High-performance liquid chromatographic-tandem mass spectrometric (HPLC-ESI MS/MS) method for the estimation of Levothyroxine in Human serum

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Abstract

A simple, sensitive and high throughput, liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method has been developed for the estimation of Levothyroxine (LT4) in human serum.The method involved protein precipitation with acetonitrile with Levothyroxine D3(IS) as an internal standard form 0.100 mL of human serum.The chromatographic analysis was achieved on a Sciex API 4000 (Zorbax Eclipse XDB C18, (4.6 X 150) mm, 5 µm) analytical column using isocratic mobile phase, consisting Acetonitrile: 0.1% Glacial Acetic Acid in water, 60:40 v/v, at a flow-rate of 0.600 mL/minute. The precursor → product ion transition for levothyroxine (m/z 777.800 → 731.700), and IS (m/z 780.700→ 734.800) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ionization mode. The method is validated over a wide dynamic concentration range of 25.000 ng/mL to 600.000 ng/mL. Matrix effect was assessed and the mean extraction recovery was greater than 65.0 % for levothyroxine and IS in stripped and unstripped human serum. The method is rugged and rapid with a total run time of 4.0 min and was used in a clinical study with 36 healthy subjects. The assay reproducibility was successfully demonstrated by reanalysis of 144 subject samples.

Keywords: Levothyroxine, HPLC-MS/MS; Protein precipitation; bioequivalence; high throughput; sensitive

Introduction

During the latter half of the nineteenth century, medical professionals increasingly recognized thyroid disease as a distinct clinical entity, marking a significant turning point in the understanding and management of endocrine disorders. This period saw pivotal advancements in the characterization of thyroid conditions. [1,2,3,4]

It took significant time for synthesized LT4 to establish itself as the primary treatment for hypothyroidism. The widespread use of thyroid extract-based products didn't notably decline until the latter part of the 1960s. This delay was primarily due to challenges in ensuring the consistency of their biological effects and their limited shelf life. [2,5] Despite the introduction of stringent regulatory standards for LT4 tablet manufacturing, which guarantee consistent dosing, desiccated thyroid products remain available for therapeutic use today. Their persistence is notable, despite a lack of compelling objective evidence demonstrating superior efficacy in managing hypothyroid symptoms.[5, 6]

Levothyroxine sodium is an FDA-approved medication available in oral and injectable forms. It's prescribed as a treatment for underactive thyroid (hypothyroidism) and various other conditions. Hypothyroidism refers to a state where the thyroid gland produces insufficient thyroid hormones.[8] Thyroid hormone is available as levothyroxine, which is biologically equivalent to your own thyroid hormone, thyroxine (T4).[5] Levothyroxine works by replacing or supplementing these hormones, which are essential for normal mental health, physical activity, and overall development, especially in children. Factors like natural deficiencies, thyroid gland damage from radiation or medications, or surgical removal can lead to low thyroid hormone levels, highlighting the importance of levothyroxine therapy in managing such conditions. [8, 9]

Various methods have been developed for quantification of LT4 and other thyroid hormones in various matrices such as human plasma, Human serum and Rat serum.[12-29]
The method presented has the highest extensive range of linearity and lowest LLOQ of 25.000 ng/ml to 600.000 ng/ml.

Research Methodology

Chemical and materials

Reference standard Levothyroxine sodium (92.09%) and internal standard Levothyroxine D3 (93.31%), were obtained from bio-organics. Glacial Acetic Acid (ACS/AR/GR grade) obtained from Merck, Acetonitrile (HPLC/ULC/MS grade) and Methanol (HPLC/LC-MS/ULC/MS grade) procured from Biosolve, Activated Charcoal powder obtained from Rankem and Sigma aldrich, Water (HPLC grade/Type I Water) was obtained from Rankem. Inhouse Blank Human Serum stored at -20ºC until used.

Liquid chromatography and mass spectrometry conditions

Chromatographic analysis was conducted on an AB SCIEX API 4000 equipped with Agilent 1290 Infinity II HPLC and Zorbax Eclipse XDB C18, (4.6 X 150) mm, 5 µm column, that was maintained at 40ºC in a column oven. The mobile phase consisted of Acetonitrile: 0.1% Glacial Acetic Acid in water, 60:40 v/v and was delivered at a flow rate of 0.600 mL/minute.

Standard stock solutions, calibration standards and quality control samples

The stock solution of Levothyroxine (0.200 mg/mL) was prepared by dissolving the accurately weighted reference standard in methanol. Calibration standards and quality control (QC) samples were prepared by spiking mixed CC intermediate and mixed QC intermediate solution respectively. Calibration curve standards were made at concentrations of 25.000, 50.000, 60.000, 120.000, 300.000, 420.000, 510.000, 600.000 ng/mL, whereas high, medium1, medium2 and low QC samples were prepared at concentrations of 450.000, 180.000, 90.000 and 75.000 ng/mL respectively. The LLOQ QC prepared at 25.000 ng/ml.

A stock solution (0.200 mg/mL) of the Levothyroxine D3 was prepared by dissolving the accurately weighted respective reference standard in methanol. Its ISTD working solution 1000.000 ng/mL was prepared by appropriate dilution of the stock/ ISTD intermediate solution in methanol. All solutions (standard stock, calibration standards and QC samples) were stored at 5 ºC until use.

Sample preparation

Treated (stripped) blank human serum was prepared as per following procedure. Weighed about 2.00000 g of Activated Charcoal powder and added into 40 mL volume of blank human

serum and vortexed to mixed. Serum were Rotated on LLE Extractor at 40 rpm for 12 hours. The serum was centrifuged at 4000 rpm at 10˚C for 90 minutes. After centrifugation the serum was filtered with cotton bids to remove charcoal and collected the stripped serum filtrate. Collected stripped serum was stored into deep freezer maintained at -20± 5°C.

Method validation procedures

The method was validated for human Serum following the ICH guideline M10 on bioanalytical method validation and study sample analysis. [29]

Levothyroxine is a synthetic T4 hormone that is biochemically and physiologically indistinguishable from the natural one, and it is administered when the body is deficient in the natural hormone. All the method validation experiments were performed in charcoal stripped and unstripped serum. Charcoal stripping of blank serum is done to remove interfering substances that could affect the accuracy and precision of levothyroxine (including endogenous thyroxine) quantification in bioanalytical assays.

A system suitability experiment was performed by injecting six consecutive injections using aqueous system suitability or extracted (Stripped) system suitability samples of levothyroxine 150.000 ng/mL and IS 1000.000 ng/mL once in a day and additionally in other occasions.

Intra-batch accuracy and precision was evaluated by replicate analysis of serum samples on same day. The analytical run consisted of calibration curve and six replicates of LLOQ QC, LQC, MQC2, MQC1, HQC samples. QC samples were prepared in treated (stripped) as well as un-treated (unstripped) human serum. Accuracy with stripped and unstripped QC samples was evaluated separately in an individual run. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three different days. The precision at each concentration level of QC samples should be ≤ 15.00% and for the LLOQ QC it should be ≤ 20.00%. [29]

The % recovery for analyte and internal standard (ISTD) was determined by comparing the mean peak area of the analyte and internal standard in 6 replicates of extracted samples against mean peak area of 6 replicates of post-extracted spiked quality control samples representing 100% recovery at high, middle and low concentrations. Extracted and post-extracted spiked samples will be prepared using stripped and unstripped human serum.

Application of method and incurred sample reanalysis.

After an overnight fasting of at least 10.00 hrs. prior to scheduled time of dosing, all subjects were administered single oral dose of one tablet of either test product (T) or one tablet of reference product (R) orally as per randomization sequence in sitting posture with 240 ± 02 mL of water at room temperature. Blood samples (4.0 mL) were collected at pre-dose -0.50, -0.25, 0.00 hours collected in morning on dosing day prior to dosing and post dose 0.50, 1.00, 1.50, 1.75, 2.00, 2.25, 2.50,2.75, 3.00, 3.25, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 18.00, 24.00, 36.00, 48.00 and 72.00 hours post dose in each study period.

Experimental

Method development

The three commonly used extraction methods are Liquid-Liquid extraction (LLE), Solid phase extraction (SPE) and protein precipitation (PPT). To develop a selective, rugged and reliable method for the estimation of levothyroxine in human serum all the extraction procedures were systematically investigated. The chromatographic and mass spectrometric conditions were suitably optimized to get the desired sensitivity, selectivity and linearity in regression curves.

Mass spectrometry

Levothyroxine was tuned in the electrospray ionization (ESI) mode with both negative and positive polarity. However, the electrospray ionization (ESI) mode with positive polarity was provided a good signal. The levothyroxine showed an intense protonated molecular ion at m/z 777.800 under positive turbo ion spray ionization. The collision-induced dissociation of this ion formed a distinctive product ion at m/z 731.700. The selected reaction monitoring (SRM), based on the m/z 777.800 -731.700 transition, was used for Levothyroxine (Fig. 01). This achieved an LLOQ of 25.00 ng/mL for Levothyroxine using a 0.100 mL serum sample and injecting 2 µL into the Agilent HPLC 1290 Infinity II coupled with AB Sciex API 4000 triple Quadrupole mass spectrometer. The most abundant and consistent common product ions in Q3 MS spectra for Levothyroxine were observed at m/z 731.700 by applying collision energy of 35 eV. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and adequate response to the analyte. A dwell time of 200 ms for the drug and its IS was adequate and accurate and precise MRMs was finalized. Furthermore, to reduce in-source fragmentation, cone

voltage, desolvation temperature and cone gas flow were fine tuned without compromising levothyroxine response.

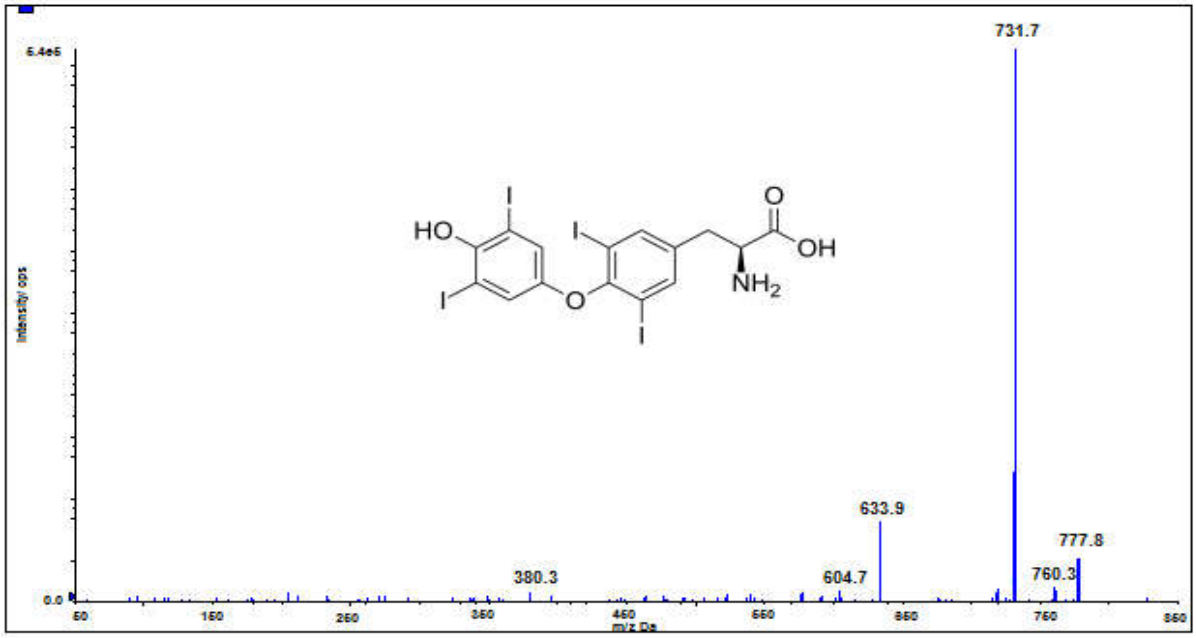


Fig. 01: Product ion mass spectra of Levothyroxine (m/z 777.8-731.7, Scan range 50-850 Da) in positive ionization mode

Optimization of extraction technique

Reported procedures for the estimation of levothyroxine in human serum have used either LLE, solid phase extraction, Protein Precipitation for sample preparation with little or no information on ion suppression or matrix interference. All three extraction procedure were performed during extraction trial. Considering the chemical structures of analytes and levothyroxine protein binding, protein precipitation finalized by using the various combinations of organic solvents like methanol, acetonitrile, acetonitrile with glacial acetic acid. The samples were precipitated with the 0.5% Glacial Acetic Acid in Acetonitrile to serum, centrifuged to separate the supernatant and gave good response and desired recovery through the extraction.

Matrix preparation

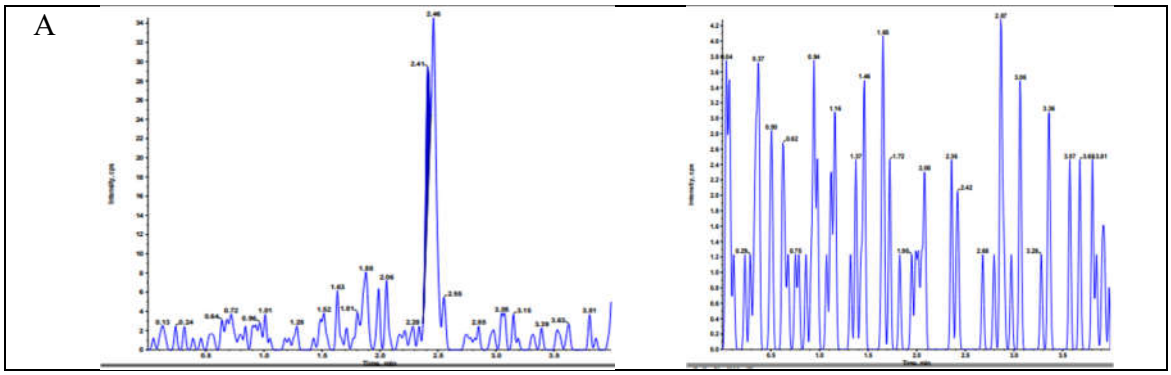
Good response and good peak shape of analyte and ISTD observed. However, inconsistent ISTD area was observed. Hence, decided to do charcoal stripping treatment of serum to avoid interference and to minimize presence of endogenous levothyroxine from matrix. The mechanism

of charcoal stripping involves adsorption, a process where molecules adhere to the surface of the charcoal. Good chromatography and consistent ISTD area observed in stripped serum.

Optimization of chromatographic conditions

To have a rugged and efficient chromatography, efforts were made to minimize matrix interference, achieve adequate run time in order to ensure high throughput and attain high sensitivity with good peak shapes. The analytical potential of three different reversed- phase columns was evaluated namely, Agilent Eclips plus C8 100, Thermo Hypurity C 18, 50*4.6, and Agilent Eclipse XBD C18, 150. Separation was tried using various combinations of 0.1% AA in ACN: 0.1%AA in Water:: 50:50, 01%GAA in water :ACN ::65:35, 60:40 0.1% GAA in waterman, on these columns to find the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. , it was required to wash out the retained interferences in the column after elution of the analyte. Hence, careful optimization of chromatography was needed. In the present work, the best chromatographic conditions as a function of analyte peak intensity, peak shape, adequate retention and analysis run time were achieved with Zorbax Eclipse XDB C18, (4.6 X 150) mm, 5 µm using Acetonitrile: Mobile Phase Buffer (0.1% Glacial Acetic Acid in water) (60:40 v/v) mobile phase and flow rate 0.600ml/min. The total chromatographic run time was 4.0 min with a retention time at about 2.40 min. The sensitivity achieved for levothyroxine was 25.00 ng/mL.

Representative MRM ion chromatograms of extracted blank human serum (double blank) at LLOQ for levothyroxine (Fig.2) demonstrated the selectivity of the method.



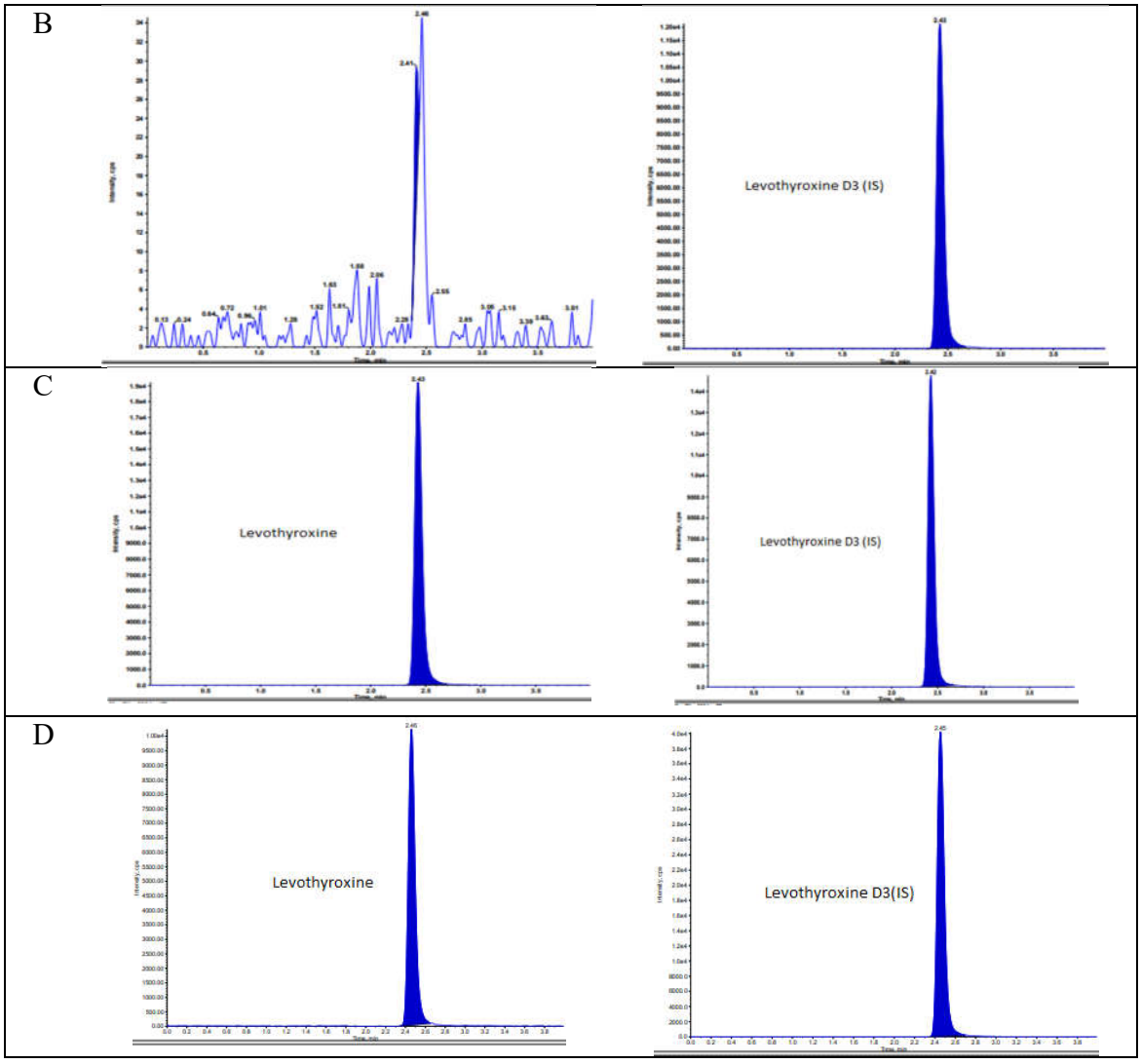


Fig.2 Representative MRM ion-chromatograms of (A) double blank (without Levothyroxine and IS), (B) blank serum with IS, (C) Levothyroxine at LLOQ and IS, and (D) subject sample at 1.00 hr after administration of Levothyroxine Sodium capsules 0.2 mg (3 x 0.2 mg).

Results and discussions

Results for method validation

For system suitability the % CV of analyte RT and ISTD RT and Area ratio was observed ≤ 2.47% for Levothyroxine. Peak Area (Signal of Levothyroxine) for system performance was observed as ≥ 10.8. Carryover evaluation was performed in each analytical run to ensure that it did not affect the accuracy and the precision of the proposed method. During the validation levothyroxine Carryover observed as ≤ 7.94 % and for IS ≤ 0.08 %.

The calibration curves were linear over the concentration range of 25.000–600.000 ng/mL Levothyroxine.

The intra-batch precision in stripped serum (CV) ranged from 0.997 to 2.61% and the accuracy was within 98.83 % and 106.37%. For the inter batch experiments in stripped serum, the precision varied from 2.46 to 3.67% and the accuracy was within 100.02 % and 106.11% for Levothyroxine (Table 1).

Table 1: Intra-batch and inter-batch precision and accuracy of Levothyroxine stripped

QC	Nominal Concentration (ng/mL)	Intra batch				QC	Nominal Concentration (ng/mL)	Inter batch			
		n	Mean concentration found (ng/mL)*	Accuracy (%)	CV (%)			n	Mean concentration found (ng/mL)↑	Accuracy (%)	CV (%)
HQC	450.000	6	473.2945	105.18	0.97	HQC	450.000	30	472.594	105.02	3.14
MQC1	180.000	6	190.2913	105.72	2.61	MQC1	180.000	30	191.006	106.11	2.46
MQC2	90.000	6	95.7317	106.37	1.68	MQC2	90.000	30	94.472	104.97	2.87
LQC	75.000	6	77.6435	103.00	1.29	LQC	75.000	30	78.036	104.05	2.67
LLOQ QC	25.000	6	24.7072	98.83	2.64	LLOQ QC	25.000	30	25.006	100.02	3.67

* Mean of six replicates at each concentration

↑Mean of six replicates of five Precision and accuracy batches

The intra-batch precision in unstripped serum (CV) ranged from 1.08 to 1.96% and the accuracy was within 98.61 % and 105.83%. For the inter batch experiments in stripped serum, the precision varied from 2.73 to 3.81% and the accuracy was within 102.52% and 106.53% for Levothyroxine. (Table 2)

Table 2:Intra-batch and inter-batch precision and accuracy of Levothyroxine unstripped

QC	Nominal Concentration (ng/mL)	Intra batch				QC	Nominal Concentration (ng/mL)	Inter batch			
		n	Mean concentration found (ng/mL)*	Accuracy (%)	CV (%)			n	Mean concentration found (ng/mL)↑	Accuracy (%)	CV (%)
HQC	498.880	6	527.9787	105.83	1.08	HQC	498.880	30	531.4524	106.53	2.76
MQC1	228.880	6	239.6132	104.69	1.22	MQC1	228.880	30	242.8346	106.10	2.73
MQC2	138.880	6	143.4888	103.32	1.30	MQC2	138.880	30	146.5818	105.55	3.07
LQC	123.880	6	127.4675	102.90	1.12	LQC	123.880	30	129.4244	104.48	3.51
LLOQ QC	73.880	6	72.8545	98.61	1.96	LLOQ QC	73.880	30	75.7384	102.52	3.81

* Mean of six replicates at each concentration

↑Mean of six replicates of five Precision and accuracy batches

The Overall % recovery (Stripped) for Levothyroxine 68.67% and 72.30% for and Levothyroxine D3. The Overall % recovery (Unstripped) for Levothyroxine 75.50% and 73.92% for Levothyroxine D3.

The autosampler stability of the spiked quality control samples maintained at 10ºC was determined up to 78.0 hrs. without significant drug loss in stripped and unstripped serum. All stability results in serum at two QC levels are shown in Table 3.

Table 3: Stability results of Levothyroxine

Stability of the Levothyroxine in human Serum (n=6)			
Storage condition	Nominal concentration (ng/mL)	Mean Stability samples(ng/mL) ± SD	% Mean Stability
Bench Top Stability (BT) for 25 Hours (Yellow Monochromatic Light) at ambient temperature in Serum(Stripped)			
HQC	450.000	443.7965± 7.99880	98.62
LQC	75.000	72.4312± 2.36815	96.57
Bench Top Stability (BT) for 25 Hours (Yellow Monochromatic Light) at ambient temperature in Serum(Unstripped)			
HQC	498.880	518.7895± 12.51118	103.99
LQC	123.880	125.7937± 2.54531	101.54
Freeze and Thaw stability 5 cycles at -20±5ºC (stripped)			
HQC	450.000	440.5548± 3.59695	97.90
LQC	75.000	74.0220± 1.47509	98.70
Freeze and Thaw stability 5 cycles at -20±5 ºC (Unstripped)			
HQC	498.880	527.0167± 9.84766	105.64
LQC	123.880	121.8567± 1.59566	98.37
Freeze and Thaw stability 5 cycles at -70±15 ºC (Stripped)			
HQC	450.000	443.7238± 5.75613	98.61
LQC	75.000	73.7113± 1.64702	98.28
Freeze and Thaw stability 5 cycles at -70±15 ºC (Unstripped)			
HQC	498.880	514.4403± 12.42708	103.12
LQC	123.880	123.6818± 2.42266	99.84
Long term stability in serum at -20±5ºC (Stripped) 97 days			
HQC	450.000	441.4543±5.19259	98.10
LQC	75.000	74.1488±2.02902	98.87
Long Term Stability in Serum at -70±15˚C (Stripped) 97 days			
HQC	450.000	444.3502±7.47154	98.74
LQC	75.000	73.3458±1.61289	97.79
Long term stability in serum at -20±5ºC (Unstripped) 96 days			
HQC	498.880	519.0187±6.97619	104.04
LQC	123.880	123.3108±1.71565	99.54
Long term stability in serum at -70±5ºC (Unstripped) 96 days			
HQC	498.880	527.016±79.84766	105.64
LQC	123.880	121.8567±1.59566	98.37

For method ruggedness, the precision (CV) and accuracy values for Levothyroxine with different columns (Stripped) ranged from 1.66 to 3.61% and 103.65 to 105.48% respectively. The precision (CV) and accuracy values for different columns (Unstripped) ranged from 1.13 to 2.96% and 106.09 to 108.46% respectively. The experiment with different analysts with stripped serum, the results ranged from 0.94 to 3.70% and 98.32 to 105.82% for precision and accuracy of Levothyroxine respectively. The experiment with different analysts with unstripped serum, the results ranged from 0.38 to 2.43% and 100.56 to 102.78% for precision and accuracy of respectively. The precision for dilution integrity of 1/2 and 1/10th dilution were 0.70% and 1.72%, and the accuracy results were 96.77% and 99.77% for Levothyroxine in unstripped serum and precision for dilution integrity of 1/2 and 1/10th dilution were 0.96% and 2.44%, and the accuracy results were 103.53% and 101.70% for Levothyroxine in unstripped serum which is well within the acceptance limits of 15% for precision (CV) and 85 to 115% for accuracy.

Application of the method and incurred sample reanalysis (ISR)

The developed method was used to estimate levothyroxine concentration in human serum samples after administration of three tablets of 3 x 0.2 mg oral dose of levothyroxine. Fig. 3 shows the mean serum concentration vs. time profile of levothyroxine in healthy subjects. The method was sensitive enough to monitor levothyroxine concentration up to 72.00 h. The mean pharmacokinetic parameters and log transformed geometric least squares mean values for C_{max} and AUC_{0-t}, under fasting conditions are summarized in Table 4. The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125%, which shows bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. Furthermore, there was no adverse event during the course of the study.

Table 4: Mean pharmacokinetic parameters and 90% CIs of natural log (Ln)-transformed parameters following oral administration of Levothyroxine sodium capsule, 0.2 mg (3 x 0.2 mg) oral dose 36 healthy adult human subjects.

Parameter	Geo LS Mean Test	Geo LS Mean Ref	Ratio (test/reference) (%)	90% confidence interval (Lower-Upper)	Intra-subject variation (%CV)
C_{max} (ng/mL)	95.49	98.66	96.78	91.90-101.93	13.20
AUC $_{0-t}$ (h ng/mL)	2892.64	2774.24	104.27	100.69-107.98	14.83

Conclusion

The HPLC-ESI-MS/MS method for quantitation of levothyroxine in human serum was developed and fully validated as per ICH M10 guidelines. A total of 1872 samples were analyzed during a period of 43 days, which included calibration, QC and subject samples and the precision and accuracy were well within the acceptable limits. The advantages of this method include high sensitivity, small sample volume for processing and short chromatographic run 4.0 min. based on dilution reliability results it is possible to extend the ULOQ to 1080.000ng/ml for stripped serum and 1128.880 ng/mL for unstripped. In addition, assay reproducibility is effectively proved by reanalysis of 144 subject samples.

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