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Highlight Article

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

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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 and 113 ng/dL (conversion factor for TT3 to SI Units is ng/dL × 0.0154 = 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 condition suggests that supplementation with T3 (as in combination therapy) may be required to optimize patient care.

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 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

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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 [Fig. 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 ng/dL × 0.0154 = 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 13C labeled internal standard, T3 13C6 (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 13C labeled internal standard in acetonitrile for deproteinization. After vortexing and centrifugation, 200 μL of supernatant was diluted with 500 μL of 0.1 M-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 (MRM) monitoring 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 (Fig. 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 and 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, Fig. 2 and vertical line, IA, Fig. 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 in-house 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 (Fig. 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)] (Fig. 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 Fig. 3 were treated with LT4. Fig. 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.

3.1. 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 °C, height 168.1 cm, and weight 84.4 kg (BMI 29.9 kg/m^2). 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 percentile, 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 8 am and 2 h post-dose at 10 am. They were found to have normalized, TT3 129 and 145 ng/dL at 8 and 10 am 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