FAST ANALYSIS OF HYDROXYLATED POLYBROMINATED DIPHENYL ETHERS AND THYROID HORMONES IN SERUM USING UPLC-MS/MS AND UPLC/Q-TOF MS

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Introduction

The hydroxylated PBDEs (OH-PBDEs) are *structural analogues* of the PBDEs, and have recently obtained special attention because they are more potent than PBDEs or methoxylated PBDEs for some endpoints [1, 2]. OH-PBDEs can disrupt thyroid hormone homeostasis, oxidative phosphorylation, and estradiol synthesis, and are neurotoxic [1, 3]. OH-PBDEs affect thyroid regulation by decreasing circulating levels of thyroid hormones, altering expression of genes that encode thyroid-regulating proteins, and reducing activity of thyroid-regulating enzymes. Furthermore, in vitro studies have shown that PBDEs and their CYP P450–mediated metabolites, hydroxylated PBDEs, can compete for binding sites on thyroid hormone transporters in serum [4](Thyroid hormones are essential hormones for regulating growth and development in humans and wildlife. The thyroid hormones main include thyroxine (T4) and 3, 3',5-triidothyronine (T3) are tyrosine-based hormones produced by the thyroid gland.Biotransformation of T4 also produces the inactive metabolite 3,5',3'-triidothyronine (rT3) [5] , which may inhibit thyroid production through feedback mechanisms[6].

A number of analytical methods have been reported for analysis of OH-PBDEs in environmental and biological samples. The most common method is measurement of their MeO-derivatives by GC–MS with electroncapture-negative ionization (ECNI) or high resolution mass spectrometry (HRMS) using diazomethane derivatization [7-8].However, the toxicity and easy explosive of diazomethane make the analysis difficult to handle. Alternative methods based on LC–MS/MS have been developed for OH-PBDEs [9-10], atmospheric pressure chemical ionization (APCI)-LC/MS/MS and electrospray ionization (ESP)-LC/MS/MS methods have been developed for the analysis of OH-PBDEs without the time consuming step of derivatization[9, 10]. The high instrumental detection limit and/or matrix effects of LC–MS/MS would contribute to the high MDLs. Consequently, a method with lower MDLs is required for detection of OH-PBDEs in environmental samples. Most clinical laboratories routinely measure free or total T4 and T3 by automated radioimmunoassay kits [11-]. LC coupled with MS in single ion monitoring mode, and tandem MS (MS/MS) in multiple reaction monitoring (MRM) mode have been used to detect total or free T4 or T3 [12, 13]. Thus, these methods all have some limitations for direct measurements of a suite of thyroid hormones. Therefore, the selectivity and sensitivity of UPLC-MS/MS methods need to be further improved to make it more efficient in OH-PBDEs analysis.

In this study we investigated the mass-spectrometric characteristics of a suite of two main thyroid hormones (T4 and T3) and OH-PBDEs and report on a method for the simultaneous analysis of these compounds in serum samples using a solid phase extraction (SPE) cleanup and UPLC-Q-TOF and UPLC-MS/MS analysis. This method can be used to measure concentration levels of target compounds and provide more knowledge on regulation and influence of hormones and OH-PBDEs present in biological samples.

Materials and methods

Chemicals. Acetonitrile and formic acid (LC-MS grade) were purchased from Merck (Whithouse Station NJ), Distilled water was filtered through a Milli-Q system (Millipore, MA). Leucine- enkephalin was from Sigma-Aldrich (St. Louis, MO). All of the standard samples were purchased from SIGMA (St Louis, MO). Fourteen stock standards of hydroxylated polybrominated diphenyl ethers, 4'-OH-BDE-17(50 µg/mL), 3'-OH-BDE-28 (50 µg/mL), 4-OH-BDE-42 (10 µg/mL), , 3-OH-BDE-47 (50 µg/mL), 5-OH-BDE-47 (50 µg/mL), 6-OH-BDE-47 (10 µg/mL), 6-OH-BDE-82 (50 µg/mL), 6-OHBDE-85 (10 µg/mL), 5'-OH-BDE-99 (10 µg/mL), 6'-OH-BDE-99 (10 µg/mL), 6-OH-BDE-140 (10 µg/mL) and 6'-OHBDE-187 (10 µg/mL) were purchased from

AccuStandard (New Haven, CT, USA). Recovery surrogate standards ^{13}C-6'-OH-BDE100 and injection internal standards 13C-6-OH-BDE47 at 50 µg/mL were purchased from Wellington (Guelph, ON, Canada).

Sample preparation. An aliquot of 0.5mL of thawed serum was placed into a 10 mL glass centrifuge tube. 120µL of protection solution (ascorbic acid, citric acid) and 1 mL of acetone were added to the centrifuge tube and mixed thoroughly and then allowed to sit for at least 30 min to deproteinate the serum. ^{13}C-6'-OH-BDE100 (50µg) were spiked into each sample. After vortex mixing, all the samples were certrifuged at 3500 rpm for 5 min at room temperature. All the supernatants and the extracts were reduced to 1.0 mL under N_2. The concentrated extracts were loaded into a SampliQ SPE cartridge. 3.0 mL of 30% methanol in water as fist wash solution, 4.0 mL of 0.1% formic acid in methanol as elution solution.

Chromatography. Chromatographic separation was performed on a 50 mm × 2.1 mm ACQUITYTM 1.7 µm BEH C_{18} column (Waters, Milford, MA) using a ACQUITYTM Ultra Performance Liquid Chromatography system (Waters, Milford MA). The column was maintained at 30 °C and sample manager at 10 °C. Two different aqueous mobile phases were used to optimize separation. The mobile phase was composed of solvent A (water) and solvent B (acetonitrile). The first solvent gradient program consisted of 50% of solvent B increased to 70% in 2 min, increasing solvent B from 70% to 90% over 4 min, continuously increasing solvent B to 100%, and returning back to 50% of solvent B 2 min, followed by a 1.0 min re-equilibration prior to the next sample injection. Another gradient program was consisted of 20% of solvent B for 0.5min, increasing solvent B from 20% to 50 % over 3 min, continuously increasing solvent B to 90% in 7min, and returning back to 20% of solvent B over 2 min, followed by a 1.0 min re-equilibration prior to the next sample injection. linear gradient of 80% A for , where A = 0.1% ammonium formate (pH=3.0 -3.5) and B = acetonitrile. The gradient duration was 10 min at a flow rate of 0.25 mL/min. The sera samples were diluted in of 1:1 distilled water/methanol; a 5µL aliquot of each sample was injected onto the column.

Mass Spectrometry. Mass spectrometry was performed on a Waters XEVO-G2QTOF (Waters MS Technologies, Manchester, U.K.) operating in both ESI^+ and ESI^- ion modes. The nebulization gas was set to 600 L/h at a temperature of 400 °C, the cone gas was set to 50 L/h, and the source temperature was set to 100 °C. A capillary voltage and a cone voltage were set to 2.5kv and 20-40 V, respectively. The MCP detector voltage was set to 2075 V. The Q-TOF MS acquisition rate was set to 0.2 s with a 0.1 s interscan delay. Tune page was used to regulate the sample cone voltage. Argon was employed as the collision gas. The scan range was from 75 to 1200 *m*/*z*. Data was collected in centroid mode. All the analyses were acquired by using the lock spray to ensure accuracy and reproducibility; leucine−enkephalin was used as the lock mass at a concentration of 2 ng/mL and a flow rate of 5 µL/min, generating an $[M + H]^+$ ion at 556.2771 Da in ESI^+ mode, and an $[M - H]^-$ ion at 554.2615Da in ESI^- mode. The lock spray frequency was set at 20 s.

Data Analysis. The raw data were analyzed by the Micromass MarkerLynx and ChromLynx applications manager (Waters, U.K.); the retention time (RT) and *m*/*z* data pair for each peak was detected by software. The ion intensities for each peak detected were then normalized, within each sample, to the sum of the peak intensities in that sample. The statistical analysis was performed by SPSS 10.0 version (SPSS, Inc., Chicago, IL).

Results and discussion

In this study, the most effective separation of fifteen OH-PBDEs and two thyroid hormones were achieved in 10 min using a ternary mixture of 5 mM ammonium formate and acetonitrile as the mobile phases (Fig. 1). In our developed method, all OH-PBDEs were separated completely in only 7 min without derivatization and no buffer was used in the aqueous mobile phase. Chang et al. [14] used acetonitrile and 0.1% formic acid in water as the mobile phases for the separation of ten OH-PBDEs after derivatization with dansyl chloride. However, thyroid hormones were separated in ammonium formate and/or ammonium acetate as aqueous mobile phase. The separation of isomer OH-PBDEs on LC chromatographic column was known to be difficult. In the published work, only 2–4 OH tetraBDE isomer were analyzed and the separating degree was not satisfied [9, 10,]. While using the improved methods in this work, five most frequently detected OH-tetraBDE isomer, including two para-hydroxylated PBDEs (4'-OH-BDE49 and 4-OH-BDE42), two meta-hydroxylated PBDEs (3-OH-BDE47 and 5-OH-BDE47) and 6-OH-BDE47 that possibly regulated hormone level in serum, can be simultaneously identified, which were shown in Fig. 1.

MS/MS parameters were also optimized by TOF-MS and TOF-MS/MS. Positive and negative ion mode was used to measure T3, T4 and all fifty OH-PBDEs. In this study, it was also found that positive ion mode has much higher sensitivity than negative ion mode for T3 and T4. This result was similar published data by LC-MS/MS [15]. Since acetonitrile tended to carbonize the corona pin of APCI ion source and reduce the sensitivity consequently, ESI in negative ion mode was selected as the most suitable ionization for OH-PBDEs. In TOF-MS scan mode, the $[M-H]^-$ base peak formed at m/z 420.7842 and 422.7860 for OH-triBDE m/z 500.6975 and 500.6966 for OH-tetraBDE, m/z 578.6075 for OH-pentaBDE, m/z 658.5180 for OH-hexaBDE and m/z 738.4265 and 740.4240 for OH-heptaBDE. Following the successful detection of the $[M-H]^-$ ion, fragmentor voltage was optimized to obtain the most intense signal. Subsequently, product ion scan mode was used to choose product ions and optimize collision energy. The typical fragment ions of analytes were consistent with the results in previous study [9, 10]. However, the formation of specific fragment ions allowed the efficient analytical method generally based on the MRM mode. Two specific MRM transitions were used for each compound, one for qualitative analysis and another for quantification. The $[M-H]^-$ precursor ion, the product ions, the chosen MRM transitions with optimized fragmentor (frag) and collision energy (CE) for each OH-PBDEs were different. In our study, fixed mass were the $[M-H]^-$ precursor ion of OH-pentaBDEs because product ion $[M-H-HBr]^-$ were not found. T3 and T4 have similar precursor ion. For higher bromianted congeners such as OH-hexaBDEsand OH-heptaBDE, product ion $[M-H-HBr]^-$ were found with higher peak purity. In addition, for lower brominated congeners including OH-triBDE and OH-tetraBDE, $[M-H]^-$, $[M-H-HBr]^-$ and $[M-H-HBr]^-$ were found in TOF-MS/MS mode. Using the developed method, OH-PBDEs and thyroid hormone in fish serum samples were studied. T3 and T4 were detected at levels with 0.8 and 4.2 ng/mL and all OH-PBDEs were not found in laboratory cultured fish.

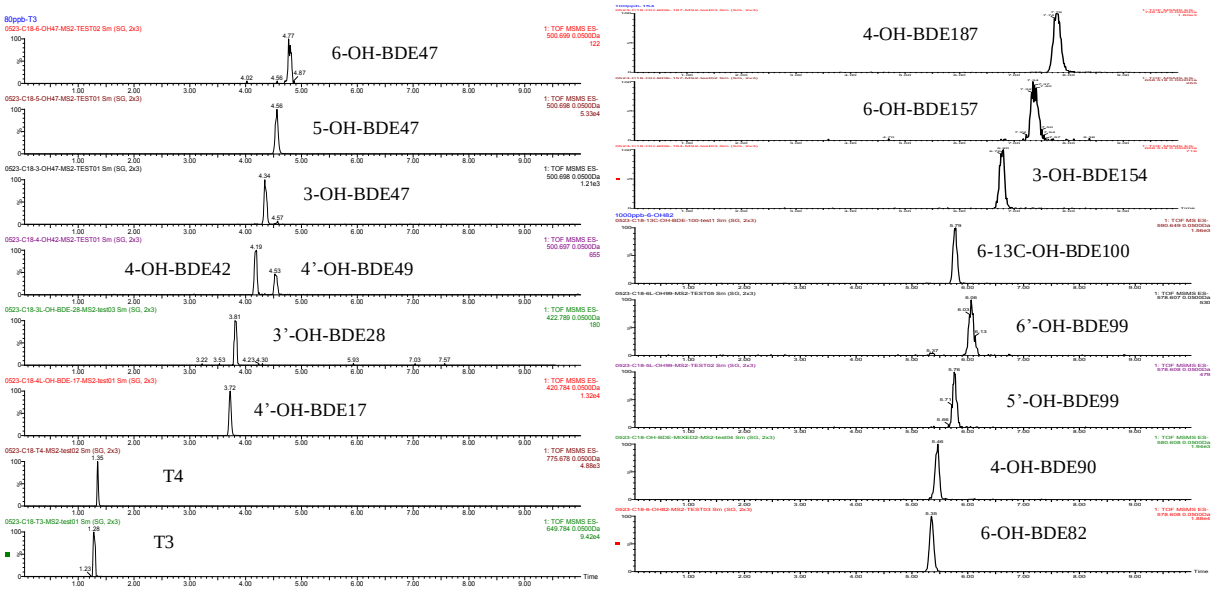

Fig. 1. Total ion current chromatographs obtained using the UPLC-Q TOF MS/MS method in negative ion mode.

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