TECHNICAL NOTE 73564

Blood serum rT3 quantitation by LC-MS/MS

Using liquid-liquid extraction following protein precipitation for research purposes

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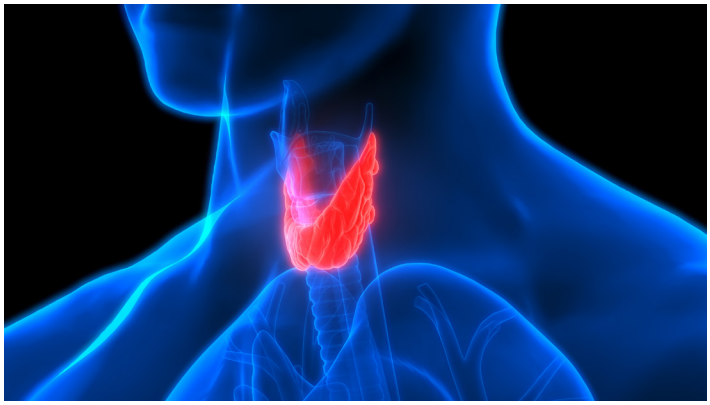
Application benefits

- Selective and sensitive quantitation of reverse triiodothyronine (rT3) in blood serum
- Reliable sample preparation and LC separation to maximize recovery and minimize interferences
- Short data acquisition window to permit high throughput using a multichannel LC-MS/MS system

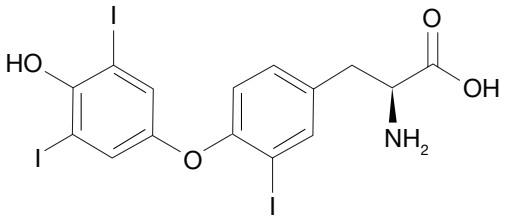
Goal

To accurately measure rT3 in blood serum samples prepared by protein precipitation followed by liquid-liquid extraction to achieve an analytical quantitation range of 5 to 60 ng/dL (0.05 to 0.6 ng/mL or 50 to 600 pg/mL) prior to UHPLC separation using a multichannel LC-MS/MS system to maximize throughput.

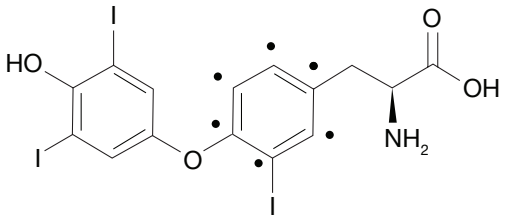


Introduction

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.



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

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

Experimental

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 (Round Rock, TX). Custom-made QCs were purchased from UTAK (Valencia, CA). Other laboratory consumables were 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 were 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 system, 50 µL of each extract were injected into a heated (60 °C) 100 × 2.1 mm Thermo Scientific™ Accucore™ aQ column (P/N 17326-102130) packed with solid-core silica particles with a C18 bonded phase and polar end caps. As shown in Table 1, a 6-minute chromatographic 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.

Table 1. Transcend LX-4 LC method for rT3

Column:	**Accucore aQ, 2.6 µm, 100 × 2.1 mm at 60 °C**
Solvent A:	**Water** + 0.1% formic acid
Solvent B:	**Methanol** + 0.1% formic acid

Step	Start	Len	Flow	Grad	%A	%B	Comments
1	0.00	0.50	0.40	Step	95.0	5.0	Inject sample, focus analytes
2	0.50	0.50	0.40	Ramp	90.0	10.0	Focus analytes, rinse away matrix
3	1.00	0.50	0.40	Ramp	50.0	50.0	Rinse away matrix, separate analytes
4	1.50	0.50	0.40	Step	50.0	50.0	Separate analytes
5	2.00	1.50	0.40	Ramp	30.0	70.0	Separate, elute analytes
6	3.50	1.00	0.40	Step	—	100.0	Wash column
7	4.50	1.50	0.40	Step	95.0	5.0	Equilibrate column
Total Method Duration: 6.00 min				Data Window Start: 4.00 min			Duration: 1.0 min

Tandem mass spectrometry

The Thermo Scientific™ TSQ Quantis™ MS/MS system was used for selected-reaction monitoring (SRM) of two positive-ion transitions for rT3 (651.8 → 605.8 *m/z* for quantitation and 651.8 → 507.9 *m/z* for conformation) and IS (657.8 → 611.8 *m/z* and 657.8 → 513.9 *m/z*), 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. A 0.2-minute negative-ion "electro-clean" transition (1) was then added to prevent adsorption of iodine oxides onto stainless steel surfaces of the source and ion optics (2). The ion source parameters and MS/MS SRM parameters are summarized in Tables 2 and 3.

Table 2. Ion source parameters

MS source parameters	Setting
Ion source	H-ESI
Spray voltages	Pos 4500 V Neg 2500 V
Source fragmentation	10 V
Sheath gas	50
Aux gas	20
Sweep gas	2
Vaporizer temperature	300 °C
Ion transfer tube temperature	300 °C

Instrument control and data processing

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

Results and discussion

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 2. After measuring rT3 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.

Table 3. SRM data acquisition parameters for rT3 and internal standard (IS)

Compound	Start (min)	End (min)	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)
rT3	0	0.8	Positive	651.75	507.7	29
rT3	0	0.8	Positive	651.75	605.6	26
rT3 IS	0	0.8	Positive	657.75	513.7	30
rT3 IS	0	0.8	Positive	657.75	611.6	20
Electro-clean	0.8	1	Negative	600	300	20
Q1 Resolution: 0.7 FWHM			Q3 Resolution: 0.7 FWHM		CID Gas 1.5 mTorr	

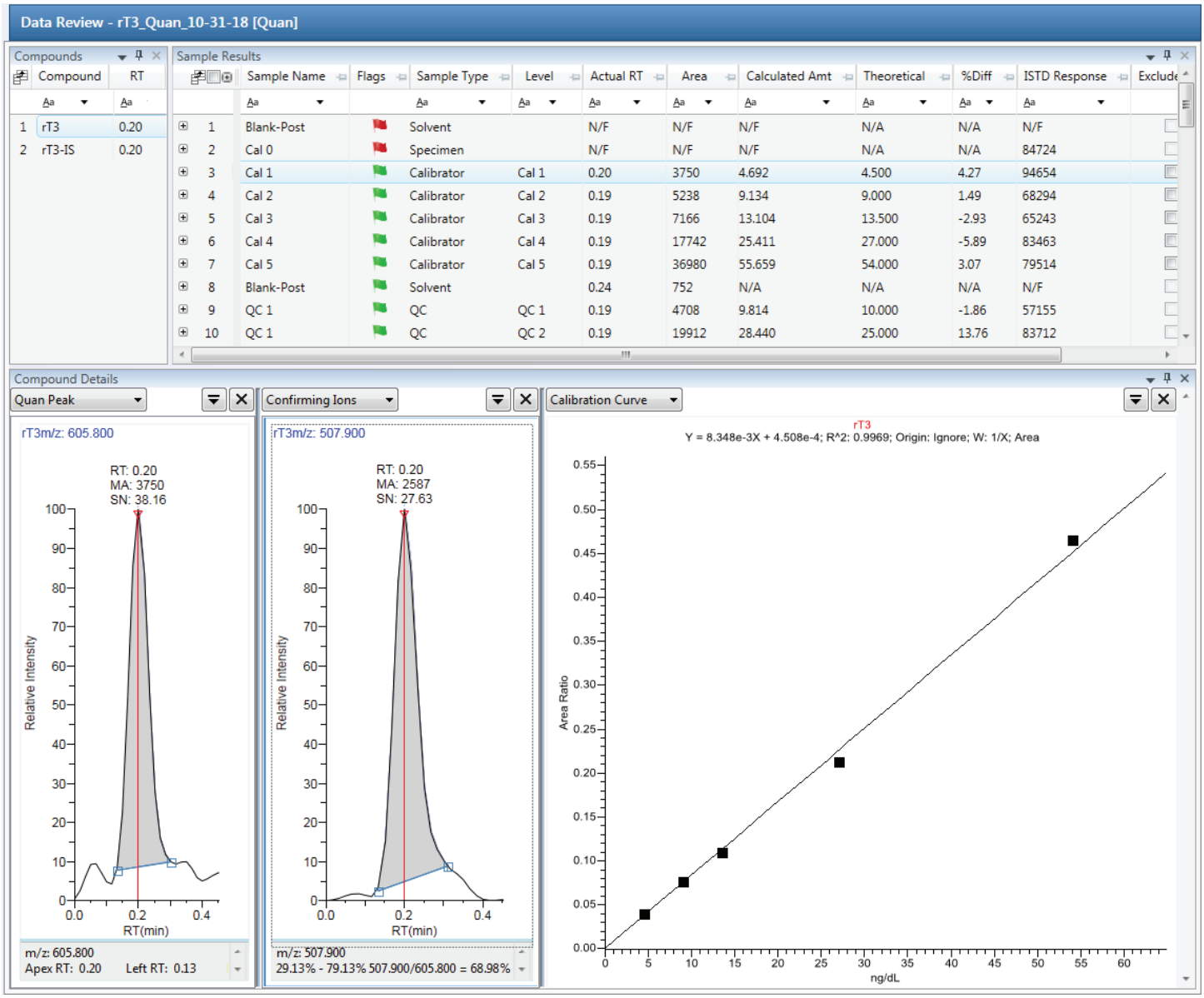


Figure 2. Typical rT3 calibrator results showing data review, chromatograms of quantifier and confirming ions of the lowest calibrator, and representative calibration curve

Throughput

Throughputs of 9, 18, or 36 injections per hour were achieved by running batches across 1, 2, or 4 channels of the Transcend LX-4 system. Running other methods on dedicated channels using common ion source parameters was achieved without noticeable differences in method performance.

In this data set, internal standard (IS) peak areas averaged 78,230 among calibrators with a 15% coefficient of variation and their IRC values averaged 40%. IS peak areas among donor serum extracts ranged from 38,190 to 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, four had rT3 peaks smaller than lowest calibrator that did not pass IRC (Figure 3).

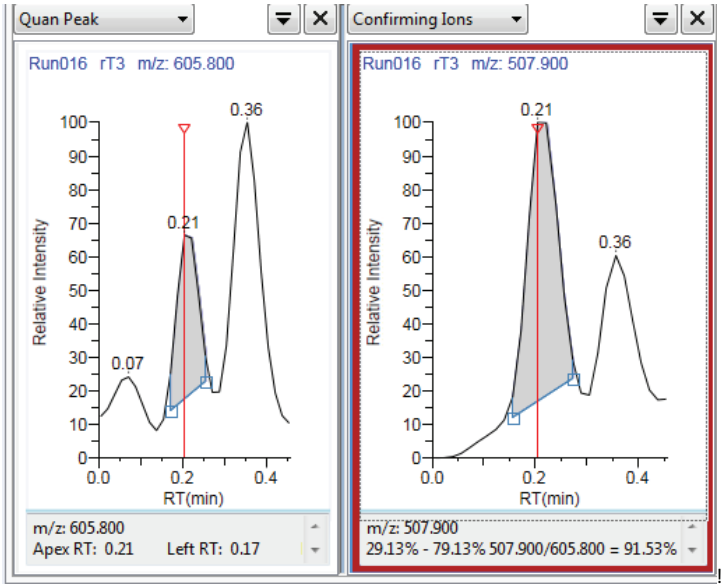


Figure 3. Example rT3 peak IRC failures

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Interference studies

rT3 was well separated from T3 and T4 among all donor sera and CAP proficiency samples that were tested. Quantitation of rT3 in CAP proficiency samples 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, which is 12% of the lower limit of quantitation (LLOQ).

Precision

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.

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. 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.

Conclusion

- Fast and reliable separation of rT3 from isobaric interferant T3 and related thyroid hormone T4 was achieved with a 6-minute LC-MS/MS method.
- Protein precipitation followed by liquid-liquid extraction successfully minimized matrix interferences while easily measuring rT3 concentrations in a range of 5 to 60 ng/dL in blood serum, the desired analytical range.
- Accurate results equivalent to reference lab results were achieved.
- Excellent inter- and intra-batch precisions with coefficients of variation less than 8% were achieved.
- Throughputs of 9, 18, or 36 injections per hour were achieved using 1, 2, or 4 channels without significant differences in accuracy or precision.
- Running multiple methods utilizing similar ion source parameters produced the same data quality as running the same method across all channels.

References

1. DiBussolo J, How to Electro-Clean APCI Sources Between Injections, Thermo Scientific Poster Note PN64408, 2016.
2. Julie Ray (ARUP Laboratories) *Recurrent Need for a Robust Method for MeasuringT3/rT3 by LC-MS/MS: An Exercise in Madness or a New Beginning for a Misused Marker?* MSACL US 2015 oral presentation.

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