A Sensitive LC/MS/MS Method for the Quantification of Free T3 and Free T4 in Serum, using a Simple Ultrafiltration Sample Preparation Procedure

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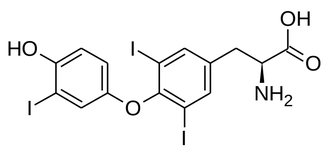
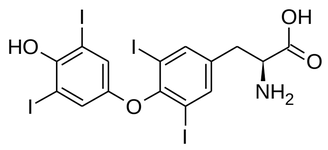
INTRODUCTION

The measurement of free thyroxine (FT4) and free 3,3´,5-triiodothyronine (FT3) in serum are important because these values can be used to help researchers assess various states of thyroid function in men, women, and children. Currently most measurements of FT3 and FT4 are performed by equilibrium dialysis immunoassay methods, which may suffer from a lack of specificity.



3,3,5-triiodothyronine (T3)



Thyroxine (T4)

Measurement by LC/MS/MS has the potential to increase the accuracy of these measurements; however sensitivity has posed a challenge due to the very low levels of circulating FT3 and FT4 in serum. Here we present a sensitive method using the AB SCIEX Triple Quad™ 6500 system, employing a simple and rapid ultrafiltration sample preparation to isolate the free T3 and free T4 prior to analysis by LC/MS/MS. Unlike earlier attempts to analyze FT3 and FT4 by LC-MS/MS, this sensitive method requires a relatively small injection volume of only 50 µL of the final sample.

We have performed a comparison study using a cohort of samples that have been previously analyzed by immunoassay, and an excellent correlation has been observed. The method LOQ was <0.5 pg/mL for both FT3 and FT4.

MATERIALS AND METHODS

Sample Preparation:

500 µL of serum was added to a polypropylene centrifugal filter, having a molecular weight cut-off (MWCO) of 10 kDa, and the sample was processed at 7500g for 60 minutes at 30° C. To 100 µL of the ultrafiltrate was added 25 µL of an internal standard solution containing T3-$^{13}C_6$ and T4-$^{13}C_6$ in water, and this was directly injected onto the LC/MS/MS system. Calibration standards were prepared in water using stock solutions obtained from Cerilliant Corporation (Round Rock, Texas). T3 and T4 were monitored in positive MRM mode, using electrospray ionization.

HPLC Conditions:

The analysis was done using a Shimadzu Prominence HPLC. Separation of the analytes was accomplished with a Phenomenex Kinetex C18 column (50x4.6, 2.6µ) at 0.6 mL/min. A 10 minute gradient allowed for the separation of T3 and T4, as well as additional interferences present in the matrix. Mobile phases were water and methanol, both containing 0.1% acetic acid. The injection volume used was 50 µL.

MS/MS Conditions:

MS/MS detection was accomplished using the AB SCIEX Triple Quad™ 6500 LC/MS/MS system equipped with IonDrive™ Turbo V source and operated in positive electrospray ionization mode. The Multiple Reaction Monitoring (MRM) mode was used, with 2 MRM transitions monitored per analyte. MRM transitions are summarized in Table 1.

AB SCIEX Triple Quad™ 6500 LC/MS/MS System

Name	Q1	Q3
T3 (1)	651.7	605.8
T3 (2)	651.7	479.0
T4 (1)	777.7	731.6
T4 (2)	777.7	604.8

Table 1. MRM Transitions for target compounds.

RESULTS

The method presented here allowed quantification of free T3 and free T4 in serum at an LOQ of <0.5 pg/mL. Figure 1 shows the S/N of each analyte at 0.5 pg/mL in a solvent calibrator. Replicate injections (n=3) of the calibration standards demonstrated excellent linearity, accuracy and precision over the concentration range from 0.5 pg/mL to 100 pg/mL of FT3 and FT4. Accuracies ranged from 95 - 108% for all concentration levels for both analytes. Linearity was >0.999 for both analytes. Measurement precision was <3% (%CV) for all concentrations, except the lowest level calibrator (0.5 pg/mL) for both analytes (19% for 0.5 pg/mL T3; 15% for 0.5 pg/mL T4).

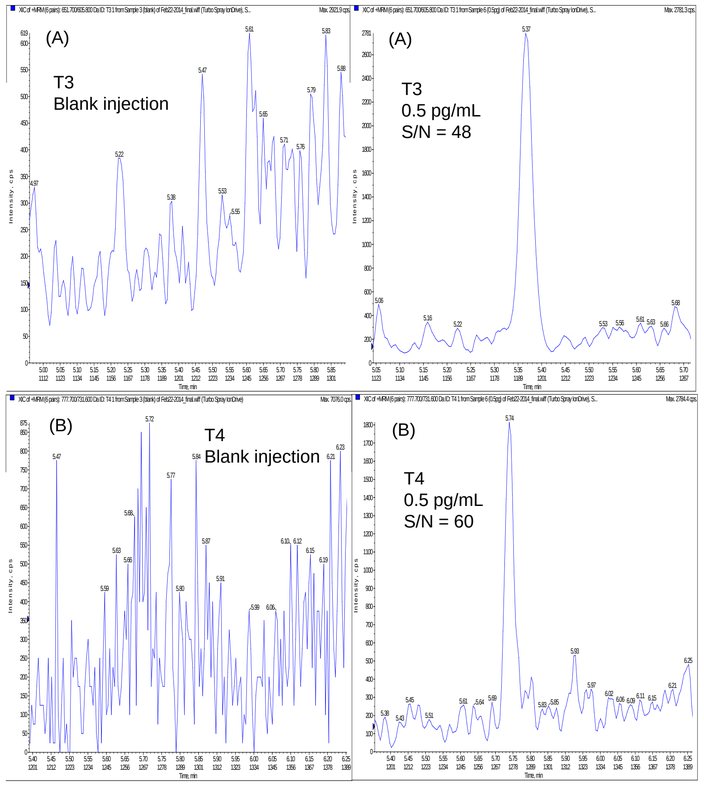


Figure 1. (A) Chromatograms showing blank (left) and LOQ in calibrator for T3 (right). (B) Chromatograms showing blank (left) and LOQ in calibrator for T4 (right).

A method comparison was performed by analyzing a series of samples that had been previously measured via immunoassay, and excellent correlation was observed. Comparison values are shown in Table 2. Representative chromatograms are shown in Figure 2, demonstrating both low and high concentrations of FT3 and FT4 measured in serum

Sample	SPH T3 (pg/mL)	LC/MS T3 (pg/mL)	Acc%	SPH T4 (pg/mL)	LC/MS T4 (pg/mL)	Acc%
1	3.3	2.7	82	10.9	13.5	124
2	1.5	1.4	93	9.3	16.1	173
3	3.3	2.3	70	10.1	10.3	102
4	3.1	3.4	110	10.1	17.3	171
5	3.1	2.6	84	7.8	7.8	100
6	3	2.3	77	12.4	14.2	115
7	2.7	1.7	63	11.7	11.5	98
8	2.6	2.2	85	10.1	13.7	136
9	3.3	2.9	88	10.1	13.8	137
10	3.8	3	79	11.7	12.9	110
11	>20.1	45	n/a	>120.4	143.9	n/a
AVERAGE			83			127

Table 2. Summary of comparison data of fT3/fT4 values obtained using immunoassay (SPH) versus the LC-MS/MS method (LC/MS).

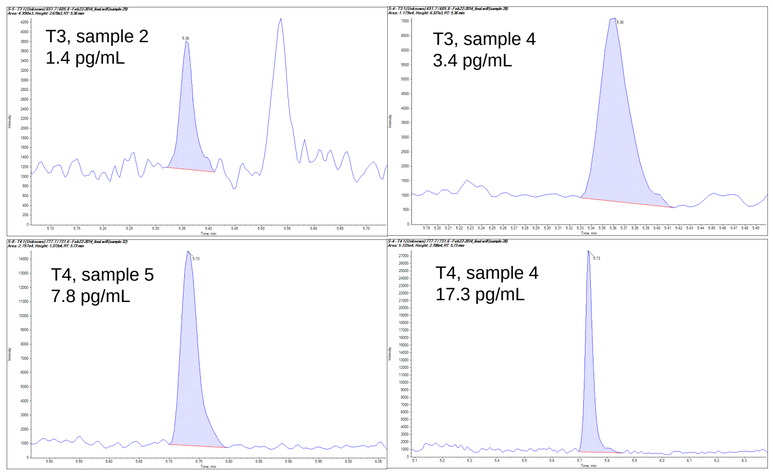


Figure 2. Representative chromatograms from serum samples. Chromatograms are labeled with the sample number and analyte concentration.

CONCLUSIONS

We have demonstrated a sensitive method for the analysis of FT3 and FT4 in serum by LC-MS/MS, which takes advantage of the simple and rapid sample preparation afforded by ultrafiltration. The method provides adequate sensitivity, accuracy and precision to allow researchers to investigate FT3 and FT4 levels in serum across the expected range of concentrations. Comparable measurements to those obtained using immunoassay were achieved. Unlike earlier MS-based methods, this method does not require a large sample injection volume, since only 50 µL of the final sample is required for injection on the LC/MS/MS system.

TRADEMARKS/LICENSING