Quantitative Analysis and Comparison of Free and Total Thyroid Hormones in Human Serum using Liquid Chromatography Triple Quadruple Mass Spectrometry with Ion Funnel Technology in Positive and Negative ESI modes.

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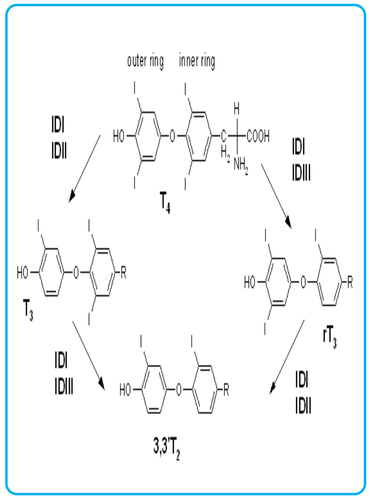
Introduction

Thyroid hormones are produced by the thyroid gland and are responsible for the regulation of metabolism, affect protein synthesis, regulate bone growth, and are involved in cell development and differentiation. Clinically relevant Thyroid hormones include Thyroxine (T4), Tri-iodo-thyronine (T3) and reverse Tri-iodo-thyronine (rT3). The major form of thyroid hormones present in blood is T4 followed by T3 which is approximately 20 times less than T4 followed by rT3 which is approximately 10 times less than T3. Diseases related to the Thyroid hormones include Hyperthyoidism, Hypothyroidism, clinical depression and neuro-developmental disorders.

A sensitive and selective analytical method is required to fully characterize and quantify the thyroids in serum. In this study, we developed methods for the analysis of Total and Free T4, T3 and rT3 in serum using an Agilent 1290 HPLC and a 6490 Mass spectrometer. We were able to separate the thyroids chromatographically one dimensionally to baseline resolution using a Poroshell 120 EC-C18 in under 6.5 minutes in both positive and negative mode.



Experimental

Standards, Calibrators and Controls

T4/T4-$^{13}C_6$-ISTD : 1mg/ml in Methanol:30% Ammonium Hydroxide (50:50) (Isosciences)
T3/T3-$^{13}C_6$-ISTD : 1mg/ml in Methanol:30% Ammonium Hydroxide (50:50) (Isosciences)
rT3/rT3-$^{13}C_6$-ISTD : 1mg/ml in Methanol:30% Ammonium Hydroxide (50:50) (Isosciences)
NIST SRM 971: Male/Female Control
Patient Samples: 5 Adult samples

Sample Preparation

Total

- 200 µl of serum sample, calibrators, controls + 400 µl Acetonitrile and 10 µl ISTD at 10 ng/ml were added to tubes and vortexed for 1 min
- 1.2 ml HPLC grade Ethyl Acetate was added and vortexed for 1 min prior to centrifugation
- Organic layer (Upper) was transferred to another tube and dried down under nitrogen at room temperature
- Reconstituted in 120 µl 75% H_2O:25% Acetonitrile

Free

- 500 µl of serum sample, calibrators, controls were added to an Amicon Centrifuge YM 30 filter unit and centrifuged at 5000 rpm for 90 minutes at room temperature
- 300 µl of supernatant had 15 µl ISTD at 10 ng/ml added and was further deproteinated with 300 µl of Acetonitrile and vortexed for 1 min prior to centrifugation.
- The supernatant was transferred to an MS vial.

Method

HPLC Conditions

Agilent 1290 Infinity HPLC series binary pump, well plate, thermostatted column compartment
Column: Agilent Technologies Poroshell 120 EC-C18, 2.7 µm, 3 x 100 mm
Column Temperature: 20 ºC (Pos)/ 45 ºC (Neg)
Injection Volume: 20 µl (Pos)/ 40 µl (Neg)
Autosampler Temperature: 4 ºC
Needle Wash: Flush port (50%Methanol:50%Water) 5 seconds
Mobile Phase A: 0.1% Acetic Acid in Water
Mobile Phase B: Acetonitrile
Flow Rate: 0.3 ml/min
Gradient: 30%B to 50%B in 5 minutes and up to 98%B for 30 seconds then 30%B for 1 minute
Total Run Time: 8 minutes (6.5 run/1.5 post-run)

Experimental and Results

MS Conditions

Agilent 6490 Triple Quadruple Mass Spectrometer

Ion mode:	Agilent Jet Stream Positive Mode	Agilent Jet Stream Negative Mode	Fragmentor 380V
Gas Temperature:	125ºC	350ºC	
Gas Flow:	16L/min	14 L/min	Cell Accelerator Voltage
Nebulizer:	55 psi	30 psi	2
Sheath Gas Temperature:	225ºC	400ºC	
Sheath Gas Flow:	11 L/min	11 L/min	
Capillary Voltage:	4000V	1500V	
Nozzle Voltage:	2000V	1000V	
Q1/Q2 Resolution:	0.7/0.7 unit	0.7/0.7 unit	
Dwell time:	50 msec	50 msec	
Delta EMV:	+500V	+500V	

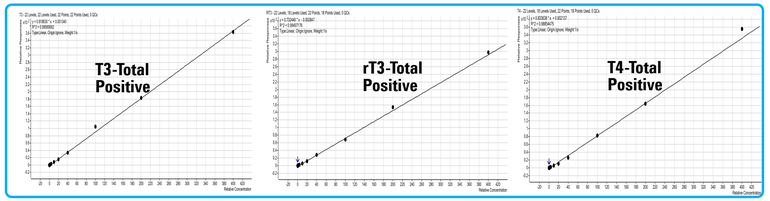
Table 1: MRM Acquisition

Compound	MRM Positive	Collision Energy (V)	RT (min)	MRM Negative	Collision Energy (V)	RT (min)
T4	777.7 > 731.7	21	5.17	775.7 > 126.9	60	4.74
	777.7 > 604.9	39	5.17	775.7 > 604.9	20	4.74
T3	651.8 > 605.8	20	4.25	649.8 > 126.8	44	3.76
	651.8 > 478.9	39	4.25	649.8 > 632.8	18	3.76
rT3	651.8 > 605.8	20	4.54	649.8 > 126.8	64	4.12
	651.8 > 507.8	19	4.54	649.8 > 478.9	22	4.12
T4-$^{13}C_6$-ISTD	783.7 > 737.7	25	5.16	781.7 > 126.9	64	4.73
T3-$^{13}C_6$-ISTD	657.8 > 611.8	19	4.25	655.8 > 126.8	40	3.75
rT3-$^{13}C_6$-ISTD	657.8 > 611.8	19	4.54	655.8 > 126.8	76	4.12

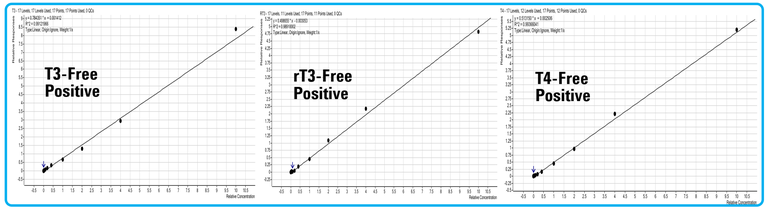
Linearity

The assay was linear over the ranges shown and positive mode was shown to be more sensitive by 5 to10 fold than negative mode. The total and free positive mode mean of coefficient determination (R^2) > 0.99 while for negative mode (R^2) > 0.98.

Compound	Linearity	LOD	Clinical Range	Compound	Linearity	LOD	Clinical Range
T4-Total Positive	1 pg/ml–1000 ng/ml	1 pg/ml	5-12.5 ug/dL	T4-Free Positive	5 pg/ml–1000 pg/ml	5 pg/ml	0.5–1.8 ng/dL
T4-Total Negative	5 pg/ml–1000 ng/ml	5 pg/ml		T4-Free Negative	10 pg/ml–1000 pg/ml	10 pg/ml	
T3-Total Positive	0.5 pg/ml–1000 ng/ml	0.5 pg/ml	14–180 ng/dL	T3-Free Positive	1 pg/ml–1000 pg/ml	1 pg/ml	2–3.5 pg/ml
T3-Total Negative	5 ng/ml–1000 ng/ml	5 pg/ml		T3-Free Negative	10 pg/ml–1000 pg/ml	10 pg/ml	
rT3-Total Positive	2.5 pg/ml–1000 ng/ml	2.5 pg/ml	10–24 ng/dL	rT3-Free Positive	5 pg/ml–1000 ng/ml	5 pg/ml	NA
rT3-Total Negative	25 pg/ml–1000 ng/ml	25 pg/ml		rT3-Free Negative	25 pg/ml–1000 ng/ml	25 pg/ml	

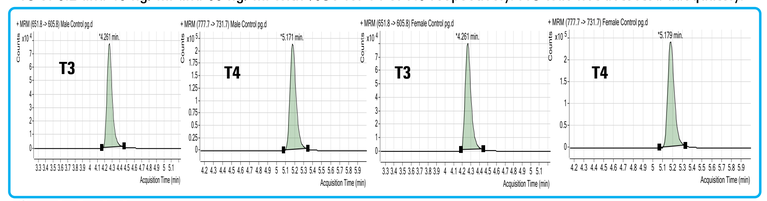


Results and Discussion



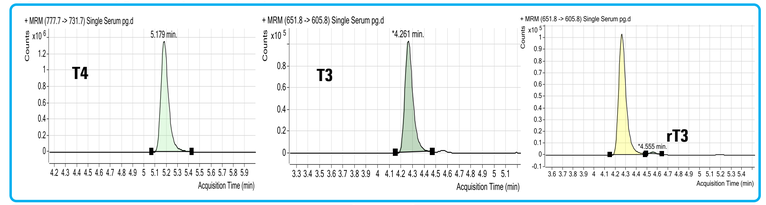
Precision, Specificity and Sensitivity

The inter–assay precision 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 964 pg/ml and 1072 pg/ml with %CV for T3 of 9.2 and 49 ng/ml and 69 ng/ml with %CV for T4 of 7.6 respectively. rT3 was not detected adequately.



Sample Analysis

The calculated mean of the 5 adult samples for Total T4, T3 and rT3 concentration was 62 ng/ml, 900 pg/ml and 86 pg/ml respectively in positive mode.



Conclusion

Baseline separation of T4, T3 and rT3 in under 8 minutes with good LOD in positive mode
Linearity (>99) of calibration curves with better accuracy, precision and reproducibility in positive mode than in negative mode
Method can achieve clinical measurement determinations for Total and Free Thyroid in positive mode
Further evaluate other sample preparation techniques for improved Free Thyroid determinations and maximize the efficiency of the method

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