Talanta 126 (2014) 163–169

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

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

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ARTICLE INFO

Article history:
Received 17 January 2014
Received in revised form
24 March 2014
Accepted 25 March 2014
Available online 29 March 2014

Keywords:
Total thyroxine
Human serum
Hollow fiber liquid-phase microextraction
Liquid chromatography–tandem mass spectrometry

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.

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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http://dx.doi.org/10.1016/j.talanta.2014.03.058
0039-9140/

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

minute (rpm) for extraction, after which the hollow fiber was then removed and the acceptor phase was drawn out and diluted with 40–50 μl of 1% formic acid in 1:4 methanol:water prior to instrumental analysis. Between samples, the syringe was washed twice with 1 M ammonia solution, twice with acetone, and twice again with 1 M ammonia solution to avoid analyte carry-over.

2.6. Sample preparation using SPE

Serum samples were pre-treated as described in Section 2.5. SPE was conducted using polymer based cation exchange cartridges (Phenomenex Strata-X-C, 100 mg/6 ml) (Torrance, CA, USA). The anion groups bonded on the polymeric surface have strong retention for cations. Analytes bearing basic groups are retained on the cartridges under acidic conditions, and released under basic conditions. For extraction, the SPE cartridge was first conditioned with 3 ml of methanol followed by 3 ml of 0.1 M hydrochloric acid. The equilibrated serum was loaded and the eluate was collected and reloaded. The cartridge was washed with 4 ml of 0.1 M hydrochloric acid followed by 4 ml of methanol. The first wash was to remove acidic or neutral polar interferences while the second wash was to remove acidic or neutral hydrophobic interferences. Low pH was maintained during the washing steps to prevent premature elution of the analytes of interest. Thyroxine was then eluted with 3 ml of 3.5% ammonia in methanol. The sample was evaporated to dryness under nitrogen at 50 °C and reconstituted with the LC mobile phase to a thyroxine concentration of about 40 ng/g for LC–MS/MS analysis.

2.7. Preparation of calibration mixtures

The working solutions of thyroxine and $^{13}C_6$-thyroxine were prepared, yielding four calibrators with ratio of analyte to internal standard ranging from 0.8 to 1.2. The mixtures were diluted with LC mobile phase to a thyroxine concentration of about 40 ng/g for LC–MS/MS analysis.

2.8. LC–MS/MS measurements

For LC, sample solutions (10 μl) were injected and separations were conducted using an isocratic mobile phase consisting of methanol/water (70:30 v/v) containing 0.1% formic acid, at a flow rate of 0.3 ml/min. The autosampler tray temperature was set at 4 °C.

Ions were generated in the positive ion mode and detected by multiple reaction monitoring (MRM). Selected ion monitoring (SIM) was also used in the method evaluation to scan for interferences with the same parameters as for MRM except it did not have Q3 mass, collision energy and collision exit potential. Nitrogen was the only gas used. Curtain gas pressure was set at 138 kPa. The ion source gas 1 and ion source gas 2 were both at settings of 310 kPa. The turbo gas temperature was adjusted to 450 °C. The ion spray voltage was set at 4500 V. The entrance potential was set at 2 V and dwell time at 200 ms for all ion pairs. For thyroxine, ion pairs 777.8/731.8 and 777.8/351.0 were monitored for quantification and confirmation, respectively. For $^{13}C_6$-thyroxine, ion pairs 783.8/737.8 and 783.8/357.0 were monitored for quantification and confirmation, respectively. The MRM parameters for the two ion pairs are summarized in Table 1.

Table 1
MRM parameters for thyroxine and $^{13}C_6$-thyroxine.

Analyte	Ion pair	Declustering potential (V)	Collision energy (V)	Collision exit potential (V)
Thyroxine	777.8/731.8	137	37	51
	783.8/357.0	137	63	23
$^{13}C_6$-thyroxine	783.8/737.8	110	38	50
	783.8/357.0	110	60	29

3. Results and discussion

3.1. Set-up of HF-LPME

In HF-LPME, the rod-like configuration involving direct attachment of the fiber to a microsyringe needle has been widely used [13,17]. Initially, the extraction method development was carried out using this configuration with a 3 cm long hollow fiber (Fig. 1A). One end of the hollow fiber was heat-sealed. The vial was capped and the sample was shaken on an orbital shaker. However, some problems were encountered with this set-up. Since one end of the hollow fiber was sealed, there was an air gap within the lumen which made it difficult to introduce as well as retrieve the acceptor phase. The small amount of acceptor phase (maximum 3 μl) injected into the lumen was lost easily through diffusion. Only about 1 μl could be successfully drawn out after extraction. At times, the sealed end of the hollow fiber broke and the acceptor phase was lost completely. Moreover, serum samples were prone to bubbles when shaken by the orbital shaker. The bubbles were generated at the surface of the hollow fiber, likely affecting the diffusion of analytes across the membrane and, consequently, the repeatability of the extractions.

To address the problem, in an alternative approach, the length of hollow fiber was increased to 7 cm and folded into a U-shape with an extension (Fig. 1B). The orbital shaker was replaced by a magnetic stirrer. The vial was capped over the extension so the stirring did not affect the hollow fiber assembly mechanically. It was easy to inject and draw out the acceptor phase with 2 open ends on the hollow fiber. The longer hollow fiber allowed more acceptor phase to be introduced so a sufficient amount was available even accounting for losses. Serum sample (0.6–1.0 ml) was intentionally topped up to 1.43 ml to create optimum contact between the hollow fiber and sample donor phase within the 1.5 ml sample vial.

3.2. Parameter optimization of HF-LPME

Experiments were carried out to optimize several parameters of the hollow fiber extraction procedure in order to achieve higher extraction efficiency as well as create a more robust and easy-to-operate procedure. Serum samples instead of pure standard solutions were used for method development as serum matrix is more complicated and more difficult to extract. The MRM transitions at m/z 777.8/731.8 for thyroxine were used for the peak area integration.

3.2.1. Ion-pairing agent

Thyroxine is a particularly challenging analyte for extraction as it has both acidic and basic moieties. It would be ionic at all pH values and may have difficulty in entering the organic phase within the hollow fiber. It has been reported that suitable ion pair reagents can be added to the sample to generate ion pairs with sufficiently hydrophobic character to effectively enter the organic phase [18,19]. Thus, the donor phase was acidified and alkyl sulfonic acid salt was added as ion-pairing agent to mediate the transport of thyroxine across the organic phase. Although pure standard solution was successfully extracted, the alkyl sulfonic acid salts caused precipitation in serum samples and resulted in very poor extraction as partial clogging of the membranes occurred.

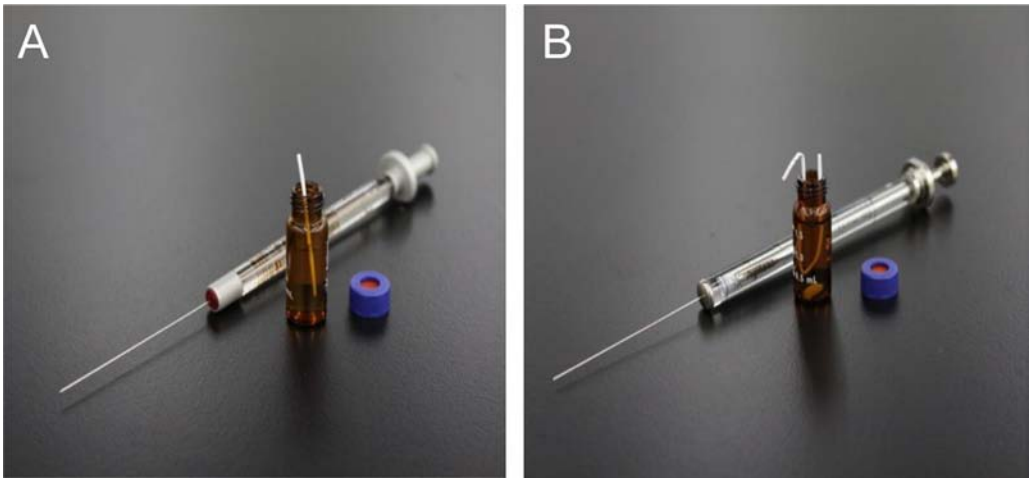

Fig. 1. HF-LPME configurations. (A) 3 cm hollow fiber (straight) and (B) 7 cm hollow fiber (U-shape with extension).

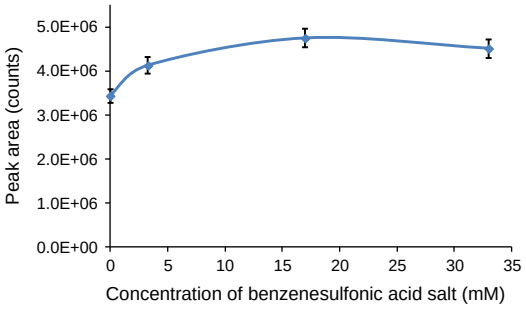

Fig. 2. Effect of concentration of benzenesulfonic acid salt.

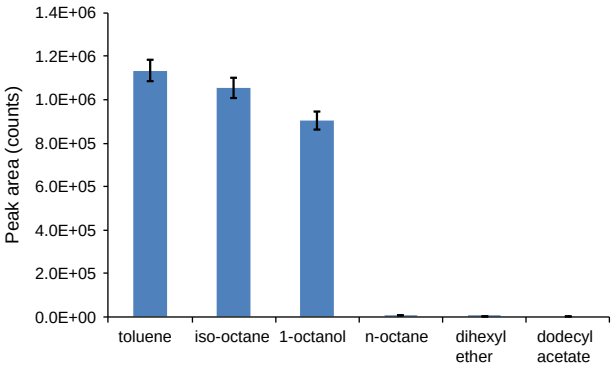

Fig. 3. Effect of different organic solvents on extraction.

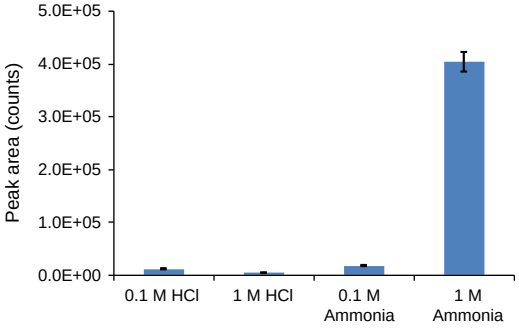

Fig. 4. Effect of acid/base concentration of acceptor phase.

A protein removal step prior to HF-LPME has been reported [20]. In our study, trifluoroacetic acid was tried for the protein precipitation but the subsequent extraction gave unsatisfactory results. Acetonitrile was then used for the protein precipitation. The organic solvent in the supernatant was evaporated before HF-LPME. Three ion-pairing agents were tested, i.e., 1-octane sulfonic acid salt, 1-hexane sulfonic acid salt, and benzenesulfonic acid salt. 1-Octanol was the immobilized organic phase and 1 M ammonia was the acceptor phase. The three ion-pairing agents gave similar results with benzenesulfonic acid salt being slightly better. The method was laborious, however, as a further evaporation step was required.

It was eventually found that when added in small amounts, ion-pairing agents did not cause serum proteins to precipitate. Different amounts of benzenesulfonic acid salt were added to the donor phase and the results are shown in Fig. 2. Nevertheless, since there was no significant improvement obtained by adding an ion-pairing agent, and it caused an unnecessary inconvenience, it was not used in the final method.

3.2.2. Organic phase

The organic phase immobilized in the wall pores of a hollow fiber needs to meet several criteria. The analyte should have good solubility in the organic solvent, the organic solvent should be immiscible with donor phase, and should have low volatility to prevent loss during the extraction. Commonly used organic solvents include long-chain alkanes, alcohols, ethers, esters and sometimes toluene [13,15,16,18].

Results from different organic solvents tested in this study are shown in Fig. 3, which indicates that the most favorable organic phases were toluene, iso-octane and 1-octanol. However, since toluene and iso-octane were more prone to drying up during the preparation of the hollow fiber for the extraction, 1-octanol was used in the final method.

3.2.3. Acid/base concentration of acceptor phase

After preconcentration in 1-octanol, the analyte was further extracted into an acceptor phase. Since 1-octanol was not suitable for injection into LC–MS/MS, a three phase HF-LPME approach was preferred. The acceptor phase could be either aqueous solution or organic solvent that was immiscible with 1-octanol. Considering the solubility of thyroxine, an aqueous buffer was chosen as the acceptor phase. Since thyroxine has both acid and amine groups, it

can be extracted into either acidic or basic buffer. Results obtained by changing the acid/base concentrations of the acceptor phase are shown in Fig. 4. Concentrations of 0.1 M and 1 M were chosen for investigation as they are commonly used [13,15,16,18,21]. As Fig. 4 shows, extraction performance was clearly most favorable when 1 M ammonia solution was used as the acceptor phase.

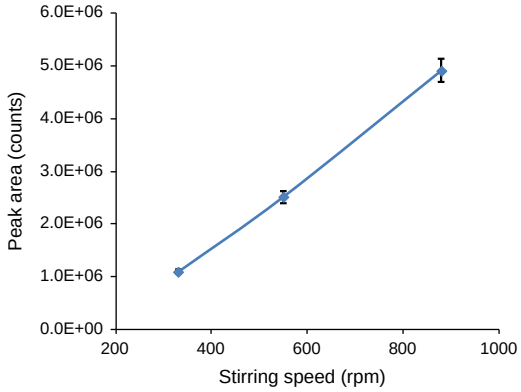

Fig. 5. Effect of stirring speed on extraction.

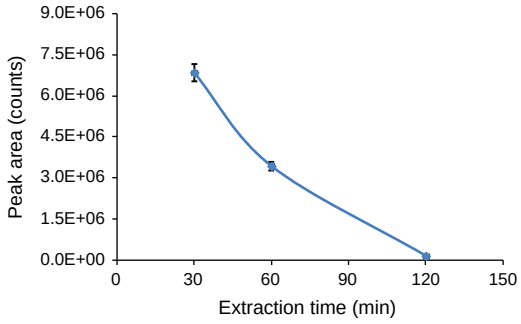

Fig. 6. Effect of extraction time on extraction.

3.2.4. Stirring speed

Samples were magnetically stirred to facilitate mass transfer. Stirring speeds ranging from between 330 and 880 rpm were evaluated. When the speed was set to above 880 rpm, the stirring could not be conducted evenly and consistently. Thus, the maximum stirring speed was maintained at 880 rpm (Fig. 5).

3.2.5. Extraction time

HF-LPME is an equilibrium-based process that is controlled by the distribution coefficients from sample solution to organic phase and from organic phase to acceptor phase [13,16,21]. A minimum extraction time is needed for the system to reach equilibrium and the amount extracted increases with time. However, if the extraction time is too long, the analyte may undergo back-diffusion and the organic phase immobilized in the membrane may be lost [21]. In this study, extraction time from 30 min to 2 h was examined. Extraction time shorter than 30 min was not investigated due to time already spent (30 min) for pre-extraction manipulation. Fig. 6 shows that an extraction time of about 30 min was most effective. Beyond 30 min, due to the reason indicated above, extraction efficiency decreased significantly.

3.3. LC–MS/MS analysis

After HF-LPME extraction, the sample was topped up to 50 µl before LC–MS/MS analysis to enable the autosampler to inject the sample 3 times. Results from the 3 injections were averaged to address instrumental drift during analysis and provide more reliable data. To check for potential interferences, samples were scanned under SIM mode to monitor the Q1 precursor ions without fragmentation. Samples prepared by SPE were also scanned for comparison. Fig. 7 shows that no interference peaks were found. HF-LPME was comparable with SPE in providing very clean extraction for serum samples, as can be seen in Fig. 7.

For measurement by LC–MS/MS, MRM transitions were monitored where the Q1 precursor ions fragmented into Q3 product ions, increasing both sensitivity and specificity. As ion pairs with lower mass were more prone to potential interference, ion pairs with higher mass were used for quantification and ion pairs with

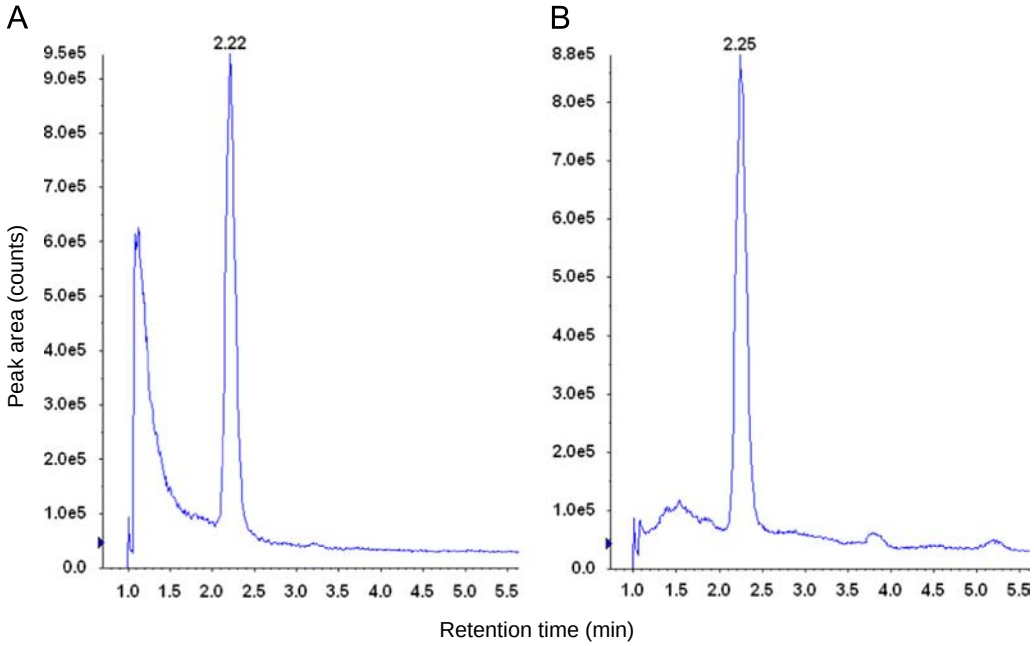

Fig. 7. SIM chromatograms at m/z 777.8 for serum sample (A) by SPE and (B) by HF-LPME.

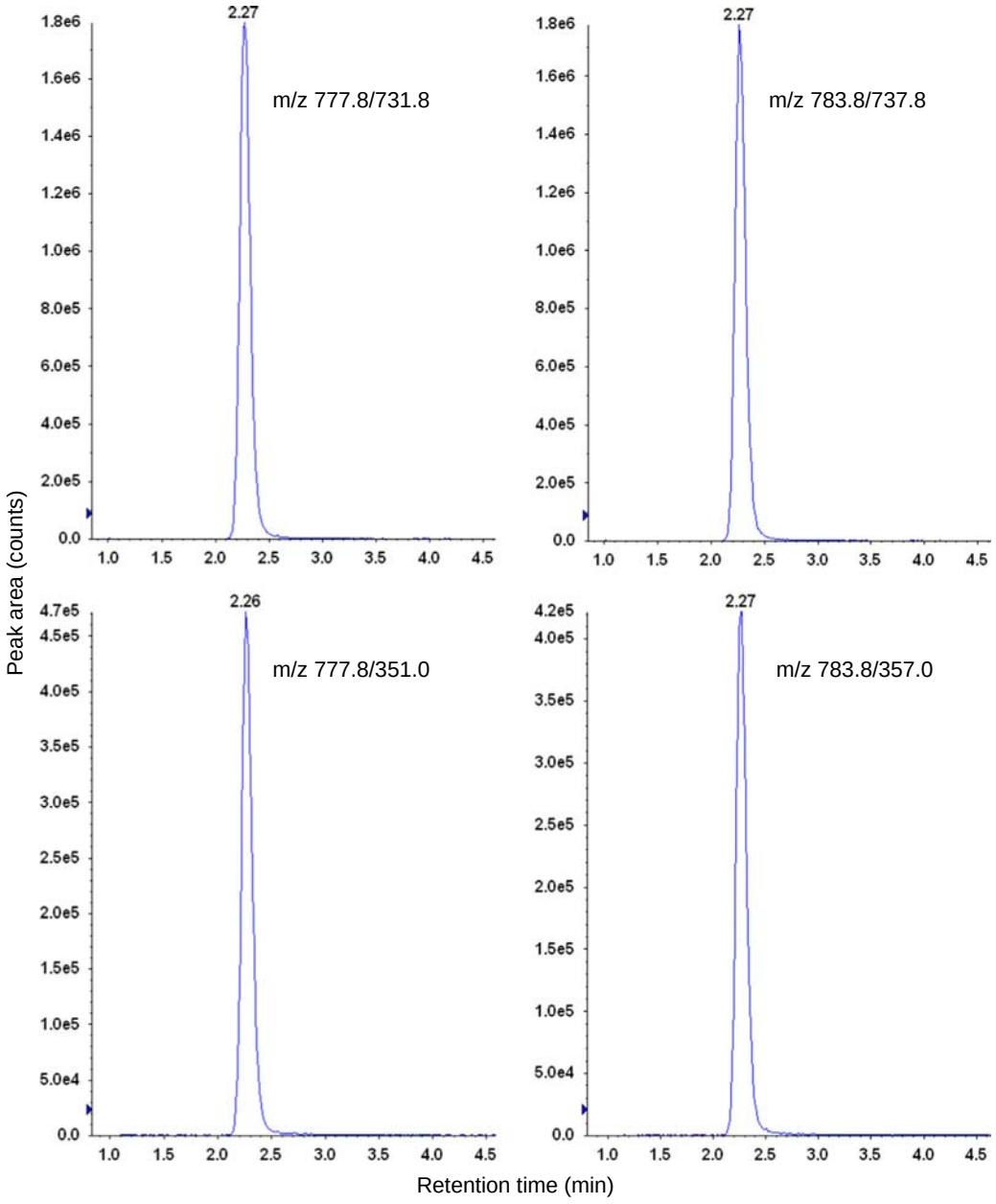

Fig. 8. Typical LC–MS/MS chromatograms for serum sample after HF-LPME.

lower mass were used for confirmation. Typical chromatograms of the analysis are shown in Fig. 8.

For the quantifying ion pair 777.8/731.8 and 783.8/737.8, the limit of detection (LOD) and the limit of quantification (LOQ) were determined to be 0.3 ng/g (three times the signal-to-noise ratio) and 1 ng/g (ten times the signal-to-noise ratio), respectively. To determine the linearity, a series of thyroxine standard solutions were prepared. The same amount of $^{13}C_6$-thyroxine was added to each solution as internal standard. The linearity range was found to be from 1 ng/g to 1000 ng/g, with $y=4.128x+0.013$ and $R^2=0.9993$.

3.4. Sample analysis using HF-LPME and SPE

Human serum from Sigma Aldrich with endogenous thyroxine was spiked with isotopically-labeled thyroxine and extracted using HF-LPME. SPE was also conducted for comparison. The results shown in Table 2 indicate that HF-LPME had very good precision with a relative standard deviation (RSD) of no more than 1.3%. This level of precision is of the same magnitude as those obtained with various SPE methods (RSDs between 0.2% and 3.0%) [8,10–12]. The difference between two ion pairs was within 1%. HF-LPME and SPE results from corresponding quantifying ion pairs agreed well with each other; the difference was less than 1.2%. An independent *t*-test was conducted to assess the bias between these two extraction methods. The calculated *t* value of 1.63 was smaller than the critical *t* value of 2.31, indicating insignificant bias

3.5. Analysis of reference materials

To further validate our newly developed method, two reference materials of human serum were obtained from Referenzinstitut für Bioanalytik, and analyzed using the developed method. The reference values of thyroxine were determined by the institute using reference methods. Both reference methods, as well as the reference laboratories, are accepted by the Joint Committee for

Table 2
Serum sample analysis using HF-LPME and SPE (concentrations are in μg/g).

	Quantification ion pair 777.8/731.8 and 783.8/737.8	Confirmation ion pair 777.8/351.0 and 783.8/357.0
HF-LPME ($n=6$)		
Average	0.0563	0.0559
SD	0.0007	0.0004
RSD (%)	1.30	0.73
Diff. between two ion pairs (%)	0.68	
SPE ($n=4$)		
Average	0.0557	0.0553
SD	0.0002	0.0005
RSD (%)	0.44	0.86
Diff. between HF-LPME and SPE (%)	1.12	1.13

Table 3
Analysis of reference materials of human serum samples (concentrations are in μg/g).

Sample no.	HM 212 03	HM 264 01
1	0.11474	0.07735
2	0.11495	0.07707
3	0.11519	0.07737
Average	0.11496	0.07726
SD	0.00022	0.00017
RSD (%)	0.19	0.22
Reference value	0.11865	0.07890
Value from the present work	0.11496	0.07726
Deviation from reference value (%)	−3.1	−2.1

Traceability in Laboratory Medicine (JCTLM) [22]. These samples were analyzed by HF-LPME–LC–MS/MS in triplicate and the results were compared with the reference values (Table 3). Our obtained values for HM 212 03 and HM 264 01 were 0.11496 μg/g and 0.07726 μg/g, with good precision of 0.19% and 0.22%, respectively. The deviation from the reference values were −3.1% and −2.1%, demonstrating good accuracy achieved by the developed method.

4. Conclusion

This work describes a method for the determination of total thyroxine in human serum based on HF-LPME coupled with LC–MS/MS analysis. HF-LPME provided direct thyroxine enrichments without the need for solvent evaporation as an extra step, as needed in SPE. This was possible since thyroxine was extracted from a relatively large sample volume and into a very small volume of acceptor solution that could be analyzed directly without any further processing. In addition, thyroxine extracts by HF-LPME were very clean even from a complex serum matrix, attributable to the protection afforded by the hollow fiber from matrix effects. The level of cleanness was comparable with that obtained by SPE. Serum samples were analyzed in replicates and the precision was very good with RSD less than 1.3%. The value obtained had no significant difference from that obtained by SPE. Furthermore, two reference materials of human serum were also analyzed. The results showed good precision with RSD around 0.2% as well as good accuracy with deviation from the reference values less than 3.2%. In contrast to the standard SPE procedure, HF-LPME provides advantages such as much lower solvent consumption, generation of little or no waste, and cost-effectiveness.

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