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Determination of thyroid hormones in mouse tissues by isotope-dilution microflow liquid chromatography–mass spectrometry method

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ABSTRACT

Thyroid hormones (THs) play a critical role in the regulation of many biological processes such as growth, metabolism and development both in humans and wildlife. In general, TH levels are measured by immunoassay (IA) methods but the specificity of the antibodies used in these assays limits selectivity. In the last decade, several analytical methods using liquid chromatography-mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS) have been developed to measure THs. These new techniques proved to be more accurate than the IA analysis and they were widely used for the determination of TH level in different human and animal tissues. A large part of LC–MS/MS methods described in literature employed between 200 and 500 mg of sample, however this quantity can be considered too high especially when preclinical studies are conducted using mice as test subjects. Thus an analytical method that reduces the amount of tissue is essential. In this study, we developed a procedure for the analysis of six THs; L-thyroxine (T4), 3,3′,5-triiodo-L-thyronine (T3), 3,3′,5′-triiodo-L-thyronine (rT3), 3,5-diiodo-L-thyronine (rT2), 3,3′-diiodo-L-thyronine (T2), 3-iodo-L-thyronine (T1) using isotope ($^{13}C_6$-T4, $^{13}C_6$-T3, $^{13}C_6$-rT3, $^{13}C_6$-T2) dilution liquid chromatography–mass spectrometry. The major difference with previously described methods lies in the utilization of a nano-UPLC (Ultra Performance Liquid Chromatography) system in micro configuration. This approach leads to a reduction compared to the published methods, of column internal diameter, flow rate, and injected volume. The result of all these improvements is a decrease in the amount of sample necessary for the analysis. The method was tested on six different mouse tissues: liver, heart, kidney, muscle, lung and brown adipose tissue (BAT). The nano-UPLC system was interfaced with a quadrupole time-of-flight mass spectrometer (Q-TOF2-MS) using the positive ion mode electrospray

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ionization. In our analytical method the instrumental calibration curves were constructed from 0 to 100 pg μL^{-1} and all of them showed good linearity ($r^2 > 0.99$). The limit of quantification was from 2.5 to 5 pg injected into the column. The method recoveries calculated using spiked mouse liver and spiked mouse muscle were between 83% and 118% (except T1 and rT2 at high concentration) with a coefficient of variation (CV) of <10% for all derivatives. The new methodology allows us to measure T4 and T3 concentrations in a range from 21 to about 100 mg and give a more extensive insight on thyroid hormone concentration in different mouse tissue.

1. Introduction

Thyroid hormones (THs) are a class of tyrosine-based hormones produced and secreted by the thyroid gland. The thyroid gland mostly produces L-Thyroxine (T4), however intracellular removal of one iodine atom, generates 3,3′,5-triiodothyronine (T3), which possesses a 100-fold higher affinity for the TH receptor than T4. The local activation of T4 into T3 is catalyzed by type two deiodinase (D2), which is primarily expressed in the hypothalamus, white fat, brown adipose tissue (BAT), and skeletal muscle [1]. In turn, T3 and T4 are inactivated by type 3 deiodinase (D3) which converts them into 3,3′-diiodo-L-thyronine (T2) and 3,3′,5′-triiodothyronine (reverse T3 or rT3), respectively. T3 can be also deiodinated into 3,5-T2 (reverse T2 or rT2) by type 1 deiodinase (D1) [2,3]. It is well known, that THs play a critical role in the regulation of key metabolic processes during normal growth as well as in adult animals and humans. They regulate metabolism primarily through actions in the brain, white fat, brown fat, skeletal muscle, liver and pancreas [1]. Therefore, it is not surprising that since a long time scientists have been interested in measuring the TH concentrations in different tissues and to relate this value with possible dysfunctions and diseases. Until recently, TH levels were measured by radioimmunoassay or immunoassay (IA)-based methods [4–6]. IA methods are fast and have high sensitivity but the lack of specificity limits selectivity [7–9]. In the last decade, several analytical methods using liquid chromatography-mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS) have been developed to measure THs since they proved to be more reliable than the IA analysis [10–16]. Recently, quite extensive analytical procedures for determination of THs in rat tissues using LC–MS/MS were reported by Kunisue et al. and Saba et al. The first group developed a methodology for the detection of six thyroid hormones (T1, rT2, T2, rT3, T3 and T4). The authors tested their procedure measuring TH concentrations in the thyroid gland, where five different thyroid hormones were quantified, and in the brain where T4 and T3 levels were measured [13]. The second group established a methodology for the simultaneous quantification of T3, T4, 3-iodothyronamine (T_1AM), and thyronamine (T_0AM) which was tested on serum and on six different tissues (heart, liver, kidney, muscle, stomach and lung) [11]. Both groups developed their procedures employing at least 500 mg of sample (except for the thyroid gland). This quantity could be sometimes too high especially if preclinical studies are conducted in mice. It is well known in fact, that a large part of animal models are based on mice instead of rats and thus an analytical method that reduces the mass of tissue needed is becoming more and more required. One of the first attempts in this direction was reported by Ackermans et al. They lowered the tissue mass to 200 mg using an ultra-performance liquid chromatography (UPLC) system instead of high-performance liquid chromatography (HPLC) [14]. Additionally, in a recent paper, it was described the possibility to improve analytical sensitivity and therefore to reduce the mass of tissue, after a derivatization step that converted T3 and T4 into butyl esters [10]. The authors tested their methodology measuring T3 and T4 on heart and liver tissue from rat and although the accuracy was not so impressive (70–75%), they were able to use as little as 50 mg of sample.

It is known that the use of nano-UPLC system greatly minimizes ion suppression and matrix effects reducing even more the sample needed for analysis. But unfortunately, the small i.d. capillaries have the tendency to become clogged limiting the use of this instrument [17]. To reduce the blocking, while retaining the advantages of UPLC vs HPLC, we decided to use our nano-UPLC system in micro configuration (μm i.d. column and microflow rate). It is in fact reported from the manufacturer that nano-UPLC apparatus can tolerate up to 10000 psi of pressure and the modern one up to 15000 psi. For the detection and quantification of THs the nano-UPLC system was interfaced with a quadrupole time-of-flight mass spectrometer (Q-TOF2-MS). Although the use of Q-TOF mass spectrometer in quantitative analysis of small molecules is rare, a recent paper showed that Q-TOF-MS can also be used for such purpose in a complex biological matrix [18].

For decades, scientists were mainly interested in the analysis of T4 and T3 the two most abundant thyroid hormones, considering the lower iodinated analogues just simply byproducts of TH metabolism. However, recent data suggest potential physiological effects of those compounds also. It has been shown that treatment with T2 and rT2 could have a mediator effect of the direct thyroid hormone regulation of energy metabolism [19]. Other studies reported that administration of 3,5-T2 could prevents lipid accumulation in the liver and muscle of rat [20]. Furthermore, in placenta where type 3 deiodinase (D3) is highly expressed and the concentration of rT3 is similar or even higher than that of T3 [21,22] the molar ratio among T4, T3 and rT3 is an important parameter linked to the fetus development [23]. Due to all these findings, an analytical procedure focused on the separation and quantification of several thyroid hormones is more suitable than one centered only on T4 and T3 measurement. In this paper, we described a procedure for the analysis of six THs (Fig. 1); L-thyroxine (T4), 3,3′,5-triiodo-L-thyronine (T3), 3,3′,5′-triiodo-L-thyronine (rT3), 3,5-diiodo-L-thyronine (rT2), 3,3′-diiodo-L-thyronine (T2), 3-iodo-L-thyronine (T1) using isotope ($^{13}C_6$-T4, $^{13}C_6$-T3, $^{13}C_6$-rT3, $^{13}C_6$-T2) dilution liquid chromatography–mass spectrometry. The aim of our work was to develop an analytical procedure in which less 100 mg of mass of tissue was needed for measuring thyroid hormone concentrations. To reach this goal, we employed a nano-UPLC system in micro configuration coupled with Q-TOF2-MS detector. Such methodology was tested in six different mouse tissues: liver, muscle, heart, kidney, lung and BAT using between 21 and 100 mg of sample. With this procedure, we could easily measure T4 and T3, whereas the levels of the other THs in the sample tested were below the detection limit. Nevertheless, compared to analogue methods described in literature, we reduced substantially the mass of tissue necessary for analysis of T4 and T3 making this procedure more suitable when only small amount of samples are available.