Quantitative Analysis of Free and Total Thyroid Hormones and Metabolites in Serum using LC-MS/MS with and without Derivatization for Clinical Research

Rory M. Doyle, Douglas McDowell, John H. Butler; Thermo Scientific, 265 Davidson Avenue, Somerset, NJ 08873

ABSTRACT

Introduction: Thyroid hormones are a diverse group of biologically active compounds that physiologically impact the body's functions. Their analysis can be challenging due to their structural similarities and ultra-low levels in serum and include thyroxine (T4), 3,3', 5-triiodothyronine (T3), and 3 ,3', 5'-triiodothyronine (rT3). A simple, sensitive and specific LC-MS/MS analytical method was developed and optimized for the quantitation of the free and total thyroid hormones and their metabolites in serum using ultracentrifugation, protein precipitation and liquid-liquid extraction sample preparation with and without derivatization. The analytical methods achieved good analyte recovery, accuracy, post-extraction cleanliness, and are capable of the low sensitivities to quantitate the thyroid hormones in serum over their dynamic range.

Methods: A Thermo Scientific™ TSQ Altis™ tandem mass spectrometer in positive and negative electrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized. 200 ml of serum was used for the analysis of the free and total thyroid hormones with and without derivatization. A Thermo Scientific™ Accucore™ C18 50 × 2.1 mm, 2.6 um column with a water:methanol mixture containing 0.1% acetic acid was used. Quantitative analysis was performed using selective reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive and negative mode due to the compounds different chemistries and structures. The accuracy of these methods were verified using standardized reference materials and the various derivatization techniques have been evaluated and optimized.

Preliminary Data: Optimal results for free and total underivatized thyroid hormones were achieved using positive electrospray ionization (ESI), and were at least 5 fold better in response than in negative mode. The sample preparation investigated and compared included protein precipitation and liquid-liquid extraction (LLE) for total thyroid where only 200 µl of serum was used. 200 µl of human serum was used for the analysis of free thyroids and the sample preparation investigated included ultracentrifugation using Amicon centrifugal units followed by a protein precipitation and liquid-liquid extraction. Good linearity and reproducibility were obtained with the concentration range from 1 pg/ml to 1000 ng/ml for the underivatized total thyroid hormones and 1 pg/ml to 1000 pg/ml for free thyroid hormones with a coefficient of determination R2>0.98 in serum. The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were determined for the free and total underivatized thyroid hormones and the analytical method demonstrates the ultra-low level pg/ml sensitivity required for free and total thyroids. Excellent reproducibility was observed for both compounds (CV < 10%) for all calibration levels and QC material. Accuracy and precision of these measurements were within the required limits (±10%). The derivatization of the free and total thyroids using butyl esterification has initially shown improved sensitivities and the analytical method is being optimized and finalized to achieve better ultra-low level detection in serum.

INTRODUCTION

The thyroid hormones are tyrosine-based hormones produced by the thyroid gland that are primarily responsible for increasing the basal metabolic rate, affect protein synthesis, regulate long bone growth and neural maturation and activity, generate or inhibit heat, and increase the sensitivity to catecholamines and enhance physical activity. The thyroid hormones are essential to proper development and differentiation of all cells and can further regulate protein, fat, and carbohydrate metabolism as well as stimulating vitamin metabolism.

In this research study, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of thyroid hormones and their metabolites that include thyroxine (T4), 3,3', 5-triiodothyronine (T3) and 3,3', 5'-triiodothyronine (rT3), The sample preparation choices were kept simple and included protein precipitation and a one step liquid-liquid extraction for the analysis of total thyroid hormones and ultracentrifugation followed by protein precipitation and a one step liquid-liquid extraction for the analysis for free thyroid hormones analysis. Further investigation as to improved analysis and detection was carried out using butyl ester derivatization of the thyroid hormones only where the one step liquid-liquid extraction employed. The methodologies were developed on a TSQ Altis tandem mass spectrometer in positive and negative electrospray ionization modes with a Vanquish HPLC system for a 6-min analytical gradient.

MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from IsoSciences, King of Prussia, PA:

Thyroxine (T4):	100 mg/mL	Thyroxine-13C6:	100 mg/mL
3,3', 5-triiodothyronine (T3):	100 mg/mL	3,3', 5-triiodothyronine-13C6:	100 mg/mL
3,3', 5'-triiodothyronine (rT3):	100 mg/mL	3,3', 5-triiodothyronine-13C6:	100 mg/mL
3,3'- diiodothyronine (3,3'-T2):	100 mg/mL	3,3'- diiodothyronine-13C6:	100 mg/mL

Reagents

The following Fisher Scientific acids, reagents, and solvents were used:

HPLC Grade Water
Methanol
Acetonitrile
Hydrogen chloride - 1-butanol solution
Acetic Acid
7N Ammonia in Methanol
Ethyl Acetate

The standards and internal standards were made up in 0.1N Ammonia in Methanol to prevent iodine migration.

Total Sample Preparation: Protein Crash

- 200 mL of Serum/HSA mixture calibrators, controls and serum sample were added to 1.5 ml Eppendorf tubes and 20 mL of Thyroid ISTD mixture at 200 ng/mL were added to each tube and vortexed briefly.
- 400 mL of Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 min at 13000 rpm.
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators were prepared in thyroid depleted serum and water (Golden West Biological, Inc., Temecula, CA).

Total Sample Preparation: Liquid-Liquid Extraction

- 200 mL of Serum/HSA mixture calibrators, controls and serum samples were added to a test tube and 20 mL of Thyroid ISTD mixture at 200 ng/mL were added to each and vortexed briefly.
- 200 mL of Acetonitrile was added to each tube and vortexed for 1 min.
- 1.2 mL of Ethyl Acetate was added to each tube and vortexed for 1 min prior to centrifugation for 10 min at 13000 rpm.
- The upper organic layer was transferred to a new test tube and dried down under nitrogen at room temperature.
- The extract was reconstituted in 200 mL of 3:1 water and methanol.
- The supernatant was transferred to an MS vial and capped.

Free Sample Preparation: Ultracentrifugation

- 400 ml of serum sample, calibrator matrix, controls was added to an Amicon™ Ultra 0.5 ml, Ultracel 10 membrane, 10 KDa Centrifugal Filter unit prior to centrifugation for 60 min at 13200 rpm at 37 °C.
- 200 ml of the filtrate was removed to a new tube and 20 ml ISTD at 1000 pg/ml were added to each tube and the calibrators were spiked with standards to the desired concentration and vortexed briefly.
- The extraction process was continued as described above for the total sample preparation-protein crash and liquid-liquid extraction protocol.

Derivatization Sample Preparation: Butyl Esterification

- The dried extracts produced following liquid-liquid extraction were derivatized using 50 µL of Butanol in 3M HCl for 15 min at 65 °C.
- Due to the acidity of the reagent, the liquid was removed by heated nitrogen flow at 40 °C.
- The samples were reconstituted in 200 µL of 3:1 water and methanol.
- The supernatant was transferred to an MS vial and capped.

The calibration curves ranged from 1 pg/mL to 1000 ng/mL and various pooled samples were used as control material.

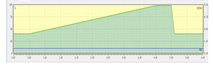
Data Analysis

The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 software, Thermo Scientific™ TSQ Altis Tune™ 2.1 software and Thermo Scientific™ Tracefinder™ 4.1 software.

HPLC Conditions

Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore C18, 50 x 2.1 mm, 2.6 mm
Column Temperature:	50 °C
Injection Volume:	20 mL
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 sec
Mobile Phase A:	0.1% Acetic Acid
Mobile Phase B:	Methanol
Flow Rate:	0.5 ml/min
Gradient:	0.0 min 60%A:40%B
	0.5 min 60%A:40%B
	4.5 min 2%A:98%B
	5.0 min 2%A:98%B
	5.1 min 60%A:40%B
Run time:	6 min



MS and Ion Source Conditions: Underivatized

TSQ Altis triple quadrupole mass spectrometer

Ion Mode:	Positive and negative electrospray (H-ESI) Mode
Vaporizer Temperature:	300 °C
Ion Transfer Tube Temperature:	275 °C
Sheath Gas:	42
Aux Gas:	15
Sweep Gas:	0
Spray Voltage:	Positive ion (V):3925 V Negative ion (V):3750 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle Time (sec):	0.8
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 sec

MS and Ion Source Conditions: Derivatized

TSQ Altis triple quadrupole mass spectrometer

Ion Mode:	Positive and negative electrospray (H-ESI) Mode
Vaporizer Temperature:	300 °C
Ion Transfer Tube Temperature:	275 °C
Sheath Gas:	42
Aux Gas:	15
Sweep Gas:	0
Spray Voltage:	Positive ion (V):2000 V Negative ion (V):2500 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle time (sec):	0.8
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 sec

Table 1. Scan parameters - SRM table.

Compound	Rt (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
3,3',5-Triiodothyronine-T3	3.31	Positive	651.84	605.7/478.9	21.9/34.4	99
		Negative	649.84	126.9/632.8	45.3/19.1	108
3,3',5-Triiodothyronine-13C6	3.31	Positive	657.67	611.7/484.9	22.1/34.4	99
		Negative	655.84	126.9//638.8	45.1/19.4	111
3,3',5'-Triiodothyronine-rT3	3.63	Positive	651.83	605.8/507.8	22.8/23.8	103
		Negative	649.98	126.9/478.9	51.9/22.1	130
3,3',5'-Triiodothyronine-13C6	3.63	Positive	657.8	611.8/513.9	22.7/37.2	104
		Negative	655.8	126.9/484.9	54.9/22.4	125
Thyroxine-T4	3.82	Positive	777.74	731.7/604.8	24.8/39.2	116
		Negative	775.31	126.9/604.8	47.3/20.3	140
Thyroxine-13C6	3.82	Positive	783.77	737.4/610.8	24.7/39.2	116
		Negative	781.54	126.9/610.9	46.1/20.6	135
3,3',5-Triiodothyronine-T3-Butyl	2.83	Positive	707.83	605.8/478.9	24.3/38.3	102
		Negative	705.79	126.9/448.7	41.9/26.2	110
3,3',5-Triiodothyronine-13C6-Butyl	2.83	Positive	713.83	611.8/484.9	18.4/38.4	98
		Negative	711.83	126.9/454.8	42.9/26.2	115
3,3',5'-Triiodothyronine-rT3-Butyl	3.16	Positive	707.85	605.8/507.9	24.4/24.6	98
		Negative	705.83	126.9/575.7	55/31.6	128
3,3',5'-Triiodothyronine-13C6-Butyl	3.16	Positive	713.85	611.8/513.9	23.9/25.1	96
		Negative	711.85	126.9/581.7	53.1/31.7	112
Thyroxine-T4-Butyl	3.17	Positive	833.75	777.8/731.8	19.8/27.3	132
		Negative	831.71	126.9/574.8	47.9/31.5	122
Thyroxine-13C6-Butyl	3.17	Positive	839.75	783.8/737.8	20.1/26.2	109
		Negative	837.75	126.9/580.8	52.8/31.4	120

RESULTS

Linearity/Sensitivity

The assays were linear over the calibration curve for the total and free thyroids in thyroid depleted serum/water mixture as shown in the table with their mean of coefficient of determinations (R2) for positive and negative mode and all sample preparation techniques. The linearity of each extraction was determined in triplicate over 3 days and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination (R2) > 98 for each sample extraction technique and the %CV for each calibration point were all <10% in order to be accepted. The analysis of total and free thyroid hormones underivatized and derivatized by positive mode electrospray using the LC and source conditions shown were found to be more sensitive than negative mode with a 5 to 10 fold difference in mass spectral response.

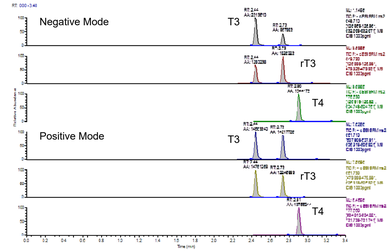
Precision/Specificity

The inter–assay precision and accuracy for total and free T4, rT3 and T3 was determined by extracting and quantifying in-house control material resulting in %CV for T4, rT3, T3 of <10% deviation form the targeted mean.

Therefore, the analytical method was determined to work best for the liquid-liquid extraction in positive mode for total and free thyroid hormones and butyl derivatization of these compounds resulted in a further 10 fold increase in sensitivity. The use of either analytical technique can achieve the laboratory required accuracy for the analysis of total and free thyroid hormones in serum.

Figure 1. Chromatograms underivatized, derivatized, negative and positive mode.

Underivatized



Derivatized

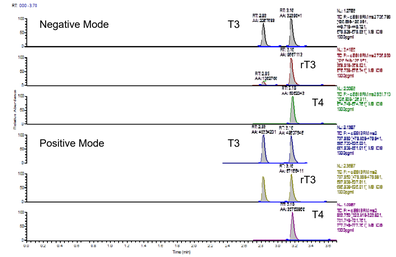


Table 2. Linearity and Sensitivity for the extraction methodology - PPT/LLE.

Compound	Linearity Total	LOQ (pg/ml)	Linearity Free	LOQ (pg/ml)
3,3',5-Triiodothyronine-PPT	5 pg/ml – 1000 ng/ml (+) 10 pg/ml – 1000 ng/ml (-)	2.5 10	5 - 1000 pg/ml (+) 10 – 1000 pg/ml (-)	2.5 10
3,3',5'-Triiodothyronine-PPT	5 pg/ml – 1000 ng/ml (+) 25 pg/ml – 1000 ng/ml (-)	5 25	5 – 1000 pg/ml (+) 25 – 1000 pg/ml (-)	5 25
Thyroxine-PPT	5 pg/ml – 1000 ng/ml (+) 10 pg/ml – 1000 ng/ml (-)	2.5 10	5 – 1000 pg/ml (+) 10 – 1000 pg/ml (-)	2.5 5
3,3',5-Triiodothyronine-LLE-No	1 pg/ml -1000 ng/ml (+) 5 pg/ml -1000 ng/ml (-)	1 5	1 – 1000 pg/ml (+) 5 – 1000 pg/ml (-)	1 5
3,3',5'-Triiodothyronine-No	2.5 pg/ml – 1000 ng/ml (+) 10 pg/ml – 1000 ng/ml (-)	2.5 10	2.5 – 1000 pg/ml (+) 10 – 1000 pg/ml (-)	2.5 10
Thyroxine-LLE-No	1 pg/ml – 1000 ng/ml (+) 5 pg/ml – 1000 ng/ml (-)	1 5	1 – 1000 pg/ml (+) 5 – 1000 pg/ml (-)	1 5
3,3',5-Triiodothyronine-LLE-Butyl	0.25 pg/ml – 1000 ng/ml (+) 2.5 pg/ml – 1000 ng/ml (-)	0.5 5	0.25 – 1000 pg/ml (+) 2.5 – 1000 pg/ml (-)	0.25 2.5
3,3',5'-Triiodothyronine-LLE-Butyl	0.5 pg/ml – 1000 ng/ml (+) 5 pg/ml – 1000 ng/ml (-)	1 5	0.5 – 1000 pg/ml (+) 5 – 1000 pg/ml (-)	0.5 5
Thyroxine-LLE-Butyl	0.25 pg/ml – 1000 ng/ml (+) 2.5 pg/ml – 1000 ng/ml (-)	0.5 5	0.25 – 1000 pg/ml (+) 2.5 – 1000 pg/ml (-)	0.25 2.5

CONCLUSIONS

- Baseline separation of thyroid hormones in 6 min with good LOQ in positive and negative mode with the butyl derivatized hormones resulting in the better LOQ levels.
- A clean serum matrix is required to achieve the desired calibration curve and LOQ as the thyroid hormones bind to proteins and albumin within serum that can result in interfering responses
- Excellent linearity of calibration curves with better accuracy, precision and reproducibility in positive mode than in negative mode by a factor of 5 fold and again the butylated hormones gave the best results in positive mode
- Derivatization of the thyroid hormones resulted in a potential 5 fold improvement in sensitivity that underivatized but also resulted a high degree of interferences demanding the use of thyroid free serum for analysis
- Further evaluate other mass spectrometer platforms such as the Thermo Scientific™ TSQ Quantis™ tandem mass spectrometer as to its suitability for the analysis of total and free butylated thyroid determinations and maximize the efficiency of the method as well as evaluate the potential for the analysis of other thyroid hormone metabolites using butylation.

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REFERENCES

1. Quantification of 11 thyroid hormones and associated metabolites in blood using isotope-dilution liquid chromatography tandem mass spectrometry. *Anal. Bioanal. Chem.* **2016,** *408* (20), 5429–42.
2. Quantitative Analysis of Thyroid Hormone Metabolites in Cell Culture Samples Using LC-MS/MS. *Eur. Thyroid J.* **2015,** *4* (Suppl. 1), 51–58.
3. Free Thyroid Hormones in Serum by Direct Equilibrium Dialysis and Online Solid-Phase Extraction–Liquid Chromatography/Tandem Mass Spectrometry. *Clin. Chem. (Washington, DC, U.S.)* **2008,** *54* (4), 642–651.
4. Development and Evaluation of a Reference Measurement Procedure for the Determination of Total 3,3′,5-Triiodothyronine in Human Serum Using Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Chem.* **2004,** *76* (17), 5092–5096
5. Determination of Six Thyroid Hormones in the Brain and Thyroid Gland Using Isotope-Dilution Liquid Chromatography/Tandem Mass Spectrometry. *Anal. Chem.* **2011,** *83* (1), 417–424.
6. Analysis of Thyroid Hormones in Serum by Liquid Chromatography Tandem Mass Spectrometry. *Anal. Bioanal. Chem.* **2010,** *397* (5), 1831–1839.

TRADEMARKS/LICENSING

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