

Short Communication

Measurement of reverse triiodothyronine levels using liquid chromatography-tandem mass spectrometry in the serum of 89 outpatients

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Abstract Background: Reverse triiodothyronine (rT3) has useful clinical applications, such as differentiating euthyroid sick syndrome from hypothyroidism. However, rT3 measurement is unavailable in the clinical setting in Japan. Therefore, we assessed the feasibility of using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for measuring serum rT3 levels in outpatients.

Method: MS/MS detection was performed in the positive ionization mode on a tandem quadrupole mass spectrometer equipped with an electrospray ionization interface. The precursor-product ion pairs of rT3 (m/z 652 \rightarrow 508) and ¹³C₆-rT3 (m/z 658 \rightarrow 514) were selected. Quantification was performed via selected reaction monitoring using peak areas. The serum from 89 patients (age, 8.1 \pm 8.1 years) who visited Aichi Medical University was collected. Patients diagnosed with thyroid disease were excluded. rT3 was extracted from 100 μ L of patient serum by liquid-liquid extraction. ¹³C₆-rT3 was used as the internal standard. Serum rT3 levels were also determined using a radioimmunoassay (RIA) kit.

Result: The lower limit of rT3 quantification was 0.02 ng/mL. The relative standard deviation and relative error were <9% and <4%, respectively, indicating an acceptable level of reproducibility. Compared with RIA, LC-MS/MS exhibited superior linearity (0.99872 versus 0.99999). rT3 levels in the patient serum samples were in the range of 0.038–0.853 ng/mL.

Conclusion: The LC-MS/MS technique reliably measured rT3 in the serum of 89 outpatients.

Key words: thyroid hormone, reverse T3, LC-MS/MS, ¹³C-labeled internal standard, quantification limit, feasibility studies

Introduction

Reverse triiodothyronine (rT3) is the inactive form of thyroid hormone that binds to its receptor upside down. Despite its inertness, rT3 is used to diagnose euthyroid sick syndrome (ESS)¹. ESS is the abnormal findings in thyroid function tests that often occurs in critical conditions, such as inflammation, myocardial infarction, starvation, sepsis, burns, trauma, surgery, malignancy, and bone marrow trans-

plantation¹. The most common hormonal pattern in ESS is a low T3 level with normal thyroxine and thyroid-stimulating hormone levels; this pattern resembles the findings in hypothyroidism¹. rT3 is extremely useful for differentiating ESS from hypothyroidism because rT3 is typically high in the former and low in the latter¹. However, rT3 measurement is unavailable in the clinical setting in Japan.

Compared with immunoassays, such as radioimmunoassay (RIA), mass spectrometry (MS) has the potential to measure the levels of multiple hormones during a single analysis with improved specificity and sensitivity, lower reagent cost, and a wide measurement range²⁻⁵. This study aimed to validate the liquid chromatography-tandem MS (LC-MS/MS) technique for measuring serum rT3 levels. The technique was applied to rT3 quantification in the serum of 89 outpatients.

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Materials and Methods

Subjects

To select patients with a wide range of serum rT3 concentrations, we collected the serum from 89 patients with various conditions who visited Aichi Medical University between June 18 and June 22, 2016. These patients ($n=89$; age, 8.1 ± 8.1 years; range, 1 month–48 years) included four adults. Patients diagnosed with thyroid disease were excluded. All samples were stored at -80°C and were analyzed in a single batch at the completion of the study. In accordance with the Ethical Guidelines for Medical and Health Research Involving Human Subjects, Chapter 5, Part 12, informed consent was not obtained because the serum used in this research had been anonymized. The study was approved by the ethics committee of Aichi Medical University (reference number 2015-H359).

Reagents and standards

rT3 and its ^{13}C -labeled internal standard ($^{13}\text{C}_6$ -rT3) were purchased from IsoSciences (King of Prussia, PA, USA). According to the manufacturer's certificate of analysis, the purity of $^{13}\text{C}_6$ -rT3 was determined to be 97% using high-performance liquid chromatography. A Kinetex C18 $2.6\text{-}\mu\text{m}$ column (00D-4462-AN) and SecurityGuard™ ULTRA cartridge (AJ0-9298) were obtained from Phenomenex (Torrance, CA, USA). Hydrophilic polyvinylidene fluoride (PVDF) membrane (SLHVR04NL) was purchased from Millipore (Billerica, MA, USA). An rT3 RIA kit (RVR-EW-125) was purchased from DIAsource ImmunoAssays SA (Louvain-La-Neuve, Belgium).

A 1-mg/mL stock solution of rT3 was prepared using 95% ethanol (EtOH) in water (v/v) containing 6mM sodium hydroxide. $^{13}\text{C}_6$ -rT3 at 40 $\mu\text{g}/\text{mL}$ concentration was prepared using 30% ammonium in methanol (MeOH; v/v). The stock solutions were stored at -30°C . Working solutions were prepared by diluting the stock solutions with 50% MeOH in water (v/v). Calibration standard solutions ranging from 0.02 to 5 ng/mL were prepared by spiking the delipidized human serum with ultra-low hormones, steroids, and other analytes (MSG 3000, Golden West Biologics, Inc., Temecula, CA, USA) with various amounts of stock solution of rT3. A labeled internal standard mixture in serum was prepared at a final concentration of 0.25 ng/mL.

Instruments

The LC-MS/MS technique was developed on a LTQ

Velos Dual Pressure Linear Ion Trap LC-MS/MS system coupled to an Accela ultra performance liquid chromatography system (ThermoFisher, Waltham, MA, USA) in the positive ion multiple-reaction monitoring mode. The Phenomenex Kinetex C18 $2.6\text{-}\mu\text{m}$ column and SecurityGuard™ ULTRA cartridge were used as the analytical and guard columns, respectively. The initial conditions for the LC gradient were 50% A [0.1% formic acid in water (v/v)] and 50% B (MeOH). The composition was changed gradually over 3 min till a composition of 20% A and 80% B was obtained. MS/MS detection was performed in the positive ionization mode on a tandem quadrupole mass spectrometer equipped with an electrospray ionization interface. The precursor-product ion pairs of rT3 (m/z 652 \rightarrow 508) and $^{13}\text{C}_6$ -rT3 (m/z 658 \rightarrow 514) were selected. The spray voltage was set at 4.0kV, and the capillary temperature was set at 450°C . The sheath gas flow rate was 10 (arbitrary units). Quantification was performed by selected reaction monitoring using peak areas.

Sample preparation

Serum samples (100 μL) from patients were spiked with $^{13}\text{C}_6$ -rT3 as the internal standard. After vortexing, 400 μL of 2% NH_4OH in EtOH (v/v) was added. The solutions were vortexed and maintained at 4°C for 1 h. The mixtures were then centrifuged at $13000\times g$ for 5 min. The supernatants were decanted into new 2.0-mL tubes, and the pellets were washed by resuspending in 100 μL of 2% NH_4OH in EtOH (v/v). The supernatants were combined, and the solution was evaporated by freeze-drying. Subsequently, the residue was reconstituted using 100 μL of 50% MeOH in water with 0.05% formic acid (v/v). After filtering using a 4-mm syringe filter and a hydrophilic PVDF membrane, a 15- μL aliquot of the resulting sample was subjected to LC-MS/MS analysis.

Method validation

The quantification limit (lower limit of quantification) was determined by injecting the serum at the concentration of rT3, ranging from 0.02 to 5 ng/mL, as serial diluted calibrators⁶. Precision was obtained as the coefficient of variation [relative standard deviation (RSD%)]. Accuracy was obtained as the relative error (RE%), which was calculated as follows: $\text{RE}\% = [(\text{measured amount} - \text{spiked amount}) / \text{spiked amount}] \times 100$.

RIA

Serum rT3 levels in the patients were also measured using an rT3 RIA kit following the manufacturer's protocol.

Statistical analysis

rT3 levels were expressed as mean±standard deviation. Data analysis and visualization were aided by the Daniel's XL Toolbox add-in for Excel, version 7.1.4, by Daniel Kraus, Würzburg, Germany (www.xltoolbox.net).

Results and Discussion

The LC-MS/MS technique for measuring serum rT3 levels was validated by evaluating a series of parameters, such as the quantification limit, linearity, precision, and accuracy. The quantification limit for rT3 was estimated to be 0.02 ng/mL with a signal-to-noise ratio of 10:1 under the LC-MS/MS conditions used in the experiment. This limit was comparable with that obtained in a previous study⁴. The calibration curve for rT3 was generated using the following regression equation: $y=0.0593077x+0.0002492$, $r^2=0.9999$, in the range of 0.02–5 ng/mL. The RSD% and RE% values of the standards were 4.76% and 2.13% at 0.02 ng/mL, 8.46% and 3.78% at 0.05 ng/mL, 2.49% and 1.11% at 0.2 ng/mL, 3.35% and 1.50% at 0.5 ng/mL, 1.92% and 0.85% at 2.0 ng/mL, and 1.56% and 0.70% at 5.0 ng/mL, respectively. At concentrations >0.02 ng/mL, all RSD% and RE% values were <10%. The values were acceptable at all concentrations.

The technique was then applied to rT3 quantification in the serum of 89 outpatients. Their serum rT3 levels were detected in the range of 0.038–0.853 ng/mL. Compared with RIA, LC-MS/MS provided superior linearity [0.99872 (RIA) versus 0.99999 (LC-MS/MS); Fig. 1]. Moderate correlation was observed between LC-MS/MS and RIA (r^2 , 0.7287; $p<0.001$).

The relationship between age and rT3 level measured using LC-MS/MS is shown in Fig. 2. The reference ranges of serum rT3 levels measured using RIA have been reported to be 0.6–2.5 ng/mL (birth to 6 days), 0.09–0.35 ng/mL (children older than 6 days), and 0.11–0.32 ng/mL (adults)⁷. These reference ranges for serum rT3 levels are comparable with our results.

Several patients with bilateral urethral stones (n=1), severe asthmatic bronchitis (n=1), influenza (n=2), and cyclic vomiting (n=1) had high rT3 levels. Elevated rT3 was observed in most patients with acute or chronic ill-

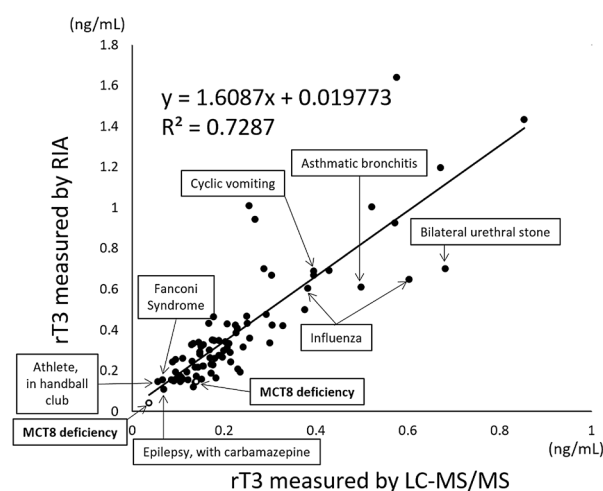


Fig. 1. Comparison of liquid chromatography–tandem mass spectrometry (LC-MS/MS) and radioimmunoassay (RIA) for determining serum reverse triiodothyronine (rT3) levels.

Arrows and boxes indicate values measured in patients with various diseases. Open circles indicate values measured in patients with monocarboxylate transporter 8 (MCT8) deficiency.

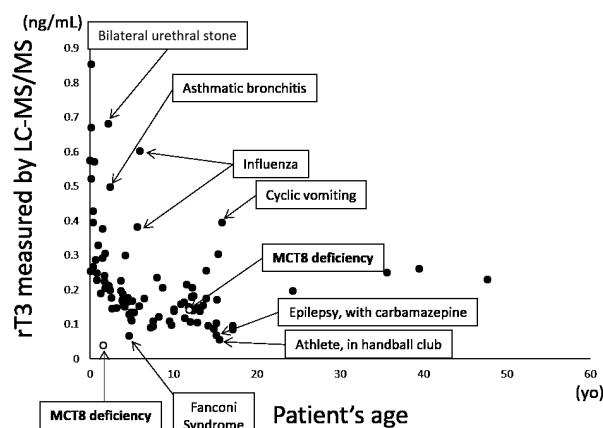


Fig. 2. Relationship between the patient age and rT3 level measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Arrows and boxes indicate values measured in patients with various diseases. Open circles indicate values measured in patients with monocarboxylate transporter 8 (MCT8) deficiency.

nesses¹. On the contrary, patients with Fanconi syndrome (n=1) and epilepsy treated with carbamazepine (n=1) had low rT3 levels. Carbamazepine, which alters thyroxine and T3 metabolism due to increased hepatic metabolism, may decrease rT3 levels¹. However, to the best of our knowledge, the reason for decrease in rT3 levels in patients with Fanconi syndrome is yet to be clarified. A high school handball player among the subjects also had a low rT3 level

(0.056 ng/mL). Aerobic exercise with adequate caloric intake can reasonably decrease rT3 levels⁸⁾.

rT3 levels were also measured in two patients with monocarboxylate transporter 8 (MCT8) deficiency, an inherited thyroid disease. MCT8 deficiency, also known as Allan-Herndon-Dudley syndrome, is characterized by the dysfunction of the transporter protein for thyroid hormone in the brain. It has been reported that low rT3 levels in patients with MCT8 deficiency do not overlap with the normal range of rT3 levels⁹⁾. In our study, a 1-year-old patient with MCT8 deficiency had an rT3 level of 0.038 ng/mL, which is clearly low. Another 10-year-old patient with MCT8 deficiency had an rT3 level of 0.141 ng/mL, which is comparable with those in other children. Further studies should be performed to determine the relationship between serum rT3 levels and illness.

Because the primary aim of this study was to examine the reliability of measuring serum rT3 levels using LC-MS/MS in the clinical setting, we will investigate the normal range of serum rT3 levels measured using LC-MS/MS in children and the relationship between abnormal rT3 levels and various diseases in future studies. In summary, we validated the LC-MS/MS technique for measuring serum rT3 levels. The technique reliably measured serum rT3 levels in 89 outpatients.

Conflict of Interest

The authors declare that we have no conflict of interest.

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