Sensitive Detection of Three Forms of Thyroid Hormone in Human Serum Using the Agilent 6490 Triple Quadrupole LC/MS System

Application Note

Clinical Research

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Abstract

A rapid method for the quantification of free and total thyroid hormones in serum was developed using an Agilent 1290 Infinity LC System coupled to an Agilent 6490 Triple Quadrupole LC/MS System with Agilent Jet Stream (AJS) technology. Sample preparation was performed using a simple filtration procedure for free concentrations and a liquid-liquid extraction for total concentrations. Excellent linearity was observed from 0.5 to 1,000 pg/mL, with run times of only 6.5 minutes.

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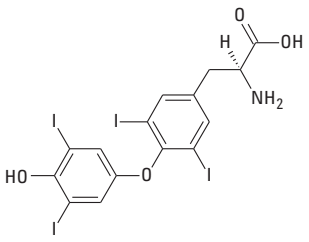
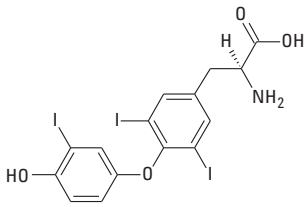
Introduction

Thyroxine (3,5,3′,5′-tetraiodothyronine or T4) is the precursor to the more active form of thyroid hormone, triiodothyronine (3,3′,5-triiodothyronine or T3). Reverse triiodothyronine (3,3′,5′-triiodothyronine or rT3), is an inactive form of the thyroid hormone (Figure 1). Most of the thyroid hormone circulating in the blood is bound to transport proteins. Only a very small fraction of the circulating hormone is free (unbound) and biologically active. Thus, measuring very low concentrations of the three forms of free thyroid hormones is essential to understanding their roles in the human body.

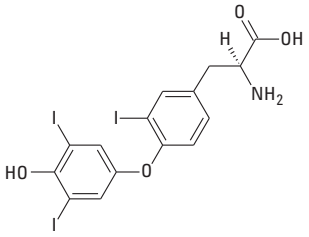
This application note describes the development of an analytical method for the sensitive and accurate determination of free and total thyroid hormones in serum using a 1290 Infinity LC System coupled to a 6490 Triple Quadrupole LC/MS with AJS technology in positive ionization mode. Using tandem mass spectrometry (MS/MS) and multiple reaction monitoring (MRM), the method is linear from 0.5 to 1,000 pg/mL, with lower limits of quantitation (LLOQ) ranging from 1 to 5 pg/mL for free, and 0.5 to 2.5 pg/mL for total thyroid hormone determination.



Thyroxine (T4)



Triiodothyronine (T3)



Reverse Triiodothyronine (rT3)

Figure 1. The three forms of thyroid hormone found in human serum.

Experimental

Reagents and standards

Stock solutions of T4, T3, rT3, and their ^{13}C-labeled internal standards (IsoSciences) were prepared at 10 µg/mL in 50:50 methanol: 30 % ammonium hydroxide, and stored at –20 °C. Working solutions were prepared by diluting stock solutions with methanol. Calibration standard solutions ranging from 0.5 to 1,000 pg/mL were prepared by spiking ultra-low hormones and steroids serum (Golden West Biologics) with various amounts of stock solution. A labeled internal standard mixture was created at 5 ng/mL in methanol. Standard reference material (SRM 971) was purchased from the National Institute of Standards and Technology (NIST).

Instruments

The method was developed on the Agilent 1290 Infinity LC System coupled to an Agilent 6490 Triple Quadrupole LC/MS system with AJS in positive ion mode. Table 1 lists the instrument conditions, and Table 2 shows the 6490 Triple Quadrupole LC/MS/MS acquisition parameters.

Table 1. LC and MS instrument conditions.

LC conditions	
Analytical column	Agilent Poroshell 120 EC-C18, 3.0 mm × 100 mm × 2.7 µm (p/n: 695975-302)
Column temperature	20 °C
Injection volume	20 µL
Mobile phase	A = 0.1 % acetic acid in water, B = Acetonitrile
Gradient	30 % B 0.0 minutes 50 % B 5.0 minutes 98 % B 5.1 minutes 98 % B 5.4 minutes 30 % B 5.5 minutes
Run time	6.5 minutes
Flow rate	0.3 mL/min
Post time	1.5 minutes
MS conditions	
Acquisition mode	Agilent JetStream, positive ionization; MRM
Sheath gas temperature	N_2; 225 °C
Sheath gas flow rate	11 L/min
Drying gas temperature	N_2; 125 °C
Drying gas flow rate	16 L/min
Nebulizer gas pressure	N_2; 55 psi
Nozzle voltage	2,000 V
Capillary voltage	4,000 V
Cell accelerator voltage	2 V
Delta EMV	500 V
Quadrupole 1/2 resolution	0.7

Table 2. Retention times and acquisition parameters for the thyroid compounds (*Qualifier ions).

Retention time (min)	Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell (msec)	Collision energy (V)
5.1	T4	777.7	731.7	50	21
5.1	T4*	777.7	604.9	50	39
5.1	T4-$^{13}C_6$	783.7	737.7	50	25
4.2	T3	651.8	478.9	50	39
4.2	T3*	651.8	605.8	50	20
4.2	T3-$^{13}C_6$	657.8	611.8	50	19
4.5	rT3	651.8	507.8	50	19
4.5	rT3*	651.8	605.8	50	20
4.5	rT3-$^{13}C_6$	657.8	611.8	50	19

Sample preparation

For the determination of free thyroid hormones, calibrators and standard reference material samples were prepared using Amicon Centrifuge YM-30 filter units. 500 µL of serum was added to an Amicon Centrifuge YM-30 filter and centrifuged at high rpm for 2 hours. 300 µL of filtrate was transfered to a clean microfuge tube along with 30 µL of internal standard and vortexed. 300 µL of acetonitrile was added, vortexed for 1 minute and centrifuged at high rpm for 10 minutes. The supernatant was transfered to a 96-well plate.

Total thyroid hormone concentrations were also determined for each of these samples using an ethyl acetate liquid-liquid extraction (LLE). A 200-µL aliquot of sample was combined with 20 µL of internal standard and 400 µL of acetonitrile in a tube and vortexed. 1.2-mL of ethyl acetate was added to the mixture which was then vortexed for 60 seconds. The tube was centrifuged at 13,000 rpm for 10 minutes, and the organic layer was transferred to a clean tube and evaporated. The dried sample was then reconstituted with 120 µL of 75:25 water:acetonitrile.

Results and Discussion

Method performance

Chromatographic separation of T3 and rT3 is critical due to common MRM transitions. Figure 2 illustrates the baseline separation obtained for the three forms of thyroid hormone obtained from either free thyroid or total thyroid analyses.

Linearities of calibration curves were excellent, with coefficients of variation (R^2) ≥0.990 from 0.5 to 1,000 pg/mL. The R^2 values were slightly higher for free thyroid determinations, versus total thyroid (Figure 3).

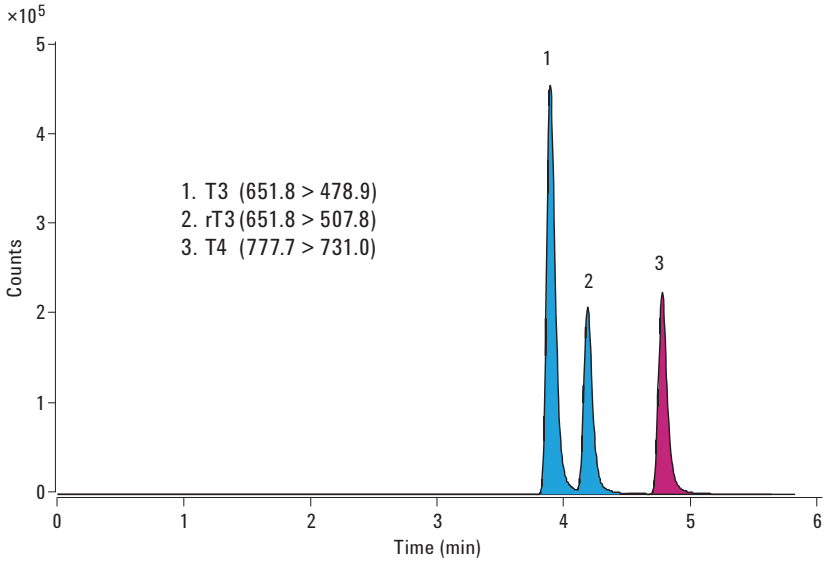


Figure 2. Chromatographic separation of the three forms of thyroid hormone extracted from human serum samples.

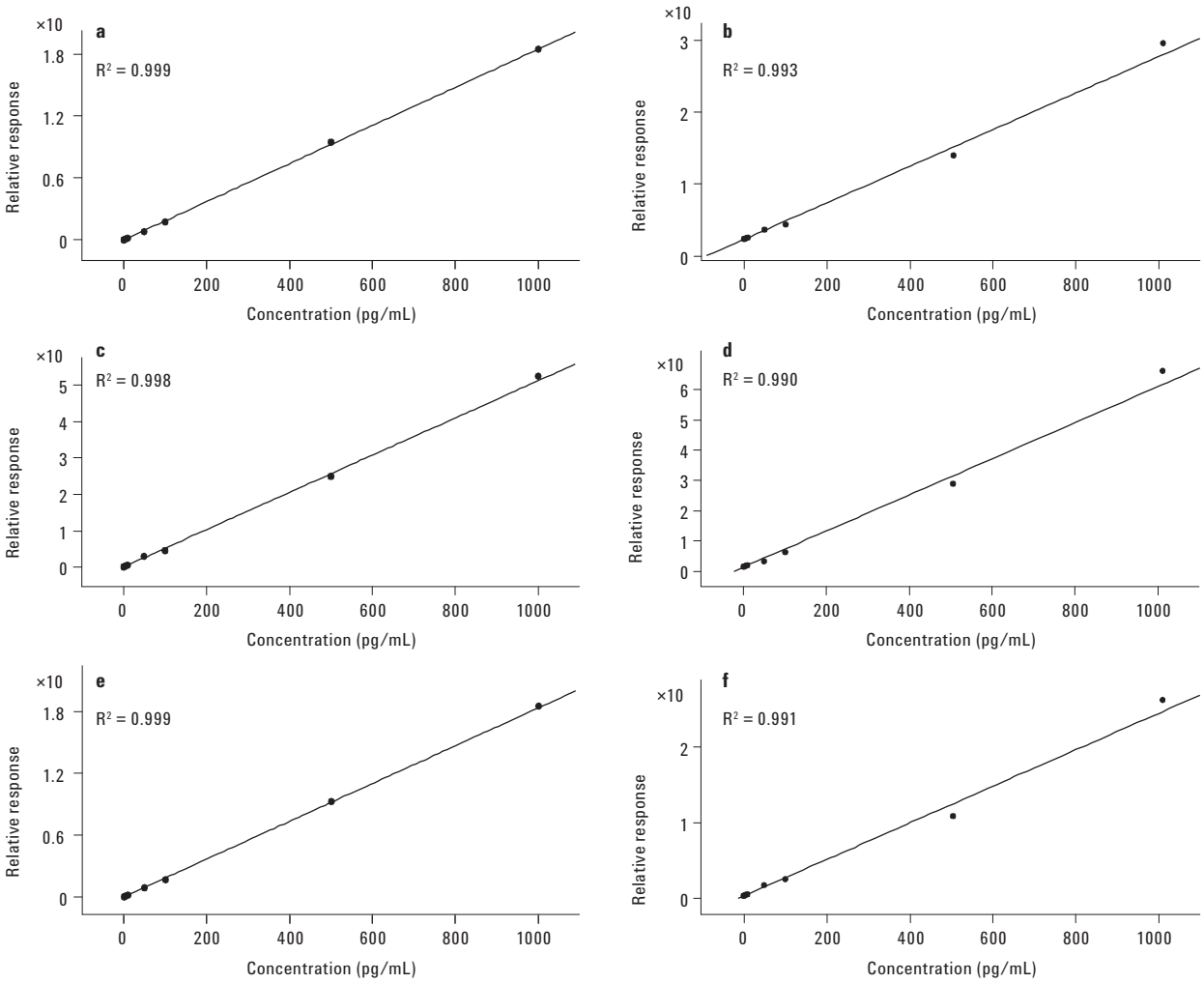


Figure 3. Calibration curves for free/total T4 (a,b), T3 (c,d), rT3 (e,f).

The method was sensitive, with LLOQ ranging from 1 to 5 pg/mL for free hormones, and from 0.5 to 2.5 pg/mL total thyroid determinations (Table 3). Chromatograms for both free and bound thyroid hormones illustrate the ability to quantitate these analytes at very low concentrations (Figure 4).

Conclusions

A sensitive and specific LC/MS/MS method has been developed for the simultaneous analysis of thyroxine (T4), 3,3′,5-triiodothyronine (T3) and 3,3′,5′-triiodothyronine (rT3) in serum. A simple filtration sample preparation for free thyroid hormones and a liquid-liquid extraction sample preparation for total thyroid hormones enables rapid determination of thyroid hormone levels down to low pg/mL levels.

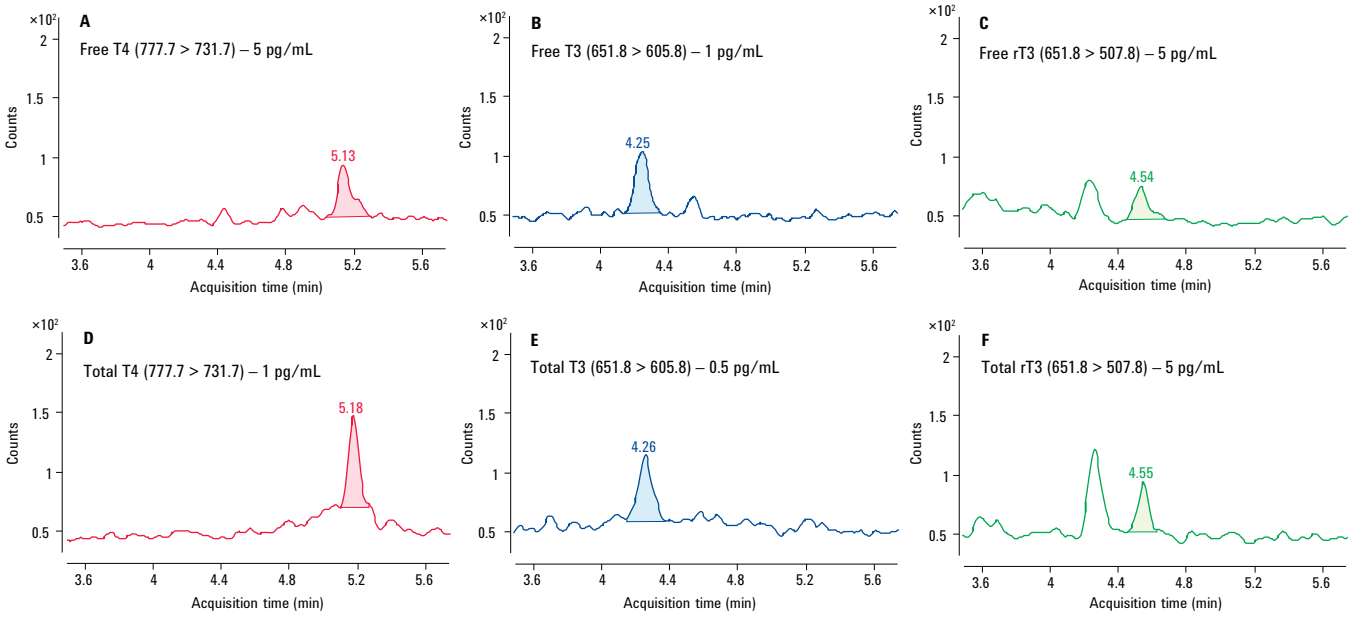


Figure 4. MRM traces for the LLOQ concentrations for free and total thyroid hormones.

Table 3. LLOQ for thyroid hormones in human serum.

Analyte	Free LLOQ (pg/mL)	Total LLOQ (pg/mL)
T4	5	1
T3	1	0.5
rT3	5	2.5

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