were reliable and consistent, indicating high method precision. This validated method was effectively applied for the quantification of Levothyroxine in pharmaceutical dosage form. Overall, the RP-HPLC technique demonstrated strong potential for routine quality control analysis of Levothyroxin

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