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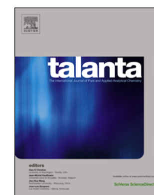

Determination of total thyroxine in human serum by hollow fiber liquid-phase microextraction and liquid chromatography–tandem mass spectrometry

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ABSTRACT

Determination of total thyroxine in human serum using hollow fiber liquid-phase microextraction (HF-LPME) has been accomplished for the first time. HF-LPME serves as an inexpensive sample pretreatment and the cleanup method that is nearly solvent-free. Thyroxine was extracted through a water immiscible organic solvent immobilized in the wall pores of a polypropylene hollow fiber into 20 μl of an aqueous acceptor phase inside the lumen of the hollow fiber. This technique produced extracts that had comparable cleanness with those obtained using solid-phase extraction (SPE). Serum samples with endogenous thyroxine were spiked with isotopically-labeled thyroxine and analyzed by liquid chromatography–tandem mass spectrometry after HF-LPME extraction. Extraction parameters including the organic phase, acid/base concentration of acceptor phase, stirring speed and extraction time were optimized. The calibration range was found to be linear over 1–1000 ng/g with the limit of detection (LOD) of 0.3 ng/g. For quantification of total thyroxine in human serum, 6 subsamples were prepared and the results indicated very good precision with a relative standard deviation of $<1.3\%$. The difference from the SPE method was less than 1.2%, with independent *t*-test showing insignificant bias. Two reference materials of human serum were analyzed, and our obtained values were compared with the reference values. The results showed very good precision with RSD around 0.2% and the deviation from the reference values were -3.1% and -2.1%. The newly developed method is precise, accurate, inexpensive, and environmentally friendly.

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1. Introduction

People of all ages and races can suffer from thyroid disease with women five times more likely than men to have thyroid problems [1,2]. In particular, abnormal thyroid function during pregnancy can affect fetal well-being [3,4]. A non-functioning thyroid gland affects one in 4000 newborns. If the problem is not corrected, the child will suffer from physical and mental retardation [5].

Clinicians use a set of thyroid functions tests to evaluate the health status of the thyroid gland. A thyroid functions test panel commonly includes the measurement of thyroid hormones such as thyroid stimulating hormone thyrotropin, thyroxine (T4) and triiodothyronine (T3). These thyroid hormones are potent regulators of cellular proliferation and metabolic rate and must be maintained within an optimal range for normal development and health. Thyroxine is the major hormone secreted by the thyroid gland and its normal serum concentration range is 60–160 nM (0.047–0.124 μg/g) [6]. Routine laboratories typically use immunoassays to determine thyroxine concentrations in human serum.

Due to the variability of different immunoassays, mass spectrometric (MS) methods have been developed for more accurate and precise measurement of thyroxine [7–12]. Most of these used solid phase extraction (SPE) for sample processing [7,8,10–12] in which analytes were eluted in organic solvent, and preconcentrated separately in an extra step, before instrumental analysis.

Abbreviations: HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; HF-LPME, hollow fiber liquid-phase microextraction; SPE, solid phase extraction; MRM, multiple reaction monitoring; SIM, selected ion monitoring; RSD, relative standard deviation.

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Hollow fiber-liquid-phase microextraction (HF-LPME) is an alternative sample pretreatment method, in which the final extractant phase is either organic or aqueous. In the latter mode, target analytes are extracted from aqueous samples into a water immiscible organic phase immobilized in the wall pores of the hollow fiber, and further into an acceptor phase present inside the lumen of the fiber which serves as a protective sheath against matrix effects [13–16]. HF-LPME has several advantages over SPE. Firstly, SPE cartridges are generally expensive, unlike the hollow fiber material. Secondly, the SPE procedure requires milliliter amounts of organic solvent while HF-LPME is nearly solvent-free (microliter volumes), and generates very little waste. The latter approach is environmentally friendly and compatible with the green chemistry concept. Thirdly, SPE results in analyte dilution so additional steps of evaporation and reconstitution are necessary while in HF-LPME, analytes are enriched and cleaned up simultaneously, and the acceptor phase can be directly analyzed without further processing.

HF-LPME has been used in a variety of matrices including serum. In serum, the most widely studied analytes are either acidic or basic analytes [13,15,16]. To the best of our knowledge, HF-LPME has not been applied to the analysis of health status markers such as thyroxine. In this study, an HF-LPME method was developed and the HF-LPME conditions were optimized for the analysis of total thyroxine in serum. Sample analysis was conducted by liquid chromatography (LC) tandem MS (MS/MS). Results were compared with those obtained by SPE.

2. Materials and methods

2.1. Materials

Methanol and acetonitrile (HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was obtained from a Milli-Q Integral system (Millipore, Milford, MA, USA) (resistivity = 18.2 MΩ-cm). Formic acid (~98%), acetic acid (≥99.85%), 1-octanol (≥99%), and 3,5-diiodo-L-tyrosine dihydrate (≥98%) were bought from Sigma Aldrich (Singapore). Hydrochloric acid (fuming 37%), ammonia solution (25%), and sodium phosphate dibasic (≥99.0%) were supplied by Merck (Singapore).

Thyroxine (IRMM-468), obtained from the European Commission – Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), was used as the calibration standard. The standard has a certified purity of 98.6 ± 0.7% after taking into consideration inorganic residues, water, ethanol and organic impurities detectable by high-performance LC and LC–MS. The isotopically-labeled internal standard, $^{13}C_6$-thyroxine was obtained from Medical Isotopes, Inc. (Pelham, NH, USA). LC–MS examination showed that the $^{13}C_6$-thyroxine contained none of the unlabeled standard thyroxine.

Accurel© PP Q3/2 polypropylene hollow fiber membrane (Membrana, Wuppertal, Germany) with an inner diameter of 600 µm, wall thickness of 200 µm and wall pore size of 0.2 µm was used for HF-LPME.

2.2. Instrumentation

Sample weighing was performed on Mettler Toledo XP205 balance with a readability of 0.01 mg and maximum capacity of 220 g (Mettler-Toledo Inc., Columbus, OH, USA). LC–MS/MS measurements were performed on a Shimadzu Prominence UFLCXR LC system comprising a CBM-20A system controller, a CTO-20AC column oven, two LC-20ADXR pumps and a SIL-20AC autosampler (Shimadzu Scientific Instruments, Columbia, MD, USA) coupled with an AB Sciex Qtrap® 5500 MS/MS instrument (AB Sciex, Foster City, CA, USA). The LC column was a Unison UK-C18 column (2.0 × 100 mm, 3-µm particle diameter) (Imtakt Corporation, Kyoto, Japan). Parallel extractions were carried out using a multi-position magnetic stirrer with heating (Fisher Scientific, Singapore). Solutions were evaporated/heated using Stuart sample concentrator with block heater (Bibby Scientific Limited, Staffordshire, UK). Centrifugation was carried out using a Sartorius Centrifuge, Sigma 3–16P (Sartorius Stedim Biotech, Aubagne, France). Serum samples were stored in ultra-low temperature freezer capable of cooling to −86 °C (Sanyo, San Diego, CA, USA).

2.3. Samples

Extraction method development was carried out on human serum IPLA-SER2 (Innovative Research, Novi, MI, USA) and human serum P2918 (Sigma Aldrich). To ensure homogeneity, the commercial serum was centrifuged to remove large particles. After mixing, the serum was distributed into smaller portions and stored at −80 °C to prevent multiple freeze-thaw cycles that could affect the concentration of the native analytes. Frozen serum samples were thawed at room temperature before analysis.

Two reference materials, lyophilized human serum HM 212 03 and HM 264 01 were obtained from Referenzinstitut für Bioanalytik (Bonn, Germany). Samples were reconstituted and analyzed on the same day.

2.4. Preparation of standard solutions

Approximately 5 mg of the thyroxine standard was accurately weighed into an amber glass vial and dissolved in 7.5 ml of methanol and 20 µl of 1 M hydrochloric acid. The stock solution was sonicated for 1 min to completely dissolve thyroxine, and vortexed to ensure homogeneity.

The final working solution was diluted with 0.05 M sodium phosphate dibasic buffer (pH 11.6) containing 50 µg/g of diiodotyrosine as a protective carrier substance. The final concentration of thyroxine in the working solution was approximately 0.35 µg/g. A working solution of isotopically-labeled internal standard, $^{13}C_6$-thyroxine, at a concentration of approximately 0.35 µg/g was prepared in the same way as the unlabeled thyroxine. All the solutions were distributed into smaller portions and stored at −30 °C to prevent multiple freeze-thaw cycles that could affect the concentration of the native analytes.

2.5. Sample preparation using HF-LPME

Serum (0.6–1.0 ml) was weighed into 2 ml amber glass vial. An appropriate amount of $^{13}C_6$-thyroxine was added to give a 1:1 mass ratio of analyte to internal standard. Each sample was acidified to approximately pH 2 with 130 µl of 1 M hydrochloric acid. Water was added to give a total sample volume of 1.43 ml. Samples were mixed well and equilibrated at room temperature for 2 h in the dark before being processed. The pre-treated samples were then subjected to HF-LPME.

A piece of hollow fiber was cut into 7 cm lengths with both ends unsealed, washed by sonicating in an acetone bath and air dried. A pair of tweezers was used to lower the hollow fiber into 1-octanol. The membrane was held for about 5–8 s to impregnate the wall pores with 1-octanol, and then sonicated in water for 2–4 s to remove excess solvent. Ammonia solution (20 µl, 1 M) was drawn into a 500 µl syringe with a bevel tip of 0.5 mm o.d. The syringe needle was tightly fitted into one end of the hollow fiber and the syringe plunger was depressed so that the lumen of the hollow fiber was completely filled with 1 M ammonia solution.

The hollow fiber was placed into the sample vial previously loaded with the sample and a 7 × 2 mm mini stir bar. The solution was stirred on a magnetic stirrer for 30 min at 880 revolutions per

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