thermoscientific

LC-MS/MS quantitative analysis of 11 total thyroid hormones and metabolites in serum for clinical research use

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ABSTRACT

Introduction: Liquid chromatography triple quadrupole mass spectrometry is suited for rapid analysis of multiple analytes of similar and different structures and physicochemical properties. Thyroid hormones are a diverse group of biologically active compounds and include- thyroxine (T4), 3,3', 5-triiodothyronine (T3), 3,3', 5'-triiodothyronine (rT3), 3,5-diiodothyronine (3,5-T2), 3,3'- diiodothyronine (3,3'-T2), 3-iodothyronine (T1), thyronine (T0), 3-iodothyronamine (T1AM), tetraiodothyroacetic acid (Tetrac), triiodothyroacetic acid (Triac), and diiodothyroacetic acid (Diac). A sensitive and specific LC-MS/MS analytical research method was developed and optimized for the quantitation of thyroid hormones and their metabolites in serum. Simple sample preparation techniques were used that included a protein crash and liquid-liquid extraction. A one dimensional chromatographic configuration achieved the required sensitivity, accuracy and robustness to measure the compounds over their relevant dynamic range.

Method: A Thermo Scientific™ TSQ Quantiva™ tandem mass spectrometer in positive and negative Electrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized. 200 µl of serum was used for the analysis of the thyroid hormones. A Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 2.6 µm 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 from donor individuals.

Results: Good linearity and reproducibility were obtained with the concentration range from 1 pg/ml to 10000 pg/ml for the thyroid hormones and metabolites with a coefficient of determination R2>0.98 or better for all drugs in the various matrices. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to the pg/ml levels of the thyroid hormones and metabolites in biological samples. Excellent reproducibility was observed for all compounds (CV < 10%) for the techniques and configurations used.

Conclusion: A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry research method was developed and verified for the simultaneous measurement of thyroid hormones and metabolites in serum. The sample preparation techniques are quick and easily applied for high throughput analysis.

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 11 thyroid hormones and their metabolites that include thyroxine (T4), 3,3', 5-triiodothyronine (T3), 3,3', 5'-triiodothyronine (rT3), 3,5-diiodothyronine (3,5-T2), 3,3'- diiodothyronine (3,3'-T2), 3-iodothyronine (T1), thyronine (T0), 3-iodothyronamine (T1AM), tetraiodothyroacetic acid (Tetrac), triiodothyroacetic acid (Triac), and diiodothyroacetic acid (Diac). The sample preparation choices were kept simple and included protein crash and a one step liquid-liquid extraction. The methodologies were developed on a Thermo Scientific™ TSQ Quantiva™ tandem mass spectrometer in positive and negative Electrospray ionization modes with a Thermo Scientific™ Vanquish™ HPLC system for a 6 minute analytical gradient.

MATERIALS AND METHODS

Standards

The following analytical reference standards and Internal standards were obtained from-

Isosciences, Inc., King of Prussia, PA-

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

Santa Cruz Biotechnology, Inc., Dallas, TX-

Thyronine (T0):	1 g
3-Iodothyronine (T1):	50 mg
3-Iodothyronamine (T1AM):	2.5 mg
3,5-Diiodothyronine (3,5-T2):	1 g
Tetraiodothyroacetic acid (Tetrac):	25 mg
Triiodothyroacetic acid (Triac):	100 mg
Diiodothyroacetic acid (Diac).	1 mg

Reagents

The following Fisher Scientific™ acids, reagents and solvents were used-

HPLC grade Water	Acetic Acid
Methanol	7N Ammonia in Methanol
Acetonitile	Ethyl Acetate

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

Sample Preparation- Protein Crash

- 200 µL of Thyroid depleted serum/water mixture calibrators, controls and serum sample were added to 1.5 ml eppendorf tubes and 20 µL of Thyroid ISTD mixture at 200 ng/mL were added to each tube and vortexed briefly
- 400 µL of Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes 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)

Sample Preparation- Liquid-Liquid Extraction

- 200 µL of Thyroid depleted serum/water mixture calibrators, controls and serum samples were added to a test tube and 20 µL of Thyroid ISTD mixture at 1000 ng/mL were added to each and vortexed briefly
- 200 µL 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 minutes 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 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 donor samples were used as control material.

Data Analysis

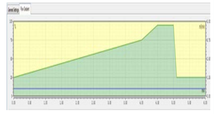
The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1 SW, Thermo Scientific™ TSQ Quantiva Tune™ 2.1 SW and Thermo Scientific™ Tracefinder™ 4.1 SW

METHOD

HPLC Conditions-

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

Column:	Accucore C18, 100 x 2.1 mm, 2.6 µm
Column Temperature:	50 ºC
Injection Volume:	10 µL
Sampler Temperature:	4 ºC
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	0.1% Acetic Acid
Mobile Phase B:	Methanol
Flow Rate:	0.5 ml/min
Gradient:	0.0 min- 75%A:25%B 4.0 min- 25%A:75%B 4.5 min- 5%A:95%B 5.0 min- 5%A:95%B 5.1 min- 75%A:25%B
Run time:	6 mins



MS and Ion Source Conditions-

TSQ Quantiva triple quadrupole mass spectrometer

Ion mode:	Positive and Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	300 ºC
Ion Transfer Tube Temperature:	275 ºC
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 secs

Table 1- Scan Parameters- SRM table

Compound	Rt (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
Thyronine- T0	1.06	Positive	274.15	215/257	18.9/10.3	55
		Negative	272.13	255.109.1	15.3/27	55
3-Iodothyronine-T1	2.01	Positive	400.08	354./256	16.8/16.9	153
		Negative	398.1	381/126.9	16.6/27.9	93
3-Iodothyronamine-T1AM	1.60	Positive	355.98	339.1/212.1	10.3/20	71
		Negative	354.07	226.1/126.9	16.9/22.6	70
3,3'-Diiodothyronine-T2	3.03	Positive	525.93	479.8/381.9	19.9/20.9	86
		Negative	523.93	126.9/506.9	38.3/20.4	99
3,3'-Diiodothyronine-13C6	3.03	Positive	531.95	514.8/485.8	15.5/20.4	86
		Negative	529.96	126.9/512.9	35.9/19.9	99
3,5-Diiodothyronine-3,5-T2	2.46	Positive	525.98	479.9/353	19.4/26.7	89
		Negative	523.96	126.9/506.9	32.2/17	102
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
Diiodothyroacetic acid -Diac	3.74	Negative	450.93	126.9/324	14/13.1	54
Triiodothyroacetic acid -Triac	4.32	Negative	576.77	126.9/449.9	30.1/18.9	76
Tetraiodothyroacetic acid-Tetrac	4.75	Negative	746.65	126.9/447.9	29.4/24.1	137

RESULTS

Linearity/Sensitivity

The assay was linear over the calibration curve for the total Thyroids in thyroid depleted serum/water mixture as shown in the table with their mean of coefficient of determinations (R^2) for positive and negative mode and for both 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 (R^2) > 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 Thyroid hormones 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 where applicable.

Precision/Specificity

The inter–assay precision and accuracy for total T4 and T3 was determined by extracting and quantifying five replicates of the NIST SRM 971 Male and Female Control resulting in mean 1.5 ng/ml and 1.4 ng/ml with %CV for T3 of 8.5 and 8.1 and 45 ng/ml and 70 ng/ml with %CV for T4 of 6.5 and 6.1 respectively. rT3 was not detected nor were the other thyroids and metabolites. Thus the results were <10% deviation form the targeted mean.

Four in house samples were extracted and the following were found within the determined calibration curves- Thyroxine, 3,5-Diiodothyronine and 3,3',5-Triiodothyronine in all four samples, 3,3',5'-Triiodothyronine and Thyronine in 3 samples, 3-Iodothyronamine and 3.3'-Diiodothyronine in samples and 3-Iodothyronine, Diiodothyroacetic acid, Triiodothyroacetic acid and Tetraiodothyroacetic acid were not found in any samples.

Therefore, the analytical method that worked best and meets the laboratory required accuracy for thyroid hormones in serum was the liquid-liquid extraction in positive mode.

Table 2- Linearity and Sensitivity for the best extraction methodology-LLE

Compound	Linearity	LOQ (pg/ml)
Thyronine	25 pg/ml – 1000 ng/ml (+) 50 pg/ml – 1000 ng/ml (-)	25 50
3-Iodothyronine	25 pg/ml - 1000 ng/ml (+) 50 pg/ml -1000 ng/ml (-)	25 50
3-Iodothyronamine	50 pg/ml – 1000 ng/ml (+) 250 pg/ml – 1000 ng/ml (-)	25 250
3-Iodothyronine	50 pg/ml – 1000 ng/ml (+) 50 pg/ml – 1000 ng/ml (-)	50 50
3,3'-Diiodothyronine	50 pg/ml – 1000 ng/ml (+) 50 pg/ml – 1000 ng/ml (-)	50 50
3,5-Diiodothyronine	50 pg/ml – 1000 ng/ml (+) 50 pg/ml – 1000 ng/ml (-)	50 50
3,3',5-Triiodothyronine	1 pg/ml -1000 ng/ml (+) 5 pg/ml -1000 ng/ml (-)	1 5
3,3',5'-Triiodothyronine	2.5 pg/ml – 1000 ng/ml (+) 10 pg/ml – 1000 ng/ml (-)	2.5 10
Thyroxine	1 pg/ml – 1000 ng/ml (+) 5 pg/ml – 1000 ng/ml (-)	1 5
Diiodothyroacetic Acid Negative	500 pg/ml – 1000 ng/ml	500
Triiodothyroacetic Acid Negative	500 pg/ml – 1000 ng/ml	500
Tetraiodothyroacetic Acid Negative	500 pg/ml – 1000 ng/ml	500

Figure 1: Chromatograms Negative Mode. * indicates compound

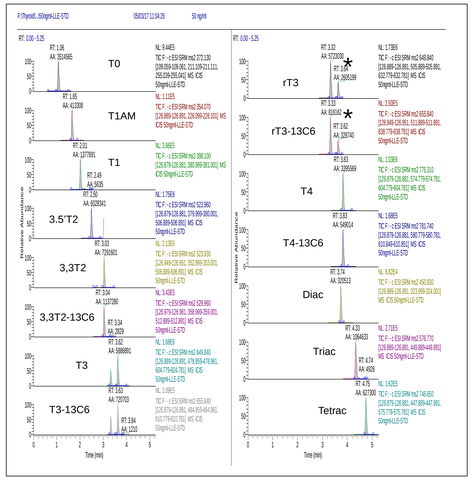
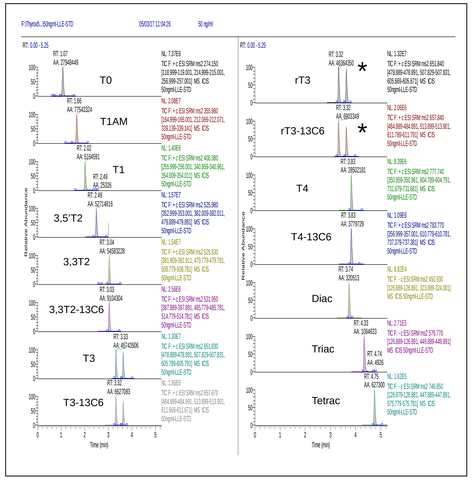


Figure 2- Chromatograms Positive Mode. * indicates compound



CONCLUSIONS

- Baseline separation of 11 thyroid hormones and their metabolites in 6 minutes with good LOQ in positive and negative mode.
- 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
- Excellent linearity of calibration curves with better accuracy, precision and reproducibility in positive mode than in negative mode where applicable
- Further evaluate other sample preparation techniques for improved total Thyroid and metabolite determinations and maximize the efficiency of the method as well as evaluate potential for the analysis of free thyroid hormones and metabolites

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REFERENCES

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