Development of a Sensitive LC/MS Assay for Measuring Thyroid Hormones T3 and T4 in Late-Fetal and Neonatal Rat Samples

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1 Introduction

Drugs and chemicals have the potential to perturb thyroid hormone homeostasis with associated deficits in neurological development and function. Guidelines published by the Organization for Economic Co-operation and Development (OECD), as well the US EPA, require the evaluation of circulating levels of T3, T4, and thyroid stimulating hormone (TSH) in fetal (Gestation Day (GD) 20) and neonatal specimens to address this risk. Sensitive assays capable of measuring T3, T4, and TSH are required and traditional electrochemiluminescent immunoassays (ECLIA) are inadequate. In response, a highly sensitive LC-MS/MS method was developed.

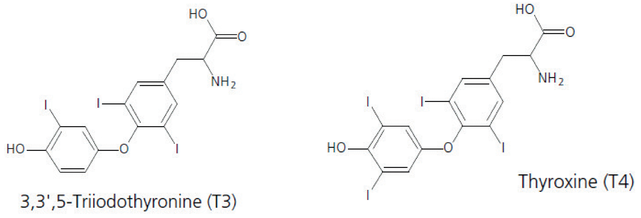

Figure 1. Chemical structure of critical Thyroid Hormones 3,3',5-Triiodothyronine (T3) and Thyroxine (T4).

2 Methods

- The analytical method is conducted on wet ice and uses a Supported Liquid Extraction (SLE) in a 96-well plate format.
- A surrogate analyte approach utilizes stable isotopically labeled T3 and T4 in adult rat serum thus having matching calibration, quality control, and sample matrices.
- Only 50µL of rat serum is required for simultaneous T3 and T4 quantitation.
- Validated range allows for multiplexed analysis of developmental and adult samples in the same analytical run.

Instrument Parameters

LC Conditions

Parameter:	
LC System:	Shimadzu Nexera®
Column:	Agilent ZORBAX RRHD Eclipse Plus 95Å Phenyl-Hexyl, 2.1 × 50 mm, 1.8-µm particle size
Mobile Phase A:	0.1% Glacial Acetic Acid in Milli-Q water
Mobile Phase B:	0.1% Glacial Acetic Acid in Acetonitrile
Sample Temperature:	4°C
Column Temperature:	50°C

MS Conditions

MS System:	API 5500
Ionization Mode:	ESI Positive Mode
IS Voltage:	5500
Temperature:	650°C

3 Results

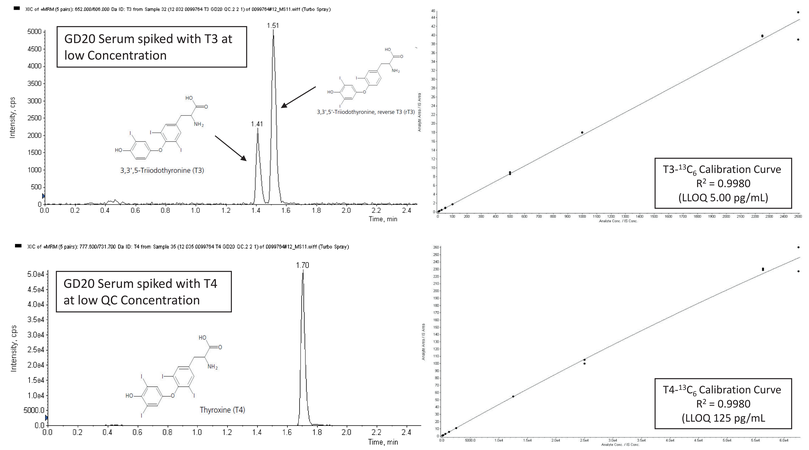

Figure 2. To the left are representative chromatograms of native GD20 rat serum quality control samples overspiked at the low QC concentration with T3 and T4, respectively. These quality control samples demonstrate the ability to quantitate extremely low concentrations of thyroid hormones in sample matrix. To the right are surrogate analyte calibration lines utilizing stable label isotope T3 and T4.

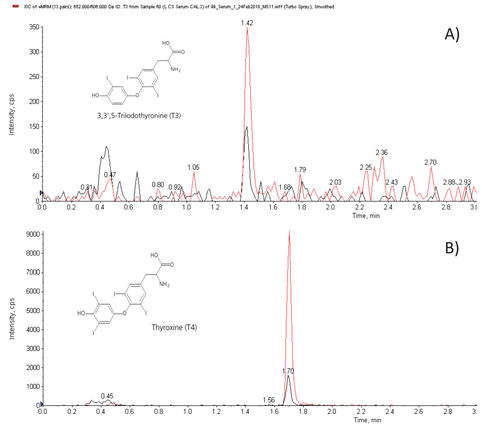

Figure 3. Thyroid hormones are monitored simultaneously for a quantitative ion (**Red Trace**) and a confirmatory ion (**Black Trace**). This adds an additional layer of identification points for species identification in agreement with several guidance documents for environmental and organic residue analyses. Shown are the quantitative and monitor ions for **A)** T3 at 10.0 pg/mL and **B)** T4 at 250 pg/mL adding confidence to identification even at extremely low sample concentrations.

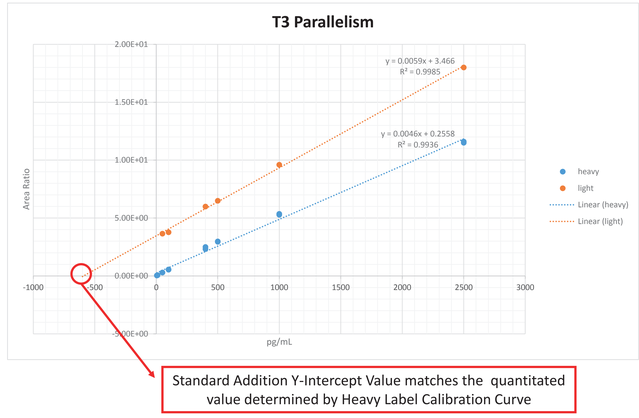

Figure 4. The analytical performance of T3 and T3-$^{13}C_6$ are compared to demonstrate parallelism and confirm the acceptability of the surrogate analyte approach in rat serum.

LC-MS/MS Assay and Performance Summary		
	T3	**T4**
LLOQ (pg/mL)	5.00	125
Range (pg/mL)	5.00 – 2,500	125 – 62,500
Inter-day % RE (%CV) at LLOQ (n=18) [^{13}C-labeled]	+5.4 (14.6)	+2.4 (11.3)
Inter-day % RE (%CV) overspiked GD20 serum (n=9/12)	+1.1 (12.4)	-10.4 (12.3)
Inter-day % RE (%CV) overspiked adult serum (n=9)	-0.8 (5.9)	+4.2 (6.2)

LC-MS/MS Assay Rat Serum Summary		
	T3 (pg/mL)	**T4 (pg/mL)**
Gestation Day 20 (Litters pooled, regardless of sex)	12.2 – 20.9	2566 – 6090
Postnatal Day 4 (litters pooled, regardless of sex)	91.3 – 273	17,200 – 35,900
Postnatal Day 13 (mixed gender)	NA	55,500 - 109,000
Adult (Female)	317 – 763	15,500 – 40,600
Adult (Male)	267 - 550	16,200 – 47,100

Figure 5. The method for T3 and T4 was validated according to guidelines for bioanalytical method validation (FDA 2001 and EMA BMV 2011). All testing results demonstrated passing criteria, allowing the method's use in supporting sample analysis.

4 Conclusions

LC-MS/MS offers sensitivity enhancements capable of determining T3 and T4 during the period of thyroid development in rats (Late gestation and early neonatal period). The low sample volume (50 µL) improves over ELISA methodology (>200 µL) to achieve T3 and T4 measurements. LC-MS/MS advances the 3Rs initiative (replace, reduce, refine) by reducing by one-third the serum volume needed as compared with conventional ELISA methods. The method was applied to determine basal levels of T3 and T4 in GD20 (16.9 – 19.8 and 2566 – 3650 pg/mL for T3 and T4, respectively) and PND 4 (131 – 202 and 17,200 – 28,500 pg/mL for T3 and T4, respectively) rat serum samples, data previously below limits of detection in traditional assays.