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Isotope dilution tandem mass spectrometric method for T4/T3

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Abstract

Background—The thyroid hormones thyroxine (T4) and 3,5,3′-triidothyronine (T3) are essential for regulating a number of biological processes, including growth, neurodevelopment, carbohydrate metabolism, oxygen consumption and protein synthesis. Immunoassays are the current methods for thyroid hormone measurement and suffer from a lack of specificity. Our objective was to simultaneously measure T4 and T3 using isotope dilution tandem mass spectrometry within a single run. To compare the results obtained by this MS/MS method with those obtained by an immunoassay procedure on the same samples (DPC Immulite for T3, Diagnostics Product, and Dade RxL Dimension for T4, Dade-Behring).

Methods—An API-3000 tandem mass spectrometer (SCIEX, Toronto, Canada) equipped with TurboIonSpray and Shimadzu HPLC system was used employing isotope dilution with deuterium-labeled internal standard (L-thyroxin-d_2). The method requires 100 μl of serum and involves addition of internal standard, precipitation of proteins with methanol and injection of the supernatant onto a C-18 column. After washing, the switch valve is activated and T4 and T3 eluted using a methanol gradient. T4 and T3 by immunoassay were performed using the Dade RxL Dimension and the DPC Immulite, respectively.

Results and conclusions—An isotope dilution tandem mass spectrometry method for the simultaneous determination of total T4 and T3 in serum is described which is accurate, specific, precise (%CVs 3.5–9.0), simple and fast (< 7 min).

Keywords

Thyroxine; Triiodothyronine; Thyroid Hormones; Tandem mass spectrometry

1. Introduction

The thyroid hormones, thyroxine (T4) and triidothyronine (T3), are important in regulating a number of biological processes, including growth and development, carbohydrate metabolism, oxygen consumption, protein synthesis and fetal neurodevelopment. Synthesis

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of all circulating T4 and a small percentage of circulating T3 occurs on thyroglobulin molecules located within the thyroid. The bulk of the T3 present in the blood is produced enzymatically via monodeiodination of T4 by specific intracellular deiodinases—enzymes present in the follicular cells and the cells of target tissues [1]. In serum drawn from healthy human subjects, total T4 is present at about 60-fold higher concentration than total T3. T4 acts as a prohormone, as the reservoir for the production of T3, the active hormone. The metabolic activity associated with thyroid hormone (TH) is initiated by T3 binding to specific nuclear receptors within target cells. Thyroid hormone concentrations in blood are essential tests for the assessment of thyroid function.

The presence of circulating iodothyronine-binding autoantibodies that interfere with total T4 and T3 RIAs is a known phenomenon [2–4]. These autoantibodies may give falsely high, or falsely low values of thyroid hormone measurements depending on the assay separation method used, and are often in discordance with the clinical features [2–4]. Direct serum-free T4 and T3 (FT4 and FT3) measurements are a way to compensate for such abnormal binding. However, technically, it is difficult to measure the free hormone concentrations since these are so low. It is easier to measure the total (free and protein-bound) thyroid hormone concentrations; total hormone concentrations are measured at nanomolar levels whereas free hormone concentrations are measured in the picomole range and to be valid, must be free from interference by the much higher total hormone concentrations.

Immunoassays (IAs) are notoriously unreliable with more and more literature published supporting their lack of specificity [5–12]. Table 1 shows the major differences reported by the College of American Pathologists program for proficiency testing of thyroid hormones that clearly illustrates the difference in specificity of the various antibodies used. For example, Table 1 shows mean results between different methods reported in the College of American Pathologists Proficiency Testing (CAP PT) Program can vary by a factor of approximately 2. Some factors such as pregnancy, estrogen therapy or genetics abnormalities in protein binding have also reportedly made IA methods for T4 and T3 diagnostically unreliable [2,3,13,14].

Currently serum total T4 (TT4) and total serum T3 (TT3) concentrations are most commonly measured by immunoassay methods. Recently some reports of quantitative measurement of T4 and T3 by HPLC, gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) were published [15–19]. All those methods required extraction, derivatization and even prior chromatographic separation that are very time-consuming [20,21]. This paper describes an isotope dilution tandem mass spectrometry method for the simultaneous determination of T4 and T3 in serum.

2. Methods

2.1. Chemicals and reagents

Standards of T4 and T3 were from Sigma (St. Louis, MO). A stable deuterium-labeled internal standard, L-thyroxin-d_2 was synthesized according to procedures described in the literature [15,16] by Dr. Tomas Class from the Chemistry Department at Georgetown University. HPLC grade methanol was purchased from VWR Scientific. All other chemicals were of analytical grade and purchased from Sigma.

2.2. Solutions and standards

Stock solutions of T3, T4 and internal standard (IS) were prepared separately to obtain concentration of 1 mg/ml for each. 40% ammonium hydroxide (v/v) in methanol was used as a solvent. The analyte stock solutions were diluted with methanol to obtain the spiking

solutions. The solutions were stored at 4 °C and could be used for several months. Standards for the calibration curve in the range of 0.325–5 ng/ml for T3 and 12.5–200 ng/ml for T4 were prepared by adding the analyses to 3% human γ-globulin (volume of spiking solution < 2% of final volume). Quality control (QC) samples (Diagnostic Product, Los Angeles, CA) at low, medium and high levels were used. A solution of 50 ng/ml d_2-T4 in methanol was used as the internal standard.

2.3. Sample preparation

Serum/plasma samples were thawed at room temperature. IS solution (150 μl) was added to aliquots of 100 μl of the serum or plasma sample. After 30 s of vortex mixing, the samples were stored for 10 min at room temperature to allow complete protein precipitation. The samples were centrifuged for 10 min at 15,000 rpm and 100 μl of supernatant was injected into the LC-MS-MS system.

2.4. LC/MS/MS conditions

An API 3000 tandem mass spectrometer (SCIEX, Toronto, Canada) equipped with TurboIonSpray and Shimadzu HPLC system was used to perform the analysis. Negative ion multiple reaction-monitoring (MRM) mode was used. The transitions to monitor were selected at m/z 650 → 127 for T3, m/z 776 → 127 for T4, m/z 778 → 127 for d_2-T4. Nitrogen served as auxiliary, curtain and collision gas. Gas flow rates, source temperature, ion spray voltages and collision energies were optimized for every compound by infusion of 1 μg/ml of the standard solutions in methanol at 20 μl/min and by flow-injection analysis (FIA) at LC flow rate. The main working parameters for the mass spectrometer are summarized in Table 2. Data processing was performed on Analyst 1.3 software package.

2.5. LC-MS-MS procedure

The procedure is based on an online extraction/cleaning of the injected samples with subsequent introduction into the mass spectrometer by using a built-in Valco switching valve. One hundred microliters of the deproteinized sample was injected onto a Supelco LC-18-DB (3.3 cm × 3.0 mm, 3.0 μm ID) chromatographic column equipped with a Supelco Discovery C-18 (3.0 mm) Guard column, where it underwent cleaning with 20% (v/v) methanol in 5 mmol/l ammonium acetate pH= 4.0 at flow rate 0.8 ml/min. After 3.5 min of cleaning the switching valve was activated, the column was flushed with water/methanol gradient at flow rate 0.5 ml/min and the samples were introduced into the mass spectrometer. The gradient parameters are shown in Table 3.

2.6. Immunoassays for T4 and T3

T4 was measured by the Dade RxL Dimension (Dade-Behring Diagnostics, Glasgow, DE) and T3 by the DPC Immulite (DPC) according to the manufacturer's specifications.

3. Results

The optimal mass spectrometer working parameters are shown in Tables 2 and 3. Replicate sera were assayed both within- and between-day at several concentrations. The within- and between-day precision data is provided in Tables 4 and 5. Recovery studies for T4 and T3 are shown in Tables 6 and 7. All results shown are the means of 8 replicates. Fig. 1 shows a typical chromatogram. Specimens were tested for T3 and T4 by both immunoassay (T3 DPC Immulite, T4 Dade Behring Dimension RxL) and by tandem mass spectrometry. Linear regression correlations (Prism) are shown in Figs. 2 and 3. The lower limit of quantitation was found to be 0.15 ng/ml for both T3 and T4. Detection limit was around 0.062 ng/ml.

4. Discussion

Evidence initially gleaned from both the CAP PT Program and pediatric reference ranges employing different immunoassays indicated the probability of lack of specificity for T4 and T3 immunoassay tests. To adequately assess this phenomenon we developed the isotope dilution tandem mass spectrometric method described in this manuscript. Serum T4 and T3 detection methods have evolved through a variety of technologies since the 1950s. Radioimmunoassay (RIA) methods to detect THs were developed in the 1970s. Serum T4 and T3 concentrations are currently measured by competitive immunoassay methods (IAs) that are mostly non-isotopic and use enzymes, fluorescence or chemiluminescence molecules as signals [22]. Table 1 clearly indicates that current IAs for T4 and T3 lack specificity and give mean results differing by a factor of approximately 2 in CAP PT programs. Total hormone assays necessitate the inclusion of a displacing agent (such as salicylate) to release the hormone from its binding proteins [23]. The displacement of hormone binding from serum proteins by such agents, together with the large sample dilution employed in modern assays, facilitates the binding of hormone to the antibody reagent.

Since T3 is 10-fold lower in concentration compared with T4 in blood it therefore presents both a technical sensitivity and precision challenge despite the use of a higher specimen volume. Although a reliable high-range T3 measurement is critical for diagnosing hyperthyroidism, a reliable normal-range measurement is also important for adjusting antithyroid drug dosage and detecting hyperthyroidism in sick hospitalized patients, in whom a paradoxically normal T3 value may indicate hyperthyroidism.

The correlation coefficient for the T4 comparisons (0.931) is significantly better than for the T3 comparisons (0.848) (Figs. 2 and 3). T3 by tandem mass spectrometry gave slightly higher results than those obtained by the DPC Immulite (Fig. 2). While this is true for children, our preliminary data for non-pregnant and pregnant women indicates a very poor correlation for T3 in both groups (r between 0.407 and 0.574). This is the subject of another manuscript.

The reasons for this are not clear but could include standardization issues, heterophilic antibodies etc. Of importance, we determined that reverse T3, which lacks a daughter ion of 127 m/z, therefore does not interfere in our tandem mass spectrometry method. Applying the tandem mass spectrometric method to CAP PT samples in the K/KN general ligand program again revealed that around 85% of the immunoassay methods gave means on samples which were lower than the means obtained by our tandem mass spectrometry method while 15% had higher means.

In conclusion, correlations between immunoassays and tandem mass spectrometry for T4 proved to be adequate except for the pregnant population, while the data for T3 was far less impressive especially during pregnancy. Recovery studies from several different sera using deuterated T4 as internal standard showed consistent (90–109%) recoveries for both T4 and T3 (Tables 6 and 7). The recovery differences found between samples were surprisingly larger for T4 than for T3. This indicates a lack of need to use deuterated T3 as the T3 internal standard. The isotope dilution tandem mass spectrometric method we developed is rapid (< 7 min), accurate (provides the true result as has been assessed by recovery studies), specific (measures only the analyte it purports to measure), precise (low %CV) and easy to perform. Its role in the routine clinical laboratory is currently being evaluated.

Acknowledgments

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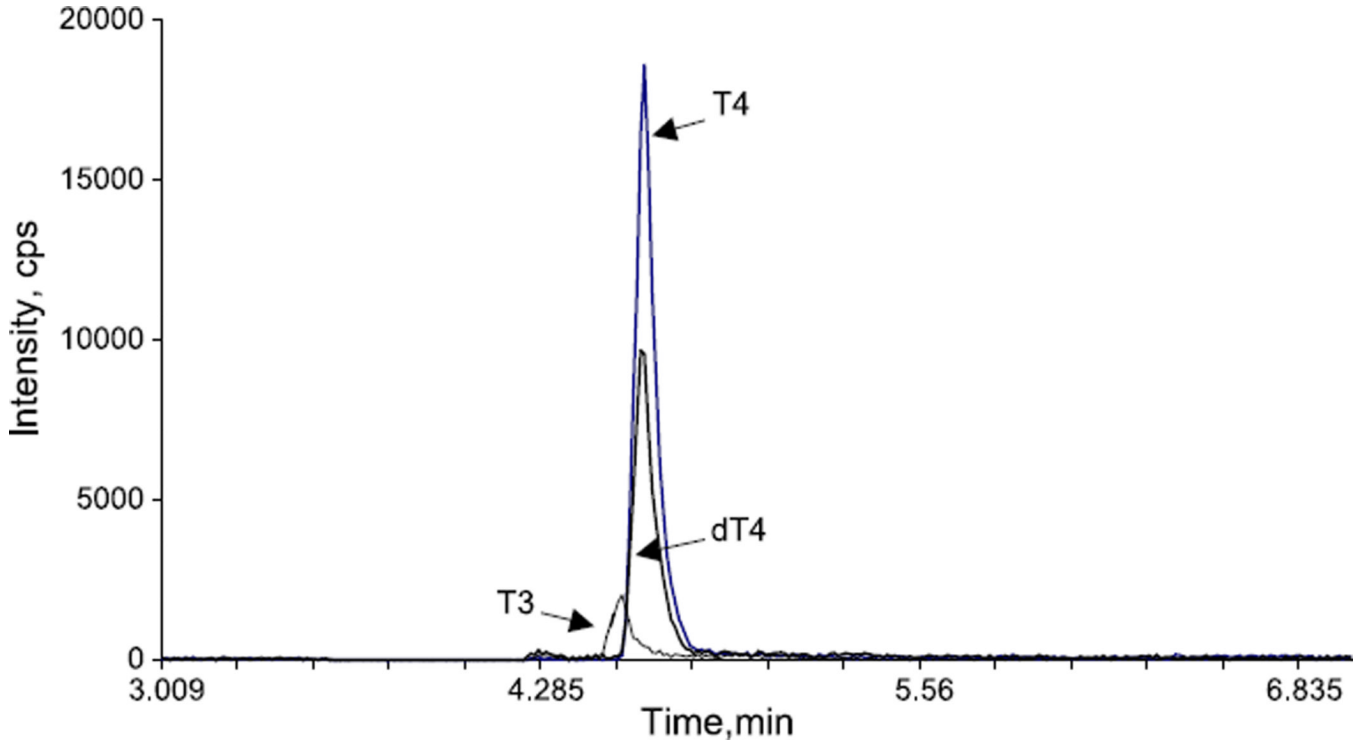

Fig. 1.
Tandem mass spectrometric chromatogram for a plasma sample. T4 *m*/*z* (776/127); D_2T4 *m*/*z* (778/127); T3 *m*/*z* (650/127).

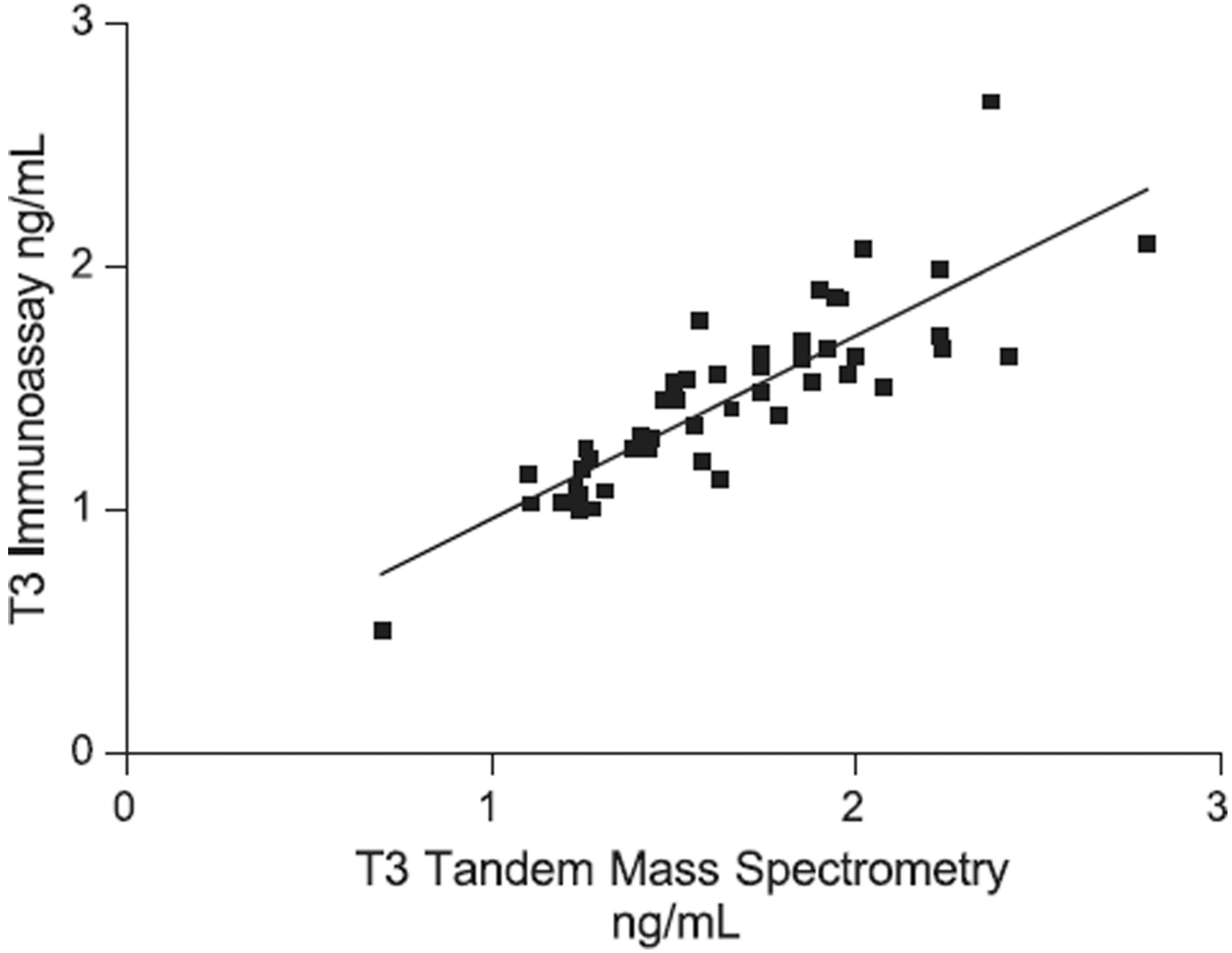

Fig. 2.
T3 measured by isotope dilution tandem mass spectrometry vs. immunoassay. IA= 0.75 MS + 0.21; $r = 0.848$; $S_{y \cdot x} = 0.1956$; $n = 49$.

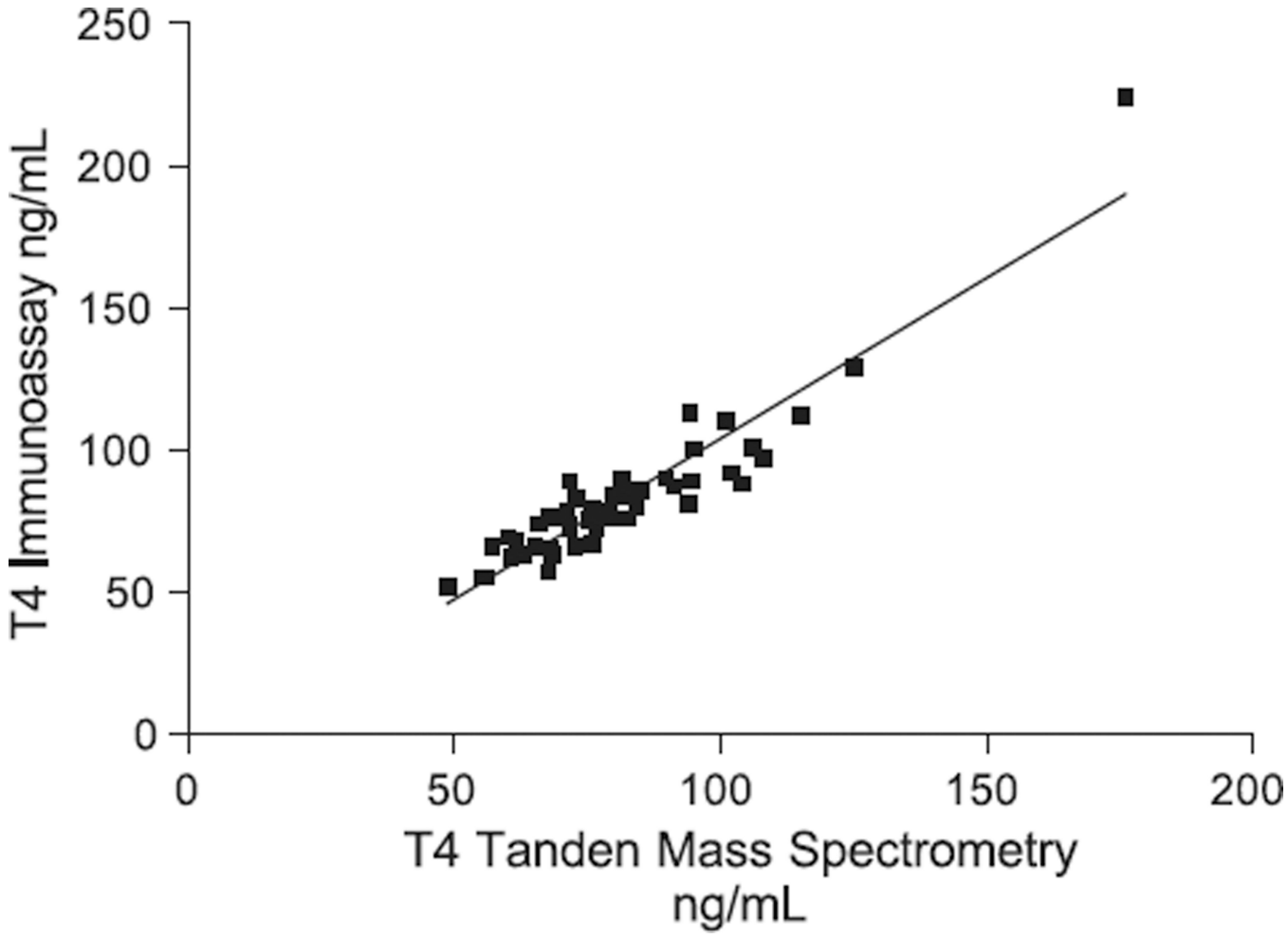

Fig. 3.

T4 measured by Isotope dilution tandem mass spectrometry vs. immunoassay. IA = 1.13 MS – 8.99; $r = 0.931$; $S_{y \cdot x} = 9.54$; $n = 50$.

Table 1

Problems with immunoassays: data acquired for samples from the CAP PT program 2003

Analyte	Mean CAP result for method giving lowest value	Mean CAP result for method giving highest value
Triiodothyronine (ng/dl)	108.5	190.2
	364.8	610.1
Thyroxine	5.64	10.09
(μg/dl)	1.64	3.65
	8.73	13.12

Table 2

Tandem mass spectrometer working parameters

Parameter	Value
Nebulizer gas (NEB)	8
Curtain gas (CUR)	10
Collision gas (CAD)	6
TurboIon Spray Heater gas	7 l/min
TurboIon Spray (IS) voltage	4500 V
Entrance Potential (EP)	7.5 V
Collision cell Exit Potential (CXP)	5 V
Source temperature	450°
Dwell time	250 m sec

Table 3

Gradient parameters

Time (min)	Methanol (%)
3.50	75
5.25	76
5.50	100
7.00	End

Table 4

Within-day precision ($n = 10$)

Analyte	Control 1			Control 2		
	Mean (ng/ml)	S.D.	CV (%)	Mean (ng/ml)	S.D.	CV (%)
T3	1.04	0.014	1.36	2.44	0.077	3.19
T4	24.1	0.437	1.81	81.2	1.502	1.85

Table 5

Between-day precision ($n = 20$, 1 run per day for 20 days)

Analyte	Control 1			Control 2			Control 3		
	Mean (ng/ml)	S.D.	CV (%)	Mean (ng/ml)	S.D.	CV (%)	Mean (ng/ml)	S.D.	CV (%)
T3	1.08	0.05	4.47	2.39	0.22	9.21	3.49	0.31	9.00
T4	24.4	1.39	5.69	76.6	3.11	4.06	116.3	4.15	3.57

Table 6

Recovery of added thyroxine (T4)

Sample no.	Added (ng/ml)	Detected mean	Added amount recovered	Recovery, %
1 (n = 8)	0	85.9	NA	NA
	10	96.7	10.8	108.0
	40	127.5	41.6	104.0
2 (n = 5)	0	62.6	NA	NA
	10	72.1	9.5	95.0
	40	98.0	35.4	90.0
3 (n = 5)	0	73.8	NA	NA
	10	84.7	10.9	109.0
	40	116	42.2	105.0
4 (n = 5)	0	58.3	NA	NA
	10	68.0	9.7	97.0
	40	95.0	36.7	92.0

NA= not applicable.

Table 7

Recovery of added triiodothyronine (T3)

Sample no.	Added (ng/ml)	Detected mean	Added amount recovered	Recovery, %
1 (n = 8)	0	1.88	NA	NA
	0.25	2.12	0.24	96.0
	1.00	2.85	0.97	97.0
2 (n = 5)	0	1.70	NA	NA
	0.25	1.96	0.26	104.0
	1.00	2.76	1.06	106.0
3 (n = 5)	0	1.56	NA	NA
	0.25	1.81	0.25	100.0
	1.00	2.62	1.06	106.0
4 (n = 5)	0	0.49	NA	NA
	0.25	0.74	0.25	100.0
	1.00	1.50	1.01	101.0

NA= not applicable.