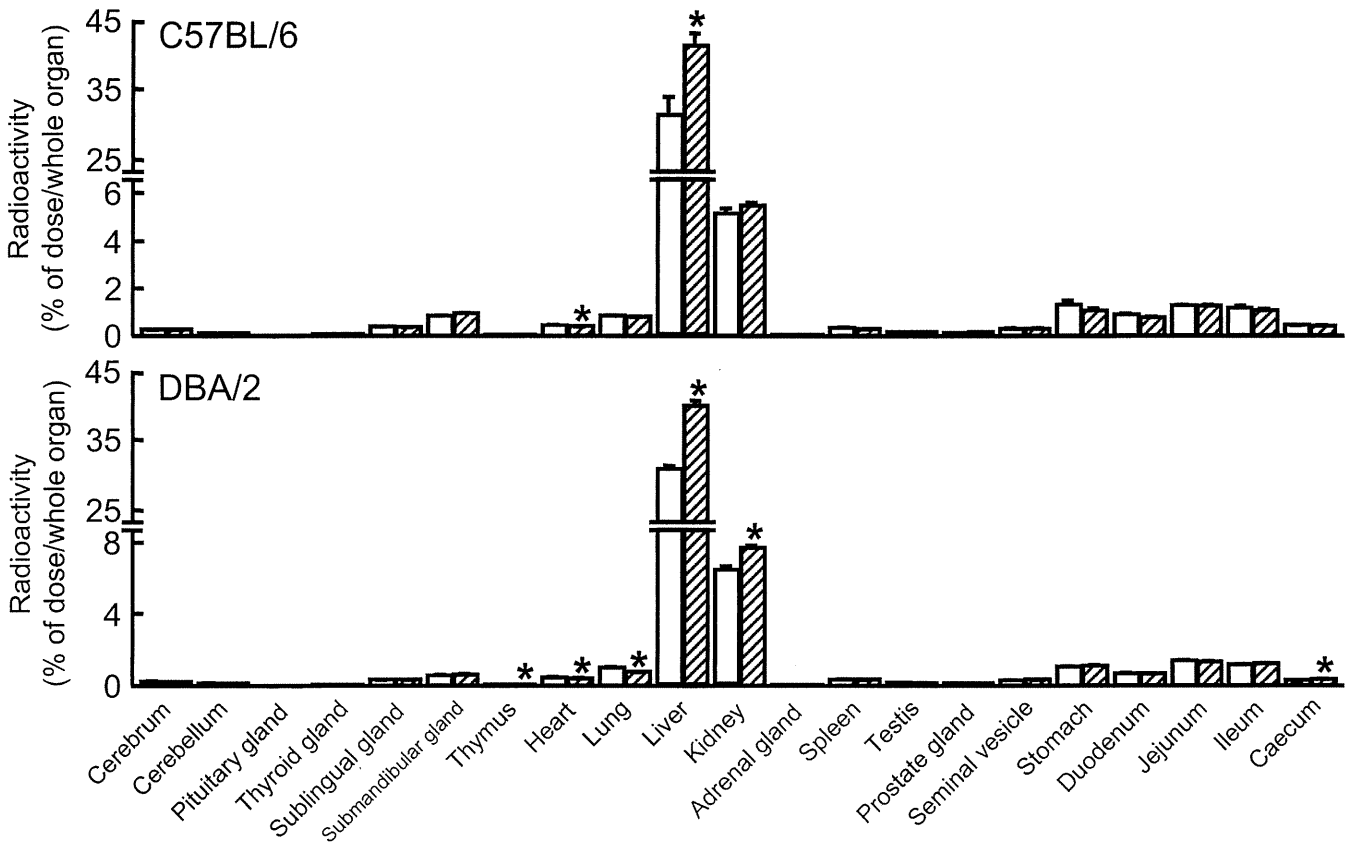

FIG. 11. Tissue distribution of [^{125}I]T_4 after administration of [^{125}I]T_4 to 4-OH-CB187-pretreated mice. Experimental protocols were the same as those described in the legend of Fig. 10. Each column represents the mean ± S.E. (vertical bars) for five to seven animals. *, $P < 0.05$, significantly different from each control. Open bars, control; hatched bars, 4-OH-CB187.

As another possible mechanism for the 4-OH-CB187-induced decrease in level of serum total T_4, TTR-associated pathway might be considered because PCB and its hydroxylated metabolites act as T_4 antagonists to TTR (Lans et al., 1993; Brouwer et al., 1998; Meerts et al., 2002; Kato et al., 2004) and because serum TTR level is closely correlated with serum thyroid hormone level (Episkopou et al., 1993). Accordingly, competitive inhibition of a T_4-TTR complex formation by 4-OH-CB187 is considered to promote a decrease in the level of serum total T_4. In the present study, both the decrease in the level of [^{125}I]T_4 bound to serum TTR and the increase in the level of [^{125}I]T_4 bound to serum albumin and TBG were confirmed in 4-OH-CB187-pretreated mice. Furthermore, 4-OH-CB187-mediated decrease in serum total T_4 and free T_4 levels occurred in wild-type and TTR-heterozygous mice but not in TTR-deficient mice. These findings indicate that 4-OH-CB187 inhibits formation of serum T_4-TTR complex and further suggest that 4-OH-CB187-induced inhibition of the T_4-TTR complex formation might lead to change in tissue distribution of T_4.

Because distribution levels of [^{125}I]T_4 to plasma and tissues after a [^{125}I]T_4 treatment are not significantly changed up to 48 h later (Oppenheimer et al., 1968), distribution levels of [^{125}I]T_4 in several tissues were examined 5 min after the [^{125}I]T_4 administration to 4-OH-CB187-pretreated mice. The results indicated that the mean total body clearance of [^{125}I]T_4 and the distribution volume of [^{125}I]T_4 to tissues were increased by 4-OH-CB187 pretreatment in both C57BL/6 and DBA/2 mice. A tissue-to-serum concentration ratio (K_p value) also increased in several tissues, especially the liver, in the 4-OH-CB187-pretreated C57BL/6 and DBA/2 mice compared with the corresponding control mice. In addition, more than 40% of the

TABLE 2

Accumulation of [^{125}I]T_4 in the 4-OH-CB187-pretreated mice livers

The radioactivity in the liver was measured at 5 min after the [^{125}I]T_4 administration. The values shown are expressed as the mean ± S.E. for five to seven mice.

Animal	[^{125}I]T_4	
	Control	4-OH-CB187
	% of dose/g liver	*% of dose/g liver*
C57BL/6	27.13 ± 1.06	35.14 ± 1.19*
DBA/2	25.25 ± 1.02	31.29 ± 1.25*

* $P < 0.01$, significantly different from each control.

TABLE 3

Liver weights after the administration of 4-OH-CB187 to mice

Animals were killed 4 days after the administration of 4-OH-CB187 (1.0 mg/kg), and the liver weight was measured. The values shown are expressed as the mean ± S.E. for five to eight animals.

Animal	Liver Weight	
	Control	4-OH-CB187
	% of body weight	*% of body weight*
C57BL/6	4.63 ± 0.29	4.71 ± 0.09
DBA/2	4.90 ± 0.17	4.92 ± 0.12

* $P < 0.001$, significantly different from each control.

[^{125}I]T_4 dosed was accumulated in the liver in 4-OH-CB187-pretreated mice.

In conclusion, the present findings show that 4-OH-CB187 possesses the ability to reduce serum thyroid hormone level in mice and further indicate that the 4-OH-CB187-mediated decrease occurs mainly through increase in accumulation (transportation from serum to liver) of T_4 in the liver. Furthermore, the present findings strongly suggest that the increased accumulation in the liver would be attributed to the 4-OH-CB187-mediated inhibition of a T_4-TTR complex formation in serum.

Acknowledgments. We thank Professor Shuichiro Maeda of the University of Yamanashi (Japan) for providing male TTR(+/−) and female TTR(−/−) mice.

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Anal. Chem. **2009**, *81*, 5942–5948

Simultaneous Determination by APCI-LC/MS/MS of Hydroxylated and Methoxylated Polybrominated Diphenyl Ethers Found in Marine Biota

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A method has been developed for the simultaneous analysis of hydroxylated and methoxylated analogs of tetrabromodiphenyl ethers (OH-tetraBDEs and MeO-tetraBDEs) and of hydroxylated and methoxylated analogs of tetrabromobiphenyl (diOH-tetraBB and diMeO-tetraBB) using high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (APCI-LC/MS/MS) in negative ion mode. Chromatographic separation was performed on a 150 mm ODS column with acetonitrile:water (9:1, v/v) in mobile phase. Multiple reaction monitoring (MRM) was performed using the precursor $[M-H]^-$ ion for hydroxylated analogs, and the $[M-Br+O]^-$ ion for tetraBDEs and tetraBB, and their methoxylated analogs. The method was validated using cod liver oil samples spiked with nine analytes (100 ng/g) for linearity ($r^2 > 0.998$), recovery (75−95%), repeatability (8−36% RSD), and sensitivity (limits of quantification (LOQ), 0.1−0.25 ng/g lipid for phenolic analytes and 6−80 ng/g lipid for neutral brominated compounds). The APCI-LC/MS/MS was applied to analyze tiger shark and bull shark liver samples, where their concentrations were up to 8 ng/g (lipid weight) for OH-BDEs, whereas they were up to 540 ng/g (lipid weight) for MeO-BDEs. The results were consistent with values determined by electron ionization (EI)-GC/MS. The first detection of 2,2′-dihydroxy-3,3′,5,5′-tetrabromobiphenyl (2,2′-diOH-BB80) by this method was in marine sponge from Micronesia. The advantage of the LC/MS/MS method over GC/MS is that it provides rapid and simultaneous determination of OH-BDEs, MeO-BDEs, and their related analogs with a single preparation step and without the involvement of chemical derivatives. Although the method provides the different LOQ ranges between hydroxylated and neutral brominated analogs, future work could apply the method to the full range of PBDE-like contaminants present in the environment and in biota tissues.

Polybrominated diphenyl ethers (PBDEs) are brominated flame retardants (BFRs) in a variety of consumer products and are widely found in the Arctic[1] as well as in household dust.[2] Recently, hydroxylated (OH-) and methoxylated (MeO-) tetrabromodiphenyl ethers (tetraBDEs) have been isolated in marine sponges,[3,4] blue mussels, and red alga.[5,6] Naturally occurring components of primary interest are 6-methoxy-2,2′,4,4′-teterabromodiphenyl ether (6-MeO-BDE47), 2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE68), 2,2′-dimethoxy-3,3′,5,5′-tetrabromobiphenyl (2,2′-diMeO-BB80), and their corresponding hydroxylated analogs. These organobromines are persistent and lipophilic and have been shown to bioaccumulate in marine biota such as fish,[7−9] mammals,[10−13] and also humans[14−16] via the food chain. Although the OH- and MeO-brominated analogs have been

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10.1021/ac9009885 CCC: $40.75
Published on Web 06/16/2009

reported to pose significant health risks such as thyroid disruptions[17–19] and cytotoxicity,[20] little is known about the source and biotransformation process of these naturally produced compounds in the environment.

So far, OH-PBDEs have been measured as their methoxylated derivatives by GC/MS using electron ionization (EI) or electron capture negative ionization (ECNI) after derivatization by diazomethane.[21–23] The derivatized OH-BDEs were then determined separately from native MeO-BDEs and PBDEs. However, the GC analysis of these phenolic compounds always required (1) a time-consuming derivatization step prior to injection, (2) careful handling of harmful/carcinogenic reactant (i.e., diazomethane), and (3) possible quantification errors since the derivatization reaction may not give quantitative results for all OH-PBDE congeners. For this reason, a method using liquid chromatography coupled to electrospray ionization tandem mass spectrometry (ESI-LC/MS/MS) has been developed. Using multiple reaction monitoring (MRM) in negative ion mode for 2′-hydroxy-2,4,4′-tribromodiphenyl ether (2′-OH-BDE28), OH-tri-BDEs were detected at the low ppb range in waste and surface waters.[24] Mas et al.[25] have also proposed an ISP-LC/MS/MS method for eight OH-PBDEs, which proved to be an efficient, robust, sensitive, and selective tool. For the other phenolic BFRs such as tetrabromobisphenol A (TBBPA), ESI-LC/MS/MS techniques have been developed[26] and applied to the analysis of sediment and sewage sludge.[27]

PBDEs and MeO-PBDEs have also been determined by GC/MS in selected ion monitoring (SIM) mode because of its high sensitivity and selectivity. As an alternate method, we previously developed an LC/MS/MS method using atmospheric pressure chemical ionization (APCI) for the determination of MeO-tetraBDEs and the related natural organohalogens that produced phenoxide $[M\text{-}Br+O]^-$ ions in negative ionization mode.[28] The optimized MRM transition enabled us to analyze MeO-tetraBDEs and halogenated bipyrroles found in marine mammals.[28] Recently, an atmospheric pressure photoionization (APPI)-LC/MS/MS method has been developed for the analysis of BFRs (PBDEs and TBBPA) and their degradation products[29,30] and applied to the analysis of eight PBDEs in housedust.[31] However, these anthropogenic and natural organobromines have been determined separately from hydroxylated analogs by GC/MS or LC/MS/MS methodologies, and, to our knowledge, no attempt has been made for the combined determination of both OH- and MeO-BDEs.

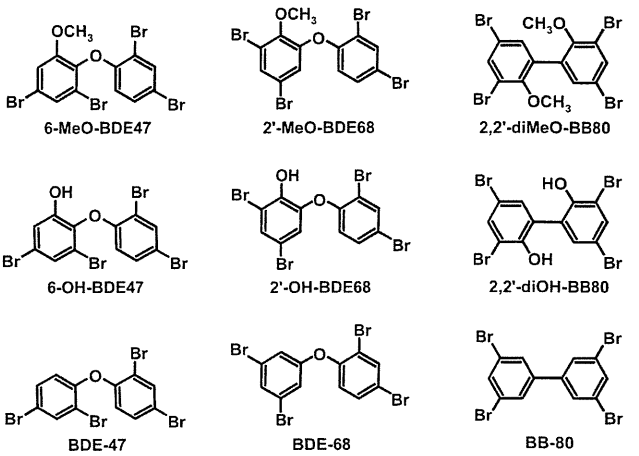

Figure 1. Chemical structures of the analyzed compounds.

The aim of the present study is to propose a simultaneous quantification method by APCI-LC/MS/MS for tetraBDEs and their relevant hydroxylated and methoxylated analogs in the environment (Figure 1). In the present study, quantification by the MRM transition in negative ion mode was validated using cod liver oil spiked at known concentration ranges of nine analytes. The method was applied for the detection of OH- and MeO-tetraBDEs in marine sponge from Palau Island, Micronesia, and for quantification in shark liver samples from Ishigaki Island, Japan. The results are compared with those obtained by GC/MS in electron ionization mode.

EXPERIMENTAL SECTION

Chemicals and Reagents. As standard materials, 2,2′-diMeO-BB80 and 4′-methoxy-2,3′,4,5′,6-pentabromodiphenyl ether (4′-MeO-BDE121) were synthesized by Dr. Göran Marsh of Stockholm University, Sweden.[12] 2,2′-diOH-3,3′,5,5′-Tetrabromobiphenyl (2,2′-diOH-BB80) was synthesized via demethylation of 2,2′-diMeO-BB80 using boron tribromide. 6-MeO-BDE47, [$^{13}C_{12}$] labeled 6-OH-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE47), and 2′-MeO-BDE68 were purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). The other native OH-tetraBDEs, MeO-tetraBDEs, 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 2,3′,4,5′-tetrabromodiphenyl ether (BDE-68), and 3,3′,5,5′-tetrabromobiphenyl (BB-80) were purchased from AccuStandard Inc. (New Haven, USA). LC/MS-grade acetonitrile (MERCK, Darmstadt, Germany) and distilled deionized water (Milli-Q reference ultrapure water purification system, Millipore Co., MA, UAS) were used as LC mobile phase solvents.

Sample Preparation. Samples of a marine sponge (Demospongeae, *Dysidea sp*) were collected from Nikko Bay in Republic of Palau, Micronesia 2005. Two species of sharks, tiger shark

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Table 1. Characterization of Target Compounds and MRM Parameters used for Quantitative Determination

compounds	chemical formula	monoisotopic mass	LC retention time (min)	major precursor ion	product ion	MRM transition (m/z)	DP(V)	CE(V)
6-OH-BDE47	$C_{12}H_6O_2Br_4$	497.7	6.7	$[M-H]^-$ $[M-C_6H_2(OH)Br_2]^-$	Br^-	500.5 → 78.9	−10	−42
2′-OH-BDE68	$C_{12}H_6O_2Br_4$	497.7	7.2	$[M-H]^-$ $[M-C_6H_2(OH)Br_2]^-$	Br^-	500.6 → 78.8	−20	−36
2,2′-diOH-BB80	$C_{12}H_6O_2Br_4$	497.7	5.2	$[M-H]^-$	Br^-	500.6 → 78.8	−60	−76
[$^{13}C_{12}$]6-OH-BDE47 (IS)	$C_{12}H_6O_2Br_4$	509.7	6.7	$[M-H]^-$	Br^-	512.4 → 78.8	−15	−46
6-MeO-BDE47	$C_{13}H_8O_2Br_4$	511.7	10.7	$[M-C_6H_2(OH)Br_2]^-$ $[M-Br+O]^-$ $[M-CH_3]^-$	Br^-	450.7 → 78.9	−35	−48
2′-MeO-BDE68	$C_{13}H_8O_2Br_4$	511.7	13.6	$[M-C_6H_2(OCH_3)Br_2]^-$ $[M-Br+O]^-$	Br^-	450.7 → 78.6	−40	−58
2,2′-diMeO-BB80	$C_{14}H_{10}O_2Br_4$	525.7	13.6	$[M-C_6H_2(OCH_3)Br_2]^-$ $[M-Br+O]^-$	$[M-Br+O-CH_3]^-$	464.7 → 449.6	−40	−18
BDE-47	$C_{12}H_6OBr_4$	481.7	12.7	$[M-CH_3]^-$ $[M-Br+O]^-$ $[M-C_6H_3Br_2]^-$	Br^-	420.7 → 78.9	−20	−50
BDE-68	$C_{12}H_6OBr_4$	481.7	16.7	$[M-Br+O]^-$ $[M-C_6H_3Br_2]^-$	Br^-	420.6 → 78.7	−40	−54
BB-80	$C_{12}H_6Br_4$	465.7	21.9	$[M-Br+O]^-$	Br^-	404.7 → 78.8	−45	−50
4′-MeO-BDE121(IS)	$C_{13}H_7O_2Br_5$	589.6	16.7	$[M-Br+O]^-$	$[M-Br+O-CH_3]^-$	530.6 → 515.7	−55	−22

(*Galeocerdo cuvier*, $n = 6$) and bull shark (*Carcharhinus leucas*, $n = 1$), were culled off the coast of Ishigaki Island, Japan 2007, and their livers were removed and stored at −20 °C prior to analysis.[9] Sponge samples were extracted with acetonitrile, and the crude extracts were filtered and subjected to an LC/MS/MS column. The clean up procedure of shark liver samples was modified by our previous method.[28] In brief, accurately weighed liver samples (2 g) were cut into small pieces and mixed with 10 volumes of anhydrous sodium sulfate. The mixtures were wet-packed with dichloromethane (DCM)/*n*-hexane (1:1, v/v) into a glass column (2 cm, i.d.). The filtered extracts were concentrated, and the lipid contents were determined gravimetrically. Portions of the lipids were spiked with two internal standards ([$^{13}C_{12}$]6-OH-BDE47 for the phenolic analytes and 4′-MeO-BDE121 for the neutral analytes), and then the lipids were removed by gel permeation chromatography (GPC) (Bio-Beads, S-X3, Bio-Rad Laboratories, CA), with elution with DCM/*n*-hexane (1:1, v/v). The eluate containing target organobromines was concentrated to dryness and dissolved in acetonitrile (100 μL). The solution was subjected to LC/MS/MS without any further purification.

To compare the accuracy of this method with that of the EI-GC/MS, the extract from a bull shark liver was further prepared. Briefly, after GPC, hexane extracts were partitioned by 0.5 M sodium hydroxide/ethanol (7:3, v/v). The organic phase was purified by silica gel column chromatography (Wako gel S-1, 1 g Wako Pure Chemical Ind. Ltd., Osaka, Japan). The aqueous layer was acidified by HCl and back-extracted with *n*-hexane/diethyl ether (9:1, v/v). The phenolic fraction was concentrated and reacted with diazomethane. Then, both neutral and phenolic fractions (as methoxylated derivatives) were separately subjected to the EI-GC/MS system under the same conditions as described previously.[28]

APCI(-)-LC/MS/MS Analysis. Analyses were carried out using a liquid chromatograph (Prominence 20A; Shimadzu Co., Kyoto, Japan) coupled to a tandem mass spectrometer (API 3200Q Trap triple-quadrupole MS/MS system; Applied Biosystems Japan Ltd., Tokyo, Japan). A reversed phase Shim-pack FC-ODS column (150 mm × 4.6 mm, i.d., 3.0 μm particle size; Shimadzu Co., Kyoto, Japan) was used. The isocratic mobile phase composition was also optimized with acetonitrile:water (9:1, v/v) at 0.5 mL/min. The column conditions were all programmed at room temperature, and the column was equilibrated for more than 2 min between runs. The samples were kept at 4 °C in an autosampler, and a volume of 10 μL of each sample was injected into the HPLC column. The data were acquired and processed using the Analyst 1.4.2 software package.

MS/MS parameters were optimized in infusion experiments using individual standard solutions and two internal standards at a concentration of 2.5 μg/mL in acetonitrile. Each solution was pumped into the APCI source through a syringe pump at a constant flow rate of 60 μL/min. The best conditions for the selected analytes were modified by previous settings.[28] First, the precursor ions were chosen from Q1 scan mode. Next, for each stable selected precursor ion, the declustering potential (DP), entrance potential (EP), and collision cell entrance potential (CEP) were all adjusted using the "Quantitative Optimization" setting. The optimized parameters, DP and CE for the MS/MS of each analyte (Figure 1), are summarized in Table 1. Full-scan data acquisition was performed by scanning from m/z 50 to 600 (Q1 scan range) in the profile mode, using a scan time of 1 s with a step size of 0.1 amu and a pause between each scan of 5 ms. To choose the fragmentation patterns of m/z (Q1) → m/z (Q3) ions for the MRM transitions, product ion scan mass spectra were recorded by collision-activated dissociation (CAD) of selected precursor ions. MRM experiments were performed using a dwell time of 150 ms.

Quality Assurance and Quality Control. A nine-point calibration curve in the concentration range of 0.3−700 ng/mL was used to determine linearity. The limits of quantification (LOQ) using APCI-LC/MS/MS were determined using a signal-to-noise ratio (S/N) of 20 (Table 2) from cod liver oil (SRM1588b, NIST, MD, USA) spiked at 50 ng of nine analytes. Repeatability (% relative standard deviation (RSD)) was evaluated by interassay variations, which were assessed by five consecutive injections of 2−20 ng/mL standard solution and by measuring the same standard solution on different days. Recoveries for nine analytes in the cod liver oil were assessed by spiking with 50 ng of each compound through the entire extraction method. All reported

Table 2. Method Recoveries, Repeatability (RSD for *N*=6, 2–20 ng/mL), and Limits of Quantitation (LOQ, *S/N*=20) for Determination of Nine Target Compounds

compounds	recovery (%)	repeatability RSD (%)	LOQ (ng/g lipid weight)
6-OH-BDE47	88	8.9	0.24
2′-OH-BDE68	92	13	0.25
2,2′-diOH-BB80	75	8.2	0.11
6-MeO-BDE47	80	20	43
2′-MeO-BDE68	80	25	7.3
2,2′-diMeO-BB80	90	20	5.7
BDE-47	85	33	80
BDE-68	95	28	na[a]
BB-80	91	36	na

[a] Not analyzed.

concentrations were calculated by comparing their peak areas relative to internal standard.

RESULTS AND DISCUSSION

LS/MS/MS Identification. The APCI(-)-LC/MS/MS mass spectra of three reference compounds, 6-OH-BDE47, 6-MeO-BDE47, and BDE-47, are shown in Figure 2. The Q1 scan for 6-OH-BDE47 exhibited two major precursor ions, $[M-H]^-$, due to deprotonation, and $[M-C_6H_2(OH)Br_2]^-$ ($C_6H_2Br_2O^-$, *m/z* 249) due to cleavage of the diphenyl ether bond. Such cluster ions were also observed for 2′-OH-2,3′,4,5′-tetrabromodiphenyl ether (2′-OH-BDE68), but not for 2,2′-diOH-BB80 (Figures S-1 and S-2). In contrast, meta-substituted OH-tetraBDEs (3-OH-2,2′,4,4′-tetrabromodiphenyl ether (3-OH-BDE47) and 5-OH-2,2′,4,4′-tetrabromodiphenyl ether (5-OH-BDE47)) showed more abundant phenoxide ion $[M-C_6H_2(OH)Br_2]^-$ as compared to ortho-substituted OH-tetraBDEs (Figure S-1), suggesting the usefulness of structural identification of OH-substituents. For both 6-MeO-BDE47 and BDE-47, the Q1 scan commonly yielded the phenoxide ion $[M-Br+O]^-$ due to a substitution reaction of brominated congeners and O_2^- in the APCI source, as observed in the APPI source in other reports.[29,31] A cluster $C_6H_2BrO^-$ ion (*m/z* 249) was commonly observed in the three mass spectra (Figure 2). For the other clusters, a $[M-CH_3]^-$ ion was characteristic for 6-MeO-BDE47 and 2,2′-diMeO-BB80 but not for 2′-MeO-BDE68 (Figures 2, S-1, and S-2). The formation of $[M-X+O]^-$ (X=halogen) has been characteristic for halogenated compounds including PBDEs in negative APCI and APPI modes[28,31] and can be useful for selective and sensitive determination of target compounds. On the other hand, the Q3 scan of $[M-H]^-$ or $[M-Br+O]^-$ exhibited the formation of Br^- ion for all analytes except for 2,2′-diMeO-BB80 and 4′-MeO-BDE121, both of which yielded a characteristic $[M-Br+O-CH_3]^-$ ion (Figures 2, S-1, S-2, and S-3).

In order to identify the naturally occurring OH- and MeO-PBDE analogs in the environment, we initially screened several marine sponges collected from Micronesia to determine whether OH-tetraBDEs could be detected by a Q1 scan. The APCI(-) mass spectra (Q1 and Q3 scans) for the phenolic analytes in *Dysidae sp.* are shown in Figure S-4, suggesting that *Dysidae sp.* is one of the possible sources of OH- and MeO-tetraBDEs (mainly 2′-OH-BDE68 and 2′-MeO-BDE68). This is the first report of detection of hydroxylated and methoxylated organobromines by LC/MS/MS, whereas MeO-tetraBDEs have been recently reported in marine mammal blubber,[12,28] shark liver,[9] and human milk[14] by GC/MS.

MRM Transition. The chemical formulas, the major precursor/product ions, and the MRM transitions with their optimized DPs and CEs for each compound are listed in Table 1. The $[M-H]^-$ ions for hydroxylated congeners and the $[M-Br+O]^-$ ions for the other brominated congeners were selected as precursor ions. For 6-OH-BDE47, 2′-OH-BDE68, and 2,2′-diOH-BB80, use of the same MRM transition ($[M-H]^-$ (*m/z* 501) → Br^- ion (*m/z* 79)) is possible because these were chromatographically separated. For the neutral brominated analytes, though the MRM transition took place at ($[M-Br+O]^- \rightarrow Br^-$), the most effective MRM transition ($[M-Br+O]^- \rightarrow [M-Br+O-CH_3]^-$) was chosen for 2,2′-diMeO-BB80 and 4′-MeO-BDE121.

HPLC Optimization. Figure 3 shows the total ion chromatogram (TIC) and the individual MRM chromatograms of the nine analytes and two internal standards. In the previous LC/MS/MS study,[28] we used isocratic methanol as a mobile phase for MeO-PBDE analyses. However, on a methanol-based ODS column, 6-OH-BDE47 and 2′-OH-BDE68 were coeluted, even if the mobile phase was modified from isocratic to gradient elution. The use of acetonitrile:water (9:1, v/v) could resolve the coelution and provide shorter retention times for all analytes (within 22 min) (Figure 3). In addition, 3-OH-BDE47 and 5-OH-BDE47 have been reported as relevant phenolic products in the Canadian Arctic marine food web[32] as well as metabolites derived from rats dosed with BDE-47.[33,34] We confirmed in this study that 6-OH-BDE47 (6.74 min) was completely separated from 3-OH-BDE47 (6.46 min) and 5-OH-BDE47 (6.49 min), both of which did not overlap with 2′-OH-BDE68 (7.21 min) in this system. Although chromatographic separation was not achieved between 2′-MeO-BDE68 (g) and 2,2′-diMeO-BB80 (h) or between BDE-68 (i) and 4′-MeO-BDE121 (j), the selectivity was resolved by using the different MRM transition and DP/CE voltages (Figure 3 and Table 1).

Method Validation. Because there is presently no reference material available for these analytes, we used cod liver samples spiked with $[^{13}C_{12}]$6-OH-BDE47 as internal standards for the determination of phenolic analytes and samples spiked with 4′-MeO-BDE121 for determination of neutral analytes. To evaluate the performance of the optimized APCI-LC/MS/MS approach for the selected compounds, recovery, repeatability, and sensitivity were examined (Table 2). No memory effects between consecutive runs at the spiking concentration (700 ng/mL) used were observed, and no traces of the studied compounds were found in blank samples. Internal standard calibration curves were linear at a range from 0.3 to 700 ng/mL ($r^2 > 0.998$) for the phenolic analytes, whereas they were linear at a range from 14 to 700 ng/mL ($r^2 > 0.995$) for neutral analytes, indicating acceptable linearity for all target compounds over the environmentally relevant concentration range. As shown in Table 2, the internal standard-corrected mean recoveries ranged from 75% (2,2′-diOH-BB80) to 95% (BDE-68). At the level of 2 ng/mL standard solution, repeatability, as measured by relative standard

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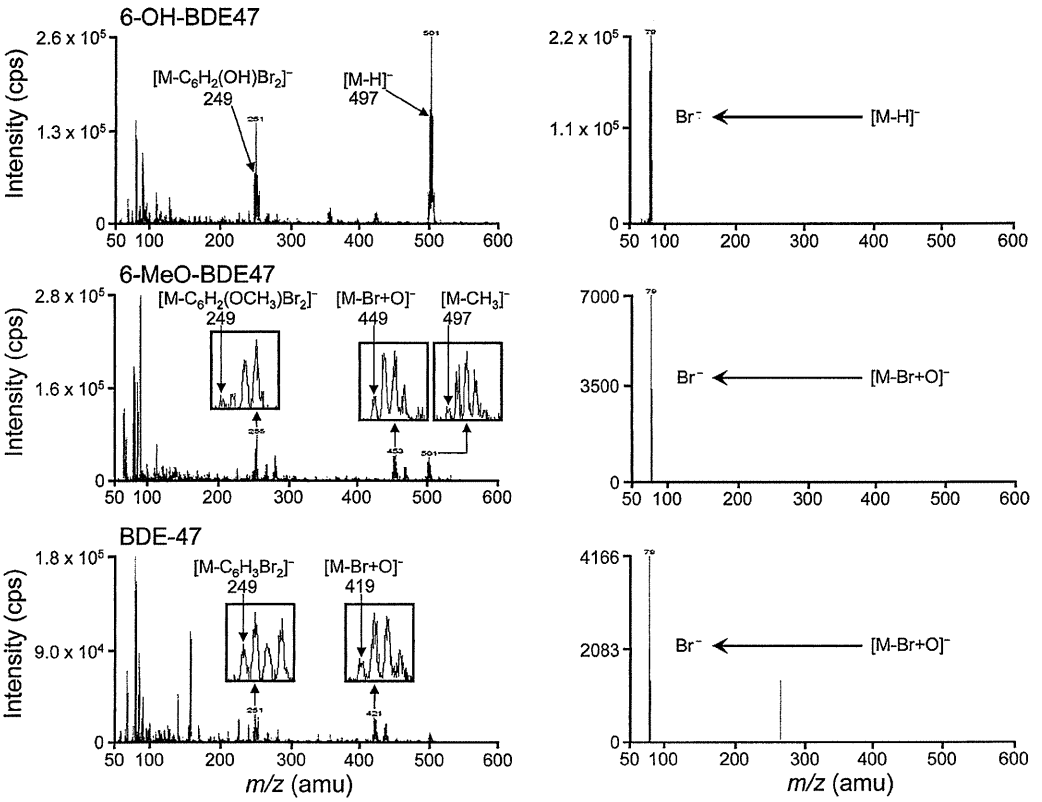

Figure 2. Precursor ion (Q1) and product ion (Q3) scans for 6-OH-BDE47, 6-MeO-BDE47, and BDE-47.

deviation (%RSD), was between 8 and 13% for phenolic analytes, and at the level of 20 ng/mL, repeatability ranged between 20 and 36% for neutral analytes. Less repeatability for MeO-BDEs and BDEs may be due to matrix effects by ion suppression, depending on the target compounds, or due to the lower ionization efficiency producing $[M\text{-}Br+O]^-$ ions by different MRM transitions of the analytes and the internal standard. We investigated the matrix effects on the signal strength of analytes by adding 50 ng of target compound to a concentrated extract of cod liver, and we found that the response varied at 25–45% RSD ($n = 4$), compared to standard solution only (data not shown). This suggests that the coelution of matrix components would interfere with the signal of an analyte. If so, the improved cleanup procedure may minimize this effect. LOQ, defined as the minimum concentration with a S/N of 20, ranged from 0.11 to 0.25 ng/g lipid weight for OH-tetraBDEs and from 5.7 to 80 ng/g lipid weight for BDE-47 and MeO-analytes (Table 2). The LOQ of the hydroxylated analytes was similar to the results for eight OH-PBDEs reported by the ISP(-)-LC/MS/MS method.[25] The values were also comparable to those established for GC/MS after derivatization in this study. As compared to phenolic analytes, 6-MeO-BDE47 and BDE-47 showed lower sensitivity by one or 2 orders of magnitude. In particular, the LOQ of BDE-47, one of the major environmentally relevant contaminants, was 80 ng/g lipid weight, corresponding to 290 pg on column ($S/N = 20$). This value is approximately 1 order of magnitude higher than the results obtained by negative APPI-LC/MS/MS method, where the LOQ for BDE-47 was reported to be 5.8 pg on column ($S/N = 3$).[31] This is due to the decreased solvent efficiency producing phenoxide ions in acetonitrile as compared with the methanol mobile phase, since acetonitrile can produce clusters that are too strongly bound to ionize the analyte sufficiently.[31,35] However, the spiking experiments using environmental samples (cod liver oil) suggested the potential usefulness of this methodology to determine the presence of phenolic organobromines in blood samples at pg/g concentrations and the presence of MeO-PBDEs and PBDEs in the marine food web at ng/g concentrations. To obtain more accurate concentrations, correction using matrix spike recovery or the method of standard addition is suggested.

The advantage of the proposed APCI method over GC/MS is that simultaneous determination of OH- and MeO-brominated analogs could be completed within 22 min after a single cleanup GPC procedure. The GC/MS method is further required for additional preparation steps including partitioning between OH- and MeO-PBDEs, derivatization of OH-PBDEs, and silica gel purification of neutral analytes. Furthermore, additional preparation for GC/MS analysis lowered the recovery (61 ± 12%, $n = 5$) of 2,2′-diOH-BB80 compared to that (75 ± 8%, $n = 4$) obtained by LC/MS/MS analysis.

Application to Marine Biota Analysis. The developed method was applied to analyze shark liver samples collected in Okinawa, Japan 2007. Typical MRM chromatograms for target compounds in marine sponge and bull shark liver are illustrated in Figure 4, where major components were identified as 2′-OH-BDE68 and 2′-MeO-BDE68. Table 3 shows the concentrations of the nine analytes in the liver of tiger shark (*Galeocerdo cuvier*) and a bull shark (*Carcharhinus leucas*) by APCI-LC/MS/MS. In the tiger shark liver, 6-OH-BDE47 could be detected in three of six samples, whereas the other phenolic compounds were quantified at levels of 0.1–0.7 ng/g lipid weight in the liver of tiger

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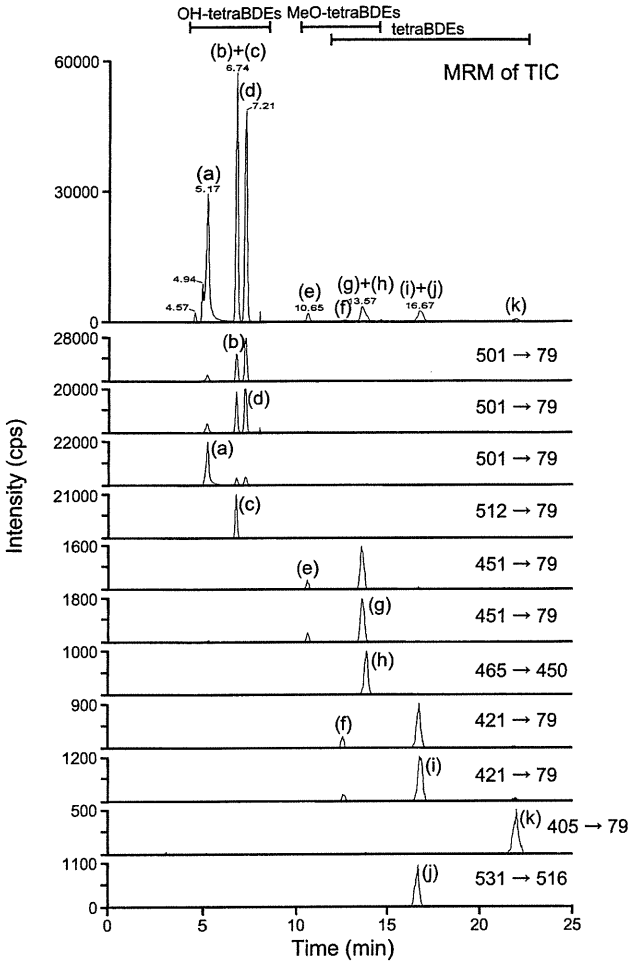

Figure 3. Total ion chromatogram (TIC) for all the MRM channels and the individual MRM chromatograms for (a) 2,2′-diOH-BB80, (b) 6-OH-BDE47, (c) [$^{13}C_{12}$]6-OH-BDE47 (IS), (d) 2′-OH-BDE68, (e) 6-MeO-BDE47, (f) BDE-47, (g) 2′-MeO-BDE68, (h) 2,2′-diMeO-BB80, (i) BDE-68, (j) 4′-MeO-BDE121 (IS), and (k) BB-80, analyzed on a 150 × 4.6 mm, i.d., Shim-pack FC-ODS column; mobile phase, acetonitrile:water (9:1, v/v) at a flow rate of 0.5 mL/min (injection volume, 10 μL) at 0.7 μg/mL standard mixture.

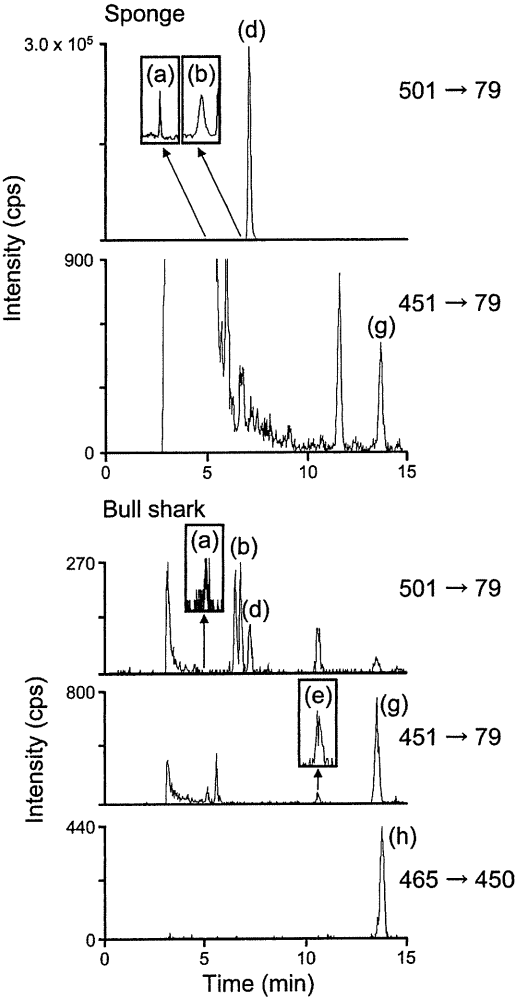

Figure 4. MRM chromatograms obtained from a sponge extract (upper) and a bull shark liver (lower). Peaks corresponding to (a) 2,2′-diOH-BB80 (501→79), (b) 6-OH-BDE47 (501→79), (d) 2′-OH-BDE68 (501→79), (g) 2′-MeO-BDE68 (451→79), and (h) 2,2′-diMeO-BB80 (465→450) were detected in both samples.

sharks ($n = 6$). The concentrations of 2′-MeO-BDE68 ranged from 65 to 212 ng/g lipid weight, which were 2 orders of magnitude higher than those of 2′-OH-BDE68 (0.2–0.7 ng/g lipid weight), and were consistent with the previous results.[9] In the bull shark liver, OH-tetraBDEs were detected at the higher levels of 6–8 ng/g lipid weight, as compared to those in tiger sharks. In contrast, the levels of 6-MeO-BDE47, 2′-MeO-BDE68, and 2,2′-diMeO-BB80 ranged from 230 to 540 ng/g lipid weight, respectively. The values were consistent with results (290–590 ng/g lipid weight) obtained by the GC/MS method. The ratios of OH-organobromines to MeO-organobromines ranged from 0.0003 to 0.034, indicating the high distribution of MeO-organobromines in lipid-rich organisms. In fact, OH-tetraBDEs have been reported to be present at concentrations 2 orders of magnitude lower than MeO-tetraBDEs in human blood or milk[15,16,36] as well as in the marine food web.[34]

The OH- and MeO-tetraBDEs investigated have been proposed to be natural organohalogens produced by cyanobacteria[6,37] and are presumably present in, e.g., sponges *Dysidea dendyi*,[35,38,39] *Dysidea herbacea*,[3] and alga *Cladophora fascicularis*[40] and *Ceramium tenuicorne*.[5] Although OH- and MeO-tetraBDEs have never been commercially produced or been reported as byproduct in industrial processes,[41] they may be present via metabolism from biota after exposure to anthropogenic PBDEs (i.e., BDE-47).[33,42] To our knowledge, this is the first report of the detection of 2,2′-diOH-BB80 and 2,2′-diMeO-BB80 in marine sponges and bull sharks. However it is unknown whether 2,2′-diOH-BB80 produced by bacteria[43] is biotransformed to the corresponding 2,2′-diMeO-BB80 present in mammals from Australia (250–4100 ng/g lipid

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Table 3. Concentration of Target Compounds in Shark Liver by MRM-LC/MS/MS and SIM-GC/MS

	tiger shark						bull shark	
number	IG07-72	IG07-69	IG07-33	IG07-97	IG07-30	IG07-06	IG07-124	
sex	male	male	male	male	female	female	male	
body length (cm)	267	275	290	305	310	319	280	
	APCI(-)-LC/MS/MS						APCI(-)-LC/MS/MS	EI-GC/MS
concentration (ng/g lipid weight)								
6-OH-BDE47	ND[a]	ND	ND	0.33	0.51	0.25	8.4	6.3
2′-OH-BDE68	0.43	0.25	0.21	0.49	0.68	0.39	6.2	6.8
2,2′-diOH-BB80	0.1	0.21	0.12	0.2	0.44	0.13	7.5	9.6
6-MeO-BDE47	259	ND	68	166	227	403	305	313
2′-MeO-BDE68	171	65	158	97	107	212	538	594
2,2′-diMeO-BB80	107	47	29	114	362	472	233	286
BDE-47	94	ND	ND	ND	173	458	399	432
BDE-68	ND	ND	ND	ND	ND	ND	ND	ND
BB-80	ND	ND	ND	ND	ND	ND	ND	ND
ratio								
6-OH-BDE47/6-MeO-BDE47	-	-	-	0.002	0.002	0.001	0.028	0.020
2′-OH-BDE68/2′-MeO-BDE68	0.003	0.004	0.001	0.005	0.006	0.002	0.012	0.011
2,2′-diOH-BB80/2,2′-diMeO-BB80	0.001	0.004	0.004	0.002	0.001	0.0003	0.032	0.034
6-MeO-BDE47/BDE-47	2.755	-	-	-	1.312	0.88	0.764	0.725

[a] Not detected (less than LOQ).

weight)[10] and from the Pacific Ocean (10−800 ng/g lipid weight).[12] The present levels determined by APCI(-)-LC/MS/MS were similar to the results obtained in sharks by GC/MS,[9] indicating that the APCI(-)-LC/MS/MS method would be acceptable for determining the environmentally relevant levels of diOH- and diMeO-BB80. Overall, the APCI method is adequate for rapid and reliable reassessment of hydroxylated and methoxylated organohalogens in archived samples, without cumbersome procedures.

CONCLUSION

An optimized APCI(-)-LC/MS/MS method for the simultaneous determination of OH- and MeO-tetraBDEs and their related analogs was developed. The advantages of the method include moderate detection limits, congener specificity using selected MRM transitions, and the use of a single cleanup procedure without derivatization. The linearity, recovery, repeatability, and sensitivity of this method were validated within acceptable ranges. The method was applied to analyze the livers of tiger sharks and bull sharks from Japanese coastal waters. The data were in excellent agreement with the values of all analytes obtained by GC/MS. Although the method provides a different sensitivity range (LOQ 0.1−0.3 ng/g lipid weight for phenolic analytes and LOQ 6−80 ng/g lipid weight for neutral brominated analogs), future work could apply this method to the full range of PBDE-like contaminants present in the environment and in biota tissues.

ACKNOWLEDGMENT

This research was financed by Grants-in Aid from the Japan Society for the Promotion of Science (B19310042, B20404006, C20510070). We are grateful to Dr. Göran Marsh (Stockholm University) for kindly providing the standard reference compounds.

SUPPORTING INFORMATION AVAILABLE

Precursor ion (Q1) and product ion (Q3) scans for 2′-OH-BDE68, 2′-MeO-BDE68, and BDE-68 (Figure S-1), precursor ion (Q1) and product ion (Q3) scans for 2,2′-diOH-BB80, 2,2′-diMeO-BB80, and BB-80 (Figure S-2), precursor ion (Q1) and product ion (Q3) scans for [$^{13}C_{12}$]6-OH-BDE47 (IS) and 4′-MeO-BDE121 (IS) (Figure S-3), and mass spectra (Q1 and Q3 scans) for 2′-OH-BDE68 and 2′-MeO-BDE68 obtained from a sponge extract (Figure S-4). This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review May 7, 2009. Accepted June 10, 2009.

AC9009885

福岡医誌　100(5)：200—209，2009

2,2',5,5'-四塩素化ビフェニル（CB52）のウサギ肝ミクロゾームによる代謝

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Metabolism of 2,2',5,5'-Tetrachlorobiphenyl (CB52) by Rabbit Liver Microsomes

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Abstract Our preceding studies have reported that 2,2',5,5'-tetrachlorobiphenyl (tetraCB)(CB52) is mainly metabolized to 3-hydroxy (OH)-metabolite by phenobarbital (PB)-inducible cytochrome P450 (P450) isoforms such as CYP2B1 and CYP2B18. In this study, the metabolism of CB52 by liver microsomes of untreated and PB-treated rabbits was investigated. Rabbit liver microsomes produced mainly 3-OH- and 4-OH-metabolites (M-1 and M-2) at an equal extent and two other metabolites (M-3 and M-4) and also that phenobarbital (PB) treatment accelerated the formation of all these metabolites. M-3 was assumed to OH-tetraCB by GC-MS. Another metabolite, M-4, was determined to 3,4-diOH-CB52 by GC-MS and ^{1}H-NMR. Addition of antiserum against CYP2B4, a constitutive and PB-inducible rabbit P450 isoform, to a microsomal incubation system resulted in almost complete inhibition of the formation of 3-OH-, 4-OH- and 3,4-diOH-metabolites. These results suggest that CYP2B4 plays an important role in CB52 metabolism in rabbit liver.

はじめに

PCBはカネミ油症の原因物質である[21]とともに世界的な環境汚染物質としても有名である．PCBは脂溶性が高いことから，経口摂取後，極めて高い効率で小腸から吸収される．PCBのうち，置換塩素数が4個以下のPCBは，肝小胞体に局在するチトクロムP450（P450）によって容易に代謝され，代謝物として主に胆汁を介して糞中に排泄されるが，塩素数が5個以上になると代謝されにくくなり，肝，脂肪組織および血液中に残留することになる[33]．PCB代謝物の主なものは，一水酸化（OH）体であるが，2次代謝物として，二水酸化（diOH）体，メチルスルホン（$MeSO_2$）体および親PCBから塩素が1個脱離したOH体などが見つかっている[20]．また，これらの代謝物の生成には，3つのP450サブファミリーに属するP450（CYP1A，CYP2AおよびCYP2B）が関

与することが明らかになっている[1)12)13)16)~18)]．このうち，CYP1AおよびCYP2AはPCB異性体のうち主に3，4，5位に塩素置換されたものを，一方，CYP2Bに属するP450は2，5位に塩素置換されたものをよく代謝し，OH体を生成する．

2,2',5,5'-四塩素化ビフェニル（tetraCB）（CB52）は，カネミ油症原因油であるカネクロール400の主成分の一つ[25)]で，フェノバルビタール（PB）型の肝酵素誘導能を有するPCB異性体の原型[36)29)]といえるものである．当研究室では，ラット，モルモットおよびハムスター肝ミクロゾーム（Ms）を用いてCB52の代謝を調べた結果，いずれの動物でも3-OH体が主代謝物であること，また，この生成はPB前処理で顕著に増加することを明らかにした[19)]．さらに，CB52代謝に関与するP450分子種についても検討を加え，ラットCYP2B1[12)]，ラットCYP2B2[12)]，モルモットCYP2B18[16)]およびハムスターP450HPB-1[18)]が3-水酸化反応を，また，ハムスターCYP2A8[17)]が4-水酸化反応をそれぞれ触媒することを明らかにした．

一方，Gardnerらは，CB52を投与したウサギ尿中から3-OH体に加え，4-OH体およびtrans-3,4-dihydro-3,4-dihydroxy体を検出している[5)]．この報告は，PCB代謝において中間体として3,4-epoxide体の存在を示唆した最初の報告である．また，この事実は，ウサギが他の実験動物と異なるPCB代謝酵素系を有することを示唆している．そこで，本研究ではウサギ肝MsによるCB52の代謝を調べるとともに，代謝に関与するP450分子種を明らかにすることを目的とした．

実　験　方　法

1．実験材料

(1) CB52および代謝物

CB52および4-OH-CB52は既報[11)17)19)]に従い，合成した．3-OH-CB52はCB52（200 mg/kg）をラット腹腔内に1回投与し，得られた糞より精製した[8)]．

(2) 動物の薬物処理および肝Msの調製

7匹の雄性日本白色種ウサギ（体重3.0~4.5 kg）を用いた．このうち4匹を未処理群，3匹をPB処理群に分け，PB処理群には飲料水として0.1%（w/v）PB水溶液を6日間自由に摂取させた．pentobarbitalによる麻酔下，ウサギ肝を摘出し，常法により肝Msを調製した[36)]．なお，動物の取り扱いは，「中村学園大学における実験動物のための指針」を遵守し，行った．

(3) ウサギ肝P450（CYP2B4）抗血清の調製

まず，PB前処理ウサギ肝Msより，1種類のP450（CYP2B4）を既報[18)16)]に準じて精製した．すなわち，PB前処理ウサギ肝（110 g湿重量）から肝Msを調製し，これをコール酸で可溶化後，ω-aminooctyl-Sepharose 4Bカラム，hydroxyapatite，DEAE-Biogel A agarose，CM-Sephadex C-50の各カラムを用いて精製し，1種類のP450分子種を得た．この精製標品は比含量10.3 nmol/mg protein，収量1.6%であった．さらにN-末端アミノ酸20個の配列を調べたところ，既報[2)]のCYP2B4と一致した．以下，抗血清を得るために，精製したCYP2B4をRibi adjuvant（RIBI ImmunoChem Research Inc.，USA）に懸濁し，3匹のモルモット背中皮下に注射した．5週間後，モルモットの頸動脈より全血を採取し，血清分離剤（栄研製）により，抗血清を得た[16)~18)]．

2．ウサギ肝Msによる代謝

ウサギ肝MsによるCB52の代謝は既報[19)]に準じて行った．すなわち，40 μM CB52あるいはそのOH代謝物（3-OHあるいは4-OH体）をNADPH生成系（0.33 mM NADP，5 mM glucose-6-phosphate（G-6-P），G-6-P脱水素酵素1 unit），6 mM $MgCl_2$，牛血清アルブミン（0.8 mg/ml）およびウサギ肝Ms（1 mg protein）を，100 mM HEPES緩衝液（pH 7.4）とともに合計1 mlとして，37℃で20分間インキュベート後，代謝物をchloroform-methanol（2：1）1 mlおよびn-hexane 4 mlで3回ずつ抽出した．抽出液は濃縮乾固し，N,O-bis-(trimethylsilyl)acetamideによるトリメチルシリル（TMS）化あるいはdiazomethaneによるメチル化後，電子捕獲型検出器付ガスクロマトグラフィー（GC-ECD），あるいは質量分析計付GC（GC-MS）に付した．代謝物の定量は，M-3を除き，それぞれCB52代謝物の検量線を用いてGC-ECDにより行った．GC-ECDの条件は次の通りである．分析機器，

ECD 付 HP5890 Series II ガスクロマトグラフ (Hewlett-Packard 製)；カラム，DB-1 fused silica キャピラリーカラム (15 m × 0.25 mm i.d., 0.33 μm 膜厚，J&W Scientific 製)；オーブン温度，200℃；注入口温度，250℃；検出器温度，250℃；キャリアーガス，N_2 (1 ml/min).

一方，代謝物の分子量は，質量分析器付 HP5890 Series II ガスクロマトグラフ (Hewlett-Packard 製) を用いて，EI モードで測定した．GC-MS 分析条件は次の通りである．カラム，DB-1 fused silica キャピラリーカラム (30 m × 0.25 mm i.d., 0.33 μm 膜厚，J&W Scientific 製)；オーブン温度，210℃；注入口温度，250℃；検出器温度，280℃；キャリアーガス，He (1 ml/min).

3．3,4-diOH-CB52 の分離精製

500 ml の大容量の反応液，すなわち，40 μM 3-OH-CB52 を基質として用い，前述のように，牛血清アルブミン (0.8 mg/ml)，NADPH 生成系，6 mM $MgCl_2$，PB 前処理ウサギ肝 Ms (500 mg protein) とともに 100 mM HEPES 緩衝液 (pH 7.4) で，37℃，1時間インキュベーションを行った．その後，100 ml の chloroform-methanol (2：1) と 400 ml の n-hexane を加えて2回抽出し，さらに diazomethane でメチル化後，HPLC により M-4 を分離精製した．HPLC 条件は次の通りである．カラム，ODS カラム (20 mm i.d. × 250 mm, YMC 製)；プレカラム，ODS プレカラム (20 mm i.d. × 50 mm, YMC 製)；流速，5 ml/min；溶離液，methanol-H_2O (9：1)．M-4 のメチル誘導体は保持時間 25.9 min に溶出され，最終的に，収量は 1.5 mg であった．M-4 のメチル誘導体の化学構造は GC-MS および ^{1}H-NMR により，3,4-dimethoxy (diMeO)-CB52 であると決定された．

MS spectrum；m/z (relative abundance, %)：354 (60, M^++4), 352 (100, M^++2), 350 (80, M^+), 335 (45, M^+-15), 307 (30, M^+-43), 292 (30, M^+-58), 272 (36, M^+-78).

^{1}H-NMR δ (ppm): 3.954(3H, s, 3 or 4-MeO), 3.983 (3H, s, 4 or 3-MeO), 7.255 (1H, s, 6-H), 7.430 (1H, d, J=2.52Hz, 6'-H), 7.523 (1H, dd, J=2.52Hz, 8.57Hz, 4'-H), 7.593 (1H, d, J=8.57Hz, 3'-H).

4．抗 CYP2B4 抗血清の添加による代謝阻害

モルモットで調製した抗 CYP2B4 抗血清を 100 mM HEPES 緩衝液 (pH 7.4) 中でウサギ肝 Ms とともに，30℃で 30 分間インキュベート後，CB52 および NADPH 生成系を添加して反応をスタートし，さらに 37℃で 20 分間インキュベートした．反応液は，上記と同様に有機溶媒で抽出し，GC-ECD に付した．

5．その他

ウサギ肝 Ms のタンパク質の定量は，Lowry ら[23]の方法を用いて行った．なお，標準タンパク質として牛血清アルブミンを用いた．P450 含量は Omura と Sato[28]の方法により測定した．SDS-ポリアクリルアミドゲル電気泳動は Laemmli[22]の方法により，また，ウェスタンブロットは Guengerich ら[7]の方法により行った．ウサギ肝 Ms 中 CYP2B4 タンパクの免疫染色は Konica immunostaining キット (生化学工業) を用いて行った．N-末端アミノ酸の測定は，Model 473A gas phase sequencer (Applied Biosystems, USA) を用いてエドマン分解法により行った[18]．

^{1}H-NMR スペクトルの測定は，500 MHz JEOL GSX-500 spectrometer (日本電子製) により行った．試料は acetone-d_6 に溶解し，また，内部標準物質として tetramethylsilane を用いた．

実験結果

1．ウサギ肝 Ms による CB52 の代謝

Fig. 1 には，ウサギ肝 Ms により生成された CB52 代謝物の TMS 誘導体のガスクロマトグラムを示す．代謝物と思われる4種類のピーク (M-1, M-2, M-3 および M-4) が，それぞれ保持時間 6.75 分，7.07 分，7.37 分および 12.77 分に観察された．標品の保持時間との比較から，M-1 および M-2 は，これまでに報告している 3-OH-CB52 および 4-OH-CB52 の TMS 誘導体であることが明らかとなった[19]．なお，両 OH 体以外に，新たに2つの代謝物ピーク (M-3 および M-4) が観察された．M-3 の TMS 誘導体は，M-2 のすぐ後ろに検出されたのに対し，M-4 の

TMS誘導体は保持時間が，他3者よりずっと長かった．

次に，M-1とM-2につき，それぞれ標品のTMS誘導体の検量線から定量を試み，既報の実験動物の結果と比較した．Table 1に示すように，未処理ウサギ肝Msにより，M-1とM-2が1.1：1でほぼ同程度生成された．未処理ウサギ肝Msでの代謝パターンおよび代謝活性の強さは，ハムスターとよく似ていた．

一方，PB前処理ウサギ肝Msでは，M-1の生成が未処理ウサギ肝Msの2.4倍に，また，M-2の生成も未処理ウサギ肝の2.4倍に増加した．このようなPB前処理によるM-1（3-OH体）の顕著な増加はラット，ハムスターおよびモルモットのいずれとも共通していたが，M-2（4-OH体）の増加に関しては，ハムスターと類似していた．

さらに，M-1とM-2の生成量の相関関係を調べた．その結果，Fig. 2のように，両代謝物の生成量は非常によく相関しており，相関係数は0.889で有意であった．この結果から，3-OH体および4-OH体は同一のP450分子種によって生

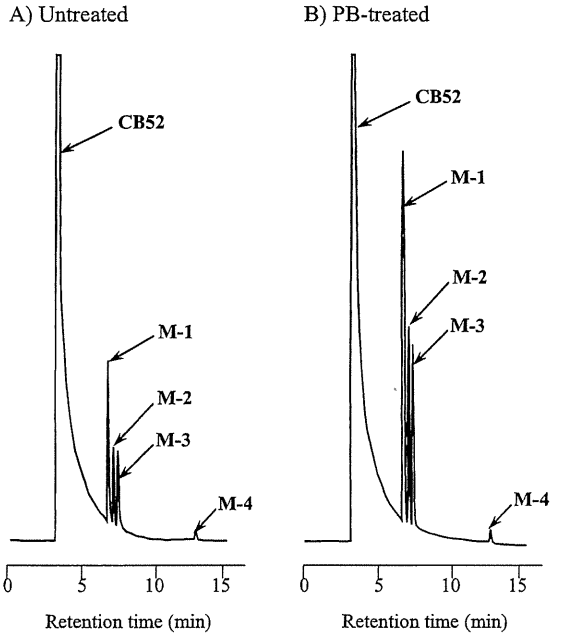

Fig. 1 Gas chromatograms of the trimethylsilylated derivatives of CB52 metabolites formed by liver microsomes of untreated (A) and PB-treated (B) rabbits.

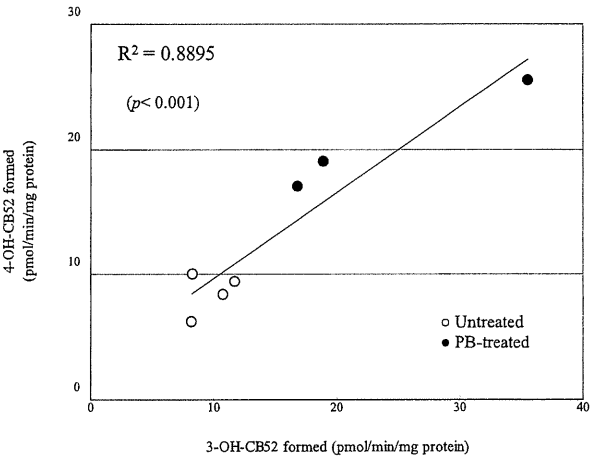

Fig. 2 Correlation of 3-OH- and 4-OH-CB52 formed by liver microsomes of untreated and PB-treated rabbits.

Table 1 Metabolism of CB52 by liver microsomes of untreated and PB-treated rabbits, rats, hamsters and guinea pigs

Animal	Treatment	No.	Metabolite formed (pmol/min/mg protein)	
			3-OH	4-OH
Rabbit	None	4	9.8 ± 1.7(100)	8.5 ± 1.7(100)
	PB	3	23.8 ± 10.2(243)	20.5 ± 4.4(241)
Rat[19]	None	4	N.D.	N.D.
	PB	4	324.0 ± 15.4	N.D.
Hamster[19]	None	4	6.3 ± 0.5(100)	5.6 ± 0.3(100)
	PB	4	20.1 ± 0.2(319)	11.1 ± 0.3(198)
Guinea pig[19]	None	4	8.4 ± 0.9(100)	N.D.
	PB	4	19.3 ± 2.2(230)	1.1 ± 0.1

N.D., not detected.
Each value represents the mean ± S.D. of three or four animals and those in parentheses are the relative ratio to the control.
Data in rats, hamsters and guinea pigs were cited from the reference (19).

成されていることが示唆された.

2. CB52 代謝物生成の経時的変化

M-1 および M-2 とともに生成された M-3 と M-4 が代謝物であるかどうかを確めるために, インキュベーション時間を 60 分間まで延ばして, これらの代謝物の経時的な増減を調べた. Fig. 3 に示すように, インキュベーション時間 10 分後までは M-1, M-2 および M-3 は, いずれもほぼ直線的に増加したが, M-1 については 30 分後から, わずかに減少が見られた. 一方, M-4 は 10 分の遅れで増加し始め, その後 60 分間わずかずつであるが増加した. これらの結果から, M-3 と M-4 はいずれも CB52 代謝物であることが示唆された.

3. M-3 と M-4 の化学構造

M-3 および M-4 の分子量を調べるため, これらを diazomethane でメチル化後, GC-MS にかけた. その結果, M-3 のメチル誘導体は分子量 320 で, 塩素 4 個を含む同位体ピークが観察されたことから, methoxy (MeO)-tetraCB であることが判明した (データ未掲載). 一方, M-4 のメチル誘導体は, 分子量 350 であり, 同様に塩素 4 個を含む同位体ピークが観察されたことから, diMeO-tetraCB であることが明らかになった (実験方法 3. 参照).

次に, M-4 の ^{1}H-NMR を測定するため, 3-OH-CB52 を基質として 500 ml の反応液を用いてインキュベーションを行った. M-4 を有機溶媒で抽出後, HPLC で分離精製した. このメチル誘導体につき ^{1}H-NMR を測定した結果, 実験方法 3. に示すように, 3-MeO および 4-MeO 基に由来する 2 本の singlet と 6 位プロトン由来の singlet が, それぞれ 3.95 ppm, 3.98 ppm および 7.26 ppm に検出された. また, 2,5-二塩素置換された芳香環の 3 つのプロトンに由来するシグナルが, 7.43 ppm (doublet), 7.52 ppm (doublet) および 7.59 ppm (singlet) に検出された. 以上の結果から, 最終的に, M-4 は 3,4-diOH-CB52 であることが明らかとなった.

4. ウサギ CYP2B4 の精製

PB 前処理ウサギ肝 Ms より, P450 分子種の精製を試みた. 最終的に, 比含量 10.3 nmol/mg protein で, 見かけの分子量 50,000 の P450 分子

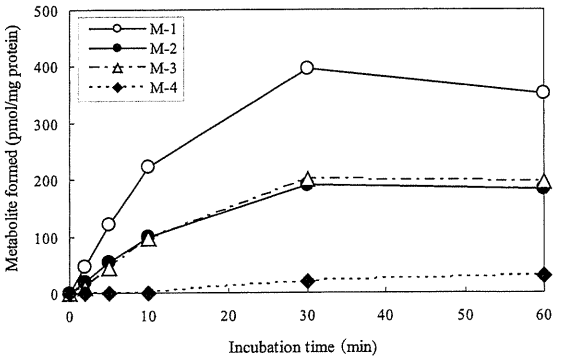

Fig. 3 Time course of CB52 metabolism by liver microsomes of PB-treated rabbits.

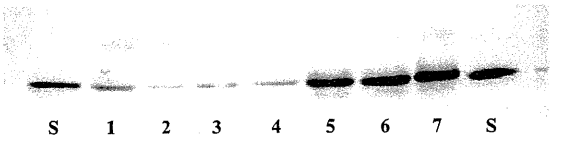

Fig. 4 Immunoblot of rabbit liver microsomes with antiserum against CYP2B4. Lanes S contain purified rabbit CYP2B4 (1 μg protein). Lanes 1-4 and 5-7 contain liver microsomes (10 μg protein each) from four untreated and three PB-treated rabbits, respectively.

Table 2 N-Terminal amino acid sequence of a rabbit P450 purified in this study

		Amino acid residue				
P450	Animal	1	5	10	15	20
This study	(rabbit)	MEFS	LLLLL	AFLAG	LLLLL	F
CYP2B4[2)]	(rabbit)	MEFS	LLLLL	AFLAG	LLLLL	F
CYP2B1[37)]	(rat)	MEPS	I LLLL	ALLVG	FLLLL	V
P450HPB-1[18)]	(hamster)	MEPS	TLLLL	TLLLS	FLVLL	V
CYP2B18[26)]	(guinea pig)	MELS	LLLFL	ALLLG	LLLLL	F

The abbreviations used are as follows : M, methionine ; E, glutamic acid ; F, phenylalanine ; P, proline ; S, serine ; L, leucine ; I, isoleucine ; A, alanine ; G, glycine ; T, threonine ; V, valine

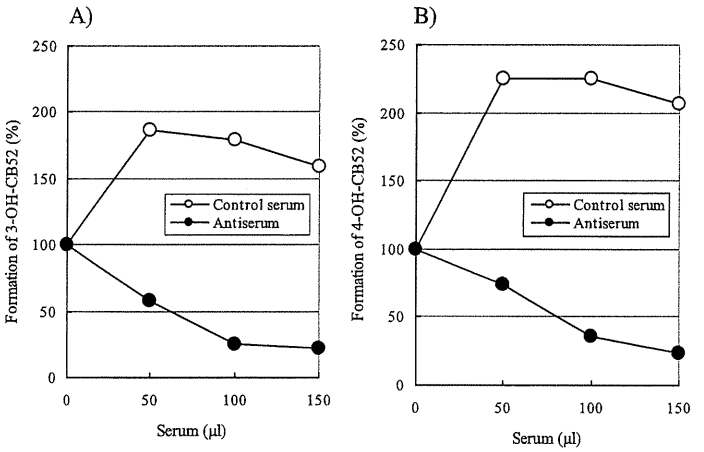

Fig. 5 Effect of antiserum against CYP2B4 on the formation of 3-OH- and 4-OH-metabolites from CB52 with liver microsomes of PB-treated rabbits. Open and closed circles indicated control serum and antiserum raised against CYP2B4, respectively. Each point represents the mean of duplicate determinations.

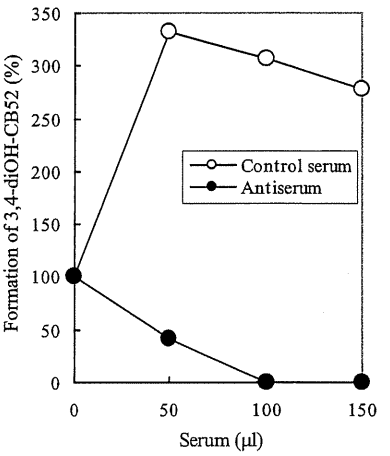

Fig. 6 Effect of antiserum against CYP2B4 on the formation of 3,4-diOH-CB52 from 3-OH-CB52 with liver microsomes of PB-treated rabbits. Open and closed circles indicated control serum and antiserum raised against CYP2B4, respectively. Each point represents the mean of duplicate determinations.

種1種類を得ることができた．本P450のN-末端アミノ酸20個を調べたところ，Table 2に示すように，これまでに報告されたPB誘導性P450のうち，CYP2B4[2]と完全に一致したことから，以下，本P450をCYP2B4とした．

本P450に対する抗血清をモルモットで作製後，これを用いて未処理およびPB前処理ウサギ肝Ms中のCYP2B4の検出を試みた．ウサギ肝MsをSDS-ポリアクリルアミド電気泳動およびウェスタンブロット後，免疫染色したところ，Fig. 4に示すように，未処理ウサギにおいて，CYP2B4タンパクと分子量が一致するタンパクバンドが1本検出された．また，PB前処理ウサギでは同じタンパクバンドが著しく増加していた．これらの事実から，これまでの報告のように，CYP2B4が常在型であり，かつPB誘導性であることが確認された．さらに，PB前処理によるCYP2B4タンパクの増加が，Table 1に示したM-1（3-OH体）およびM-2（4-OH体）の生成活性の増加とよく相関していることから，両代謝物の生成にCYP2B4が関与していることが示唆された．

5．抗CYP2B4抗血清添加によるCB52代謝阻害

CB52代謝におけるCYP2B4の関与の程度を明らかにするため，抗CYP2B4抗血清を用いて代謝阻害を試みた．その結果，Fig. 5に示すように，PB前処理Msによる M-1（3-OH体）およびM-2（4-OH体）の生成は抗血清150 μlの添加で，いずれも約90%が阻害された．また，M-3とM-4の生成も，定量的ではないものの，本抗血清の添加により強く阻害された（データ未掲載）．

次に，3-OH-CB52から3,4-diOH-CB52への2次代謝に及ぼす抗CYP2B4抗血清の添加効果を調べた．基質として3-OH体を用い，PB前処理Msによる代謝を調べた．その結果，Fig. 6に示すように，抗血清100 μlの添加により，3,4-diOH体の生成は完全に阻害された．この結果から，CYP2B4が3-OH体から3,4-diOH体への酸化反応にも，大きく関与していることが示唆された．

考　　察

ウサギ肝MsによるCB52の代謝を調べたところ，ウサギ肝は，他の動物とかなり異なる代謝パターンを有することが明らかとなった．すなわち，代謝物として3種類のOH体と3,4-diOH体が生成されたが，主代謝物の3-OH体と4-OH体の生成比はいづれの群でもほぼ1：1であった．これまでに，PCBの水酸化機構として次の2つの経路が考えられている．1つはベンゼン環のC-H結合に酸素原子が挿入される経路（直接水酸化）で，Prestonらは，ラットにおけるCB52の3-水酸化反応が，PB誘導性P450による直接水酸化で進行していることを示した[30)31)]．もう1つは，代謝中

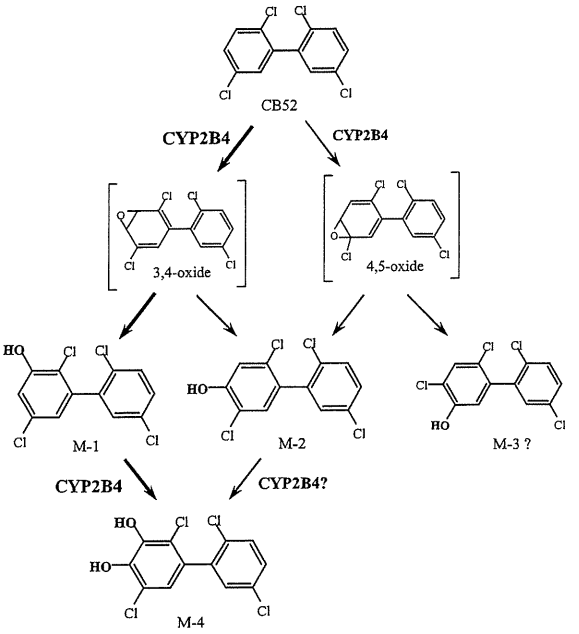

Fig. 7 Postulated metabolic pathways of CB52 in rabbit liver.

間体の3,4-あるいは4,5-epoxide体を経由し，さらに塩素原子のNIH転位を伴って進行する経路で，MC誘導性P450が主に関与している．コプラナーPCBの3,3',4,4'-tetraCB（CB77）[35]や3,3',4,4',5-pentachlorobiphenyl（CB126）[15]などの4-水酸化反応がこの経路で進行しているといわれている．一方，Forgueら[3,4]はCB52の3,4-epoxide体の単離に成功し，さらにこれが開裂すると，3-OH体および4-OH体へと変換することを報告した．今回のウサギの場合，どの経路であるか明確ではないが，3-OH体と4-OH体がほぼ同程度生成される事実から，代謝中間体として3,4-epoxide体を経由していると考えられる．また，CB52と関連した2,5-二塩素置換ベンゼンを有するPCB代謝物を調べると，ほ乳動物組織中で必ず3-$MeSO_2$体と4-$MeSO_2$体の両方が検出されている[14,10]．これらのことから，ウサギ肝におけるCB52代謝は，中間体として3,4-epoxide体を経由していると思われる．Fig. 7にウサギ肝におけるCB52の推定代謝経路を示す．

今回，3-OH体および4-OH体以外に，もう1つ別のOH体（M-3）が生成された．現在のところ，分子量以外は不明であるが，GCの保持時間が3-OH体と4-OH体より長いことから，5位の塩素原子が4位に転位した代謝物かもしれない．そうであれば，M-3は5-OH-2,2',4,5'-tetraCBであると考えられるが，この点は今後の課題である．

CYP2B4抗血清を用いた代謝阻害実験により，CYP2B4が3-OH体，4-OH体，3,4-diOH体およびM-3の生成すべてに関与することが明らかとなった．当研究室の一連の研究では，CYP2Bサブファミリーに属する，ラットCYP2B1[12]およびCYP2B2[12]，モルモットCYP2B18[16]およびハムスターP450HPB-1[18]は3-水酸化反応のみを触媒した．一方，4-水酸化反応を触媒するのはハムスターCYP2A8[17]だけであったが，最近，McGrawとWallerにより，2,2',4,5,5'-pentachlorobiphenyl（CB101）の4'-水酸化酵素として，ヒトCYP2A6が報告された[24]．これらの事実は，CYP2B4が他の動物P450酵素と異なる水酸化機構を有すること，さらにウサギ肝においてCYP2B4が最も重要なCB52代謝酵素であることを示している．

最近，我々はCB101の3'-あるいは4'-OH体から3',4'-diOH体への代謝をラット，ハムスターおよびモルモット肝Msを用いて調べ，その結果，親CB101から3'-あるいは4'-OH体を経由して最終的に3',4'-diOH体が生成されることを明らかにした[27]．このことは，親PCBからdiOH体への代謝過程で，3位および4位へ2度連続して直接水酸化が起こることを示唆している．本研究でも3-OH-CB52を用いて3,4-diOH体への代謝を調べたところ，比較的容易に3,4-diOH体が生成され，さらにCYP2B4が大きく関与していた（Fig. 6）．これらを考え合わせると，CYP2B4の場合には，3,4-epoxide体を経由する経路と直接水酸化の両方の経路で3,4-diOH体を生成しているかもしれない．

CB52の毒性は，CB77, CB126および3,3',4,4',5,5'-hexachlorobiphenyl（CB168）などのコプラナーPCBに比べはるかに弱い[36]．そのため，毒性等価係数は設定されていない．しかしながら，代謝的に毒性が増強される例がいくつか報告されている．Stadnickiらは培養細胞の増殖に及ぼす影響を調べ，CB52の3,4-epoxide体がCB52より強い阻害活性を示した[34]．また，Haraguchiらはラットにおいて4-メチルチオ（MeS)-CB52が親CB52に比べ体重増加をより強く抑制したり，肝薬物代謝酵素をより強く誘導することを報告した[9]．さらに最近では，PCBのdiOH体が女性ホ

ルモン様作用[6]を示したり，細胞毒性[32]を有することが報告されている．今後は，CB52代謝物の3,4-diOH体の毒性評価も重要な研究課題の1つとなろう．

総　括

1．ウサギ肝Msによる CB52の代謝を調べた結果，これまでに報告されたラット，モルモットおよびハムスターと異なり，3-OH体と4-OHがほぼ同程度で生成された．これらの生成はPB前処理により，いずれも未処理の2.4倍に増加した．なお，これら以外に2つの代謝物が生成され，1つはOH-tetraCBであること，もう1つは3,4-diOH-CB52であることが明らかになった．

2．モルモットから調製されたCYP2B4抗血清を用いてウサギ肝Msを免疫染色したところ，CYP2B4は常在型であり，PB誘導性であることが確認された．PB前処理によるCYP2B4タンパクの増加は，主代謝物の3-OH体および4-OH体の増加とよく一致していた．

3．CYP2B4抗血清を用いて，CB52の代謝阻害を試みたところ，3-OH体と4-OH体の生成は抗血清100 μlの添加で，いずれも90%前後が強く阻害された．また，3-OH-CB52から3,4-diOH体への代謝に及ぼす添加効果を調べたところ，抗血清100 μlの添加で，3,4-diOH体の生成は完全に阻害された．

4．以上の結果から，ウサギ肝CYP2B4は，CB52代謝のすべての経路で大きく関与していることが示唆された．

謝　辞

本研究は厚生労働科学研究費（食品の安心・安全確保推進研究事業）および日本学術振興会科学研究費補助金（基盤研究(B)，No. 20404006 原口浩一；基盤研究(C)，No. 20510070 加藤善久）に一部負うものである．ここに記して謝意を表します．

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(Received for publication March 25, 2009)

Chemosphere 79 (2010) 314–319

Contents lists available at ScienceDirect

Chemosphere

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

Levels of perfluorooctane sulfonate and perfluorooctanoic acid in female serum samples from Japan in 2008, Korea in 1994–2008 and Vietnam in 2007–2008

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ARTICLE INFO

Article history:
Received 21 October 2009
Received in revised form 8 January 2010
Accepted 16 January 2010
Available online 10 February 2010

Keywords:
Perfluorooctane sulfonate
Perfluorooctanoic acid
Serum
Long-term trend
East Asia

ABSTRACT

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have recently received attention owing to their widespread contamination in the environment. One of major manufacturers, 3M Company voluntarily phased out PFOS production in 2002. We measured the PFOS and PFOA concentrations in serum samples from Japan (Sendai, Takayama and Osaka), Korea (Busan and Seoul) and Vietnam (Hanoi) to evaluate the possible effects of the phase-out on the serum levels. There were spatial differences in both the serum PFOS and PFOA concentrations. The serum PFOS concentrations (ng mL^{-1}) evaluated as the geometric mean (geometric standard deviation) in 2007–2008 ranged from 4.86 (1.45) in Sendai, Japan, to 9.36 (1.42) in Busan, Korea. The serum PFOA concentrations ranged from 0.575 (2.32) in Hanoi, Vietnam, to 14.2 (1.73) in Osaka, Japan. Historically archived samples collected from Korea in 1994–2008 revealed that the serum PFOA concentrations increased by 1.24-fold in Busan from 2000 to 2008 and 1.41-fold in Seoul from 1994 to 2007. On the other hand, the serum PFOS concentrations did not change from 1994 to 2007/2008. The serum PFOS levels in Japan in 2008 were significantly decreased compared with previously reported values (22.3–66.7% of the values in 2003/2004). However, the serum PFOA levels showed a clear decline from 2003 to 2008 in a high-exposed area, Osaka, but not in low-exposed areas in Japan. The trends toward decreases were not uniformly observed in Asian countries, unlike the case for the United States, suggesting that local factors associated with the production and introduction histories in each country overwhelm the effects of the phase-out.

1. Introduction

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have recently received attention owing to their widespread contamination in the environment, wildlife and humans (Kannan et al., 2001; Fromme et al., 2009). In 2002, after 50 years of production, 3M Company phased out their manufacture of PFOS (Renner, 2001). On the other hand, DuPont and several manufacturers in Japan produce and continue to use PFOA for the manufacture of fluoropolymers (Hogue, 2004; Japan Fluoropolymers Industry Association, 2005). Possible precursors to PFOA have also been used for various applications (van Zelm et al., 2008). PFOS has been regulated or issued guidelines at various levels by different governments, including those of the United States (Significant New Use Rules, United States Environmental Protection Agency, 2000), Canada (Schedule 1 of CEPA 1999, Environment Canada, 2006) and the European Union (Directive 76/769/EEC, European Commission, 2006). In the Stockholm Convention on Persistent Organic Pollutants (POPs), it was decided that PFOS should be listed in Annex B of the convention (UNEP, 2007; Wang et al., 2009). The Environmental Protection Agency of the United States (2006) launched a stewardship program and manufacturers have committed to reduce PFOA emissions voluntarily.

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0045-6535/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2010.01.027