Reverse Triiodothyronine (rT3) Quantification in Blood Serum for Research Purposes by LC-MS/MS using Liquid-Liquid Extraction following Protein Precipitation

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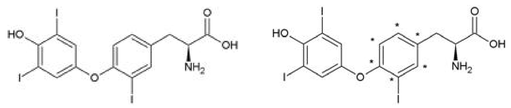
OVERVIEW

Reverse triiodothyronine (rT3), was quantified from 5 to 60 ng/dL in blood serum subjected to protein precipitation followed by liquid-liquid extraction. Separation of rT3 from triiodothyronine (T3) was achieved by aqueous-to-methanol gradient elution through a heated reversed-phase LC column. The six-minute research method had a 0.5-minute data window to prevent elution of T3 into the MS/MS and permit throughputs from 9 to 36 injections per hour using a 4-channel LC-MS/MS system with excellent precision and correlation of donor-specimen results with those of a reference laboratory.

INTRODUCTION

Reverse triiodothyronine (rT3), shown in Figure 1, is an inactive isomer of the most potent thyroid hormone triiodothyronine (T3). Both are made from thyroxine (T4). For scientists studying the metabolic consequences of starvation and critical illness, we report an LC-MS/MS research method that offers robust, reliable quantitation of rT3 from 5 to 60 ng/dL in blood serum after protein precipitation (PPT) and liquid-liquid extraction (LLE). We quantified rT3 in donor blood serum samples using a 4-channel liquid chromatography (LC) system coupled to a triple-stage quadrupole (TSQ) mass spectrometer (MS/MS) with a heated electro-spray ionization (HESI) source.

Figure 1. rT3 and its $^{13}C_6$ internal standard



Reverse Triiodothyronine (rT3)
Formula: $C_{15}H_{12}I_3NO_4$
Mono-isotopic mass: 650.79

Reverse Triiodothyronine-$^{13}C_6$ (rT3-IS) internal standard
Formula: $^{13}C_6C_9H_{12}I_3NO_4$
Mono-isotopic mass: 656.93

MATERIALS AND METHODS

Consumables

Fisher Scientific™ Optima™ solvents were used for LC mobile phases and wash solutions, as well as for preparations of calibrators, quality controls (QCs) and donor blood serum samples. rT3 and rT3-$^{13}C_6$ analytical reference standards were purchased from Cerilliant Corporation (Round Rock, TX). Custom-made QCs were purchased from UTAK Laboratories Inc. (Valencia, CA). Other laboratory consumables were purchased from Thermo Fisher Scientific.

Sample Preparation

Each calibrator level was made in a diluent of 1% bovine serum albumin in phosphate-buffered saline. To precipitate proteins and extract analytes, 125 µL aliquots of specimens (calibrators, quality controls and donor serum samples) were vortexed with 250 µL of water, 600 µL of acetonitrile and 200 µL of methanol containing rT3-$^{13}C_6$ internal standard (IS), shown in Figure 1. 1.2 mL of ethyl acetate was added and vortexed for 1 minute. After centrifugation (5,000 RFC for 5 minutes), 2 mL of the upper organic layer of each were transferred to respectively-labeled glass tubes and dried with nitrogen flow at 70°C. The residue of each tube was reconstituted with 150 µL of 25% acetonitrile in water and transferred to its corresponding well of a microtiter plate, which was placed in the autosampler drawer cooled to 10°C.

Liquid Chromatography

Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 LC system, 50 µL of each extract were injected into a heated (60°C) 100 x 2.1 mm Thermo Scientific Accucore™ aQ column packed with solid-core silica particles with a C18 bonded phase and polar end caps. As shown in Figure 2, a 6-minute mobile phase gradient from 5% methanol in water containing 0.1% formic acid to 100% methanol separated rT3 and IS from T3 and other interfering compounds and eluted them into the heated ESI source of the MS/MS system.

Tandem Mass Spectrometry

The Thermo Scientific™ TSQ Quantis™ MS/MS system was used for selected-reaction monitoring (SRM) of two transitions for rT3 (651.8 > 605.8 for quantitation and 651.8 > 507.9 for conformation) and IS (657.8 > 611.8 and 657.8 > 513.9), which occurred within a 0.8-minute data window. Ion ratios were calculated from peak areas measured by these transitions to help verify peak purity. The MS/MS data acquisition method is summarized in Figure 3.

Instrument Control & Data Analysis

Thermo Scientific™ TraceFinder™ with Aria™ MX software was used to control the Transcend LX-4 LC and TSQ Quantis MS/MS systems, submit batches to desired channels, as well as analyze data and report results.

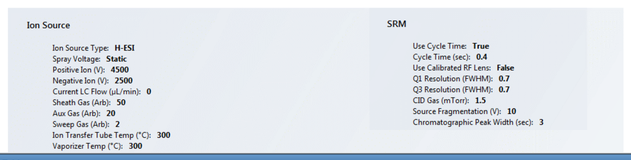
Figure 2. Transcend LX-4 LC method for rT3

Column: Accucore aQ, 2.6 µm, 100 x 2.1 mm at 60ºC Solvents A: Water, B: Methanol, both with 0.1% formic acid

Start	Len	Flow	Grad	%A	%B	S/D	Col	Comments
0.00	0.50	0.40	Step	95.0	5.0	Elute	---->	Load & focus samples
0.50	0.50	0.40	Ramp	90.0	10.0	Elute	---->	Focus analytes & rinse away matrix
1.00	0.50	0.40	Ramp	50.0	50.0	Elute	---->	Rinse away matrix & separate analytes
1.50	0.50	0.40	Step	50.0	50.0	Elute	---->	Separate analytes
2.00	1.50	0.40	Ramp	30.0	70.0	Elute	---->	Separate & elute analytes
3.50	1.00	0.40	Step	-	100.0	Elute	---->	Wash column
4.50	1.50	0.40	Step	95.0	5.0	Elute	---->	Equilibrate column

Total Method Duration: 6:00 min Data Window Start: 4.00 min Duration: 1.0 min

Figure 3. TSQ Quantis MS/MS data acquisition method for rT3



SRM Table

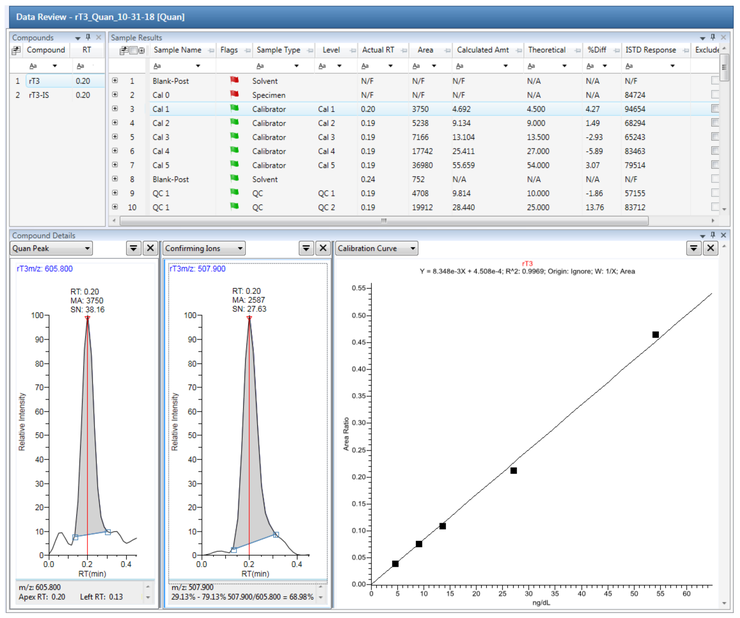
Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	Min Dwell Time (ms)	RF Lens (V)
rT3	0	0.8	Positive	651.75	507.7	29	98.7	190
rT3	0	0.8	Positive	651.75	605.6	26	98.7	190
rT3 IS	0	0.8	Positive	657.75	513.7	30	98.7	190
rT3 IS	0	0.8	Positive	657.75	611.6	20	98.7	190
Electro-clean	0.8	1	Negative	600	300	20	399.271	200

Results

Typical Quantitation Performance

Each channel of the Transcend LX-4 – TSQ Quantis system consistently achieved linear quantitation of rT3 in extracted calibrators from 5 to 60 ng/dL as ion ratio confirmation (IRC) values averaged 55%. Typical rT3 results for calibrators and QCs are shown in Figure 4. rT3 was measured in both QCs over 22 days. QC 1 had a mean of 9.4 ng/dL with 16.8% CV and QC 2 had a mean of 28.8 with 7.9% CV.

Figure 4. Typical rT3 calibrator results

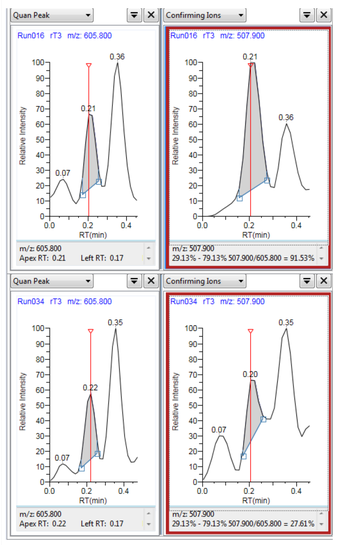


In this data set, summarized in Table1, internal standard (IS) peak areas averaged 78,230 among calibrators with a 15% coefficient of variation and their IRC confirmation values averaged 40%. IS peak areas among donor serum extracts ranged from 38,190 and 71,520 with an average recovery of 69%, which indicated moderate ion suppression. However, all IS peak IRC values were between 36% and 45%, indicating that the IS adequately compensated for matrix effects. Out of 25 donor serum extracts shown in Table 1, four had rT3 peaks that did not pass IRC (Figure 5).

Table 1. Typical rT3 and IS peak measurements

rT3_Quan_10-31-18		rT3			rT3 IS		
File Name	rT3 (ng/dL)	RT (min)	Peak Area	Ion Ratio	RT (min)	Peak Area	Ion Ratio
Pre-Blank	0.0	N/F	0	NaN	N/F	0	NaN
Cal 0	0.0	N/F	0	NaN	0.11	84724	0.42
Cal 1	4.7	0.20	3750	0.69	0.20	94654	0.37
Cal 2	9.1	0.19	5238	0.54	0.19	68294	0.41
Cal 3	13.1	0.19	7166	0.42	0.19	65243	0.37
Cal 4	25.4	0.19	17742	0.57	0.19	83463	0.39
Cal 5	55.7	0.19	36980	0.49	0.19	79514	0.41
Post-Blank	0.0	0.24	752	0.00	N/F	0	NaN
QC 1a	9.8	0.19	4708	0.50	0.19	57155	0.40
QC 2a	28.4	0.19	19912	0.52	0.19	83712	0.39
3033798	11.1	0.19	4799	0.51	0.19	51647	0.39
3038449	13.4	0.20	7272	0.56	0.19	64693	0.40
3042093	10.3	0.21	4585	0.47	0.21	52906	0.40
3039912	16.1	0.20	9412	0.39	0.21	69935	0.39
3036590	7.6	0.21	2290	0.92	0.21	35673	0.40
3038355	7.8	0.22	2546	0.34	0.22	39007	0.38
3047841	16.4	0.21	6502	0.34	0.21	47360	0.44
3032124	4.3	0.22	1635	0.74	0.20	45381	0.41
3031082	12.4	0.21	8326	0.40	0.21	80120	0.39
3031166	14.4	0.21	8648	0.52	0.21	71519	0.41
3030930	11.3	0.20	3798	1.05	0.21	40038	0.36
3042748	8.2	0.21	3290	0.81	0.21	48044	0.38
3029459	15.3	0.21	9395	0.47	0.21	73227	0.40
3045364	10.4	0.22	3653	0.72	0.22	41964	0.36
3035459	7.9	0.21	3910	0.55	0.21	58591	0.42
3033241	10.9	0.20	6363	0.54	0.21	69331	0.38
3035526	10.8	0.22	5798	0.66	0.21	63731	0.41
3032156	14.2	0.22	5429	0.43	0.21	45807	0.41
3042212	9.4	0.22	4300	0.68	0.21	54507	0.42
3028509	7.1	0.20	2369	0.28	0.22	39854	0.38
3046188	17.2	0.21	6578	0.48	0.21	45589	0.44
3035764	12.1	0.21	5964	0.62	0.21	58809	0.39
3036845	10.6	0.22	6064	0.65	0.21	67938	0.36
3037301	12.8	0.21	6056	0.57	0.21	56657	0.40
3044592	9.2	0.20	3629	0.33	0.21	46768	0.37
3047444	17.3	0.22	5527	0.52	0.22	38194	0.45
QC1b	8.3	0.22	6121	0.53	0.21	87000	0.41
QC2b	27.9	0.20	17972	0.49	0.21	77137	0.41

Figure 5. Example rT3 peak IRC failures



Interference studies

rT3 was well separated from T3 and T4 among all donor sera, and CAP proficiency samples were tested, resulting in measurements that were within 10% of expected values.

The selectivity/specificity performance of this rT3 LC-MS/MS research method was evaluated by 1:1 dilution of normal donor sera with lipemic, icteric and hemolyzed sera. Accurate quantitation of rT3 in lipemic dilutions required manual integration of Quan and Confirm peaks. Accurate quantitation in hemolyzed dilutions was not achieved. Accurate rT3 concentrations were easily measured among icteric dilutions. Therefore, this method is not recommended for hemolyzed and lipemic serum samples.

Carryover, measured in solvent blanks immediately after injections of Cal 5 (56 ng/dL) among 10 batches, averaged 0.3 ng/dL and never exceeded 0.6 ng/dL (less than 1% carryover).

Precision

As shown in Tables 2a and 2b, intra- and inter-batch precisions among 20 replicate injections from three pools (low, medium and high rT3 levels) were less than 6% and 8% coefficient of variation (CV), respectively.

Table 2. Intra- and inter-batch precision results

Intra-batch results (ng/dL)			
Run	Low	Medium	High
1	14.1	27.1	51.0
2	15.4	27.3	57.5
3	15.7	28.5	56.3
4	15.3	28.5	49.4
5	13.6	26.6	49.0
6	15.8	27.0	54.8
7	15.6	28.2	48.5
8	15.5	27.4	56.4
9	14.4	24.8	56.5
10	14.6	26.4	53.6
11	15.6	28.0	51.0
12	14.8	27.5	53.6
13	15.4	26.1	51.1
14	16.3	25.8	50.2
15	17.4	27.5	54.7
16	14.9	27.2	55.1
17	14.6	25.6	52.7
18	16.6	26.4	56.4
19	16.1	26.0	56.3
20	16.0	28.1	49.5
Mean:	15.4	27.0	53.2
StdDev:	0.9	1.0	3.0
% CV:	5.8	3.8	5.6

Inter-batch results (ng/dL)				
Run		Low	Medium	High
Day 1	1	15.7	39.5	43.9
	2	15.4	36.9	46.6
	3	16.3	37.7	43.3
	4	16.5	38.7	44.1
	5	16.4	37.8	47.3
Day 2	1	17.0	40.3	49.4
	2	14.9	38.6	47.8
	3	15.0	38.5	51.0
	4	15.2	35.5	49.3
	5	15.1	35.1	48.4
Day 3	1	16.5	36.9	47.7
	2	14.4	35.4	47.2
	3	17.5	36.9	47.9
	4	17.3	35.6	47.9
	5	18.0	35.2	45.5
Day 4	1	16.9	38.2	47.3
	2	16.4	33.6	45.6
	3	18.8	33.6	45.5
	4	15.5	34.0	47.5
	5	19.0	32.8	41.5
Mean:		16.4	36.5	46.7
StdDev:		1.3	2.1	2.3
% CV:		7.8	5.8	4.9

Accuracy assessment

Comparison of LC-MS/MS quantitation of rT3 in 60 donor blood serum samples between a reference lab and our research lab showed excellent correlation, as summarized in Table 3. rT3 values ranged from 5.8 to 56.2 ng/dL. Only 1 out of 54 results exceeded the 20%.difference limit with a 20.6% difference. On average, the two methods differed by 2%, a small positive bias by our lab.

Table 3. Comparison of donor-serum rT3 results (ng/dL) between reference (Ref Lab) and research lab (BRL)

Sample	Ref Lab	BRL	% Diff	Sample	Ref Lab	BRL	% Diff	Sample	Ref Lab	BRL	% Diff
1	13.7	13.8	0.7	21	5.8	6.6	13.8	41	10.8	9.5	-12.0
2	15.7	15.0	-4.5	22	13.0	13.1	0.8	42	13.4	14.6	9.0
3	15.5	16.1	3.9	23	15.9	16.9	6.3	43	13.3	14.2	6.8
4	15.1	13.5	-10.6	24	21.2	23.2	9.4	44	13.2	12.3	-6.8
5	17.5	19.9	13.7	25	12.6	13.2	4.8	45	14.7	13.8	-6.1
6	15.5	14.6	-5.8	26	13.0	14.3	10.0	46	13.2	13.0	-1.5
7	13.7	11.4	-16.8	27	15.6	16.2	3.8	47	18.5	20.9	13.0
8	7.8	8.8	12.8	28	13.8	12.8	-7.2	48	14.9	13.8	-7.4
9	7.5	7.8	4.0	29	14.5	13.8	-4.8	49	16.7	17.1	2.4
10	15.3	17.8	16.3	30	15.5	17.0	9.7	50	18.7	17.3	-7.5
11	23.7	25.4	7.2	31	14.7	12.5	-15.0	51	29.2	30.5	4.5
12	12.5	13.5	8.0	32	9.7	9.8	1.0	52	17.9	18.2	1.7
13	10.2	11.7	14.7	33	12.5	13.9	11.2	53	9.5	10.1	6.3
14	14.0	14.7	5.0	34	9.7	11.7	20.6	54	9.0	10.8	20.0
15	18.7	20.0	7.0	35	10.4	9.8	-5.8	55	11.8	10.9	-7.6
16	7.5	8.3	10.7	36	15.4	17.2	11.7	56	20.3	17.7	-12.8
17	16.5	17.0	3.0	37	11.9	11.3	-5.0	57	14.7	17.5	19.0
18	12.0	13.6	13.3	38	15.1	15.1	0.0	58	56.2	48.3	-14.1
19	12.5	13.2	5.6	39	10.9	10.1	-7.3	59	15.1	12.1	-19.9
20	12.4	12.4	0.0	40	17.2	18.0	4.7	60	12.4	11.6	-6.5

CONCLUSIONS

Robust, reliable and sensitive quantification of rT3 in donor blood serum samples prepared by protein precipitation followed by liquid-liquid extraction was achieved using this research method with a four-channel LC-MS/MS system. We demonstrated:

- Desired analytical range from 5 to 60 ng/dL easily achieved
- Accurate results virtually equivalent to reference lab results
- Excellent inter- & intra-batch precisions with coefficients of variation less than 8%
- Carryover less than 1%
- Throughputs of 9, 18 or 36 injections per hour from a 1-, 2- or 4-channel UHPLC system
- Multi-channeling with other LC-MS/MS methods utilizing similar ion-source conditions

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TRADEMARKS/LICENSING