Measurement of Thyroxine (T4), Triiodothyronine (T3) and Reverse Triiodothyronine (rT3) by Liquid Chromatography with Online Sample Cleanup-Tandem Mass Spectrometry in Negative Mode

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Introduction

Liquid chromatography and triple quadrupole mass spectrometry (LC-MS/MS) has become an essential tool for small molecules quantitation due to its high sensitivity and specificity, excellent reproducibility and the ability to perform simultaneous analysis of multiple analytes. The accurate and precise measurement of thyroid hormones in blood is imperative in thyroid function monitoring. Quantitation of thyroid hormones in serum or plasma is challenging due to their low levels under normal physiological conditions. We have developed a sensitive and reliable LC-MS/MS method on Agilent 6490 QQQ that utilizes JetStream ionization source and dual ion funnel technologies. These innovative technologies enable more efficient ion generation and ion sampling that result in the sensitive detection of Thyroxine (T4), Triiodothyronine (T3) and Reverse Triiodothyronine (rT3) in serum. We also explore the online sample cleanup to minimize the workload of sample preparation

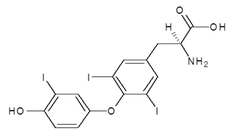

Figure 1. Triiodothyronine (T3)

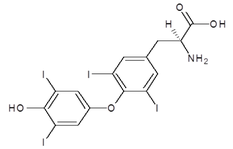

Figure 2. Thyroxine (T4)

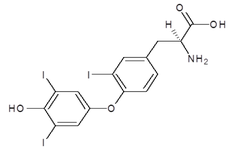

Figure 3. Reverse Triiodothyronine (rT3)

Sample Preparation

Charcoal stripped serum (Golden West MSC 4000) was spin filtered at 3000 x g for about 2 hours at 4 degree using Amicon Ultra Spin Filter 10K (MWCO 10K). This is to remove thyroid hormone binding proteins, HSA and other larger proteins. Analytical standards of thyroid hormones were purchased from Cerilliant Company at 100 ug/mL in 0.1 N ammonium hydroxide methanol. T4, T3 and rT3 were diluted to 100 ng/mL in acetonitrile. They are further diluted at 1:1000 in spin filtered serum to obtain highest spike-in concentration at 100pg/mL. The 100pg/mL spiked hormones as starting concentration were diluted serially with spin filtered serum to 50pg/mL, 20pg/mL, 10pg/mL, 5pg/mL, 2pg/mL, 1pg/mL.

Mass Spec Parameters

MS analysis was performed with an Agilent 6490 Triple Quadrupole Mass Spectrometer. A short hexabore capillary allows up to 10x more ion sampling. The dual ion funnel technology efficiently removes gas and neutrals while capturing ions.

Ionization Source:	AJS
Ion mode:	Negative
Gas temperature:	150 °C
Drying gas (nitrogen):	14 L/min
Nebulizer gas pressure:	40 psi
Sheath gas (nitrogen):	375 °C
Sheath gas flow:	10 L/min
Capillary voltage:	3000V
Nozzle voltage:	1750V
Fragmentor voltage	**380V**
Dwell time	50ms
Funnel RF	LP=160V HP=170V

Compound	Prec Ion	Prod Ion	CE (V)
Triiodothyronine (T3)	649.8	126.9	50
Thyroxine (T4)	775.8	126.9	35
r-Triiodothyronine (r-T3)	649.8	604.6	32
r-Triiodothyronine (r-T3)	649.8	462.8	32

Table 1. MRM Transitions

LC Configuration and Condition

Chromatographic separation is achieved by using one 1260 binary pump and a guard cartridge for online sample cleanup (pump and column 1 in figure 4) and one 1290 UHPLC binary pump and an analytical column (Pump and Column 2 in Figure 4).

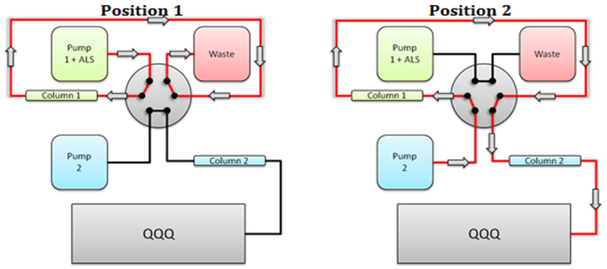

Figure 4. online sample cleanup configuration

Components	Parameters
Analytical Column Trapping Column	Agilent Poroshell 120 EC-C18 2.1 x 50 mm, 2.7µm Agilent Eclipse Plus C18 2.1x12.5mm, 3.5µm Cartridge
Column Temp	Room temp
Autosampler Temp Injection Volume	4°C 100 µL
Needle Wash	Flush port for 5 seconds
Pump 1 (Trapping)	A: H_2O + 0.01% Formic Acid B: Methanol 0.6mL/min
Pump 2 (Analysis)	A: H_2O + 0.01% Formic Acid B: Acetonitrile : Isopropanol=75:25 (v/v) 0.4mL/min
Pump 1 Gradient	5%B during 0.6min, 98%B at 2.5min, hold at 98%B until 4.6min, 5%B at 4.7min
Pump 2 Gradient	10% B during 0.6min, 55%B at 4.0min, 98%B at 4.1min, hold at 98%B until 5.5min, 10%B at 5.6min
Stop Time	6min
TCC Valve Switching	Position 1 at 0min, Position 2 at 0.6min, Position 1 at 3min

Table 2. LC Parameters

Results and Discussion

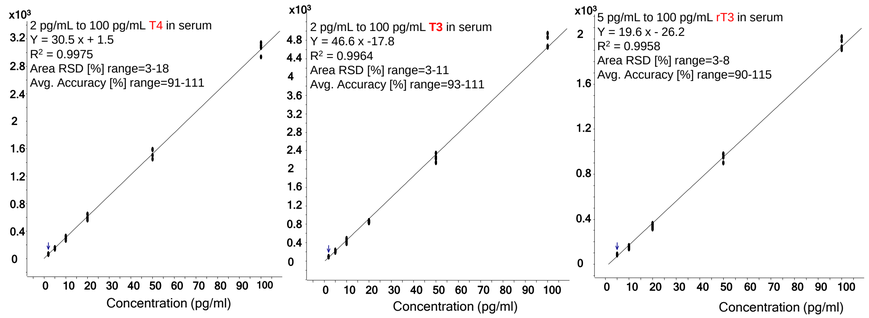

Figure 5. Calibration curves for free (a) T3, (b) T4 and (c) rT3 from 2pg/ml to 100pm/ml.

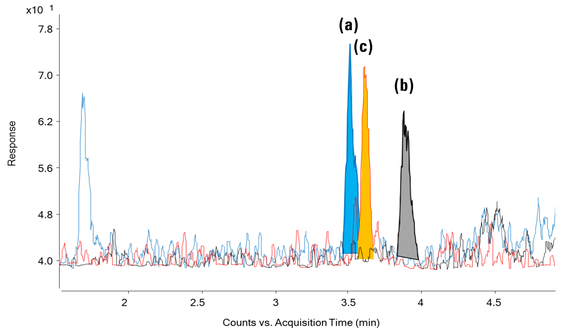

Figure 6. Free (a) T3, (b) T4 and (c) rT3 at a concentration of 2pg/ml

Conclusions

A sensitive LC-MS/MS method has been developed for free thyroid hormones measurement with Agilent 6490 QQQ that comprises JetStream ion source and dual ion funnel technologies. The limit of detection (LOD) for thyroid hormones is 1pg/mL for T3 and 2pg/mL for T4 and rT3. The limit of quantitation (LOQ) of thyroid hormones is 2pg/mL for T3 and T4, 5pg/mL for r-T3. Online sample cleanup configuration is effective in reducing the workload of sample preparation and is practical for high volume analysis.