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Validation of a novel *in vitro* assay using ultra performance liquid chromatography–mass spectrometry (UPLC/MS) to detect and quantify hydroxylated metabolites of BDE-99 in rat liver microsomes

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ABSTRACT

The purpose of this study was to develop and validate an ultra performance liquid chromatography–mass spectrometry (UPLC/MS) method to investigate the hepatic oxidative metabolism of 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), a widely used flame retardant and ubiquitous environmental contaminant. Hydroxylated metabolites were extracted using liquid-to-liquid extraction, resolved on a C_{18} column with gradient elution and detected by mass spectrometry in single ion recording mode using electrospray negative ionization. The assay was validated for linearity, accuracy, precision, limit of quantification, range and recovery. Calibration curves were linear ($R^2 \geq 0.98$) over a concentration range of 0.010–1.0 μM for 4-OH-2,2′,3,4′,5-pentabromodiphenyl ether (4-OH-BDE-90), 5′-OH-2,2′,4,4′,5-pentabromodiphenyl ether (5′-OH-BDE-99) and 6′-OH-2,2′,4,4′,5-pentabromodiphenyl ether (6′-OH-BDE-99), and a concentration range of 0.0625–12.5 μM for 2,4,5-tribromophenol (2,4,5-TBP). Inter- and intra-day accuracy values ranged from −2.0% to 6.0% and from −7.7% to 7.3%, respectively, and inter- and intra-day precision values ranged from 2.0% to 8.5% and from 2.2% to 8.6% ($n = 6$), respectively. The limits of quantification were 0.010 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and 0.0625 μM for 2,4,5-TBP. Recovery values ranged between 85 and 100% for the four analytes. The validated analytical method was applied to identify and quantify hydroxy BDE-99 metabolites formed *in vitro*. Incubation of BDE-99 with rat liver microsomes yielded 4-OH-BDE-90 and 6′-OH-BDE-99 as major metabolites and 5′-OH-BDE-99 and 2,4,5-TBP as minor metabolites. To our knowledge, this is the first validated UPLC/MS method to quantify hydroxylated metabolites of PBDEs without the need of derivatization.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are man-made chemicals that have been used as flame retardants on a variety of consumer products since the 1970s. PBDEs are highly susceptible to release during use, disposal, and recycling of PBDE-containing products. As a result, PBDEs have become globally distributed in the environment [1]. PBDEs are lipophilic, relatively persistent and bioaccumulate in humans and wildlife species. A marked increase in total PBDE concentrations has been observed in human and wildlife tissues over the last three decades [2] and is of concern because of the potential toxicity of PBDEs.

2,2′,4,4′,5-Pentabromodiphenyl ether (BDE-99) is the major congener in the widely used commercial penta-brominated diphenyl ether (penta-BDE) mixture that was added to polyurethane foams, textiles, upholstery, epoxy resins and paints [2]. Although the penta-BDE mixture was banned by the European Union and its manufacture was discontinued in the United States in 2004 [3], BDE-99 is a dominant congener in biotic and abiotic matrices [2,4]. In vertebrates, metabolism and excretion are the primary routes of elimination of BDE-99 and other PBDEs. Hydroxy-BDEs (OH-BDEs) have been identified in samples from wildlife and humans [5–7], but few studies have examined the hepatic biotransformation of BDE-99. In studies involving mice and rats, BDE-99 was shown to be oxidatively metabolized to a number of hydroxy metabolites, few of which were structurally characterized [8–10]. Incubation of BDE-99 with human hepatocytes resulted in the formation of 2,4,5-tribromophenol (2,4,5-TBP),

Abbreviations: 2,4,5-TBP, 2,4,5-tribromophenol; 2′-OH-BDE-28, 2′-OH-2,4,4′-tribromodiphenyl ether; 4-OH-BDE-90, 4-OH-2,2′,3,4′,5-pentabromodiphenyl ether; 4-OH-CB-121, 4-OH-2′,3,4′,5,6′-pentachlorobiphenyl; 5′-OH-BDE-99, 5′-OH-2,2′,4,4′,5-pentabromodiphenyl ether; 6-OH-BDE-47, 6-OH-2,2′,4,4′-tetrabromodiphenyl ether; 6′-OH-BDE-99, 6′-OH-2,2′,4,4′,5-pentabromodiphenyl ether; BDE-99, 2,2′,4,4′,5-pentabromodiphenyl ether; OH-BDEs, hydroxy-polybrominated diphenyl ethers; PBDEs, polybrominated diphenyl ethers.

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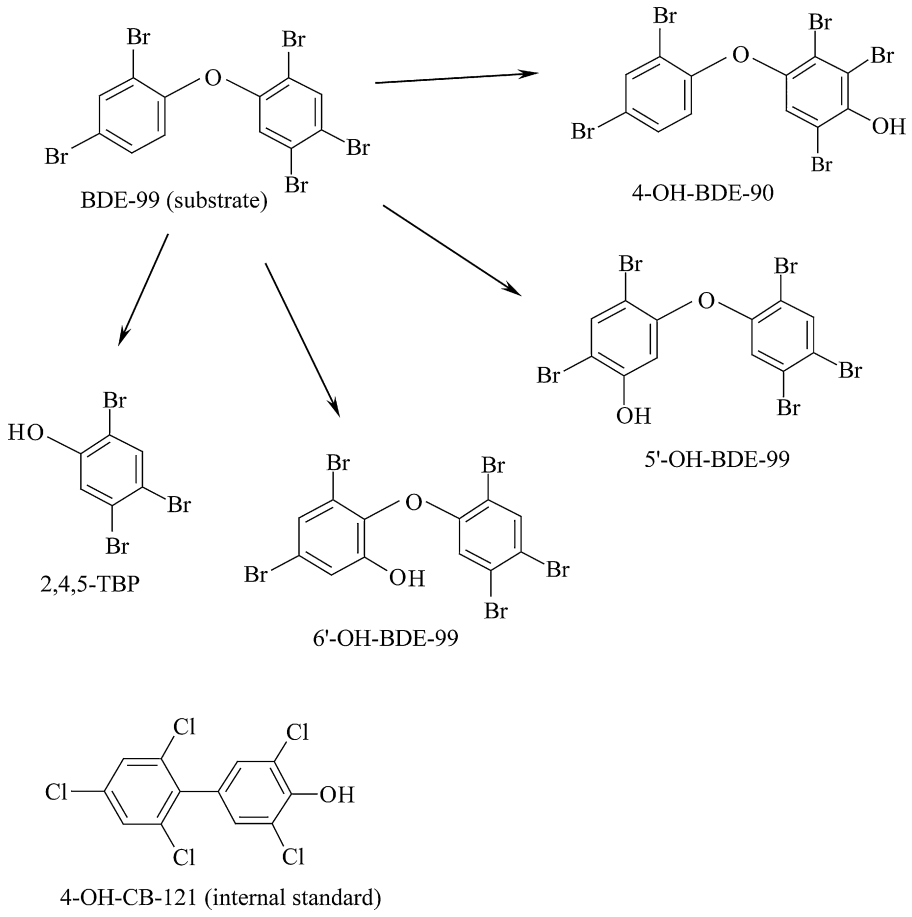

Fig. 1. Chemical structures of BDE-99 and four possible hydroxy metabolites (4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, and 2,4,5-TBP). The structure of the internal standard (4-OH-CB-121) is also shown.

5′-OH-2,2′,4,4′,5-pentabromodiphenyl ether (5′-OH-BDE-99) and an unknown OH-penta-BDE [11].

Gas chromatography–mass spectrometry (GC/MS) or GC-electron capture detector (GC/ECD) methods have been used to monitor tissue levels of PBDEs and OH-BDEs in humans and wildlife [5–7] and to investigate the formation of hydroxy metabolites of PBDEs *in vivo* [10,12,13] and *in vitro* [11]. GC/MS and GC/ECD methods exhibit high sensitivity for PBDEs and OH-BDEs. However, analysis of OH-BDEs by GC-based methods requires derivatization, which involves extensive sample preparation, use of harmful derivatizing agents such as diazomethane, and the possibility of introducing errors in the quantification of OH-BDEs due to incomplete derivatization. Liquid chromatography–mass spectrometry (LC/MS) is a sensitive analytical technique that is widely used for the separation and quantification of hydroxy metabolites of diverse xenobiotic compounds. Hydroxy metabolites can be efficiently ionized by the ionization techniques associated with LC/MS systems and thus do not require derivatization. A recent study reported the development of a LC/MS method for the analysis of OH-tri- and OH-tetra-BDEs but not of OH-penta-BDEs in biological and abiotic matrices [14]. The method was not validated and only partially characterized and there is no evidence that it can be applied to *in vitro* metabolism studies. A validated assay involving sample preparation and a sensitive analytical method designed for the investigation of the oxidative metabolism of PBDE congeners *in vitro* is not presently available.

The purpose of the present study was to develop and validate an ultra-performance LC/MS (UPLC/MS)-based *in vitro* assay for the quantification of hydroxy metabolites of BDE-99. BDE-99 can be oxidatively biotransformed into several hydroxy metabolites, but standards for only four metabolites are commercially available. Using 4-OH-2,2′,3,4′,5-pentabromodiphenyl ether (4-OH-BDE-90), 5′-OH-BDE-99, 6′-OH-2,2′,4,4′,5-pentabromodiphenyl ether (6′-OH-BDE-99), and 2,4,5-TBP (Fig. 1), a sensitive and selective analytical method without derivatization was developed, validated, and applied to investigate the oxidative biotransformation of BDE-99 by rat liver microsomes.

2. Experimental

2.1. Chemicals

The following standards, 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, 2,4,5-TBP and 4-OH-2′,3,4′,5,6′-pentachlorobiphenyl (4-OH-CB-121) (10.0 μg/mL in acetonitrile, 99% grade purity or higher), were purchased from AccuStandard (New Haven, Connecticut, US). BDE-99 (neat, 97.7% purity) was obtained from Chiron (Trondheim, Norway). Magnesium chloride, sucrose, formic acid, nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Methanol, methyl tert-butyl ether, hexane, isopropanol, sodium hydroxide, hydrochloric acid, and mono- and di-basic potassium phosphate were obtained from Fisher Scientific (Ottawa, Ontario, Canada). Hydrochloric acid and all organic solvents were of HPLC-grade or higher. Ultra pure water was prepared in our laboratory using a Milli-Q Synthesis system (Millipore, Billerica, MA, US).

2.2. Animal treatment and preparation of hepatic microsomes

Adult male Long Evan rats (body weight range: 160–190 g) were purchased from Charles River Laboratories (Montreal, Que-

bec, Canada). Rats were housed as previously described [15] and cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. Liver microsomes were prepared from pooled liver homogenates as reported previously [16] and aliquots were stored at −80 °C. Protein concentration was measured by the method of Lowry et al. [17] using bovine serum albumin as a standard.

2.3. Preparation of stock solutions

A stock solution of metabolite standards containing 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99 (at 1.25 μM), and 2,4,5-TBP (at 15.62 μM) was prepared in methanol and stored in an amber vial (final volume was 5.0 mL). A second stock solution was prepared by diluting an aliquot of the first stock solution 10 fold using methanol. After preparation, the vials were capped, vortex-mixed vigorously for 1 min, and stored at −20 °C. A separate stock solution of the internal standard (IS, 4-OH-CB-121, 75.0 μM) was prepared in methanol and stored at −20 °C. Two solutions were prepared for system suitability tests. The first solution (SST1) contained 2,4,5-TBP (0.250 μM), 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 (0.025 μM) and IS (3.0 μM) in methanol. The second solution (SST2) contained 2,4,5-TBP (5.0 μM), 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 (0.500 μM) and IS (3.0 μM) in methanol. For SST1 and SST2, aliquots of 100 μL each were stored in HPLC vials at −20 °C. Each aliquot was removed from storage before use and was used once.

2.4. Preparation of calibration standard and quality control samples

Calibration standard (CS) and two quality control (QC) samples were prepared by mixing 0.50 mg of rat hepatic microsomal protein, 50 mM phosphate buffer (50 mM KPO_4, 3 mM $MgCl_2$, pH 7.4) and an appropriate volume of one of the two stock solutions prepared in a final volume of 1.0 mL. Final metabolite concentrations in the CS samples were 0.010, 0.050, 0.100, 0.250, and 1.0 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and 0.0625, 0.125, 0.500, 1.25, 2.50, and 12.50 μM for 2,4,5-TBP. Final metabolite concentrations in the QC-Low sample were 0.025 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and 0.250 μM for 2,4,5-TBP (QC-Low). Final metabolite concentrations in the QC-High sample were 0.500 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and 5.0 μM for 2,4,5-TBP (QC-High). CS and QC samples were prepared on ice.

2.5. Sample preparation

CS and QC samples and blank samples, which contained only rat hepatic microsomes and phosphate buffer, were extracted using a liquid-to-liquid extraction technique. Tubes were incubated at 37 °C for 15 min in a shaking water bath. After incubation, 1.0 mL of ice-cold 0.5 M sodium hydroxide was added to each tube, and tubes were capped and inverted twice to mix the contents. IS was added to each tube (10.0 μL of 75.0 μM stock solution, final concentration of 3.0 μM), the tubes were vortex-mixed vigorously for 30 s and then heated in a water bath at 70 °C for 10 min. After cooling to room temperature, 2.0 mL of 6.0 M HCl were added to each tube followed by 1.0 mL of isopropanol. The tubes were vortex-mixed vigorously for 1 min and 2.0 mL of a mixture of methyl-*tert*-butyl ether:hexane (1:1, v/v) was then added to each tube. Tubes were vortex-mixed vigorously for 1 min and spun in a centrifuge at 2500 rpm for 5 min. The top organic layer was transferred to a clean set of tubes and the extraction procedure was repeated two more times. The organic phases from each extraction of the same sample were pooled and dried under a gentle flow of nitrogen. The residue was reconstituted in 250 μL of methanol, vortex-mixed vigorously for 10 s, and filtered through a 0.45 μm polytetrafluoroethylene membrane into a 300 μL HPLC vial.

2.6. UPLC/MS conditions

A UPLC/MS system was used to detect and quantify the analytes of interest and the IS. The UPLC/MS consisted of a Waters Acquity UPLC Sample Manager and a Waters Acquity UPLC Binary Solvent Manger connected to a Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with a combined Electrospray (ES) and Atmospheric Pressure Chemical Ionization (APCI) probe (Waters, Milford, MA, USA). Chromatographic separation was achieved with a Waters Acquity UPLC BEH C_{18} (2.1 mm × 100 mm, 1.7 μm) column, which was maintained at 50 °C. The autosampler tray temperature was 4 °C and the injection volume was 5.0 μL. The mobile phase was composed of solvent A (water containing 0.1% formic acid) and solvent B (methanol containing 0.1% formic acid). Solvents were filtered through 0.22 μm filters (Millipore Durapore Membrane Filters, 0.22 μm GV, Billerica, US). A gradient was used to resolve the hydroxy metabolites. Gradient elution was as follows: solvent A:solvent B (35:65, v/v) from 0 to 7 min at a flow rate of 0.2 mL/min, followed by a linear increase to solvent A:solvent B (15:85, v/v) from 7 to 27 min at a flow rate of 0.2 mL/min. At 27.1 min, solvent B was increased to 100% and flow rate was increased to 0.3 mL/min and maintained for 2 min. The column was then re-equilibrated with solvent A:solvent B (35:65, v/v) for 3 min at a flow rate of 0.3 mL/min. The total analysis time was 32 min.

The mass spectrometer was operated in negative electrospray ionization mode (ESI−) using selected ion recording (SIR) at a capillary voltage of 3 kV, cone voltage of 40 V, source temperature of 120 °C, desolvation temperature of 400 °C, and desolvation gas flow of 1005 L/h. The analytes of interest were identified by comparison of their mass-to-charge ratio (m/z) and retention time values with those of authentic standards: m/z 328.7 for 2,4,5-TBP, m/z 578.5 for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and m/z 340.8 for 4-OH-CB-121. MassLynx v. 4.1 software was used to control the UPLC/MS system.

2.7. Assay validation

The assay was validated for selectivity, limit of quantification (LOQ), accuracy, precision, linearity, range, and recovery. The performance of the UPLC/MS system was monitored using SST1 and SST2 samples. Each day, an aliquot of SST1 and one of SST2 was removed from storage, thawed, and injected as the first two samples. Chromatographic parameters such as relative retention time (RRT), capacity factor (k'), and resolution (R) were monitored for each analyte in both SST1 and SST2 aliquots. Values of these chromatographic parameters were calculated as reported by Rely [18]. Acceptance criteria were RRT = 0.26 ± 0.05, 0.90 ± 0.05, and 0.97 ± 0.05 for 2,4,5-TBP, 4-OH-BDE-90, and 5′-OH-BDE-99, respectively; k' values higher than 5 for all the analytes; and an R value higher than 2 between 5′-OH-BDE-99 and 6′-OH-BDE-99 peaks.

Selectivity was assessed by comparing the chromatograms obtained from blank and spiked rat liver microsome samples for the presence of interfering peaks with m/z and retention time values overlapping those of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, 2,4,5-TBP, and 4-OH-CB-121. Selectivity was determined at the LOQ values of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and 2,4,5-TBP, and at 3.0 μM for 4-OH-CB-121.

The LOQ of each analyte was assessed by preparing a calibration curve and five replicates of the three lowest CS samples. LOQ was determined as the lowest CS that met the following acceptance criteria: signal/noise (S/N) ratio at least 5 times the blank

response, accuracy (percent deviation, %Dev) within ± 20% of the nominal concentration, and precision (percent relative standard deviation, %RSD) not exceeding 20%. The S/N ratio was determined using MassLynx v. 4.1 software using the peak-to-peak method.

Accuracy was calculated as %Dev between the mean measured concentration ($n = 6$) and the nominal concentration. Precision was expressed as %RSD. Accuracy and precision values were determined in QC-Low and QC-High samples. Freshly prepared QC-Low and QC-High samples in six replicates were analyzed on the same day to determine the intra-day accuracy and precision values, and on six consecutive days to determine the inter-day accuracy and precision values. Acceptance criteria were as follows: intra- and inter-day %Dev within ± 15% of the nominal concentration and %RSD not exceeding 15%.

Linearity of the calibration curves was assessed using the coefficient of determination (R^2) values and the reproducibility (%RSD) of the mean slope values ($n = 6$). Calibration curves were constructed by plotting the analyte to internal standard peak area ratios (*Y*-axis) against the corresponding analyte nominal concentrations (μM, *X*-axis). Calibration curves were constructed using linear regression analyses with the weighting factor of $1/X^2$ to improve accuracy in the lower concentration range of the curve. Acceptance criteria were mean $R^2 > 0.95$ and %RSD of the mean slope <15%.

The range was defined as the linear section of the calibration curve where the CS samples were determined accurately and precisely. Acceptance criteria were $R^2 > 0.95$ for each calibration curve, %Dev within ± 15% of the nominal concentration, and %RSD not exceeding 15%.

Recovery was determined in QC-Low and QC-High samples. Recovery was calculated by comparing the peak area of spiked microsomal samples with that of unextracted standards (i.e. SST1 and SST2) at the same corresponding nominal concentrations. For each analyte, recovery was determined at two concentrations per day for six consecutive days. Acceptance criterion was a mean recovery between 80% and 120%.

The dataset generated was tested for presence of outliers using Dixon's Q test with a 95% confidence interval ($\alpha = 0.05$). We intended to exclude outliers from the dataset. However, no outliers were found.

2.8. Method application

Rates of formation of hydroxy BDE-99 metabolites in rat liver microsomes were determined using reaction mixtures, which contained 50 mM phosphate buffer, 0–1.0 mg of hepatic microsomal protein, and 50 μM BDE-99 (0.02 mL of a 2.50 mM solution in methanol) in a final volume of 0.99 mL. After pre-incubation in a shaking-water bath at 37 °C for 5 min, reactions were initiated by addition of 0.01 mL of 100 mM NADPH solution (1.0 mM final concentration) to each tube. Reactions were stopped after 0–30 min by addition of 1.0 mL of ice-cold 0.5 M sodium hydroxide. Blank samples, which contained only rat hepatic microsomes and phosphate buffer, negative control samples (devoid of substrate, NADPH, or microsomes), and CS and QC samples were routinely included in each assay. Extraction and quantification of the metabolites were performed as described in Sections 2.5 and 2.6. Quality assurance was assessed by determining the calibration curve linearity (R^2 values) using CS samples and by determining accuracy, precision, and recovery using QC-Low and QC-High samples. The performance of the UPLC/MS system was assessed using SST1 and SST2 samples. Samples were prepared in duplicate for each assay and experiments were performed three or more times on separate days.

3. Results and discussion

3.1. Optimization of UPLC/MS parameters

Two ionization techniques, APCI and ES, were compared for sensitivity of detection of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and 2,4,5-TBP with the MS set in SIR mode. ES operated in negative mode (ES-) produced a higher signal for the hydroxylated metabolites than APCI and BDE-99 could not be ionized by either ionization technique. Flow injection analysis with individual standards (0.500 μM in methanol) was used to determine the molecular ions $[M\text{-}H]^-$ and optimal cone voltage values. The most intense signal for 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and 2,4,5-TBP was obtained at 40 V. Source temperature, desolvation temperature, and desolvation gas flow values were also optimized (values reported in Section 2.6). To determine if the sensitivity of the analytical method could be improved, multiple reaction monitoring (MRM) was assessed starting with the SIR conditions. Product ion scans of the molecular ions of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and 2,4,5-TBP were performed at different collision energy values. The main product ion was a bromine fragment (m/z 79 and m/z 81) with its highest intensity at a collision energy of 40 eV. The sensitivity of the MRM method was lower than that of the SIR method (results not shown) and thus, the SIR method was used for subsequent analyses.

Separation of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and 2,4,5-TBP was achieved using a Waters Acquity UPLC BEH C_{18} (2.1 mm × 100 mm, 1.7 μm) column. Mobile phase composition was optimized using mixtures of water and acetonitrile or water and methanol. A mixture of water and methanol containing 0.1% formic acid yielded the best peak shapes and the highest peak area counts and was selected for chromatographic separation. Formic acid (0.1%) was added to enhance ionization of the compounds analyzed. The best resolution of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and 2,4,5-TBP was achieved using isocratic elution with methanol:water (65:35, v/v) for the first 7 min followed by a linear gradient to 85% methanol:15% water (from 7 to 27 min) at a flow rate of 0.2 mL/min.

3.2. Choice of the internal standard

A hydroxylated pentachlorobiphenyl (i.e. 4-OH-CB-121) was chosen as the internal standard because (i) 4-OH-CB-121, 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99 have the same total number of halogens, including two ortho halogens, (ii) 4-OH-CB-121 has one hydroxy group and similar physicochemical properties as OH-penta-BDEs, and (iii) 4-OH-CB-121 is not present in Aroclor mixtures [19] and is not likely to be present in wildlife samples, or may be present only at trace levels [20,21]. Lastly, we preferred not to use an OH-penta- or OH-tetra-BDE to avoid potential interference with possible hydroxy metabolites of BDE-99 that may be formed in rat liver microsomes.

3.3. Optimization of sample preparation

To achieve efficient extraction of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, and 2,4,5-TBP from the biological matrix of interest (i.e. rat liver microsomes) and to minimize the possibility of spurious peaks that could interfere with the peaks of interest, various steps of the sample preparation protocol were optimized. Of the various organic solvents (acetone, dichloromethane, hexane) and mixtures tested, three extractions with methyl-tert-butyl ether:hexane (2 mL, 1:1, v/v) yielded the best recovery rates for 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, and 2,4,5-TBP (≥85%) and did not produce spurious interfering peak(s). Addition of extra centrifugation steps to separate microsomes from the supernatant

or use of methanol or acetone in place of sodium hydroxide to terminate the reaction in hepatic microsomes did not improve recovery of the analytes of interest.

3.4. Assay validation

3.4.1. System suitability and UPLC/MS performance

An aliquot of SST1 and one of SST2 were analyzed at the beginning of every batch analysis. The system suitability samples met the acceptance criteria for RRT, k', and R values with every batch analyzed and no batch had to be discarded (data not shown).

3.4.2. Selectivity and LOQ

Comparison of chromatograms obtained with blank and spiked rat liver microsomes showed no interfering peaks at the retention times of 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, and 2,4,5-TBP (Fig. 2). The LOQ concentration was 0.010 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and was 0.0625 μM for 2,4,5-TBP (Table 1). The LOQ value of the current method for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99 is similar to the LOQ values reported for the analysis of OH-tri-BDEs and OH-tetra-BDEs using LC/MS/MS and GC/MS methods [14,22].

3.4.3. Accuracy and precision

Inter-day and intra-day accuracy and precision values are reported in Table 2. The assay accuracy and precision values were within the acceptance criteria. Ranges of intra-day and inter-day precision values (−7.7 to 7.3% and −2.0 to 6.0%, respectively) are consistent with those obtained by Mas et al. [14] for the determination of OH-tri-BDEs and OH-tetra-BDEs. Precision values reported by Mas et al. [14] were determined at a single concentration (i.e. 75 pg/μL, approximately 0.150 μM for OH-tetra-BDEs) and using stock solutions of the metabolites. Our experimental design differed in that precision and accuracy were determined, two QC concentrations were used and the biological matrix of interest (i.e. rat liver microsomes) was included. Therefore, our experimental design allowed us to evaluate the accuracy, precision, and sensitivity of the assay when it is applied to unknown samples of rat liver microsomes.

3.4.4. Linearity and range

The assay was linear over a concentration range of 0.010–1.0 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and a concentration range of 0.0625–12.5 μM for 2,4,5-TBP (Table 3). Accuracy and precision values for CS samples met the acceptance criteria with the exception of two CS samples for 2,4,5-TBP, which resulted in slightly higher %Dev values (i.e. 20% vs 15%). As a result, the assay range was 0.010–1.0 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and 0.0625–12.5 μM for 2,4,5-TBP (Table 3).

3.4.5. Recovery

Large recovery values were consistently obtained across the assay range for 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, and 2,4,5-TBP (Table 4). The recovery values determined in the present study are greater than those obtained previously with similar biological matrices using GC/MS or GC/ECD analysis. For example, recovery values of >67% and approximately 65% were obtained for 2′-OH-2,4,4′-tribromodiphenyl ether (2′-OH-BDE-28) and 6-OH-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47), respectively, with rat liver microsome samples and a recovery value of 50 ± 7% was obtained for 6-OH-BDE-47 with human hepatocytes [11,22,23]. Furthermore, analysis of OH-BDEs in derivatized extracts of beluga whale liver and human milk samples using GC/MS methods resulted in lower (61 ± 13% for 2′-methoxy-BDE-28; [24]) or highly variable (57–102% for OH-tri- and OH-tetra-BDEs; [25]) recovery rates, respectively. The data suggest that the sample preparation protocol used in the present assay coupled with UPLC/MS analysis yields high and reproducible recoveries for 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99 and 2,4,5-TBP.

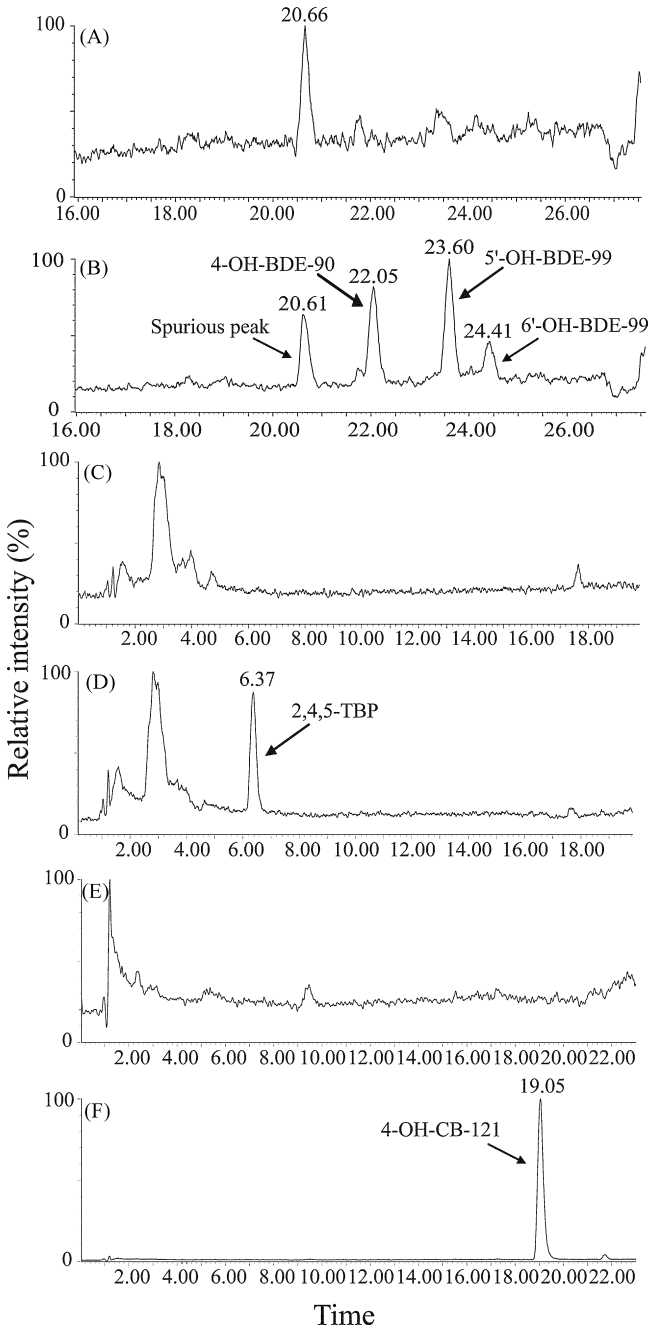

Fig. 2. Representative chromatograms of blank samples (A,C,E) and rat liver microsomes (B,D,F) spiked with 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99 (A,B), 2,4,5-TBP (C,D) and IS (E,F) at the LOQ values: 0.010 μM for 4-OH-BDE-90, 5′-OH-BDE-99 and 6′-OH-BDE-99, and 0.0625 μM for 2,4,5-TBP. IS was present at 3.0 μM.

3.5. Method application

The validated assay allowed us to investigate the oxidative metabolism of BDE-99 in liver microsomes from adult male rats. Four hydroxy metabolites were detected and identified by comparison with authentic standards. The four metabolites were 4-OH-BDE-90, 5′-OH-BDE-99, 6′-OH-BDE-99, and 2,4,5-TBP. No other metabolites or unidentified peaks were observed. Two hydroxy metabolites of BDE-99 (4-OH-BDE-90 and 6′-OH-BDE-99)

Table 1
Limits of quantification of hydroxy metabolites of BDE-99 using the proposed assay[a].

Metabolites	Concentration	Retention time (min)	Blank S/N	S/N[b]	%RSD	%Dev
2,4,5-TBP	0.0625 μM	6.4	≤3.0	38 ± 3.7	7.7	2.5
4-OH-BDE-90	0.010 μM	22.1	≤3.0	22 ± 3.8	14	4.8
5′-OH-BDE-99	0.010 μM	23.6	≤3.0	28 ± 4.3	8.0	7.8
6′-OH-BDE-99	0.010 μM	24.4	≤3.0	8.6 ± 2.1	11	1.8

[a] $n = 5$, 5 replicates per sample on the same day.
[b] Mean ± SD.

Table 2
Inter- and intra-day precision (%RSD) and accuracy (%Dev) values determined with QC-Low and QC-High samples.

Metabolites	QC samples	Nominal concentration (μM)	Measured concentration (μM)[c]	%RSD	%Dev
Inter-day[a]					
2,4,5-TBP	QC-Low	0.25	0.25 ± 0.013	5.0	0.67
	QC-High	5.0	4.9 ± 0.26	5.3	1.8
4-OH-BDE-90	QC-Low	0.025	0.027 ± 0.002	8.5	6.0
	QC-High	0.50	0.50 ± 0.025	5.0	0.57
5′-OH-BDE-99	QC-Low	0.025	0.025 ± 0.002	8.0	1.3
	QC-High	0.50	0.50 ± 0.010	2.0	0.47
6′-OH-BDE-99	QC-Low	0.025	0.025 ± 0.002	7.6	2.0
	QC-High	0.50	0.52 ± 0.020	3.9	3.5
Intra-day[b]					
2,4,5-TBP	QC-Low	0.25	0.24 ± 0.014	5.7	3.3
	QC-High	5.0	5.0 ± 0.19	3.8	−0.80
4-OH-BDE-90	QC-Low	0.025	0.027 ± 0.002	8.6	7.3
	QC-High	0.50	0.46 ± 0.010	2.2	7.7
5′-OH-BDE-99	QC-Low	0.025	0.027 ± 0.002	7.1	6.0
	QC-High	0.50	0.48 ± 0.014	3.0	3.5
6′-OH-BDE-99	QC-Low	0.025	0.025 ± 0.001	5.9	0.67
	QC-High	0.50	0.50 ± 0.029	5.7	0.83

[a] $n = 6$, 1 replicate per day for 6 days.
[b] $n = 6$, 6 replicates on the same day.
[c] Mean ± SD.

were detected and quantified after an incubation time of 5 min (Fig. 3A), suggesting that 4-OH-BDE-90 and 6′-OH-BDE-99 were major metabolites. Longer incubation times were needed to quantify 5′-OH-BDE-99 and 2,4,5-TBP, due to the lower formation rates of these two metabolites and the lower sensitivity of the assay for 2,4,5-TBP (0.0625 μM) than for OH-penta-BDEs (0.010 μM). The effect of varying protein concentration (0–1.0 mg/mL) on the rates of formation of hydroxy metabolites of BDE-99 was also investigated using a substrate concentration (BDE-99) of 50 μM and an incubation time of 10 min. Two hydroxy metabolites of BDE-99 (4-

Table 3
Calibration curve parameters: accuracy (%Dev), precision (%RSD), and linearity (slope, R^2) values determined with CS samples[a,b].

Metabolites	Nominal concentrations (μM)	Measured concentration (μM)[c]	%RSD	%Dev		Slope	R^2
2,4,5-TBP	0.063	0.075 ± 0.012	16	20	Mean	0.31	0.98
	0.125	0.15 ± 0.020	13	22	SD	0.021	
	0.50	0.60 ± 0.048	8.0	20	%RSD	6.5	
	1.25	1.4 ± 0.10	7.3	14			
	2.50	2.6 ± 0.090	3.3	4.6			
	12.5	11 ± 0.83	7.4	−11			
4-OH-BDE-90	0.010	0.010 ± 0.002	16	1.7	Mean	0.98	0.99
	0.050	0.050 ± 0.004	6.7	8.1	SD	0.13	
	0.10	0.11 ± 0.010	9.6	12	%RSD	13	
	0.25	0.27 ± 0.011	4.3	6.1			
	1.0	0.86 ± 0.092	11	−14			
5′-OH-BDE-99	0.010	0.010 ± 0.001	6.4	6.8	Mean	1.1	0.99
	0.050	0.051 ± 0.002	4.6	2.7	SD	0.11	
	0.10	0.110 ± 0.007	6.5	6.7	%RSD	10	
	0.25	0.26 ± 0.013	5.0	4.2			
	1.0	0.90 ± 0.090	10	−10			
6′-OH-BDE-99	0.010	0.010 ± 0.001	11	2.2	Mean	0.59	0.99
	0.050	0.050 ± 0.003	5.7	−1.2	SD	0.092	
	0.10	0.110 ± 0.009	8.7	8.6	%RSD	15	
	0.25	0.27 ± 0.014	5.3	6.4			
	1.0	0.96 ± 0.092	9.6	−4.4			

[a] $n = 6$, 1 replicate per day for 6 days.
[b] Y-intercept values were between -2.00×10^{-4} and 1.17×10^{-2}.
[c] Mean ± SD.

Table 4
Recovery values (mean ± SD) determined with QC-Low and QC-High samples[a].

Metabolites	QC-Low	QC-High
2,4,5-TBP	97 ± 16%	100 ± 7.5%
4-OH-BDE-90	94 ± 6.9%	97 ± 12%
5′-OH-BDE-99	97 ± 9.7%	100 ± 7.1%
6′-OH-BDE-99	85 ± 9.7%	85 ± 5.2%
Internal standard		
4-OH-CB-121	118 ± 12%	

[a] $n = 6$, 1 replicate per day for 6 days.

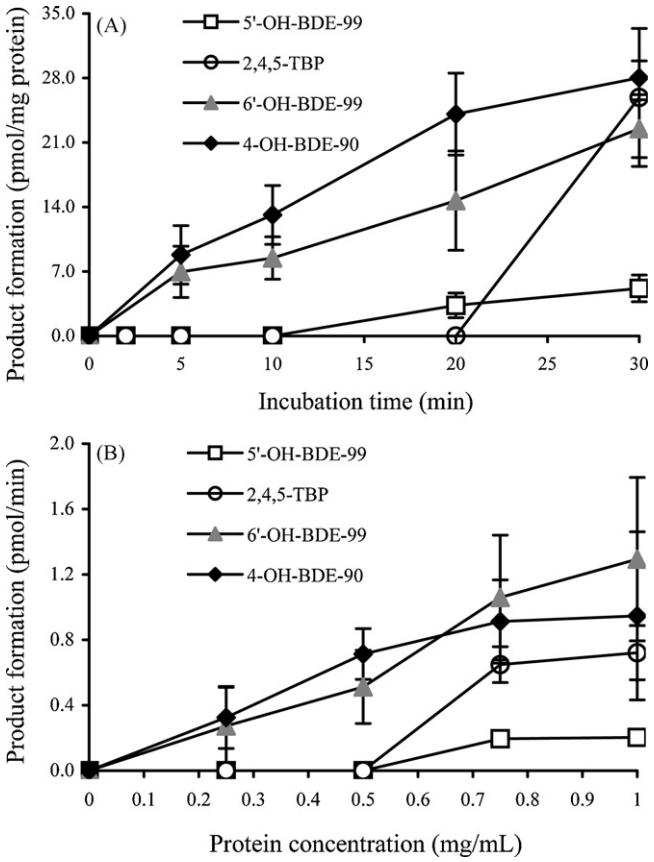

Fig. 3. Effect of incubation time (0–30 min, 0.5 mg protein/mL) and protein concentration (0–1.0 mg/mL, 10 min) on rates of formation of hydroxy metabolites of BDE-99 using liver microsomes prepared from adult male rats. Data points are the mean ± SD of three separate experiments.

OH-BDE-90 and 6′-OH-BDE-99) were detected and quantified at the lowest protein concentration tested (0.25 mg/mL, Fig. 3B). Higher protein concentrations were needed to quantify 5′-OH-BDE-99 and 2,4,5-TBP, confirming that these are minor hydroxy metabolites of BDE-99.

4. Conclusion

A novel, sensitive and selective UPLC/MS method was developed and validated to investigate the oxidative metabolism of BDE-99 in rat liver microsomes. The method allowed us to quantify formation of four hydroxy metabolites of BDE-99 without the need for derivatizing the metabolites as required using GC-based assays [5–7,10–13,25]. Our assay also represents an improvement compared to a previous LC/MS method [14] because analytes were spiked into the matrix of interest rather than determined in stock solutions, LOQ and precision were assessed more thoroughly, and additional validation criteria such as accuracy and selectivity were included. The UPLC/MS method was successfully applied to identify and quantify formation of hydroxy metabolites of BDE-99 by rat liver microsomes in a time- and protein-dependent manner rather than monitoring depletion of BDE-99 [22]. The experimental conditions used, which involved a relatively small amount of liver microsomal protein and short incubation times, suggest that our method is more metabolically responsive compared to previous *in vitro* investigations of BDE-99 metabolism [26]. The UPLC/MS method will now be applied to further characterize the oxidative metabolism of BDE-99 by determining the kinetic parameters of hydroxy metabolite formation and identifying the cytochrome P450 enzymes involved. Liver microsome samples from rats, human and wildlife species will be used to elucidate the role of oxidative metabolism of BDE-99 as a determinant of its bioaccumulation.

Conflict of interest

All the authors declare no actual or potential conflict of interest with people or organizations involved in the present study.

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