Identification of Hydroxylated Octa- and Nona-Bromodiphenyl Ethers in Human Serum from Electronic Waste Dismantling Workers

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Previous studies have reported high serum concentrations of polybrominated diphenyl ethers, especially decabromodiphenyl ether (BDE-209), in the residents of an electronic waste (e-waste) dismantling site in Guiyu town, South China. In the present study, human serum samples in this region were collected and pooled for the identification of hydroxylated diphenyl ethers (OH-PBDEs). Three OH-PBDEs, including two hydroxylated octabromodiphenyl ethers (OH-octaBDEs, 6-OH-BDE196 and 6-OH-BDE199) and one hydroxylated nonabromodiphenyl ether (OH-nonaBDE, 6′-OH-BDE206), were first structurally identified. Identification was done by coeluting a mixture of synthetic authentic standards with the methylated OH-PBDEs from the pooled samples using two gas chromatography columns with different polarities. The results were supported by full scan mass spectrometric data in electron capture negative ionization mode. All three OH-PBDE metabolites had hydroxy groups substituted in the *ortho* position. These results indicate that hydroxylated higher brominated diphenyl ethers such as OH-octaBDEs and OH-nonaBDEs can accumulate in human blood. The results suggest that higher brominated diphenyl ethers could be oxidatively metabolized into OH-PBDEs in humans. Because low brominated OH-PBDEs can also be detected in abiotic media, further investigations are needed to determine the presence of higher brominated OH-PBDEs in the environment in this region.

Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of additive flame retardants used in textiles, furniture, electronic appliances and electrical goods. Commercial mixtures of PBDEs are mainly composed of three formulations: penta-, octa-, and deca-BDEs (*1*). Because of their persistence, bioaccumulation, and potential toxicity, the European Union have initiated regulations to phase out the production and usage of penta- and octa-BDE technical mixtures, and the United States voluntarily phased out the production of penta- and octa-PBDE formulations. This voluntary ban has been extended recently to include the deca-PBDE formulation. The Stockholm Convention also recently designated penta- and octa-BDE technical mixtures as persistent organic pollutants. At present, the deca-BDE, BDE-209, is the only product allowed to be used, and it comprises approximately 80% of the world market demand for brominated diphenyl ethers (*2*). Assessments of the ecological and human health risks of BDE-209 are critical in deciding whether or not this chemical should be banned from production and use. Several agencies are now conducting further reviews of BDE-209, focusing on its toxicokinetics, degradation, and neurotoxic effects (*3*).

Although the toxicity of PBDEs is not fully understood, some toxic effects such as thyroid toxicity have been documented for hydroxylated PBDEs (OH-PBDEs) in laboratory experiments (*4, 5*). OH-PBDEs have been detected in blood samples from rats and mice and from fishes, birds, and mammals following exposure to PBDE mixtures (*6*−*13*). Marsh et al. (*6*) identified six tetra-OH-PBDEs and three tri-OH-PBDEs in rats after exposure to BDE-47. Malmberg et al. (*9*) tentatively identified sixteen OH-PBDEs in rat blood after exposure to equimolar doses of seven environmentally relevant PBDEs. In addition to in vivo and in vitro animal studies, OH-PBDEs have also been detected in pooled serum from children living or working at a municipal waste disposal site in Managua, Nicaragua (*14*) and in blood samples from pregnant women and newborn babies in the United States. The results of these studies suggest that oxidative metabolism might occur in humans (*15*). However, OH-PBDEs can also be acquired from sources other than biotransformation in biota and humans. Ueno et al. (*16*) recently reported that dibromo- to hexabromo-OH-PBDEs in abiotic media from the Great Lakes region were likely to be byproducts of atmospheric OH radical reactions or the result of sewage treatment plant effluents, and *ortho* OH-substituted OH-PBDEs have also been identified as naturally occurring formulations in marine organisms such as sponges, tunicates, and algae (*17, 18*).

Because of its high molecular weight and high hydrophobicity, BDE-209 was assumed to have low bioavailability. The calculated apparent half-life in rats and humans ranged from 2−15 days (*19, 20*). However, BDE-209 has been detected in several free-living animals, including birds (*21*−*23*), and has also been found in the blood of occupationally exposed and unexposed individuals (*24*−*27*). The results of several in vivo laboratory studies have indicated that BDE-209 might be biotransformed into hexa- to nona-BDEs via a reductive debromination pathway (*28*−*31*). Sandholm et al. detected 13 phenolic metabolites in rat plasma after exposure to BDE-209. They further tentatively identified two major metabolites as octa- and nona-OH-PBDEs (*20*). However, to the best of our knowledge, no other studies have reported the structural identification of octa- and nona-OH-PBDEs. This study formed part of our ongoing research into organic contaminants at electronic waste (e-waste) recycling sites in China. Our previous studies indicated that e-waste dismantling workers had much higher concentrations of PBDEs, especially decabromodiphenyl ether (BDE-209), than those previously reported in occupationally exposed workers (*26, 27*). The aim of the current study was to identify the structure of the OH-PBDEs in pooled serum samples from the e-waste dismantling workers (Guiyu, South China). It was performed

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by coelution of a mixture of synthetic standards and methylated OH-PBDEs in the serum samples using three gas chromatographic columns with different polarities. The synthetic procedures of three identified OH-PBDEs were also firstly reported.

Experimental Section

Chemicals and Materials. ^{13}C-labeled decabromodiphenyl ether (BDE-209) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Solvents and other chemicals used for the analysis of blood samples were *n*-hexane (pesticide grade; Fluka, Germany), methyl *tert*-butyl ether (MTBE) and 2-propanol (HPLC grade; Sigma-Aldrich, Germany), silica gel (63−200 μm; Merck, Germany), sulfuric acid (99.9%; Sigma-Aldrich, Germany), and hydrochloric acid (37%; Sigma-Aldrich, Germany). Anhydrous sodium sulfate and potassium chloride (analysis grade; Xilong Chemical Factory of Guangdong, China) were stored in sealed containers after baking at 450 °C. The three methoxylated polybrominated diphenyl ethers 3,4,5,6-tetrabromo-2-(2,3,4,6-tetrabromophenoxy)anisole (6-MeO-BDE199), 3,4,5,6-tetrabromo-2-(2,3,5,6-tetrabromophenoxy)anisole (6-MeO-BDE199) and 3,4,5,6-tetrabromo-2-(2,3,4,5,6-pentabromophenoxy)anisole (6′-MeO-BDE206) were prepared as follow.

Sample Extraction and Cleanup. A total of 27 mL serum samples were collected from six e-waste dismantling workers in Guiyu town, Shantou City, Guangdong Province. About eighty percent of the families in Guiyu town are engaged in recycling work using primitive methods (including chipping and melting plastics without proper ventilation, burning coated wire to recover copper, removing electronic components from printed circuit boards, and burning unsalvageable materials in open air). Signed consent was obtained from all participants prior to sampling. Blood samples were taken from each volunteer by medical professionals using BD Vacutainer serum tubes (with clotting agent and polymer separator). Samples were then centrifuged, frozen immediately, and stored at −20 °C until analysis.

The methods used for sample extraction and cleanup have been described previously (*14, 15, 32*). Briefly, the pooled sera was in a Teflon separating funnel and denatured using hydrochloric acid (6M, 1 mL) and 2-propanol (6 mL). The samples were then extracted three times with a 1:1 hexane MTBE mixture (8 mL). The combined organic extracts were washed with an aqueous potassium chloride solution (1%), and the solvent was reduced to dryness for gravimetric lipid weight determination.

The extracts were redissolved in hexane, and the phenolic and neutral compounds were separated using an aqueous potassium hydroxide solution (0.5 M in 50% ethanol). The aqueous phase was re-extracted three times with hexane. After acidification of the aqueous phase using hydrochloric acid (0.5M, 2 mL), the phenolic compounds were extracted three times with a 9:1 hexane:MTBE mixture. The phenolic fractions were derivatized with an excess of diazomethane solution at room temperature overnight, then further cleaned with sulfuric acid/impregnated silica gel (silica/sulfuric acid 2:1 by weight; 1 g) using 15 mL of hexane:dichloromethane (1:1, vol/vol) as the eluent. The final extracts were concentrated to 20 μL under a gentle nitrogen stream, and a total of 1 ng ^{13}C-BDE-209 was added before injection.

Instrument Analysis. Serum samples were identified using an Agilent 7890 series gas chromatograph coupled to an Agilent 5975C mass spectrometer (GC/MS). Both electron ionization (EI) and electron capture negative ionization (ECNI) modes were used for full scan analysis. The mass spectrometer was scanned from 70−1000 *m*/*z*. ECNI selected ion monitoring was simultaneously used, and the following ions were monitored: *m*/*z* 79 and 81 for Br, and 498.8 for ^{13}C-labeled BDE-209. Three different types of columns were used for identification: DB-5-HT MS [15 m × 250 μm internal diameter (i.d.); 0.10 μm film thickness; J&W Scientific, Folsom, CA], DB-17 MS (15 m × 250 μm i.d.; 0.25 μm film thickness; J&W Scientific), and SP-2331 (15 m × 250 μm i.d.; 0.20 μm film thickness; Supelco, Bellefonte, PA). Manual injection (1 μL) was performed in the pulse splitless mode with a purge time of 2.0 min. The GC oven temperature program was set as follows: held at 110 °C for 5 min, 20 °C/min to 200 °C, held for 4.5 min, and then 7.5 °C/min to 305 °C, held for 16 min.

Synthesis of 6-MeO-BDE196, 6-MeO-BDE199, and 6′-MeO-BDE206. *General Comments.* The ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker Avance 500 spectrometer, and their chemical shifts were referenced to the signal at $\delta_{H/C}$ 7.26/77.0 ppm of $CHCl_3$ ($CDCl_3$ impurities) relative to TMS. Electron ionization mass spectra (EI-MS) were obtained using an Agilent 5975B mass spectrometer connected to a Agilent 6890N gas chromatograph, equipped with a DB-5 MS capillary column (J&W Scientific, 12 m × 250 μm i.d.; 0.25 μm film thickness) using helium as the carrier gas and an electron energy of 70 eV. High performance liquid chromatography (HPLC) purification was carried out on a Waters Alliance 2695 equipped with a Waters 2996 PDA detector and a Waters sunfire C_{18} reversed-phase column (250 mm × 4.6 mm, 5 μm). Methanol and water were used as the mobile phase.

The starting material, 5-amino-2-methoxyphenol, was purchased from Alfa Aesar (Tianjin, China), and 5-fluoro-2-nitroaniline was purchased from FWD Chem (Shanghai, China). All solvents and other chemicals were analytical reagents. Silica gel column chromatography was carried out using 100−200 mesh silica. All organic phases were dried with anhydrous sodium sulfate prior to being concentrated in a rotary evaporator. The final products, 6-MeO-BDE196, 6-MeO-BDE199, and 6′-MeO-BDE206, were all further purified as authentic reference standards by HPLC.

Synthetic Procedure. The synthetic schemes for 6-MeO-BDE196, 6-MeO-BDE199, and 6′-MeO-BDE206 are shown in Figure 1. In brief, they were initiated by coupling 5-fluoro-2-nitroaniline with 5-amino-2-methoxyphenol in dimethylacetamide using potassium carbonate to yield 5-(5-amino-2-methoxyphenoxy)-2-nitroaniline **1**, which is similar to the method by Marsh et al. (*33*). This compound was then tetrabrominated in the positions *ortho* and *para* to the amino groups to give 2,4-dibromo-3-(3-amino-2,4-dibromo-6-methoxyphenoxy)-6-nitroaniline **2**. Bromination was performed in acetic acid at 80 °C because of the presence of an electron-withdrawing nitro group and high steric hindrance caused by three bromine atoms and a methoxy group in the *ortho* positions to the diphenyl ether bond. The two amino groups were then converted to bromine atoms to give 3,4,5-tribromo-2-(2,3,6-tribromo-4-nitrophenoxy)anisole **3** by diazotization with $HBr/NaNO_2$ and the Sandmeyer reaction using CuBr (*34*). Thereafter, the reduction of the nitro group with iron produced 3,4,5-tribromo-2-(2,3,6-tribromo-4-aminophenoxy)anisole **4** in good yields; however, the reaction with Pd/C or Raney nickel led to products of debromination (*35*). Compound **4** was further brominated in the position *ortho* to the amino group at room temperature in dichloromethane to produce 3,4,5-tribromo-2-(2,3,5,6-tribromo-4-aminophenoxy)-anisole **5.** 3,4,5-Tribromo-2-(2,3,4,6-tetrabromophenoxy)anisole **6** was synthesized from compound **4** by diazotization and the Sandmeyer reaction. 3,4,5-Tribromo-2-(2,3,4,5,6-pentabromophenoxy)anisole **8** was similarly obtained from compound **5**. However, an unexpected product, 3,4,5-tribromo-2-(2,3,5,6-tetrabromophenoxy)-anisole **7**, in which the amino group was converted to a hydrogen atom, was detected by GC-MS in this reaction in similar amounts to **8**. We speculated that this could be attributed to the highly hindered nature of the structure

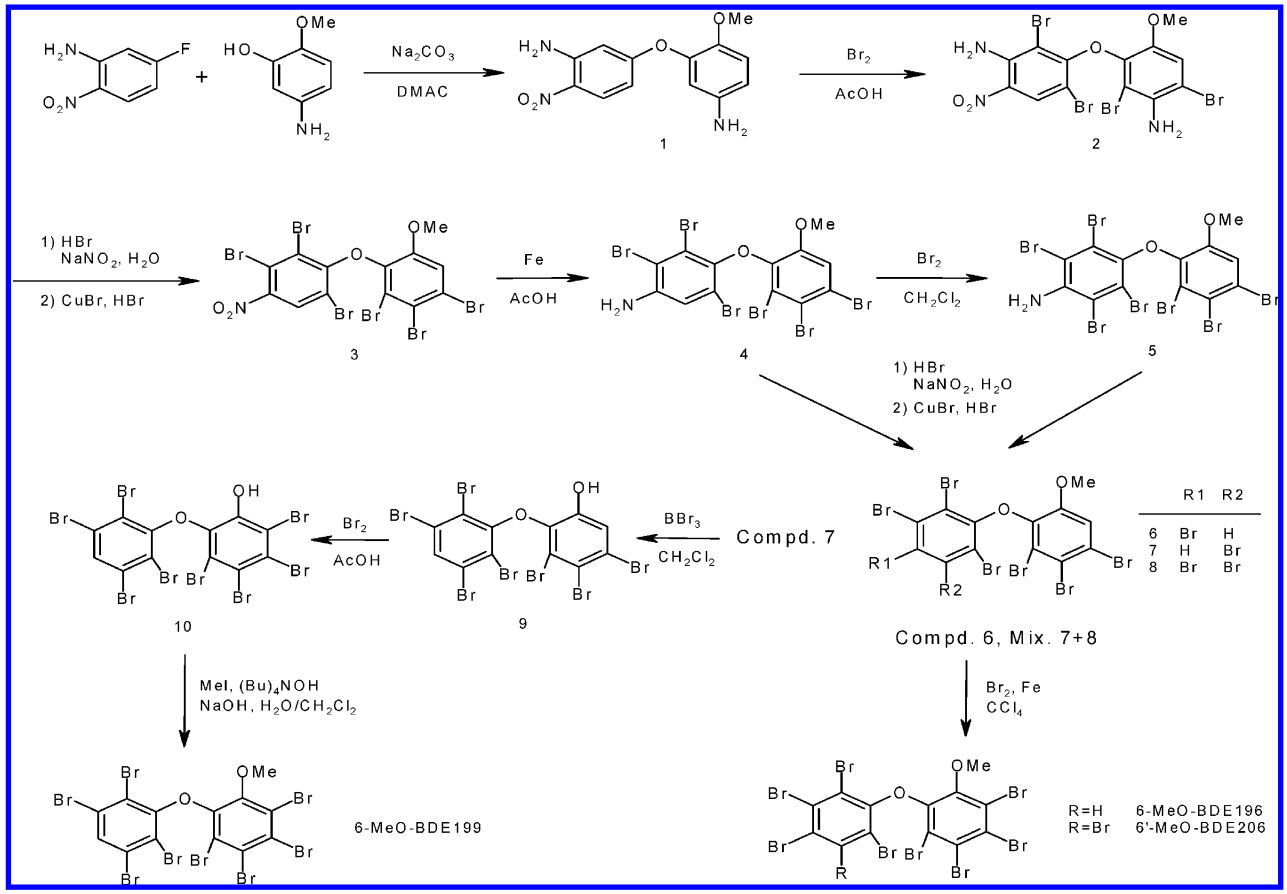

FIGURE 1. Synthetic schemes for 6-MeO-BDE196, 6-MeO-BDE199, and 6′-MeO-BDE206.

caused by the two bromine atom substituents *ortho* to the amino group. It is possible that the Sandmeyer reaction could be used as a deamination reaction for an amino group with high steric hindrance like that in compound **5**. Compounds **7** and **8**, which were not isolated as pure compounds, were both converted to the desired product 6′-MeO-BDE206 through bromination in CCl_4 with iron as the catalyst (*36*). Likewise, the target product 6-MeO-BDE196 was prepared from compound **6** by bromination. Compound **7**, isolated from the mixture of **7** and **8** by HPLC, was demethylated to 3,4,5-tribromo-2-(2,3,5,6-tetrabromophenoxy)phenol **9** with BBr_3 (*33*), which was brominated to give 3,4,5,6-tetrabromo-2-(2,3,5,6-tetrabromophenoxy)-phenol **10**. It was then remethylated to produce the target molecule 6-MeO-BDE199 (*33*). The details of the reaction conditions for each step and nuclear magnetic resonance (NMR) and mass spectra data for the intermediate products and final MeO-PBDEs are listed in the Supporting Information.

Results

Three authentic standards (6-OH-BDE-196, 6-OH-BDE-199 and 6′−OH-BDE-206) were synthesized and characterized at Shanghai University. After purification by HPLC (>95% purity), they were moved to the lab in the Guangzhou Institute of Geochemistry for identification. Before determination of OH-PBDEs in the human serum sample, two control experiments were conducted to see if the sample treatment procedures produced any OH-PBDE compounds or OH-PBDEs as a result of laboratory matrix contamination. A total of 20 ng ^{13}C-labeled BDE-209 was spiked into fetal bovine serum, and the sample cleanup procedures and determination methods were performed as described in the Experimental Section. No OH-PBDEs were produced during the sample cleanup procedures, which was consistent with the results of previously published studies that used similar cleanup procedures for the determination of OH-PBDEs (*13*−*15*). Laboratory blanks using fetal bovine serum were also treated and analyzed. OH-PBDEs were undetected or below the limit of detection, except for a minor amount of BDE-209 (<40 pg).

To identify OH-PBDEs in human blood, we pooled six serum samples collected from the e-waste dismantling workers in Guiyu town, and the phenolic fraction was isolated and analyzed using GC/ECNI-MS. Figure 2 shows the typical selected-ion monitoring (SIM) chromatogram in the pooled serum sample in ECNI mode, recorded by ion m/z: 79, 81. More than 20 brominated phenols were observed in the phenolic fraction. However, they could not be structurally identified because of the lack of authentic standards. The concentrations of brominated phenols were also not sufficient to allow full scan analysis by GC/EI-MS. However, it was possible to analyze the three most abundant peaks (marked as a, b and c in Figure 2) from the fragment ions in full scan GC/ECNI-MS (Figure 3), and their retention times were compared with those of PBDEs on the same column. Unlike the low brominated MeO-PBDEs (*37*, *38*), the phenoxide ions were predominant for the MeO-PBDEs. As shown in Figure 3, the fragment ion $[M-C_6H_{0-1}Br_{4-5}]^-$ (m/z 438.6) gave the number of bromine atoms in the methoxylated phenyl ring; there were four bromine atoms for all peaks a, b, and c. The fragment ion $[M-C_6Br_4OCH_3]^-$ gives the number of bromine atoms in the nonmethoxylated phenyl rings. Ion m/z 408.6 in peaks a and b of Figure 3 indicates that peaks a and b had four bromine atoms. Ion m/z 486.6 in Figure 3c showed peak c had five bromine atoms (*11*). The mass spectra of the three peaks in ECNI mode (Figure 3) also matched those of the synthetic standards (Figure 4). It is notable that the fragment

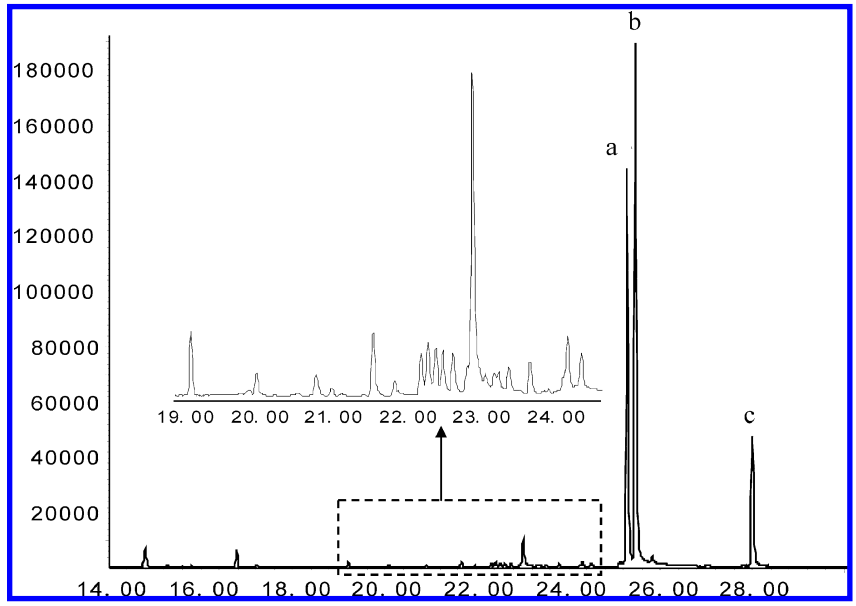

FIGURE 2. Typical selected-ion monitoring (SIM) chromatogram of methylated OH-PBDEs in pooled sera from electronics dismantling workers in ECNI mode, recorded by ion *m/z*: 79, 81.

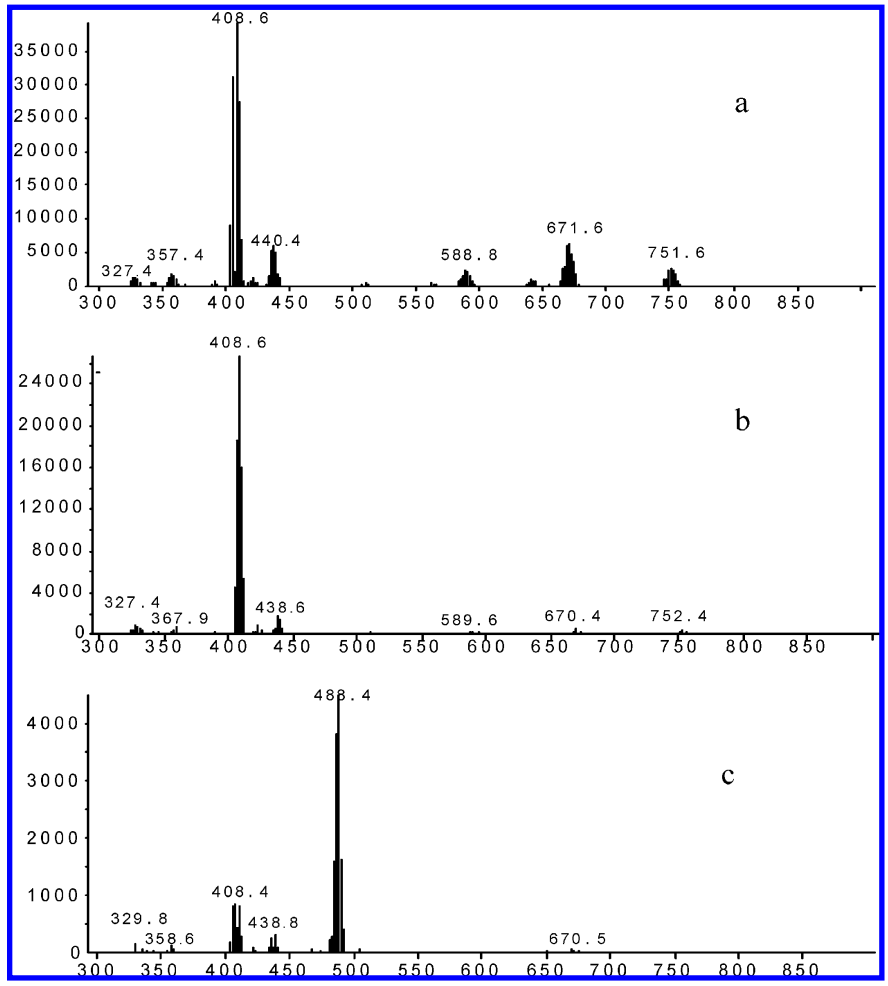

FIGURE 3. Mass spectra of three dominant methylated OH-PBDEs in pooled sera from electronics dismantling workers in ECNI mode.

ion characterization in this study significantly different with those reported for low brominated MeO-PBDEs. More synthetic standards are needed for further investigation. Additional supporting evidence was provided by the retention times, when compared with those of octa- and nona-BDEs. According to previous reports, all the eluted PBDEs on a DB-5 column after BDE-197 were octa- to deca-bromodiphenyl ethers (*39*). In our test, the retention time of BDE-197 was 24.58 min. The retention times of peaks a, b, and c in the phenolic fraction were 25.81, 25.99, and 28.65 min, respectively, which were significantly later than the rentention time for BDE-197. Taken together, these results suggest that

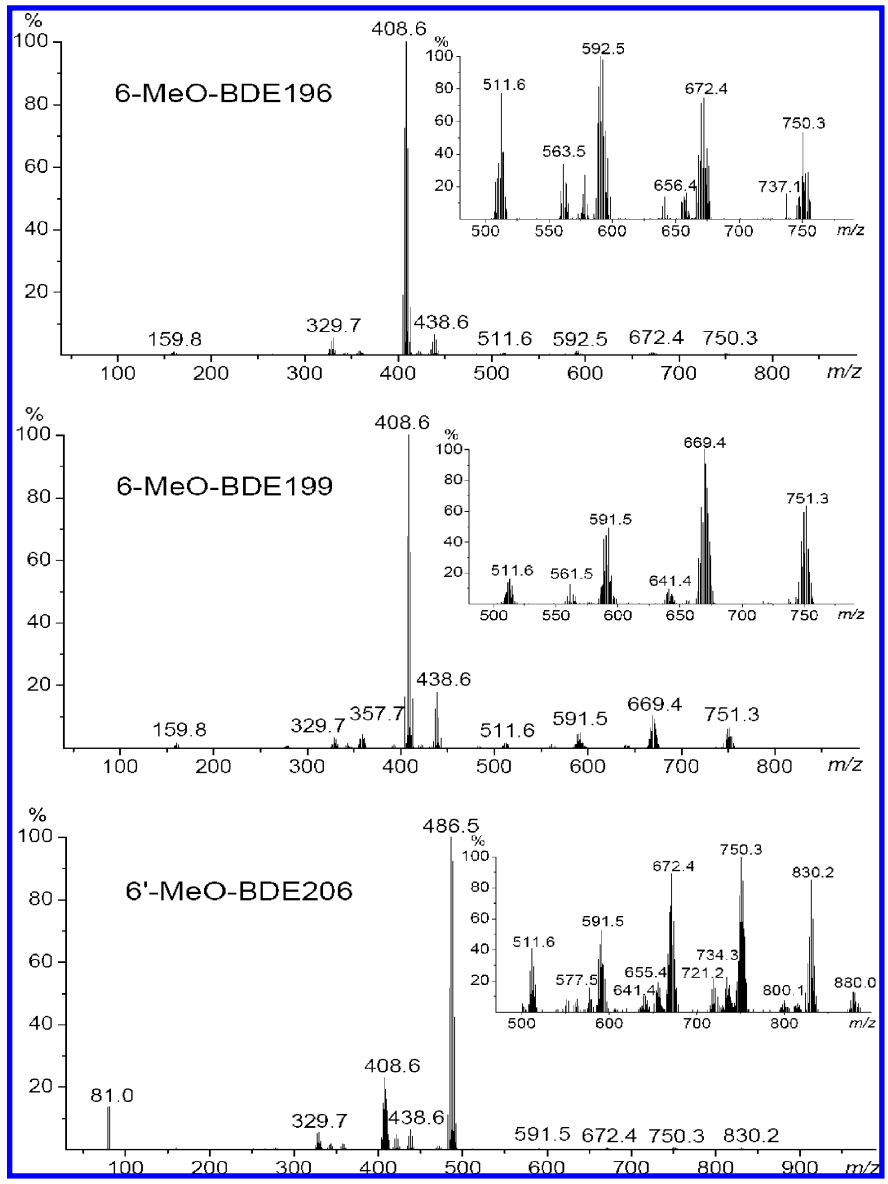

FIGURE 4. Mass spectra of synthetic 6-MeO-BDE196, 6-MeO-BDE199, and 6′-MeO-BDE206 in ECNI mode.

these three peaks represented two OH-octaBDEs and one OH-nonaBDE.

The final structural identification was conducted by coelution of a mixture of three synthetic standards and methylated OH-PBDEs in the serum samples using three gas chromatographic columns with different polarities (DB-5 nonpolar, DB-17 medium polar, and SP-2331 high polar). The relative retention time (RRT) values versus ^{13}C-labeled BDE-209 were also calculated to provide additional supporting evidence. On the SP-2331 column, 6′-MeO-BDE206 failed to elute despite increasing the oven temperature to 310 °C. However, the results of the coelution experiment (Figure 5) showed that peaks a, b, and c in the serum sample eluted with the three synthetic standards, with the same retention times on the DB-5 (Figure 5) and DB-17 columns. The similar RRTs of the three peaks in the pooled sera versus ^{13}C-labeled BDE-209 were all $< \pm 0.001$, compared with those of the synthetic standards. We therefore concluded that the human serum samples contained 6-OH-BDE199, 6-OH-BDE196, and 6′-OH-BDE206.

Discussion

Several research groups have performed in vivo experiments to investigate the metabolism of BDE-209 over the past decade (*20, 28−30, 40, 41*). Although BDE-209 has low bioavailability and a short half-life, many previous studies have found that BDE-209 could accumulate in human blood and milk as well as in animals such as rats, fishes, and birds. They have also determined that it could further debrominate to produce lower PBDEs, including penta- to nona-BDEs (*21−27, 42*). OH-PBDEs have also been tentatively detected following exposure to BDE-209 in vivo experiments. Riu et al. (*40*) dosed pregnant Wistar rats with ^{14}C-radiolabeled BDE-209 and found one octa-OH-PBDE in the rat livers, using NMR. Sandholm et al. (*20*) reported thirteen OH-PBDEs in rat plasma, and the major phenolic metabolites were characterized as one OH-octaBDE and one OH-nonaBDE. A similar study by Mörck et al. (*41*) identified two OH-hexaBDEs and six hydroxymethoxy-BDEs, with between five and nine bromines, in rat feces. However, no OH-PBDEs or debrominated metabolites were observed in human hepatocytes following in vitro exposure to BDE-209 (*43*). This result suggests that BDE-209 was either not metabolized, or that OH-PBDEs covalently bound to cellular lipids and/or proteins were not recovered during the extraction process, or that the exposure time was not long enough for BDE-209 to diffuse into the cell to be metabolized.

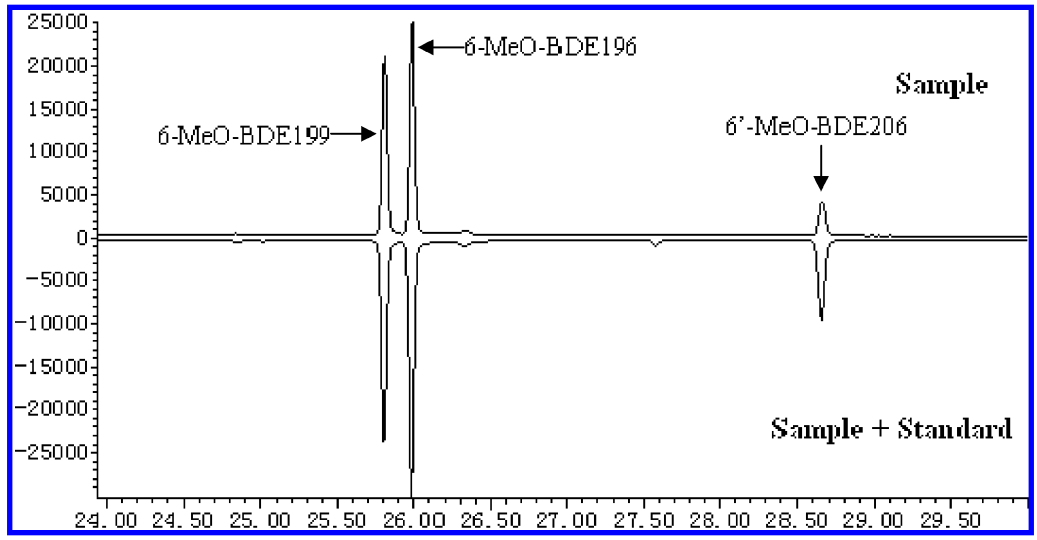

FIGURE 5. GC/MS chromatogram of coelution of three synthetic standards and pooled sera on DB-5 column in ECNI mode.

Recently, OH-PBDEs have been identified in pooled human blood samples taken from children at a municipal waste disposal site in Managua, Nicaragua, and in blood samples from pregnant women and newborn babies in the United States (*14, 15*). The OH-PBDEs detected in these studies were mainly OH-tetraBDEs and OH-pentaBDEs. This might be explained by the fact that both these study groups were exposed to high concentrations of lower brominated diphenyl ethers such as BDE-47, BDE-99, and BDE-100. The identification of OH-PBDEs with six or seven bromines is also partially limited by a lack of authentic standards. Because BDE-209 has a short serum half-life of approximately 15 days (*21*), the concentrations of BDE-209 might only reflect recent exposure, and it would therefore be more difficult to produce metabolites of BDE-209, compared to tetra- and penta-BDEs. In this study, two OH-octaBDEs (6-OH-BDE199, 6-OH-BDE196) and one OH-nonaBDE (6′-OH-BDE206) in the pooled serum samples from the e-waste dismantling workers were structurally identified using the synthetic reference standards. Our previous studies (*26*) showed that the median levels of BDE-209 in sera from people in this region were 50−200 times higher than those previously reported for occupationally exposed populations. Levels of higher brominated diphenyl ethers in the serum were significantly higher than those of the deca formulation, suggesting that BDE-209 could metabolically debrominate into octa- and nona-BDEs. The results of this study further suggest that higher brominated diphenyl ethers could be oxidatively metabolized into OH-PBDEs and accumulate in human blood serum. Thuresson (*44*) also found similar peak pattern of three identified OH-PBDEs in this study when they performed an investigation on occupational exposure to polybrominated diphenyl ethers. Because low brominated OH-PBDEs have also been detected in abiotic environmental media (*16*), further investigations are needed to determine whether or not higher brominated OH-PBDEs also occur in the environmental media in this region. If so, this could act as another source of human exposure.

It was notable that the hydroxyl group in all of the identified OH-PBDEs in this study was substituted in an *ortho* position. Results from laboratory animal studies using PBDEs showed that OH-PBDE metabolites were dominated by hydroxyl groups in the *meta* or *para* positions, whereas the *ortho* position originated from both metabolic and natural formation (*6, 7, 9*). The OH-PBDEs in the human blood samples differed from those detected in rat experiments. 6-OH-BDE47 and 6-OH-BDE99 were the most abundant metabolites in blood samples from pregnant women and newborn babies in United States (*15*), and 6-OH-BDE47 was also detected at comparatively high concentrations in blood from children at a municipal waste disposal site in Managua, Nicaragua (*14*). Qiu et al. (*15*) suggested that the differences could be explained by differences in cytochrome P450 enzyme expression between humans and mice. The P450 superfamily includes many subfamilies, based on amino acid sequence identities, and each P450 subfamily exhibits different selectivity in oxidation of the halogenated phenyl ring.

To summarize, the results of this study showed that higher brominated diphenyl ethers could be oxidatively metabolized into OH-octaBDE and OH-nonaBDE in human serum, following continuous long-term exposure to high concentrations of BDE-209, as seen in e-waste dismantling workers in Guiyu town, South China. However, further studies of the oxidative metabolism of BDE-209 in humans and biota are required, as more than 10 OH-PBDEs in human serum were unable to be identified because of a lack of authentic OH-PBDE standards. This presents an obvious obstacle to understanding the metabolic mechanisms of BDE-209 in humans and biota. Further efforts are needed to synthesize higher brominated OH-PBDEs to aid further identification. The toxicity and potential biological effects of higher brominated OH-PBDEs also need to be urgently investigated.

Acknowledgments

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Supporting Information Available

Details of the reaction conditions for each step and nuclear magnetic resonance (NMR) and mass spectra data for the intermediate products and final OH-PBDEs. This material is available free of charge via the Internet at http://pubs.acs.org.

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