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Determination of free thyroid hormones in animal serum/plasma using ultrafiltration in combination with ultra-fast liquid chromatography-tandem mass spectrometry

Rumi Tanoue, Imari Kume, Yasuo Yamamoto, Kohki Takaguchi, Kei Nomiyama, Shinsuke Tanabe, Tatsuya Kunisue*

Center for Marine Environmental Studies (CMES), Ehime University, 2-5, Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan

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ABSTRACT

Thyroid hormones (THs), which mainly consist of 3, 3′, 5-triiodo-L-thyronine (T3) and L-thyroxine (T4), play a critical role in regulating biological processes such as growth and metabolism in various animal species. Thus, accurate measurement of T3 and T4, especially physiologically active free (protein-unbound) forms, in serum/plasma is needed for the evaluation of TH homeostasis. However, such high-precision determination of free THs is lacking for non-human species. The present study aimed to develop a highly sensitive and reliable liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the determination of six free THs in serum/plasma, which is applicable to not only humans but also non-human species. Two different physical separation steps, ultrafiltration (UF) and equilibrium dialysis (ED), were examined to obtain the free TH fraction. Several experimental conditions were carefully optimized and validated for UF or ED using the commercially available bovine serum. As a result, UF at 1100 × g and 37 °C for 30 min with a 30 kDa ultrafiltration device (Centrifree YM-30, Millipore) yielded excellent precision (CV: <10%). The optimized ED step also yielded high precision (CV: <10%) and the measurement values were approximately equal to those of UF, but at least 16 h were required to reach equilibrium. Thus, UF combined with LC–MS/MS was finally chosen, in terms of the time needed for the measurement. Acceptable accuracy (recovery: 70%–110%) and intra- and inter-day precision (CV: <10% and <12%, respectively) were obtained, when triplicate analyses in three different days were conducted using the bovine serum. The developed analytical method was successfully applied to the determination of free THs in serum/plasma samples of humans, cats, and dogs. Furthermore, comparison with free T4 concentrations measured by a common immunoassay method evidently indicated that the ultrafiltration-LC–MS/MS method developed in this study can increase the specificity and accuracy of TH measurement.

1. Introduction

Thyroid hormones (THs), which mainly consist of 3, 3′, 5-triiodo-L-thyronine (T3) and L-thyroxine (T4), are responsible for regulating metabolism and maintaining energy balance [1]. THs also play a critical role in fetal and child normal growth and neurodevelopment [2]. T4 and approximately 20% of T3 produced daily are synthesized in the thyroid gland. These are secreted into the bloodstream and carried to peripheral tissues [3]. The remaining 80% of T3 is formed from the T4 in the target tissues by iodothyronine deiodinases type I and type II [3]. T3 is physiologically active and regulates expression of genes involved in the biological processes described above, by binding to thyroid hormone nuclear receptors [4].

Recent epidemiological studies have shown alterations in blood TH levels by human exposure to environmental pollutants such as polychlorinated biphenyl (PCB) [5,6], polybrominated diphenyl ether (PBDE) [5,6], perchlorate [7,8], phthalates [9], bisphenol A [9], and triclosan [10]. In addition, some studies on wildlife [11–13] and companion animals [14] have also suggested that higher levels of such environmental pollutants in the blood or house dust were involved in the variation in circulating TH levels. Indeed, *in vivo* and *in vitro* studies have reported the disruption of thyroid function by administration of these chemicals [5,15–20]. Given that thyroid

* Corresponding author.
E-mail address: kunisue.tatsuya.ew@ehime-u.ac.jp (T. Kunisue).

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system is evolutionarily and highly conserved across vertebrate taxa [21], the above observations indicate that a significant number of vertebrate species can be potentially threatened by exposure to thyroid disrupters.

When discerning thyroid dysfunction associated with disease, chemical exposure, and other disturbing factors, the concentrations of thyroid-stimulating hormone (TSH), T4, and T3 in serum/plasma are routinely measured. Reverse T3 (rT3), also known as 3, 3′, 5′-triiodo-L-thyronine, which is formed from T4 by iodothyronine deiodinases type III, is not normally measured, because it is believed that rT3 is biologically inactive and eliminated from the body in a short time [4,22]. However, significantly higher concentration ratios of rT3 to T3 (rT3/T3) have been observed in patients with hyperthyroidism [23] or renal failure [24], compared to those in the normal subjects, suggesting that the rT3/T3 ratio can also be a useful indicator for diagnosing some disorders associated with thyroid function. For measuring the serum/plasma TH levels, immunoassay (IA) methods such as radioimmunoassay (RIA) and electrochemiluminescence immunoassays have been commonly used to evaluate TH homeostasis. IA methods are sensitive, but they pose several technical challenges, including a lack of adequate specificity and accuracy resulting from immunochemical cross-reactivity [12,25–27]. Biomolecules in the blood, which can react with antibodies used in IAs, vary by species and interfere with assay performance; i.e., they can falsely depress or enhance TH measurement values through non-specific reactions with anti-T3 or anti-T4 animal antibodies. Although many IA kits are commercially available to measure TH levels in serum/plasma of human beings and a limited number of non-human species, these kits have not been fully validated for the measurement values in non-human species. Accordingly, TH measurement using liquid chromatography-tandem mass spectrometry (LC–MS/MS) has recently attracted attention as a novel advanced method which is superior to the conventional IAs, regarding selectivity, specificity, and validity [28–34]. Our group has recently demonstrated that the developed LC–MS/MS method was a valid and superior assay for the TH measurement in wildlife species. Indeed, we found a weak or no correlation between serum T4 or T3 levels measured using LC–MS/MS and IA methods in Baikal seals, whereas TH levels in human serum were strongly correlated between both assays [12]. Furthermore, when the relationship between concentrations of THs in sera and dioxins, which are disrupters of TH homeostasis, in blubber tissues was examined, a significantly negative correlation was found for serum T4 levels measured by the LC–MS/MS, but not for those measured by IA. Our results suggested that TH measurement by IA methods can lead to misinterpretation of the potential effects on TH homeostasis in wildlife and concluded that the LC–MS/MS method is more reliable, particularly when TH homeostasis in non-human species are examined by comparing with the levels of environmental pollutants and other disrupting factors.

More than 99% of THs circulation in the bloodstream binds to transport proteins such as thyroxine-binding globulin (TBG), albumin, and transthyretin (TTR); i.e., approximately 0.03% of the total blood T4 and 0.3% of the total blood T3 are present as free (protein-unbound) form [35]. Circulating free THs, which are regulated by the hypothalamic-pituitary-thyroid axis, can enter target cells, followed by physiological responses. Therefore, it has been widely accepted that the measurement of free TH levels can be a more important element for properly assessing the thyroid function, because total TH levels (protein bound + unbound THs) can be affected by the fluctuation in transport protein concentrations or binding capacities.

For the measurement of free THs, equilibrium dialysis (ED) combined with IAs is still employed as a common method. The ED is a traditional procedure to physically separate serum/plasma into two fractions, an equally-divided protein-unbound fraction and a protein-bound + another unbound fraction, prior to quantitative analysis of free THs. Alternatively, Soldin et al. developed an analytical method [29] for the determination of free THs in human serum/plasma by using ultrafiltration (UF) in combination with LC–MS/MS and already applied this method to clinical examinations for the accurate diagnosis [36]. While ED step is time-consuming (e.g., it requires 24 h) and technically demanding, the UF step has the advantage of rapid separation (around 30 min) without dilution with phosphate-buffered saline which is unfit for LC–MS/MS analysis. Thus, further expansion of usage of UF combined with LC–MS/MS analysis would be expected, but accuracy and robustness of the UF step under laboratories have not been fully validated [37]. Several factors affect the binding of THs to transport proteins such as TBG and TTR in the blood; e.g., temperature, blood pH, and fatty acid concentration [38–41]. Also, temperature-dependent and pH-dependent structural transitions of binding proteins may vary between species. To the best of our knowledge, there is no study examining these possible factors, which can affect free TH levels for non-human species when using the UF method. The present study aimed to develop a highly sensitive and reliable LC–MS/MS method for the determination of six free THs [T4, T3, rT3, 3,5-diiodo-L-thyronine (3,5-T2), 3,3′-diiodo-L-thyronine (3,3′-T2), and 3-iodo-L-thyronine (3-T1)] in serum/plasma, which is applicable in not only humans but also non-human species (e.g., wildlife and companion animals). An isotope-dilution method using stable isotope labeled internal standards (ISs: T4-$^{13}C_6$, T3-$^{13}C_6$, and rT3-$^{13}C_6$) was employed for the quantification. To assess the reliability, the developed analytical method was subsequently applied to the determination of free THs in sera of pet cats and dogs, and their free T4 concentrations were compared with values measured by a common IA method.

2. Materials and methods

2.1. Chemicals and materials

Native standards of T4 (purity: 98.6%), T3 (purity: 95.5%), rT3 (purity: 96.5%), 3,5-T2 (purity: ≥98%), and 3,3′-T2 (purity: ≥99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and 3-T1 (purity: 95%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). ISs of T4-$^{13}C_6$ (purity: 97.9%), T3-$^{13}C_6$ (purity: 95.5%), and rT3-$^{13}C_6$ (purity: 97.8%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Liquid chromatography–mass spectrometry grade methanol and acetic acid were purchased from Wako Chemicals (Osaka, Japan). Ultrapure water was obtained using a Direct-Q3 water purification system (Merck Millipore, Japan). Oasis HLB cartridges (30 mg, 1 mL) and Oasis HLB cartridges (10 mg, 1 mL) were purchased from Waters (Milford, MA, USA). SampliQ OPT cartridges (60 mg, 3 mL) and SampliQ OPT cartridges (30 mg, 1 mL) were purchased from Agilent Technologies (Palo Alto, CA, USA). The following centrifugal filter devices: Centrifree YM-30 (Merck Millipore, Japan), Amicon Ultra 0.5 (Merck Millipore, Japan), and Nanosep Omega (Pall Corporation, Japan), were purchased. Rapid Equilibrium Dialysis (RED) Device Inserts and a reusable base plate were purchased from Thermo Scientific (Waltham, MA, USA). Two different lots of bovine calf sera (described as bovine (no. 1) and bovine (no. 2) in this article) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pooled each human and feline plasma was purchased from Innovative Research (Novi, MI, USA) and Aviva Systems Biology (San Diego, CA, USA), respectively. Serum samples of pet cats (n = 19) and dogs (n = 20), which were collected at surgical treatments, were kindly supplied by veterinary clinics in Osaka, Kumamoto, and Hokkaido, Japan, and Aveiro, Portugal, during 2012–2015. All serum/plasma samples were stored at −80 °C until chemical analysis.

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