Thyroid Hormones in Rat Brain: Optimization of Procedures using Strong Anion Exchange Solid Phase Extraction with LC-MS/MS

Jermaine Ford[1]*, Cal Riutta[1,2], Katie O'Shaughnessy[1,2], Patricia A. Kosian[1], Iman Hassan[1], Mary Gilbert[1]
[1]U.S. Environmental Protection Agency, ORD, RTP/MED; [2]U.S. Department of Energy, ORISE, Oak Ridge, TN
Jermaine Ford | ford.jermaine@epa.gov | 919-541-9768

Introduction

- Thyroid hormones [3,3',5 triiodo-L-thyronine (T3), 3,3',5'-triiodo-L-thyronine (rT3) and thyroxine (T4)] are important for brain growth and development.
- Quantitative models for developmental neurotoxicity would be advanced by the determination of thyroid hormone levels in the brain.
- LC-MS/MS is a sensitive and selective analytical chemistry technique for the detection and quantification of chemicals.
- Methods have been developed to quantify thyroid hormones in brain tissue using a combination of solid-liquid extraction and solid-phase extraction.
- **Objective: Due to the complexity of brain tissue and the presence of endogenous thyroid hormones, we developed an analytical method using $^{13}C_6$ labeled thyroid hormones as surrogates to examine the matrix effects from brain tissue.**

Standards and Reagents

Primary Standards
L-Thyroxine (T4)
3,3',5'-Triiodo-L-thyronine (rT3)
3,3',5-Triiodo-L-thyronine (T3)

Surrogate Standards
L-Thyroxine-13C6 (T4-$^{13}C_6$)
3,3',5'-Triiodo-L-thyronine-$^{13}C_6$ (rT3-$^{13}C_6$)
3,3',5-Triiodo-L-thyroinine-$^{13}C_6$ (T3-$^{13}C_6$)

Internal Standards
L-Thyroxine-$^{13}C_{12}$ (T4-$^{13}C_{12}$)
3,3',5-Triiodo-L-thyronine-$^{13}C_{12}$ (T3-$^{13}C_{12}$)

Reagents
Hexane
Ethyl Acetate (EtOAc)
Dichlormethane (DCM)
Isopropanol (IPA)
Formic Acid

Reagents
Chloroform ($CHCl_3$)
Methanol (MeOH)
Acetonitrile (ACN)
Calcium Chloride ($CaCl_3$)
Dichloromethane (DCM)
Ammonium Hydroxide (NH_4OH)
Water
6-propyl-2-thiouracil (PTU)

Sample Preparation Methods

Five grams of Long Evans Rat Whole brain was homogenized in 20 mL of 10 mM 2-6-propyl-2-thiouracil in Methanol. Aliquots of 800 µL were transferred into clean low bind 15-mL falcon tubes. Samples were fortified with $^{13}C_6$ isotopically labeled surrogates and $^{13}C_{12}$ isotopically labeled T3 and T4 internal standards before extraction. Liquid-liquid extraction was performed using a series of chloroform and calcium chloride steps. The aqueous extracts were combined and reduced for the following SPE procedures below.

Solid-liquid extraction used for solid-phase extraction evaluation. Method adapted from Morreale de Escobar et al. *Endocrinology*, 117:1891 (1985).

Old Biotage Evolute Express CX 60mg (n=3)
Sample Pre-treatment: Add equal volume of 2% Formic Acid:ACN (60:40) v/v
Condition: MeOH
Equilibrate: Water
Load: Pre-treated Sample
Wash 1: 3 mL 50mM NH4OAC, pH 6
Wash 2: 3 mL 2% Formic Acid (aq)
Wash 3: 3 mL Methanol
Elute: 3 X 0.8 mL 5% NH_4OH in MeOH

New Biotage Evolute Express CX 60mg (n=3)
Sample Pre-treatment: Add equal volume of 2% Formic Acid:ACN (60:40) v/v
Condition: MeOH
Equilibrate: Water
Load: Pre-treated Sample
Wash 1: 2 mL 4% Formic Acid
Wash 2: 2 mL 70:30 Hexane/EtOAc
Wash 3: 2 mL 2% Acetic Acid in ACN
Elute: 3 X 0.8 mL 78:20:2 DCM:IPA:NH_4OH

Biotage Evolute Express AX 60 mg (n=3)
Sample Pre-treatment: Add equal volume of 5% NH_4OH:ACN (1:1) v/v
Condition: MeOH
Equilibrate: Water
Load: Pre-treated Sample
Wash 1: 2 mL 50mM NH4OAC, pH 9
Wash 2: 2 mL Methanol
Wash 3: 2 mL 2% Formic Acid in DCM
Elute: 1 mL MeOH

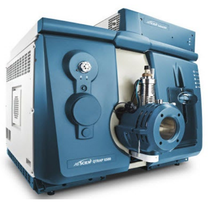
Biotage Evolute Express AX 100 mg (n=3)
Sample Pre-treated: Add equal volume of 5% NH_4OH:ACN (1:1) v/v
Condition: MeOH
Equilibrate: Water
Load: Pre-treated Sample
Wash 1: 2 mL 50mM NH4OAC, pH 9
Wash 2: 2 mL Methanol
Wash 3: 2 mL 2% Formic Acid in DCM
Elute: 1 mL MeOH

Solid-phase extraction using strong cation exchange (CX) and strong anion extraction (AX) cartridges. The affects of AX sorbent mass was also evaluated. The major difference between CX and AX is the additional formic acid in DCM wash.

Instrument Conditions

UPLC Parameter	
Column	Accucore RP-MS column, 100 mm x 2.1 mm, 2.6 µm
Column Temp	50° C
Sample Temp	5° C
Injection Volume	10 µL
Flow Rate	0.5 mL/min
Mobile Phase A	0.1% Acetic Acid in Water
Mobile Phase B	Methanol

AB Sciex 6500+ Qtrap Mass Spectrometer Parameters	
Ionization Mode	ESI Positive
Curtain Gas (CUR)	20
Collision Gas (CAD)	Medium
Ion Spray Voltage (IS)	5000
Temperature (TEM)	550
Ion Source Gas 2 (GS2)	60
Ion Source Gas 1 (GS1)	60
MRM Detection Window (sec)	45
Target Scan Time (sec)	2



Q1 Mass (Da)	Q2 Mass (Da)	Retention Time	ID	DP	EP	CE	CXP
651.8	605.8	3.65	T3.1	91	10	30	40
651.8	478.9	3.65	T3.2	99	10	47	32
651.8	507.8	3.65	T3.3	100	10	30	35
657.8	611.7	3.65	$^{13}C_6$-T3	100	10	32	40
663.8	617.8	3.65	$13C_{12}$-T3.IS	100	10	32	40
651.8	605.8	3.99	rT3.1	91	10	30	40
651.8	478.9	3.99	rT3.2	99	10	47	32
651.8	507.8	3.99	rT3.3	100	10	30	35
657.8	611.7	3.99	$^{13}C_6$-rT3	100	10	32	40
777.6	731.7	4.14	T4.1	113	10	35	45
777.6	633.7	4.14	T4.2	95	10	34	45
777.6	604.7	4.14	T4.3	120	10	55	45
783.5	737.7	4.14	$^{13}C_6$-T4	100	10	38	40
789.7	743.6	4.14	$^{13}C_{12}$-T4.IS	100	10	36	40

Results: Chromatograms

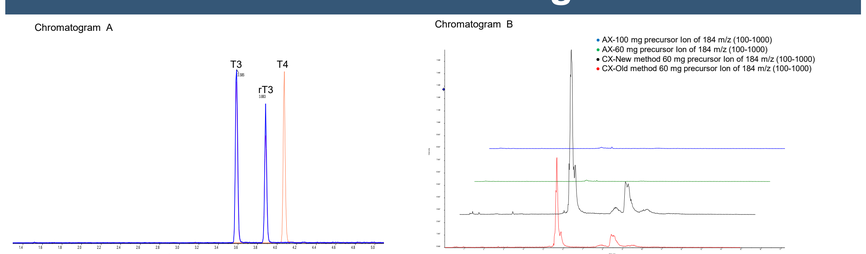


Figure 1. A. Representative chromatogram of unlabeled thyroid hormones. B. Investigation of phospholipids reduction from brain matrix using precursor ion scanning (184 m/z product ion) from each solid-phase extraction cartridge. AX-60 mg and AX-100 were both sufficient in reducing phospholipids in the final extract while the CX cartridges had phospholipid breakthrough.

Results: Calibration Comparisons

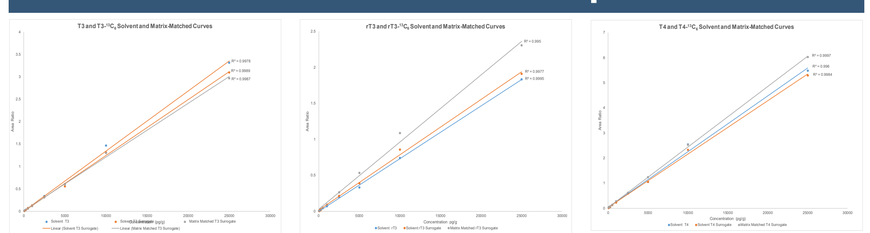


Figure 2. Calibration curve comparisons for solvent curves (unlabeled and $^{13}C_6$ isotopically labeled thyroid hormones) and $^{13}C_6$ isotopically labeled thyroid hormone matrix-matched curve (extracted on AX-60 mg cartridge). Calibration range 25 pg/mL - 25,000 pg/g. Measured calibration points were within ± 80% of their predicted value. The coefficient of determination, r^2 for all curves was ≥ 0.995. The closeness of the surrogate matrix-matched curve to the surrogate solvent curve is also an indication of reduced matrix effects. Also, it indicates that a solvent-based curve is sufficient for quantification due to the presence of endogenous thyroid hormone in the brain matrix.

Method Validation Results

Recovery and Matrix Effects Results

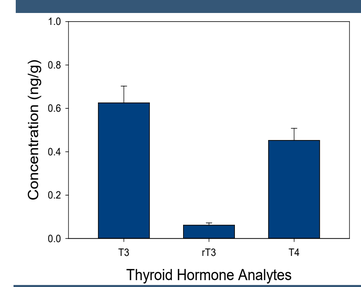
	T3-$^{13}C_6$	rT3-$^{13}C_6$	T4-$^{13}C_6$
Recovery %	46	41	35
Matrix Effects % (ME)	99	110	112
Process Efficiency (%)	45	45	37

Table 1. Pre and Post spikes samples spiked at 1,000 pg/mL based on AX 60 mg cartridge. Ideal recovery should be > 80%, the recoveries here are less due to the analyte loss during the evaporation process. If ME ~ 100%, there is no matrix effect. ME >100%, an ion-suppression occurs, and if ME <100%, ion-enhancement occurs. The PE should be ~100% is affected by the recovery. Therefore the recovery will need to be reevaluated after optimization of the evaporation process.

	Intraday (n=5)		Interday (n=15)		
	Measured (ng/g)	% RSD*	Measured (ng/g)	% RSD*	% Recovery
T3-$^{13}C_6$	4.75 ± 0.088	1.85	4.74 ± 0.220	4.65	94.9 ± 4.4
rT3-$^{13}C_6$	5.02 ± 0.589	11.7	5.14 ± 0.744	14.5	102 ± 14.9
T4-$^{13}C_6$	5.09 ± 0.146	2.87	5.00 ± 0.293	5.85	100 ± 5.9

Table 2. Precision and Accuracy were evaluated by comparing pre and post spiked samples fortified at 5.00 ng/g of $^{13}C_6$ labeled thyroid hormones. The samples were processed using the Evolute AX 60 mg cartridge with the additional wash step. Accuracy and precision values on both intra- and inter-day were found to be within the accepted limits of relative standard deviation (RSD*) <20% and recoveries >80%.

Method Applicability



- The new method was demonstrated using Long Evans rat postnatal day 2 telencephalon.
- Six control samples were analyzed on the same day for T3, rT3, and T4.
- The % RSD for each analyte was less than the acceptable range of < 20%. Resulting in a very robust and stable extraction procedure.

Conclusions and Future Directions

- The Biotage Evolute AX 60 mg cartridges successfully reduced the amount of phospholipid carryover and matrix effects from the solid-liquid brain extraction as demonstrated by phospholipid precursor scans.
- 2% formic acid in dichloromethane wash solution significantly reduced the number of phospholipids in the final extract as indicated in Figure 3.
- Although total process efficiency is ~50%, using $^{13}C_6$ isotopically labeled thyroid hormones is an excellent quality control parameter to track the recovery per sample based on the method validation study's acceptable range of 80 – 120 %.
- Also, using $^{13}C_{12}$ isotopically labeled thyroid hormones as internal standards compensate for any loss in the analytical process due to matrix effects or sample loss.
- A method detection limit study will be conducted as well as an attempt to improve overall process efficiency.