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Analysis of hydroxylated polybrominated diphenyl ethers (OH-BDEs) by supercritical fluid chromatography/mass spectrometry

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ABSTRACT

Hydroxylated polybrominated diphenyl ethers (OH-BDEs), which have anthropogenic and natural origins, have exhibited neurotoxic and endocrine disrupting effects in humans and wildlife. Therefore, there is an increased interest in the analysis of these compounds in biological matrices in order to assess their potential toxicological risks. Analysis of OH-BDEs is conventionally completed using liquid chromatography/mass spectrometry (LC/MS), or by gas chromatography/mass spectrometry (GC/MS) after derivatization. Issues with resolution in separating congeners have limited the analysis of OH-BDEs *via* LC/MS, with published methods only able to include 13 congeners in the analysis. On the other hand, while GC/MS analysis can analyze more OH-BDE congeners, derivatization of OH-BDEs to convert them to GC amenable compounds adds to sample preparation time and limits the column lifetime due to trace residues of highly reactive derivatization agents entering the column. Herein we report the development of a supercritical fluid chromatography/mass spectrometry (SFC/MS) method for the analysis of 22 OH-BDE congeners. Instrument limits of detection for the developed method ranged from 2 to 106 fg injected on column, which is lower than previously optimized LC/MS and GC/MS methods. The developed SFC/MS method was successfully applied towards the analysis of *in vitro* metabolism samples and human serum samples to demonstrate its applicability with different biological matrices.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have been used since the 1970s as additive flame retardants in numerous consumer products including electronics, textiles, and furniture. Produced as three technical mixtures (penta-, octa-, and deca-BDE), nearly 70,000 metric tons of PBDEs were consumed globally at their peak [1]. However, in 2009, the Stockholm Convention classified the lower brominated PBDEs (tetra-, penta-, hexa-, and hepta-) as persistent organic pollutants (POPs), which resulted in an international ban in the production and use of the penta-BDE and octa-BDE technical mixtures [2]. The deca-BDE formulation was recently proposed to be included in the list of POPs [3], and its use has been phased out in the United States at the end of 2013 [4]. Nevertheless, varying regulations worldwide have permitted the continued use of deca-BDE in consumer products [5,6]. Despite the halt in production and the current regulations on the use of PBDEs, these compounds are continuously introduced into the environment through disposal of older goods and electronic wastes. The persistent and hydrophobic nature of PBDEs has resulted in their presence and accumulation in humans, wildlife, sediments, particulates, and dust [7,8].

In vitro studies on the human metabolism of PBDEs have shown that PBDEs undergo biotransformation *via* cytochrome P450 (CYP) 2B6 into their hydroxylated metabolites (OH-BDEs) [9–11]. The oxidative metabolism of PBDEs into OH-BDEs has also been observed in plants [12,13] and animals [14–16]. Furthermore, OH-BDEs are known to originate from natural sources, particularly in marine environments [17–19]. Biotransformation of PBDEs and exposure to naturally occurring OH-BDEs is of concern because studies have revealed that OH-BDEs have a greater neurotoxic and endocrine disrupting potential than their PBDE counterparts [20–23]. These significant findings highlight the importance of developing fast, accurate, sensitive, and robust analytical techniques for the analysis of OH-BDEs.

The separation and analysis of OH-BDEs are commonly accomplished by liquid chromatography/mass spectrometry (LC/MS), or by gas chromatography/mass spectrometry (GC/MS) following a derivatization procedure [18,24–36]. Applications of LC/MS towards the analysis of OH-BDEs have utilized atmospheric

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pressure chemical ionization (APCI) [24,25], atmospheric pressure photoionization (APPI) [26], and electrospray ionization (ESI) sources [27–31]. The OH-BDEs have been analyzed in either positive or negative ionization modes in LC/MS with detection limits reaching sub-pg levels [30]. Detection of OH-BDEs by GC/MS has also been achieved at sub-pg levels using electron ionization (EI) [32,33] and electron capture negative ionization (ECNI) [34–36] sources.

Many OH-BDEs are isobaric positional isomers; therefore, chromatographic separation is crucial in their identification and quantification. Due to the limited chromatographic resolution in LC, previously published LC/MS methods have only analyzed up to 13 OH-BDE congeners [24–31]. In addition, the inherently low ionization efficiencies of OH-BDEs in the ESI(−) mode necessitates derivatization with dansyl chloride to achieve desired detection limits [28]. Compared to LC/MS, analysis by GC/MS offers higher resolution, however prior derivatization of OH-BDEs into GC amenable compounds is required. Common derivatization procedures for GC/MS analysis of hydroxylated compounds included methylation of OH-BDEs using either diazomethane [35] or (trimethylsilyl)diazomethane [10,11], and silylation using either a combination of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) [36] or *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) [32]. Not only do derivatization reactions require the use of potentially dangerous or toxic chemicals, but excess derivatization agents need to be cleaned-up before GC/MS analysis, adding additional steps in the sample preparation. Failure to remove excess derivatization agents shortens the GC column lifetime significantly due to their high reactivity that damages the stationary phase.

Supercritical fluid chromatography with mass spectrometry (SFC/MS) is an attractive technique for the analysis of OH-BDEs because it provides many of the same advantages that LC/MS and GC/MS offer, without the limitations associated with derivatization. Furthermore, SFC/MS offers quicker analysis times and reduced solvent consumption. High diffusivity of analytes in SFC, similar to GC, results in sharper chromatographic peaks and greater chromatographic resolution than LC [37]. SFC/MS has been explored in the separation and analysis of both lipophilic and hydrophilic compounds. Recently, SFC has been utilized to separate fatty acids [38], urinary metabolites [39], pesticides [40], amines [41], and hydroxylated compounds [42]. Furthermore, SFC/MS was previously utilized for the analysis of phosphate flame retardants in polyurethane foams [43]. To our knowledge, no SFC/MS analysis has been performed on PBDEs or PBDE analogs, such as OH-BDEs.

The objectives of this study were to (1) develop and optimize a method for the trace analysis of 22 OH-BDEs using SFC with MS detection under multiple reaction monitoring (MRM) mode (SFC/MS/MS), (2) apply the developed method towards the analysis of PBDE metabolites from *in vitro* samples of recombinant CYP2B6 incubated with 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100), and (3) apply the method to the analysis of OH-BDEs in human serum. Furthermore, the analysis of human serum samples by SFC/MS/MS was compared with previous results obtained from GC/MS/MS analysis.

2. Materials and methods

2.1. Chemicals and materials

Individual standards of 2′-OH-BDE-3, 2′-OH-BDE-7, 3′-OH-BDE-7, 4′-OH-BDE-17, 3′-OH-BDE-28, 4-OH-BDE-42, 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4′-OH-BDE-49, 2′-OH-BDE-68, 6-OH-BDE-82, 6-OH-BDE-85, 6-OH-BDE-87, 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, 3-OH-BDE-100, 6-OH-BDE-140, 3-OH-BDE-154, and 6-OH-BDE157 were purchased from AccuStandard (New Haven, CT). Isotopically-labeled standards of $^{13}C_{12}$-6-OH-BDE-47 and $^{13}C_{12}$-6′-OH-BDE-100 were obtained from Wellington Laboratories (Guelph, ON, Canada). Structures of the 22 OH-BDEs can be found in the Supporting Information (SI; Fig. S1). Neat BDE-47 was purchased from Chem Service (West Chester, PA) and neat BDE-100 was purchased from AccuStandard for *in vitro* metabolism studies. Recombinant CYP2B6 was obtained from BD Biosciences (San Jose, CA).

2.2. SFC/MS/MS optimization

Chromatographic separation was completed on an ACQUITY UPC^2 system (Waters Corporation, Milford, MA). Several UPC^2 columns were tested during the optimization process including: (1) an ACQUITY UPC^2 HSS C18 SB (2.1 × 150 mm, 1.8 μm); (2) an ACQUITY UPC^2 HSS C18 SB (3.0 × 100 mm, 1.8 μm) and ACQUITY UPC^2 HSS C18 SB (2.1 × 100 mm, 1.8 μm) in series; and (3) an ACQUITY UPC^2 Torus Diol (2.1 × 100 mm, 1.7 μm). Mobile phase compositions consisted of varying gradients of food grade carbon dioxide and isopropanol (IPA). Injection volumes, flow rates, and column temperatures differed based on the columns tested and ranged from 2 to 5 μL, 0.7 to 0.9 mL/min, and 25 to 50 °C, respectively.

Mass spectrometric analysis of OH-BDEs was conducted using the quadrupole time-of-flight (Q-ToF) SYNAPT G2-Si and the tandem quadrupole Xevo TQ-S systems (Waters Corporation, Milford, MA). Both systems were utilized during optimization of separation conditions, but ultimately, the Xevo TQ-S system was chosen for quantitation purposes due to the lower detection limits necessary for trace analysis. Individual standards of the 22 OH-BDE compounds were used to optimize the MS conditions and the ESI source parameters to obtain maximum signal intensities. For quantitative work, the optimum parameters for the ESI used a capillary voltage of 2 kV, a source offset of 50 V, a source temperature of 100 °C, and a desolvation temperature of 500 °C. Precursor ions, product ions, and collision energies for all 22 OH-BDEs are listed in Table 1.

2.3. Application in the analysis of BDE-47 and BDE-100 metabolites

Recent studies have shown CYP2B6 to be the primary enzyme responsible for the metabolism of BDE-47 and BDE-100 to OH-BDEs [10,11]. To demonstrate the applicability of the SFC/MS/MS method in the analysis of OH-BDEs, 5 μM BDE-47 and 5 μM BDE-100 were individually incubated with 0.040 nmol CYP2B6 for 40 min for *in vitro* metabolism to occur; the metabolites were extracted as previously reported [10,11]. Briefly, the assay was conducted in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.4 and initiated with 1 mM nicotinamide adenine dinucleotide phosphate (NADPH). The test samples were quenched using an ice bath and kept frozen until extraction. Before extraction, proteins were denatured by adding 500 μL of 6 M hydrochloric acid and samples were spiked with 10 ng of $^{13}C_{12}$-6-OH-BDE-47 as surrogate to check for recovery. Samples then underwent a liquid-liquid extraction (LLE) using two 3 mL additions of hexanes/dichloromethane solutions. Sample cleanup was performed with 2 mL of concentrated sulfuric acid and extracts were evaporated to dryness. Samples were reconstituted in 50 μL methanol. $^{13}C_{12}$-6′-OH-BDE-100 was used as an internal standard.

2.4. Application in the analysis of human serum samples

The presence of OH-BDEs has recently been reported in human serum samples [32,35,36]. To test the applicability of the SFC/MS/