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Is measurement of TT3 by immunoassay reliable at low concentrations? A comparison of the Roche Cobas 6000 vs. LC-MSMS

Likhona Siphe Masika¹, Zhen Zhao¹, and Steven John Soldin^{1,2,*}

¹Department of Laboratory Medicine, National Institutes of Health, 10 Center Drive Bethesda, MD, USA

²Department of Medicine, Georgetown University, 3900 Reservoir Rd NW, Washington DC, USA

Abstract

Objectives—Thyroid dysfunction is a common medical condition affecting an estimated 30 million people in the US alone. Employing gold standard Liquid chromatography-tandem mass spectrometry (LC-MSMS) methods we have examined the extent of inaccuracy of Immunoassay (IA) measurement for total T3 (TT3) at low, normal and high concentrations.

Design and Methods—268 TT3 Roche Cobas 6000 immunoassay TT3 values (covering the low, normal, and high ranges) were compared with LC-MSMS results.

Results—At TT3 concentrations between 50–113 ng/dL (Conversion factor for TT3 to SI Units is $\text{ng/dL} \times 0.0154 = \text{nmol/L}$), $n=122$, LC-MSMS values were lower than immunoassay with 72% found to be below the 2.5th percentile by LC-MSMS compared to 27% for immunoassay. Strikingly 45% of the patients classified, as normal TT3 by immunoassay were defined as lower than the 2.5th percentile by LC-MSMS. Only 38 of the 122 patients with low T3's were not receiving T4. In this latter group all of whom had TSH's >3.7 mIU/L, 74% of results by LC-MSMS were below the 2.5th percentile while only 21% were below the 2.5th percentile by IA. The clinical consequences of these inaccuracies may affect whether dosing with T4 or combination of T4 with T3 is selected for treatment. Finally the correlation of TT3 with TSH was far superior when TT3 was measured by LC-MSMS. A typical case which demonstrates our message is included.

Conclusion—T3 being the active hormone needs to be reliably measured and if the patient has low TT3 and hypothyroid symptoms persist; treatment with T3 should be considered. A typical case report is included to illustrate the problems of inaccurate immunoassay results for TT3. Measurement of TT3 by immunoassay at low concentrations is less than optimal and often provides the clinician with a normal result when the LC-MSMS method and the patient's clinical

Corresponding author and person to whom requests should be addressed: Steven John Soldin, PhD, DABCC, Department of Laboratory Medicine, National Institutes of Health, 10 Center Drive Bethesda, MD, USA, Phone: Fax: soldinsj@cc.nih.gov.

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condition suggests that supplementation with T3 (as in combination therapy) may be required to optimize patient care.

Keywords

Immunoassay; Mass Spectrometry; FDA

1. Background

Thyroid dysfunction is a common medical condition affecting an estimated 30 million people in the USA. Thyroxine (T4) is the major pro-hormone secreted by the thyroid gland. Triiodothyronine (T3) is the biologically active hormone that binds to the nuclear thyroid receptors. T4 is converted into T3 by several deiodinases present in many tissues in order to initiate signaling and gain biological activity. In the US, the Endocrine Society's preferred approach to the treatment of hypothyroid individuals is with synthetic levothyroxine (LT4) alone [1]. Despite documentation of variable LT4 to T3 conversion, there has long been evidence that about 20% of patients require combined T3/LT4 treatment, based on evidence that many patients still have hypothyroid symptoms and in these patients TT3 concentrations are often low by Liquid chromatography-tandem mass spectrometry (LC-MSMS) when treated with LT4 alone. It has recently been suggested that this T3 deficiency may be associated with failure to fully reverse the symptoms of hypothyroidism [2–5]. Moreover the approach of LT4 alone therapy has also been questioned by thought leaders in the field. Dr. Wartofsky states *"Perhaps 20% of hypothyroid patients treated with T4 alone continue to complain of symptoms suggesting thyroid hormone deficiency"*[3]. Hypothyroid patients receiving LT4 alone do often have both normal TSH and T4/FT4 by LC-MSMS but 20–30% of them still have symptoms of hypothyroidism. Wartofsky goes on to advise that T4/T3 combination therapy be considered in these cases. "Do no harm" is not just part of the Hippocratic oath, it needs to be remembered and implemented in practice! For these reasons the accurate and reliable measurement by mass spectrometry of TT3 together with FT4 and FT3 may better reflect the thyroid hormone status and guide physicians to a more accurate diagnosis and treatment. Jonklaas et al [6] studied athyreotic individuals receiving only monotherapy with T4. TT3 was measured by both IA and LC-MSMS using the Siemens Vista analyzer. With IA, all the results in the low range were above the 2.5th percentile and so the conclusion was reached that T3 administration was not necessary and only monotherapy with T4 was needed. However, contradicting this conclusion is the TT3 data measured by mass spectrometry [Figure 4] [6]. Here >50% of the results were below the 2.5th percentile indicating that in more than 50% of this population T3 combination therapy should be considered. As there are 20 million people in the US with hypothyroidism it becomes pivotal that reliable mass spectrometric equilibrium dialysis or ultrafiltration methods be used to quantify FT4, FT3 while TT3 is also more reliably measured by LC-MSMS than by IA. Our laboratory has published FT4, FT3 methods all of which correlate well with both log TSH and the patient's clinical condition. [4,6–12]. This manuscript compares the Roche IA method for TT3 with LC-MSMS. In patients not receiving T4, LC-MS/MS TT3 results correlated well with TSH.

2. Patients and methods

2.1 Participants

The TT3 study was a prospective study of samples received at the NIH Clinical Center, department of laboratory medicine (DLM) from February 2015 to October 2015 for the measurement of TT3. Samples were selected for inclusion in the study to reflect a spectrum of normal, low, and high TT3. In total we included 268 samples for analysis. The study was approved by the Institutional Review Board of the NIH (Clinical Protocol number 93-CC-0094).

2.2 Test Method

Blood samples were collected in plastic lithium heparin tubes (Greiner Bio-One, Belgium). Samples were processed according to the usual laboratory procedures for TT3. We measured TT3 by electrochemiluminescence immunoassay on Roche Cobas 6000 analyzer on the day of collection (Reference Interval 80 – 200 ng/dL)(Conversion factor for TT3 to SI Units is $\text{ng/dL} \times 0.0154 = \text{nmol/L}$). The plasma was removed and stored in cryogenic vials at -80°C until LC-MSMS analysis. TT3 measurements with LC-MSMS were performed using an Agilent 6460 triple-quadrupole MS coupled with an ESI Agilent Jet Stream ionization source and Agilent 1200 Infinity series HPLC system using isotopic dilution with ^{13}C labeled internal standard, T3 $^{13}\text{C}_6$ (Reference Interval 80–178 ng/dL). Sample preparation was performed as described in “Isotope dilution tandem mass spectrometric method for TT4/TT3” [7,8] with minor modifications. Briefly, 100 μL of sample was added to 150 μL of ^{13}C labeled internal standard in acetonitrile for deproteinization. After vortexing and centrifugation, 200 μL of supernatant was diluted with 500 μL of 0.1M-ammonium acetate in de-ionized water and 200 μL was injected onto an Agilent Eclipse XDB-C8 cartridge column. After washing, the switch valve was activated and the analyte was eluted with a water/methanol (containing 0.01% formic acid) gradient into the MS/MS system. Quantification by multiple reaction mode monitoring (MRM) analysis was performed in positive mode [7]

2.3 Statistical Analysis

We conducted statistical analysis using GraphPad Prism version 6 (Graph Pad Software, California).

3. Results

The linear regression equation between LC-MSMS and IA for TT3 measurement varied at different concentrations with significant changes between slopes and intercepts (Figure 1A, B and C). The correlation improves as concentration increases. As our biggest concern was for the 20 million hypothyroid people in the USA we focused our in depth analysis on individuals with IA TT3 between 50–113 ng/dL (the low concentration data). Using the published lower cutoff of 80 ng/dL for TT3 by LC-MSMS [20] and IA (manufacturer plus in-house studies)[horizontal line, LC-MSMS, Figure 2 and vertical line, IA, Figure 2], 72% ($n=122$) of the low TT3 group was found to be below the 2.5th percentile by LC-MSMS compared to 27% defined by IA. The inhouse study for the IA reference interval was done

using 44 healthy people to assess manufacture's claim and was found to be acceptable on the EP Evaluator. Strikingly, 45% of the patients classified as normal TT3 by IA would otherwise be defined as lower than the 2.5th percentile by LC-MSMS (Figure 2, grey box). In patients not receiving T4, TT3 was statistically significantly lower ($P<0.0001$) when measured by LC-MSMS than by IA at high TSH values [>3.7 mIU/L (95th percentile)] (Figure 3)[similar to Jonklaas et al [6,]] resulting in a higher number of patients classified with T3 deficiency and a far superior correlation with TSH. None of the patients in Figure 3 were treated with LT4. Figure 3 demonstrates that IA classified only 22% below the 2.5th percentile while LC-MSMS classified 73%. The following case report is one of many and is a good illustration of the problems discussed above and requiring TT3 measurement by LC-MSMS.

Case report

Our case involves a 56-year-old, Caucasian female, with a three-year history of hypothyroidism. She presented with lethargy, loss of energy difficulty in losing weight, constipation, muscle weakness and dry skin. She has been treated with LT4 (50 mcg/day) for the past 12 months without improved symptoms. She is very compliant with LT4 doses and was referred to us for further testing. On examination, BP 126/91, heart rate 73, respiration 18, temperature 35.8 degrees Celsius, height 168.1 cm, and weight 84.4 kg (BMI 29.9 kg/m²). The thyroid was neither enlarged nor tender. No abnormal nodules were palpated. The rest of the examination was unremarkable except for elevated cholesterol of 219 mg/dL (Desirable < 200 mg/dL).

After treatment with T4, the patient had a normal TSH (2.65 mIU/L) and thyroxine-binding globulin (TBG). Thyroid peroxidase antibodies (ATA) and thyroglobulin antibodies (ATG) were below detection limits. Both LC-MSMS and IA methods were performed to determine the status of her thyroid hormones. LC-MSMS and IA methods both showed high T4's (>11.7 mcg/dL) and normal FT4's (LC-MSMS/IA 1.9/1.6 ng/dL respectively) However, the patient had a low TT3 of 82 ng/dL (3rd percentile) and FT3 2.1 pg/mL (4th percentile) when measurements were performed by the gold standard LC-MSMS method. In contrast, IA TT3's and FT3's were in the 25th percentile and the 20th percentiles, respectively (in this study the performance of IA methods has been demonstrated to be poor and provide falsely elevated results at the low concentrations). The low TT3 and FT3 by LC-MSMS more readily explain the unresolved symptoms of hypothyroidism even with the treatment of LT4 and the normal TSH [20]. The patient was started on additional BID dosing with 12.5 µg T3 and after 10 days all her symptoms of hypothyroidism were alleviated and cholesterol had dropped from 219 to 194 mg/dL. Her after treatment LC-MSMS thyroid hormones were measured pre-dose at 8am and 2 h post dose at 10am. They were found to have normalized, TT3 129 and 145 ng/dL at 8 and 10am respectively. FT4 and FT3 were 1.3,1.4 ng/dL (1.3–2.4 nd/dL) and 3.8,4.6 pg/mL (1.5–6.2 pg/mL) at 8 and 10 am respectively.

4. Discussion

The findings for TT3 [4,6,7] should be of great concern as many clinicians depend on the results generated by the most widely used methods (IA) to make a diagnosis and to treat and

monitor their patients. Since correct diagnosis leads to appropriate treatment, the importance of accuracy of TT3 measurements at the lower concentrations cannot be over emphasized. We are now assessing the TT3 results by LC-MSMS whenever patients continue to have signs and symptoms of hypothyroidism despite their treatment with LT4. These patients often have normal TSH's and T4/FT4 (T4 therapy normalizes their TSH) but may still complain of hypothyroid symptoms. This may be due to deiodinase polymorphisms [16,17]. Autosomal deficiencies in many of the deiodinases exist and it has been estimated that approximately 40–60% of the world's population is heterozygous for a deiodinase deficiency [18, 19] these patients may benefit from T3/T4 combination therapy. We now use our previously published LC-MSMS TT3 methods [7,8] to assess how well it correlates with an immunoassay for TT3 (Roche Cobas 6000) and which assay correlates better with TSH. Previous studies have demonstrated that TT3 measurement by LC-MSMS is more accurate and specific than that by IA [4,6,12,13]. It has also been shown that the relationship between thyroid hormones (TT3, TT4, FT3, FT4) measured by LC-MSMS agrees better with both log TSH and clinical symptoms than they do when measurements are performed by IA [4,6,9,13–15] This is especially true for low values which are frequently below the 2.5th percentile for LC-MSMS and normal by IA. [6,15]. The analytical bias between LC-MSMS and IA is attributed to the non-specific binding of IA especially at the low concentrations. The background to this work is that in recent years it has been recognized that T3 is the active thyroid hormone with T4 now recognized as the pre-hormone. This raises new issues and suddenly accurate and reliable measurement of T3 is important as is its relationship with TSH and the patient's clinical condition.

In this manuscript we have demonstrated that TT3 measured by LC-MSMS differs significantly from the Roche IA results and most importantly it is the LC-MSMS data that has a far superior correlation with TSH. It should also be emphasized that 79% of low range TT3's are called normal by IA while 74% are called low by Mass Spectrometry. Accurate TT3 measurements by LC-MSMS will improve diagnosis and treatment of hypothyroidism suggesting that some of these patients would benefit by adding T3 to their current dosing regimen (require combination therapy).

FDA issues

The IAs used in this and other studies were approved by the FDA. However, the FDA 510(k) review standard is substantial equivalence of a new device to a legally marketed device without assessing whether any of them are “clinically useful”. In this case, there needs to be a good correlation of FT4, FT3 and TT3 with log TSH (and the results need to correlate with the patient's clinical condition.) This has been demonstrated for equilibrium dialysis and ultrafiltration methods followed by either LC-MSMS or IA. In hypothyroidism FT4, FT3 and TT3 are mostly in the low range and despite FDA approval none of the direct analogue IA methods correlate well with TSH [4,14,15]. Furthermore the IA TT3 measurements have been shown in this and our previous JAMA manuscript [6] to miss most patients with low TT3's. Finally both the Siemens IA used in [6] and the Roche Cobas 6000 assay used in this manuscript have poor correlation with TSH at low concentrations and consequently miss many patients with low TT3's.

5. Conclusions

This difference between gold standard LC-MSMS methods and direct IA methods is apparent for thyroid hormone concentrations in the low ranges. For example, in the low range, IAs give false normal results in about 50% of FT3 and TT3 and 65% of FT4 results [4,6,8,9,15]. This together with our previous studies showing falsely high FT3, TT3, and FT4 results by FDA approved IAs further compounds the current problems for physicians attempting to diagnose and treat their patients appropriately. Hypothyroidism affects around 20 million people in the USA alone. 4–6 million of these may be sub-optimally treated on T4 monotherapy when symptoms of hypothyroidism persist. It is true that the majority of patients on LT4 will not require T3 dosing. It is nevertheless important to accurately measure TT3 hormone in order to identify the many patients that may require T4/T3 combination therapy even though TSH may have been normalized via the T4 treatment. According to Wartofsky [3] this group accounts for about 20% of the hypothyroid population.

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Highlights

- QUESTION: Can LC-MSMS measurement of TT3 help facilitate the diagnosis of hypothyroidism?
- We focused on patients with immunoassay values between 50–113 ng/dL
- 74% of individuals not taking T4 were below the 2.5th percentile by LC-MSMS. This compares to 21% by IA.
- Combined T4/T3 dosing needs to be considered in about 20% of hypothyroid patients
- As T3 is the active hormone it is important that physicians be provided with accurate LC-MSMS analysis of TT3.

Figure 1.

Correlation between TT3 by IA and LC-MSMS. At low concentration $Y = 0.68 \cdot X + 14.3$, $r = 0.67$, $n = 122$ (A). Normal concentration $Y = 0.797 \cdot X + 8.7$, $r = 0.74$, $n = 100$ (B). High concentration $Y = 0.789 \cdot X + 5.4$, $r = 0.90$, $n = 46$ (C). (Conversion factor for TT3 to SI Units is $\text{ng/dL} \times 0.0154 = \text{nmol/L}$)

Figure 2.

Correlation between TT3 by IA and LC-MSMS at low concentrations Horizontal line LC-MSMS, vertical line IA, using the published lower cutoff of 80 ng/dL. Grey box indicates 45% of patients classified as above lower cutoff (80 ng/dL) by IA and below lower cutoff by LC-MSMS. (Conversion factor for TT3 to SI Units is $\text{ng/dL} \times 0.0154 = \text{nmol/L}$)

Figure 3. TT3 by IA and LC-MSMS at TSH values >3.7 mIU/L (95th percentile) in patients not receiving T4. $P < 0.0001$. (Conversion factor for TT3 to SI Units is $\text{ng/dL} \times 0.0154 = \text{nmol/L}$)