Table 3
Concentrations of PFCAs in breast milk samples.

Sampling site		Concentration (pg mL^{-1})						
		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	∑PFCAs
Japan	n > MDL(%)	28(93.3)	27(90.0)	20(66.7)	28(93.3)	5(16.7)	10(33.3)	30(100.0)
Kyoto	Median	89(<40–194)A*	31(<10–72)A*	17(<15–65)A*	35(<10–100)A*	<10(<10–29) n.s.	<10(<10–91)AB*	184(50.3–413.5)A*
	Mean	93.5 ± 43.7	32.1 ± 17.2	21.3 ± 15.0	36.6 ± 21.8	<10	15.2 ± 20.6	194.5 ± 83.6
	GM(GSD)	82.7(1.7)	26.5(2.0)	16.9(2.0)	30.4(2.0)	<10	<10	176.7(1.6)
	P90	173	62	44	65	22	36	315
Korea	n > MDL(%)	24(80.0)	20(66.7)	4(13.3)	22(73.3)	4(13.3)	15(50.0)	28(93.3)
Seoul	Median	62(<40–173)B*	15(<10–41)B*	<15(<15–19)B*	19(<10–51)B*	<10(<10–41) n.s.	10(<10–43)A*	114(<10–283.9)B*
	Mean	64.5 ± 33.7	14.7 ± 9.3	<15	19.6 ± 13.1	<10	16.8 ± 13.5	118.8 ± 50.9
	GM(GSD)	55.5(1.8)	11.9(2.0)	<15	15.3(2.2)	<10	11.7(2.4)	109.7(1.5)
	P90	106	29	15	42	11	40	189
China	n > MDL(%)	19(63.3)	21(70.0)	4(13.3)	17(56.7)	3(10.0)	7(23.3)	28(93.3)
Beijing	Median	51(<40–122)B*	15(<10–47)B*	<15(<15–29)B*	15(<10–47)B*	<10(<10–25) n.s.	<10(<10–43)B*	84(<10–200.8)B*
	Mean	51.6 ± 30.6	15.3 ± 9.6	<15	16.0 ± 12.9	<10	<10	87.8 ± 54.9
	GM(GSD)	43.0(1.9)	12.6(2.0)	<15	11.7(2.3)	<10	<10	68.8(2.2)
	P90	103	27	18	42	10	22	164

MDL: method detection limit; GM: geometric mean; GSD: geometric standard deviation; P90: 90th percentile.
* Medians among different sites differ significantly ($p < 0.05$, Steel–Dwass test). For example, the letters A and B indicate that the corresponding values differ significantly at $p < 0.05$, while A and A or B and B indicate that the corresponding values do not differ significantly.

Table 4
Factor analysis among PFCAs.

	Initial solution		Varimax rotated	
	F1	F2	F1	F2
Eigenvalue	2.60	1.14		
Cumulative contribution (%)	43.3	62.3		
Eigenvector				
PFOA	0.387	−0.511	**0.818**	−0.135
PFNA	0.472	−0.375	**0.857**	0.060
PFDA	0.480	−0.020	**0.668**	0.390
PFUnDA	0.518	0.261	**0.563**	**0.677**
PFDoDA	0.114	0.430	−0.086	0.488
PFTrDA	0.340	0.587	0.135	**0.822**
*Factor score (mean ± SD)**				
		Beijing	−0.5 ± 0.6^{B}	−0.2 ± 0.7
		Kyoto	0.9 ± 1.1^{A}	0.2 ± 1.4
		Seoul	−0.4 ± 0.6^{B}	0.1 ± 0.8

* Means among countries differ significantly ($p < 0.05$, Steel–Dwass test). For example, the letters A and B indicate that the corresponding values differ significantly at $p < 0.05$, while A and A or B and B indicate that the corresponding values do not differ significantly.

were no significant differences ($p > 0.05$). Regarding the total PFCAs in the milk samples, PFOA accounted for 48%, 54%, and 61% in Japan, Korea, and China, respectively. Among the long-chain PFCAs, odd-numbered PFCAs were more frequently detected than even-numbered PFCAs, except for PFDA in Japan.

PFOA was only significantly correlated with PFNA (ρ coefficient:>0.4) (Supplemental Table 1). There were also significant correlations between PFNA and PFUnDA, PFDA and PFUnDA, and PFUnDA and PFTrDA (ρ coefficients: >0.4). In general, the PFCA concentrations showed strong correlations between PFCAs of similar (i.e. adjacent) chain lengths.

The factor analysis revealed that two potential factors, F1 and F2, accounted for 43.3% and 19.0% of the total variance (with eigenvalues of >1), respectively (Table 4). After varimax rotation, F1 indicated higher eigenvectors for PFOA, PFNA, PFDA, and PFUnDA, while F2 had positive eigenvectors for PFUnDA and PFTrDA. The mean factor scores of each sampling site are also shown in Table 4. Although the F1 score was higher in Kyoto than in the other two sites ($p < 0.05$, Steel–Dwass test), there were no significant differences in the F2 scores among all the sampling sites ($p > 0.05$, Kruskal–Wallis test).

3.2. PFCA concentrations in commercially available infant formulas in Japan and China

The PFCA concentrations in the infant formulas are shown in Table 5. PFOA, PFNA, and PFDA were frequently detected in both Japan and China, but there were no significant differences between the two countries. PFUnDA was detected at 40.7 pg mL^{-1} in one sample in Japan. PFDoDA and PFTrDA were not detected in any of the formula samples. Compared with the breast milk samples,

Table 5
Concentrations of PFCAs in infant formulas.

Sampling site	Sample No.	Concentration (pg mL^{-1})[a]						
		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	∑PFCAs
Japan	1	<20	<5	<7	<5	<5	<5	<5
	2	35.8	27.0	<7	<5	<5	<5	62.8
	3	30.8	8.0	12.1	<5	<5	<5	50.9
	4	<20	8.6	11.5	<5	<5	<5	20.1
	5	22.5	92.0	19.8	40.7	<5	<5	175.0
	Meant ± SD	21.8 ± 11.8	27.6 ± 37.2	10.1 ± 6.9	10.1 ± 17.1	<5	<5	66.4 ± 65.6
China	1	35.4	50.4	14.0	<5	<5	<5	99.7
	2	<20	15.2	<7	<5	<5	<5	15.2
	3	37.1	12.2	12.9	<5	<5	<5	62.2
	4	29.9	11.6	13.9	<5	<5	<5	55.4
	Meant ± SD	28.1 ± 12.4	22.4 ± 18.8	11.1 ± 5.1	<5	<5	<5	61.5 ± 29.3

[a] A 4-mL aliquot of each infant formula was analyzed.

the PFOA levels were 4-fold and 2-fold lower in the formula samples in Japan and China, respectively. The total PFCA concentrations in the infant formulas were lower than those in the breast milk samples in Japan ($p < 0.05$, Kruskal–Wallis test), but not in China ($p > 0.05$, Kruskal–Wallis test).

3.3. Relationships between the PFCA levels and the participants' characteristics

To evaluate the influence of the participants' characteristics on the PFCA concentrations in the human breast milk samples, Spearman's correlation analyses were performed (Supplemental Table 2). PFDoDA was positively correlated with the mother's age in Korea ($p < 0.05$) and PFNA was negatively correlated the mother's age in China ($p < 0.05$). However, these correlations were not consistent among the three countries. In several epidemiological studies (Steenland et al., 2010), the PFC concentrations in the cord blood or maternal pregnancy serum were reported to be associated with the child birth weight. In our study subjects, the correlations between the PFCA concentrations and the child birth weights were not significant. The lactation period was also examined for correlations with PFCAs in the milk samples. PFDA was correlated with the lactation period in Japan ($p < 0.05$), but not in Korea. Among the PFCAs, there were no clear trends in the correlation coefficients. Although consumption of fish was one of the sources of exposure to PFCAs, no significant associations were observed between the PFCA levels in the milk samples and the fish intake ($p > 0.05$). Non-smoking mothers in Japan had relatively higher PFCAs levels than other mothers, but the difference was not significant ($p > 0.05$). The PFCA levels in the milk samples were compared between non-drinking mothers and other mothers. The PFTrDA and PFNA levels were lower in non-drinking mothers in Japan and Korea ($p < 0.05$, Mann–Whitney test).

3.4. Daily intake estimation and hazard assessment for infants

The tolerable daily intake (TDI) for PFOA was established to be 1500 ng kg body weight^{-1} d^{-1} by the Scientific Panel on Contaminants in the Food Chain requested by the European Food Safety Authority (EFSA, 2008). The average breast milk consumption rate and body weight for 1-year-old infants were assumed to be 600 g d^{-1} and 7.3 kg, respectively (Schecter, 1994). Based on these assumptions, the daily intakes of PFCAs by 1-year-old infants were estimated (Supplemental Table 3). For the infant formulas, the calculated mean levels were only 0.1–0.2% of the TDI. Meanwhile, the calculated levels for the human breast milk samples (means: 0.3–

Table 6
Comparisons of the PFCA concentrations in human breast milk with reported data (pg mL^{-1}).

Country	Region	Year	*n*		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	Reference
Japan	Kyoto	2010	30	Mean	93.5	32.1	21.3	36.6	<10	15.2	This study
				Range	<40–194	<10–72	<15–65	<10–100	<10–29	<10–91	
	Hokkaido	NA	51	Mean	89	35					Nakata et al. (2009)
				Range	<12–339	<4–150					
	Ehime	1999	24	Mean	77.7						Tao et al. (2008b)
				Range	<42.5–170	<8.82–23.9					
Korea	Seoul	2010	30	Mean	64.5	14.7	<15	19.6	<10	16.8	This study
				Range	<40–173	<10–41	<15–19	<10–51	<10–41	<10–43	
		2007	17	Mean	41						Kim et al. (2011)
				Range	<43–77	<8.8	<18	<24	<13		
China	Beijing	2008–2009	30	Mean	51.6	15.3	<15	16.0	<10	<10	This study
				Range	<40–122	<10–47	<15–29	<10–47	<10–25	<10–43	
	Zhoushan	2004	19	Mean	106.3	18.1	7.2	19.1			So et al. (2006)
				Range	47–210	6.3–62	3.8–15	7.6–56			
	12 provinces	2007	1237	Mean	116.0	16.2	9.9	37.6			Liu et al. (2010)
				Range (24 pooled samples)	<14.15–814	6–76	<1.44–63	<1.30–196			
Vietnam	Hanoi, Ho Chi Minh	2000, 2001	40	Range	<42.5–89.2	<8.82–10.9					Tao et al. (2008b)
Cambodia	Phnom Penh	2000	24	Range	<42.5–132	<8.82–12.3					Tao et al. (2008b)
Philippines	Quezon	2000, 2004	24	Range	<42.5–183	<8.82–25.0					Tao et al. (2008b)
Malaysia	Penang	2003	13	Range	<42.5–90.4	<8.82–14.9					Tao et al. (2008b)
Indonesia	Jakarta, Purwakarta	2001	20	Range	<42.5	<8.82–135					Tao et al. (2008b)
India	Chidambaram Kolkata, Chennai	2002, 2004, 2005	39	Range	<42.5–335	<8.82					Tao et al. (2008b)
USA	Unknown	2003	2	Range	<200						Kuklenyik et al. (2004)
	Massachusett	2004	45	Mean	43.8	7.26					Tao et al. (2008a)
				Range	<30.1–161	<5.2–18.4					
Sweden	Uppsala	2004	12	Range	<209–492	<5–20	<8	<5			Kärrman et al. (2007)
		1996–2004	9	Range (Pooled annual composite milk sample)	<209	<5–28	<8	<5			
Germany	NA	2006	38	Range (Archived samples + 19 fresh samples)	201–460						Völkel et al. (2008)
	North Rhine Westphalian	NA	203	Range	25–610						Bernsmann and Furst (2008)
Spain	Tarragona	2007	10	Range	<500	<30	<60	<30	<30		Kärrman et al. (2010)
	Barcelona	2008	20		<15.2–907	<11.5	<85.5–1095				Llorca et al. (2010)

0.5% of the TDI; 90th percentiles: 0.6–0.9% of the TDI) were higher than those for the infant formulas. As of 2011, there is no established TDI for PFCAs that are longer than PFOA.

4. Discussion

In the present study, we first demonstrated contamination of human breast milk with PFDoDA and PFTrDA in Asian countries. Simultaneously, we confirmed similar long-chain PFCA profiles in East Asian breast milk samples, as previously reported (Liu et al., 2010, 2011; Kim et al., 2011). A characteristic PFCA composition was observed for PFUnDA and PFTrDA (both odd-numbered PFCAs) with residual PFDoDA and PFDA (both even-numbered PFCAs). These findings indicated that odd-numbered PFCAs predominated over even-numbered PFCAs in East Asian breast milk samples. The PFCAs with longer chains than PFOA reached 47% of the total PFCAs for the average of the three countries. This finding suggests that infants are exposed to not only classical PFOA but also long-chain PFCAs in East Asia. Indeed, a factor analysis demonstrated two potential factors, F1 and F2, as sources of PFCAs. F1 had loading on medium-chain PFCAs, of which the factor score was significantly higher in Kyoto than in Beijing or Seoul. Kyoto is located in the Hanshin area, where there is a large emission source of PFOA and its related by-products (Niisoe et al., 2010). Thus, F1 may represent a local emission source of PFCAs. On the other hand, F2 had strong associations with long-chain PFCAs. The factor scores for F2 in the three large cities did not differ, suggesting that there are similar sources of long-chain PFCAs (>C10) in the three counties. Therefore, PFCA (C10–C13) exposure through the breast milk is likely to commonly occur in East Asian countries. We are the first to document this possibility.

The sources of long-chain PFCAs are still unknown. Odd-numbered PFCAs predominated in the PFCAs in this study. As previously reported (Harada et al., 2011), odd-numbered PFCAs also predominated in serum samples collected from Asian women. A review by Prevedouros et al. (2006) indicated that odd-numbered PFCAs have been manufactured in Japan via oxidation of fluorotelomer olefins. Industrial application of these odd-numbered PFCAs might contribute to the pattern of PFCAs in breast milk samples collected from East Asian women. Although FTOHs are possible precursors of PFCAs, biodegradation of FTOHs preferentially yields even-numbered PFCAs (Fasano et al., 2009). Therefore, FTOHs are unlikely to be the main exposure source for Asian populations. Further investigations into the sources and exposure routes are needed to predict the future trajectory of these PFCA levels.

Although data concerning the PFC levels in human breast milk are not as abundant as those in blood samples, we can still find several reports for PFCs in human breast milk from Asia, the United States, and Europe. The related data are summarized in Table 6. In Japan, the PFOA levels in three regions were comparable (Tao et al., 2008b; Nakata et al., 2009). In Korea, PFOA had a higher value in the present study compared with earlier research in Seoul (Kim et al., 2011) (mean: 63.8 *vs.* 41 pg mL^{-1}, range: 14.7–172.1 *vs.* 21–77 pg mL^{-1}). This increase may be consistent with the increasing trend in the PFOA level in serum samples by 1.27-fold from 2000 to 2007 in Korea (Harada et al., 2010).

In China, the concentrations of PFOA in Zhoushan ranged from 47 to 210 pg mL^{-1} (So et al., 2006) and in 12 different provinces of China, the mean PFOA level was 116 pg mL^{-1} (Liu et al., 2010). The PFOA levels showed large variations within China, although the other PFCAs were comparable among two previous studies and this study. In Southeast Asian developing countries, most of the milk samples did not contain detectable PFCAs (Tao et al., 2008b), which might result from differences in industrialization. In the United States and European countries, PFOA and PFNA were detected in human breast milk samples, but long-chain PFCAs were not observed (Kuklenyik et al., 2004; Kärrman et al., 2007, 2010; Bernsmann and Furst, 2008; Tao et al., 2008a; Völkel et al., 2008; Llorca et al., 2010). The occurrence of long-chain PFCAs in East Asian countries is likely to be a fingerprint of the sources of exposure.

Infant formulas were also evaluated in this study. The compositions of PFCAs in the infant formulas were different from those in the breast milk samples. In Japan, the levels of PFCAs in the infant formulas were lower than those in the breast milk samples. These findings probably reflect differences in the bioaccumulation potential between humans and cows.

In our study, we found no evident relationships between the mother's characteristics and the PFCA concentrations. Although there were statistically significant differences for some of the PFCAs, no consistent trends were observed among the three countries.

The estimated daily intakes of PFOA were much lower than the TDI in this study. These observations may indicate that the health risks for PFOA intake from breast milk and infant formulas are limited. However, infants have different susceptibilities to adults with regard to their dynamic growth and developmental processes (Sly and Flack, 2008). In addition, the toxicokinetics and toxicities of long-chain PFCAs are still unclear, although these PFCAs comprised 48% of the total PFCAs in this study. These uncertainties necessitate more comprehensive toxicological studies on long-chain PFCAs, including PFOA.

The limitations of this study are the sample sizes and the sample selection method. It should be noted that these findings were based on a relatively small number of non-randomly selected volunteer samples. Moreover, the sampling times for the Chinese donors were uncertain, although it is known that the profiles of chemicals may change during the lactation period. Considering these limitations, a future extended study is required for confirmation of these findings,

In conclusion, various PFCAs were detected in human breast milk samples from East Asian countries. Further studies are needed to evaluate the exposure to long-chain PFCAs and the health risks in infants.

Acknowledgments

This study was mainly supported by Special Coordination Funds for Promoting Science and Technology (No. 1300001) sponsored by the Japan Science and Technology Agency, and a Grant-in-Aid for Health Sciences Research from the Ministry of Health, Labour and Welfare of Japan (H21-Food-003). The funding bodies had no roles in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have no financial conflicts of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.10.035.

References

Bernsmann, T., Furst, P., 2008. Determination of perfluorinated compounds in human milk. Organohalogen Compd. 70, 718–721.

EFSA, 2008, Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. EFSA J. 653, 1-131. <http://www.efsa.europa.eu/en/efsajournal/pub/653.htm>.

EPA, US, 2006. 2010/15 PFOA Stewardship Program. <http://www.epa.gov/oppt/pfoa/pubs/pfoastewardship.htm>.

Fasano, W.J., Sweeney, L.M., Mawn, M.P., Nabb, D.L., Szostek, B., Buck, R.C., Gargas, M.L., 2009. Kinetics of 8–2 fluorotelomer alcohol and its metabolites, and liver

glutathione status following daily oral dosing for 45 d in male and female rats. Chem. Biol. Interact. 180, 281–295.
Fromme, H., Tittlemier, S.A., Volkel, W., Wilhelm, M., Twardella, D., 2009. Perfluorinated compounds–exposure assessment for the general population in Western countries. Int. J. Hyg. Environ. Health 212, 239–270.
Hansen, K.J., Clemen, L.A., Ellefson, M.E., Johnson, H.O., 2001. Compound-specific, quantitative characterization of organic fluorochemicals in biological matrices. Environ. Sci. Technol. 35, 766–770.
Harada, K.H., Koizumi, A., 2009. Environmental and biological monitoring of persistent fluorinated compounds in Japan and their toxicities. Environ. Health Prev. Med. 14, 7–19.
Harada, K.H., Yang, H.R., Moon, C.S., Hung, N.N., Hitomi, T., Inoue, K., Niisoe, T., Watanabe, T., Kamiyama, S., Takenaka, K., Kim, M.Y., Watanabe, K., Takasuga, T., Koizumi, A., 2010. Levels of perfluorooctane sulfonate and perfluorooctanoic acid in female serum samples from Japan in 2008, Korea in 1994–2008 and Vietnam in 2007–2008. Chemosphere 79, 314–319.
Harada, K.H., Hitomi, T., Niisoe, T., Takanaka, K., Kamiyama, S., Watanabe, T., Moon, C.S., Yang, H.R., Hung, N.N., Koizumi, A., 2011. Odd-numbered perfluorocarboxylates predominate over perfluorooctanoic acid in serum samples from Japan, Korea and Vietnam. Environ. Int. 37, 1183–1189.
Haug, L.S., Thomsen, C., Becher, G., 2009. Time trends and the influence of age and gender on serum concentrations of perfluorinated compounds in archived human samples. Environ. Sci. Technol. 43, 2131–2136.
Ihaka, R., Gentleman, R., 1996. R: a language for data analysis and graphics. J. Compd. Graph. Stat. 5, 299–314. <http://www.R-project.org>.
Kärrman, A., Domingo, J.L., Llebaria, X., Nadal, M., Bigas, E., van Bavel, B., Lindstrom, G., 2010. Biomonitoring perfluorinated compounds in Catalonia, Spain: concentrations and trends in human liver and milk samples. Environ. Sci. Pollut. Res. Int. 17, 750–758.
Kärrman, A., Ericson, I., van Bavel, B., Darnerud, P.O., Aune, M., Glynn, A., Lignell, S., Lindstrom, G., 2007. Exposure of perfluorinated chemicals through lactation: levels of matched human milk and serum and a temporal trend, 1996–2004, in Sweden. Environ. Health Perspect. 115, 226–230.
Kärrman, A., Harada, K.H., Inoue, K., Takasuga, T., Ohi, E., Koizumi, A., 2009. Relationship between dietary exposure and serum perfluorochemical (PFC) levels–a case study. Environ. Int. 35, 712–717.
Keller, J.M., Calafat, A.M., Kato, K., Ellefson, M.E., Reagen, W.K., Strynar, M., O'Connell, S., Butt, C.M., Mabury, S.A., Small, J., Muir, D.C., Leigh, S.D., Schantz, M.M., 2010. Determination of perfluorinated alkyl acid concentrations in human serum and milk standard reference materials. Anal. Bioanal. Chem. 397, 439–451.
Key, B., Howell, R., Criddle, C., 1997. Fluorinated organics in the biosphere. Environ. Sci. Technol. 31, 2445–2454.
Kim, S.K., Lee, K.T., Kang, C.S., Tao, L., Kannan, K., Kim, K.R., Kim, C.K., Lee, J.S., Park, P.S., Yoo, Y.W., Ha, J.Y., Shin, Y.S., Lee, J.H., 2011. Distribution of perfluorochemicals between sera and milk from the same mothers and implications for prenatal and postnatal exposures. Environ. Pollut. 159, 169–174.
Koizumi, A., Harada, K.H., Inoue, K., Hitomi, T., Yang, H.R., Moon, C.S., Wang, P., Hung, N.N., Watanabe, T., Shimbo, S., Ikeda, M., 2009. Past, present, and future of environmental specimen banks. Environ Health Prev Med. 14, 307–318.
Koizumi, A., Yoshinaga, T., Harada, K., Inoue, K., Morikawa, A., Muroi, J., Inoue, S., Eslami, B., Fujii, S., Fujimine, Y., Hachiya, N., Koda, S., Kusaka, Y., Murata, K., Nakatsuka, H., Omae, K., Saito, N., Shimbo, S., Takenaka, K., Takeshita, T., Todoriki, H., Wada, Y., Watanabe, T., Ikeda, M., 2005. Assessment of human exposure to polychlorinated biphenyls and polybrominated diphenyl ethers in Japan using archived samples from the early 1980s and mid-1990s. Environ. Res. 99, 31–39.
Kuklenyik, Z., Reich, J.A., Tully, J.S., Needham, L.L., Calafat, A.M., 2004. Automated solid-phase extraction and measurement of perfluorinated organic acids and amides in human serum and milk. Environ. Sci. Technol. 38, 3698–3704.
Lau, C., Butenhoff, J.L., Rogers, J.M., 2004. The developmental toxicity of perfluoroalkyl acids and their derivatives. Toxicol. Appl. Pharmacol. 198, 231–241.
Liao, C., Wang, T., Cui, L., Zhou, Q., Duan, S., Jiang, G., 2009. Changes in synaptic transmission, calcium current, and neurite growth by perfluorinated compounds are dependent on the chain length and functional group. Environ. Sci. Technol. 43, 2099–2104.
Liu, J., Li, J., Zhao, Y., Wang, Y., Zhang, L., Wu, Y., 2010. The occurrence of perfluorinated alkyl compounds in human milk from different regions of China. Environ. Int. 36, 433–438.
Liu, J., Li, J., Liu, Y., Chan, H.M., Zhao, Y., Cai, Z., Wu, Y., 2011. Comparison on gestation and lactation exposure of perfluorinated compounds for newborns. Environ. Int. 37, 1206–1212.
Llorca, M., Farre, M., Pico, Y., Teijon, M.L., Alvarez, J.G., Barcelo, D., 2010. Infant exposure of perfluorinated compounds: levels in breast milk and commercial baby food. Environ. Int. 36, 584–592.
Matsubara, E., Harada, K., Inoue, K., Koizumi, A., 2006. Effects of perfluorinated amphiphiles on backward swimming in Paramecium caudatum. Biochem. Biophys. Res. Commun. 339, 554–561.
Nakata, A., Saito, K., Iwasaki, Y., Ito, R., Kishi, R., Nakazawa, H., 2009. Determination of perfluorinated compounds in human milk and evaluation of their transition from maternal plasma. Bunseki Kagaku 58, 653–659.
Niisoe, T., Harada, K.H., Ishikawa, H., Koizumi, A., 2010. Long-term simulation of human exposure to atmospheric perfluorooctanoic acid (PFOA) and perfluorooctanoate (PFO) in the Osaka urban area, Japan. Environ. Sci. Technol. 44, 7852–7857.
Olsen, G.W., Mair, D.C., Reagen, W.K., Ellefson, M.E., Ehresman, D.J., Butenhoff, J.L., Zobel, L.R., 2007. Preliminary evidence of a decline in perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) concentrations in American Red Cross blood donors. Chemosphere 68, 105–111.
Prevedouros, K., Cousins, I.T., Buck, R.C., Korzeniowski, S.H., 2006. Sources, fate and transport of perfluorocarboxylates. Environ. Sci. Technol. 40, 32–44.
Schecter, A., 1994. Dioxins and Health. Plenum Press, New York.
Sly, P.D., Flack, F., 2008. Susceptibility of children to environmental pollutants. Ann. New York Acad. Sci. 1140, 163–183.
So, M.K., Yamashita, N., Taniyasu, S., Jiang, Q., Giesy, J.P., Chen, K., Lam, P.K., 2006. Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. Environ. Sci. Technol. 40, 2924–2929.
Steenland, K., Fletcher, T., Savitz, D.A., 2010. Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). Environ. Health Perspect. 118, 1100–1108.
Tao, L., Kannan, K., Wong, C.M., Arcaro, K.F., Butenhoff, J.L., 2008a. Perfluorinated compounds in human milk from Massachusetts. USA Environ. Sci. Technol. 42, 3096–3101.
Tao, L., Ma, J., Kunisue, T., Libelo, E.L., Tanabe, S., Kannan, K., 2008b. Perfluorinated compounds in human breast milk from several Asian countries, and in infant formula and dairy milk from the United States. Environ. Sci. Technol. 42, 8597–8602.
Upham, B.L., Deocampo, N.D., Wurl, B., Trosko, J.E., 1998. Inhibition of gap junctional intercellular communication by perfluorinated fatty acids is dependent on the chain length of the fluorinated tail. Int. J. Cancer 78, 491–495.
Van Zelm, R., Huijbregts, M., Russell, M., Jager, T., Van de Meent, D., 2008. Modeling the environmental fate of perfluorooctanoate and its precursors from global fluorotelomer acrylate polymer use. Environ. Toxicol. Chem. 27, 2216–2223.
Völkel, W., Genzel-Boroviczeny, O., Demmelmair, H., Gebauer, C., Koletzko, B., Twardella, D., Raab, U., Fromme, H., 2008. Perfluorooctane sulphonate (PFOS) and perfluorooctanoic acid (PFOA) in human breast milk: results of a pilot study. Int. J. Hyg. Environ. Health 211, 440–446.
von Ehrenstein, O.S., Fenton, S.E., Kato, K., Kuklenyik, Z., Calafat, A.M., Hines, E.P., 2009. Polyfluoroalkyl chemicals in the serum and milk of breastfeeding women. Reprod. Toxicol. 27, 239–245.
Wang, T., Wang, Y.W., Liao, C.Y., Cai, Y.Q., Jiang, G.B., 2009. Perspectives on the inclusion of perfluorooctane sulfonate into the Stockholm convention on persistent organic pollutants. Environ. Sci. Technol. 43, 5171–5175.
Wolf, C.J., Takacs, M.L., Schmid, J.E., Lau, C., Abbott, B.D., 2008. Activation of mouse and human peroxisome proliferator-activated receptor alpha by perfluoroalkyl acids of different functional groups and chain lengths. Toxicol. Sci. 106, 162–171.

Toxicology and Applied Pharmacology 263 (2012) 323–329

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

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

Comparative study on 2,2′,4,5,5′-pentachlorobiphenyl-mediated decrease in serum thyroxine level between C57BL/6 and its transthyretin-deficient mice

Yoshihisa Kato [a,*], Sekihiro Tamaki [b], Koichi Haraguchi [c], Shin-ichi Ikushiro [d], Masashi Sekimoto [b], Chiho Ohta [e], Tetsuya Endo [f], Nobuyuki Koga [e], Shizuo Yamada [b], Masakuni Degawa [b]

[a] Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Sanuki, Kagawa 769-2193, Japan
[b] School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
[c] Daiichi College of Pharmaceutical Sciences, Fukuoka 815-8511, Japan
[d] Faculty of Engineering, Toyama Prefectural University, Toyama 939-0398, Japan
[e] Faculty of Nutritional Sciences, Nakamura Gakuen University, Fukuoka 814-0198, Japan
[f] Faculty of Pharmaceutical Sciences, Health Science University of Hokkaido, Hokkaido 061-0293, Japan

ARTICLE INFO

Article history:
Received 28 April 2012
Revised 5 July 2012
Accepted 6 July 2012
Available online 16 July 2012

Keywords:
2,2′,4,5,5′-Pentachlorobiphenyl
Polychlorinated biphenyl
TTR-deficient mice
Thyroxine
Transthyretin
UDP-glucuronosyltransferase

ABSTRACT

The relationships between the changes in the levels of serum total thyroxine (T_4), serum T_4-transthyretin (TTR) complex, and accumulation of T_4 in tissues by 2,2′,4,5,5′-pentachlorobiphenyl (PentaCB) were examined using wild-type C57BL/6 (WT) and its TTR-deficient (TTR-null) mice. The constitutive level of serum total T_4 was much higher in WT mice than in TTR-null mice. In WT mice 4 days after a single intraperitoneal injection with PentaCB (112 mg/kg), serum total T_4 level was significantly decreased along with a decrease in serum T_4–TTR complex, and the levels of serum total T_4 in the PentaCB-treated WT mice were almost the same to those in PentaCB-untreated (control) TTR-null mice. In addition, a slight decrease in serum total T_4 by PentaCB treatment was observed in TTR-null mice. Furthermore, clearance of [^{125}I]T_4 from the serum after [^{125}I]T_4-administration was promoted by the PentaCB-pretreatment in either strain of mice, especially WT mice. On the other hand, accumulation level of [^{125}I]T_4 in the liver, but not in extrahepatic tissues, was strikingly enhanced in the PentaCB-pretreated WT and TTR-null mice. Furthermore, in both strains of mice, PentaCB-pretreatment led to significant increases in the steady-state distribution volume of [^{125}I]T_4 and the concentration ratio of the liver to serum. The present findings demonstrate that PentaCB-mediated decrease in serum T_4 level occurs mainly through increase in accumulation level of T_4 in the liver and further indicate that the increased accumulation of T_4 in the liver of WT mice is primarily dependent on the PentaCB-mediated inhibition of serum T_4–TTR complex formation.

Introduction

Toxicities of polychlorinated biphenyls (PCBs), such as body weight loss, impairments of reproductive and immune systems, teratogenicity, carcinogenicity and the endocrine disruption, have been intensively studied over the last 30 years (Brouwer et al., 1999; Masuda, 2009; Safe, 1990). Spectra of the toxicities are different between species of animals (McConnell, 1989; Safe, 1994), and the species difference is considered to occur through the difference in their metabolism of PCBs (Duignan et al., 1987, 1988).

Decreases in serum thyroid hormones including thyroxine (T_4) by Aroclor 1254 (Barter and Klaassen, 1994) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Schuur et al., 1997) in rats have been reported. Likewise, some of the penta- and hexa-chlorinated biphenyls are reported to decrease the level of serum thyroid hormones together with increase in the activity of hepatic drug-metabolizing enzymes in rats (Ness et al., 1993; Van Birgelen et al., 1995). We have also reported that 2,2′,4,5,5′-pentachlorobiphenyl (PentaCB) and 2,2′,3,3′,4,6′-hexachlorobiphenyl decrease the level of serum total thyroxine (T_4) in both rats and mice (Kato et al., 2001). As a possible mechanism for PCB-mediated decrease in serum thyroid hormone (T_4), increases in hepatic T_4-UDP-glucuronosyltransferases (T_4-UGTs), especially UGT1A1 and UGT1A6 (Visser, 1996), responsible for thyroid hormone metabolism have been proposed (Barter and Klaassen, 1994; Craft et al., 2002; Van Birgelen et al., 1995).

However, we have demonstrated that decreases in level of serum total T_4 in rats and mice by PCBs, including a commercial PCB Kanechlor-500 (KC500), 2,2′,4,5,5′-pentachlorobiphenyl (PentaCB), and 3,3′,4,4′,5-pentachlorobiphenyl (CB126), occur through the

Abbreviations: PCB, polychlorinated biphenyl; PentaCB, 2,2′,4,5,5′-pentachlorobiphenyl; T_4, thyroxine; T_3, triiodothyronine; TTR, transthyretin; TSH, thyroid-stimulating hormone; UGT, UDP-glucuronosyltransferase; HPLC, high-performance liquid chromatography; TBG, thyroxine binding protein; WT, wild-type.

* Corresponding author at: Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, 1314-1 Shido, Sanuki, Kagawa 769-2193, Japan. Fax: +81 87 894 01 81.
E-mail address: kato@kph.bunri-u.ac.jp (Y. Kato).

0041-008X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2012.07.004

increase in accumulation of T_4 in several tissues, especially the liver, rather than the increase in hepatic T_4-UGT activity (Kato et al., 2004, 2007, 2010a). Furthermore, we have confirmed that KC500-mediated decreases in the serum T_4 level in hamsters and guinea pigs occur mainly through increase in the accumulation (transportation from serum to tissues) of T_4 in the liver (Kato et al., 2010b). As a possible mechanism for the PCB-mediated accumulation in the liver, serum transthyretin (TTR)-related pathway is considered, because TTR plays an important role in the maintenance of serum total T_4 level (Episkopou et al., 1993) and because PCB and its hydroxylated metabolites show inhibitory effects on the formation of a serum T_4–TTR complex (Brouwer et al., 1998; Kato et al., 2004; Lans et al., 1993; Meerts et al., 2002).

In the present work, therefore, the relationships among the changes in the levels of serum total T_4, serum T_4–TTR complex, and accumulation of T_4 in tissues by PentaCB, a main component of KC500 (Haraguchi et al, 2005), were examined using wild-type [TTR(+/+)] C57BL/6 (WT) and its TTR-deficient [TTR(−/−)] (TTR-null) mice.

Materials and methods

Chemicals. 2,2′,4,5,5′-Pentachlorobiphenyl (PentaCB) was synthesized by using the Cadogan coupling reactions (Cadogan, 1962). The purity of the compound was >99% when analyzed by gas chromatography. Panacete 810 (medium-chain triglycerides) was purchased from Nippon Oils and Fats Co. Ltd. (Tokyo, Japan). The [^{125}I]T_4 (greater than 95% radiochemical pure as determined by HPLC, specific activity: 150 μCi/μg T_4), radiolabeled at the 5′-position of the outer ring, was obtained from Perkin Elmer Life and Analytical Sciences (Waltham, MA). All the other chemicals used herein were obtained commercially at the highest grade of purity.

Animal treatments. Male C57BL/6 mice (19–30 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). TTR-deficient TTR(−/−) (TTR-null) mice (19–26 g) were generated by a homologous recombination method as described previously (Episkopou et al., 1993). Male TTR-heterozygous TTR(+/−) mice were backcrossed to C57BL/6 [wild-type, TTR(+/+)] female mice for eight generations. The genotype of each pup was determined based on the presence of the mutant TTR allele by polymerase chain reaction with genomic DNA taken from the tail. Male wild-type C57BL/6 and TTR-null mice were housed three or four per cage with free access to commercial chow and tap water, maintained on a 12-h dark/light cycle (8:00 AM to 8:00 PM light) in an air-controlled room (temperature, 24.5 ± 1 °C, humidity, 55 ± 5%), and handled with animal care under the guidelines of the University of Shizuoka (Shizuoka, Japan). The administration protocol of PentaCB was determined on the basis of the data concerning dose- and time-dependent actions of PCBs obtained in our previous studies (Kato et al., 2004, 2005). Briefly, mice received a single intraperitoneal injection of PentaCB (342 μmol (112 mg)/kg) dissolved in Panacete 810 (5 ml/kg). Control animals were treated with vehicle alone (5 mg/kg).

In vivo study. Mice were killed by decapitation 4 days after the administration of PentaCB (112 mg/kg) or a vehicle alone. The liver was removed and weighed. Hepatic microsomes were prepared according to the method of Kato et al. (1995) and stored at −85 °C until use. Blood was collected from each animal between 10:30 and 11:30 AM. After clotting at room temperature, serum was separated by centrifugation and stored at −50 °C until use.

Analysis of serum hormones. Levels of total T_4, total triiodothyronine (T_3), and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay using Total T4 kit (Diagnostic Products Corporation; Los Angeles, CA), T-3 RIABEAD (Dainabot Co., Ltd, Tokyo, Japan), and the rTSH [^{125}I] Biotrak assay system (GE Healthcare UK, Ltd., Little Chalfont, Buckinghamshire, UK), respectively.

Western blot analysis. The amount of hepatic microsomal protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Western blot analyses for microsomal UGT isoforms were performed by the method of Luquita et al. (2001) using a specific antibody against rat UGT1A1 (Ikushiro et al., 1995, 1997). Mouse Ugt1a1, which corresponds to rat UGT1A1, was measured by use of an ECL detection kit (GE Healthcare UK, Ltd), and the level of Ugt1a1 protein was determined densitometrically with LAS-1000 (Fuji Photo Film. Co., Ltd., Tokyo, Japan).

Ex vivo study. At 4 days after treatment with PentaCB (112 mg/kg), the mice were anesthetized with saline solution (2 ml/kg) containing sodium pentobarbital (25 mg/ml) and potassium iodide (1 mg/ml). The femoral artery was cannulated (polyethylene tube SP8, Natsume Inc., Tokyo, Japan) and primed with heparinized saline (33 units/ml), and then animal's body was warmed to 37 °C. Fifteen minutes later, the mice received a single iv injection of 1.5 μCi [^{125}I]T_4 (0.1 ml) dissolved in saline containing 10 mM NaOH and 1% normal mouse serum.

Clearance of [^{125}I]T_4 from serum. Clearance of [^{125}I]T_4 from serum was measured according to the method of Oppenheimer et al. (1968). In brief, after the administration of [^{125}I]T_4, a portion (0.08 ml) of blood was sampled from the artery at the indicated times, and serum was prepared and stored at −50 °C until use. An aliquot (15 μl) of serum was used for determination of the level of [^{125}I]T_4 by a gamma-counter (Cobra II Auto-Gamma 5002; Perkin Elmer Life and Analytical Sciences), and the assay was performed in duplicate.

Analysis of [^{125}I]T_4 bound to serum proteins. The levels of serum [^{125}I]T_4-thyroxine binding globulin (TBG), [^{125}I]T_4–albumin, and [^{125}I]T_4–TTR complexes were determined according to the method of Davis et al. (1970). In brief, serum was diluted in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, and 30% glycerol, and the diluted serum was subjected to electrophoresis on 4–20% gradient native polyacrylamide gels PAG Mid "Daiichi" 4/20 (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). The electrophoresis was performed at 4 °C for 11 h at 20 mA in the 0.025 M Tris buffer (pH 8.4) containing 0.192 M glycine. The human albumin and TTR, which were incubated with [^{125}I]T_4, were also applied on the gel as templates. After the electrophoresis, a gel was dried and radioautographed for 20 h at room temperature using Imaging Plate 2040 (Fuji Photo Film Co., Ltd, Japan). The levels of [^{125}I]T_4–TBG, [^{125}I]T_4–albumin, and [^{125}I]T_4–TTR in serum were determined by counting the corresponding gel fractions identified from Bio Imaging Analyzer (BAS-2000II IP Reader, Fuji Photo Film Co., Ltd, Japan).

Tissue distribution of [^{125}I]T_4. Tissue distribution of [^{125}I]T_4 was performed according to the modified method of Oppenheimer et al. (1968). In brief, at 5 min after administration of [^{125}I]T_4 to PentaCB-pretreated mice, blood was sampled from abdominal aorta. Then, the cerebrum, cerebellum, pituitary gland, thyroid gland, sublingual gland, submandibular gland, thymus, heart, lung, liver, kidney, adrenal gland, spleen, pancreas, testis, prostate gland, seminal vesicle, stomach, duodenum, jejunum, ileum, caecum, brown fat, skeletal muscle, bone marrow, skin, spinal cord, and fat were removed and weighed. Radioactivities in serum and the tissues were determined by a gamma-counter (Cobra II Auto-Gamma 5002; Perkin Elmer Life and Analytical Sciences), and amounts of [^{125}I]T_4 in the tissues were calculated as ratios to the amount in serum.

Determination of PentaCB and its hydroxylated metabolites in the serum. The extraction of PentaCB and its hydroxylated metabolites from the serum was performed by the method of Haraguchi et al. (1998). The identification of these chemicals was carried out on GC/MS system (GC-17A, QP-5000, Shimadzu, Japan) with a DB-5 capillary column

(60 m×0.25 mm, i.d.). The temperature program was as follows: 100 °C, 2 min, 100–250 °C at 20 °C/min, and 250–280 °C at 2 °C/min (Mimura et al., 1999). The amounts of PentaCB and its hydroxylated PentaCB metabolites were determined with GC/ECD (GC-14A, Shimadzu, Japan) using an internal standard of 2,2′,3,4′,5,5′,6-heptachloro-4-[^{13}C] biphenylol. The quantitation limits of the hydroxylated metabolites of PentaCB were 5 ng/ml serum.

Statistics. The data obtained were statistically analyzed according to the Student's *t* test or Dunnett's test after analysis of variance. In addition, clearance of [^{125}I]T_4 from serum and the binding level of [^{125}I]T_4 bound to serum proteins were statistically analyzed according to Newman–Keuls' test after analysis of variance. The pharmacokinetic parameters of [^{125}I]T_4 were estimated with noncompartmental methods as described previously (Tabata et al., 1999).

Results

Serum hormone levels

Constitutive levels of serum total T_4 and total T_3 in TTR-null mice were 34 and 55% of the corresponding levels in WT mice, respectively (Fig. 1). In WT mice, PentaCB-treatment resulted in a marked decrease in the level of serum total T_4, while a slight decrease was observed in PentaCB-treated TTR-null mice. Furthermore, no significant changes in the levels of serum total T_3 and TSH by PentaCB were observed in either strain of mice (Fig. 1).

Hepatic T_4-UGT enzymes

The effects of PentaCB on the amount of hepatic microsomal Ugt1a1, a major T_4-UGT enzyme, in WT and TTR-null mice were examined. The amounts of Ugt1a1 protein in both strains of mice were significantly increased by PentaCB treatment (Fig. 2).

Clearance of [^{125}I]T_4 from serum

After an intravenous administration of [^{125}I]T_4 to the PentaCB-pretreated WT and TTR-null mice, their serum concentrations of [^{125}I]T_4 were measured at the indicated times (Fig. 3). Concentrations of serum [^{125}I]T_4 in PentaCB-untreated (control) TTR-null mice 5–120 min after the [^{125}I]T_4-administration were 57–65% of those in the corresponding WT mice.

PentaCB-pretreatment clearly enhanced the clearance of [^{125}I]T_4 from the serum in WT mice. Within 5 min after the administration, concentration of serum [^{125}I]T_4 in PentaCB-pretreated WT mice was about 60% of the control level, and the significant decreases maintained up to 120 min later. The level at 5 min was almost the same as that of control TTR-null mice, and the time-dependent decrease pattern in WT mice during 5–120 min was similar to that in TTR-null mice. On the other hand, significant decreases in the concentration of serum [^{125}I]T_4 in PentaCB-pretreated TTR-null mice were observed only at 5–30 min.

The serum pharmacokinetic parameters of the [^{125}I]T_4 estimated from the data (Fig. 3) were summarized in Table 1. The mean total body clearance of [^{125}I]T_4 and steady-state volume of distribution in TTR-null mice were higher than those in WT mice. Significant increase in the mean total body clearance of [^{125}I]T_4 by the PentaCB-pretreatment was observed in either WT or TTR-null mice. Likewise, the steady-state volumes of distribution in the PentaCB-pretreated WT and TTR-null mice were increased to 2.1 and 1.3-fold over the corresponding control mice, respectively.

Serum proteins bound to [^{125}I]T_4

The effects of pretreatment with PentaCB on the binding of [^{125}I]T_4 to serum proteins, such as TBG, albumin, and/or TTR, were examined in WT and TTR-null mice (Fig. 4). The levels of [^{125}I]T_4 bound to TBG and albumin in PentaCB-untreated (control) TTR-null mice were significantly higher than those in control WT mice. In WT mice, PentaCB-pretreatment resulted in not only decrease in the level of [^{125}I]T_4–TTR complex but also increase in the level of [^{125}I]T_4–albumin complex at all the times examined. Significant increase in [^{125}I]T_4–TBG complex

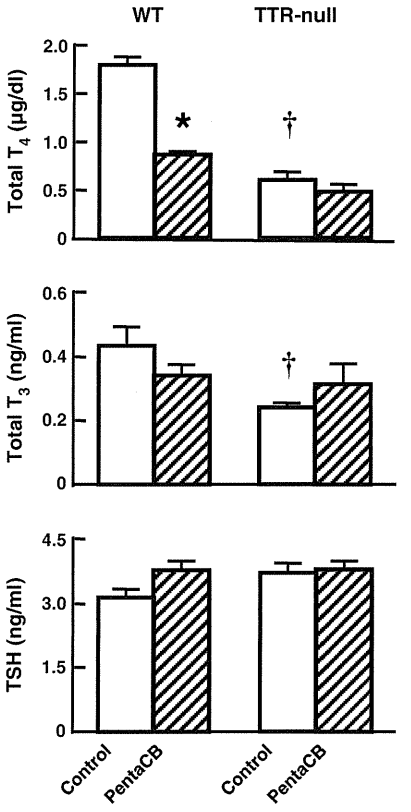

Fig. 1. Effects of PentaCB on the levels of serum total T_4, total T_3, and TSH in WT and TTR-null mice. Animals were killed 4 days after administration of PentaCB (112 mg/kg), and levels of serum thyroid hormones were measured, as described in Materials and methods. Each column represents the mean±S.E. (vertical bar) for five to six animals. *P<0.05, significantly different from each control. †P<0.05, significantly different from control WT mice.

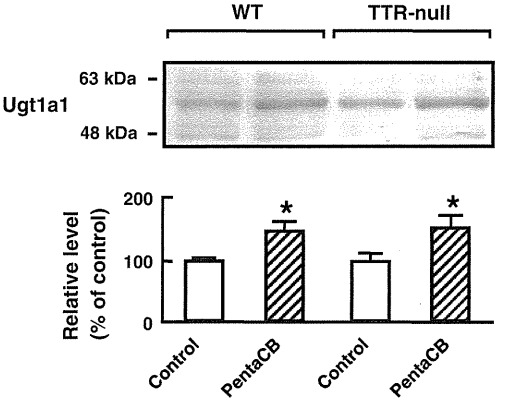

Fig. 2. Effects of PentaCB on the levels of hepatic microsomal Ugt1a1 in WT and TTR-null mice. The bands corresponding to Ugt1a1 in Western blot profiles were densitometrically quantified, as described in Materials and methods. The data are represented as the mean±SE (vertical bar) for four animals. *P<0.05, significantly different from each control.

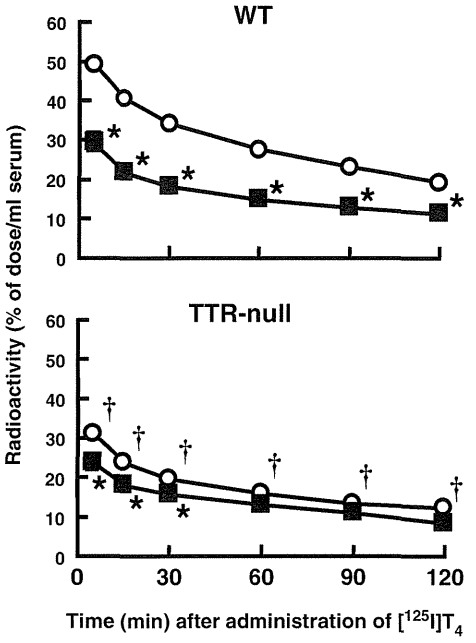

Fig. 3. Effects of PentaCB pretreatment on the clearance of [^{125}I]T_4 from serum in WT and TTR-null mice. A portion of [^{125}I]T_4 (15 μCi/ml) was intravenously administered to mice pretreated with PentaCB or vehicle alone (control). The amounts of serum [^{125}I]T_4 were measured at the indicated times after the [^{125}I]T_4 administration. Each point represents the mean ± S.E. (vertical bars) for five to six animals. *P<0.01, significantly different from each control. †P<0.001, significantly different from control WT mice. —○—, control; —■—, PentaCB.

was also observed at 120 min. In PentaCB-pretreated TTR-null mice, the increase in the level of [^{125}I]T_4–albumin and the decrease in the level of TBG complexes were observed only at 120 min.

Tissue distribution of [^{125}I]T_4

Effects of PentaCB-pretreatment on the tissue-to-serum concentration ratio (Kp value) and the distribution level of [^{125}I]T_4 in various tissues after the administration of [^{125}I]T_4 were examined in WT and TTR-null mice. In PentaCB-untreated (control) mice, Kp values of the several tissues examined were higher in the TTR-null mice than those of the corresponding tissues in WT mice (Fig. 5). In addition, in either strain of mice, the Kp value was the highest in the liver among the tissues examined. Hepatic Kp values in control WT and TTR-null mice were 0.47 and 1.20, respectively.

PentaCB-pretreatment resulted in significant increases in Kp values in the various tissues, especially the liver, in either strain of mice. Hepatic Kp values in the PentaCB-pretreated WT and TTR-null mice became 1.6 and 2.0, respectively (Fig. 5), and the hepatic accumulation level of

Table 1
Pharmacokinetic parameters for [^{125}I]T_4 after administration of [^{125}I]T_4 to PentaCB-pretreated mice.

Mice	Pretreatment	Mean total body clearance × 100 (ml/min)	Distribution volume (ml)
WT	Control	1.51 ± 0.08	2.36 ± 0.05
	PentaCB	2.17 ± 0.21*	4.86 ± 0.45*
TTR-null	Control	2.32 ± 0.07†	3.92 ± 0.23†
	PentaCB	3.17 ± 0.29*	5.15 ± 0.45*

The pharmacokinetic parameters for [^{125}I]T_4 were calculated from the data in Fig. 3 by the noncompartmental methods as described previously (Tabata et al., 1999). The values shown are expressed as the mean ± S.E. for five to six mice.
* Significant differences from the strain-matched control: P<0.05.
† Significant differences between control WT and TTR-null mice: P<0.001.

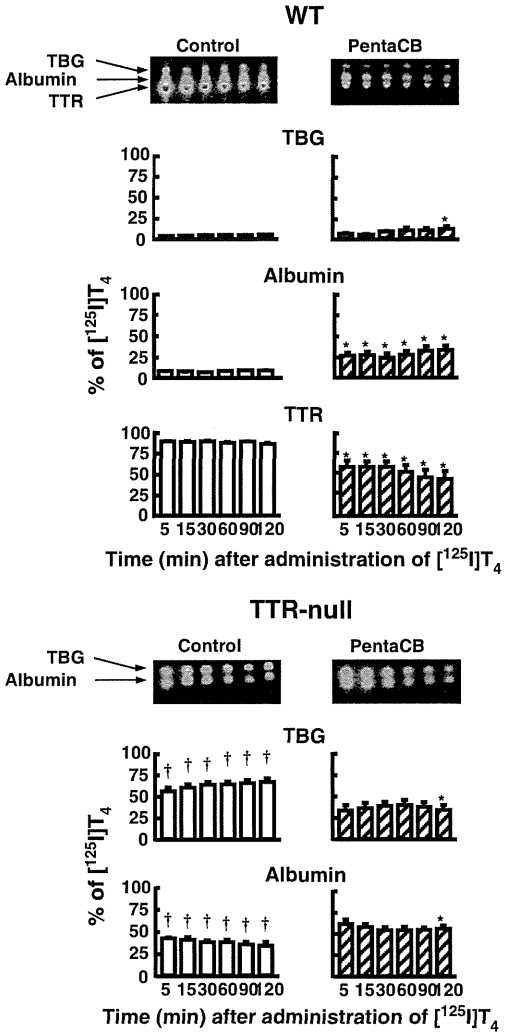

Fig. 4. Effects of PentaCB on the amounts of the [^{125}I]T_4 bound to serum proteins in WT and TTR-null mice. The amounts of the [^{125}I]T_4 bound to serum proteins 5 min after [^{125}I]T_4-administration were assessed by the method, as described in Materials and methods. Each column represents the mean ± S.E. (vertical bar) for four to six animals. *P<0.05, significantly different from each control. †P<0.05, significantly different from control WT mice.

[^{125}I]T_4 in TTR-null mice was 1.4-times higher than that in WT mice (Fig. 6).

Consequently, more than 52% and 55% of the [^{125}I]T_4 dose was accumulated in the liver of the PentaCB-pretreated WT and TTR-null mice, respectively (Fig. 6). The accumulation levels per gram of the liver in the PentaCB-pretreated WT and TTR-null mice significantly increased, as compared with those in the corresponding control mice (Table 2). In addition, no significant increases in the liver weight (Table 3) and in the accumulation level of T_4 in extrahepatic tissues by PentaCB (Fig. 6) were observed in either WT or TTR-null mice.

Hydroxylated PCB metabolites in serum

PentaCB was administered to WT and TTR-null mice, and 4 days after the administration, PentaCB and its hydroxylated metabolites in each serum were analyzed (Table 4). Serum concentrations of PentaCB were 0.258 and 0.366 μg/ml in the WT and TTR-null mice, respectively. In both strains of mice, two mono-hydroxylated metabolites (3-OH-PentaCB and 4-OH-PentaCB) were detected. One di-hydroxylated metabolite,

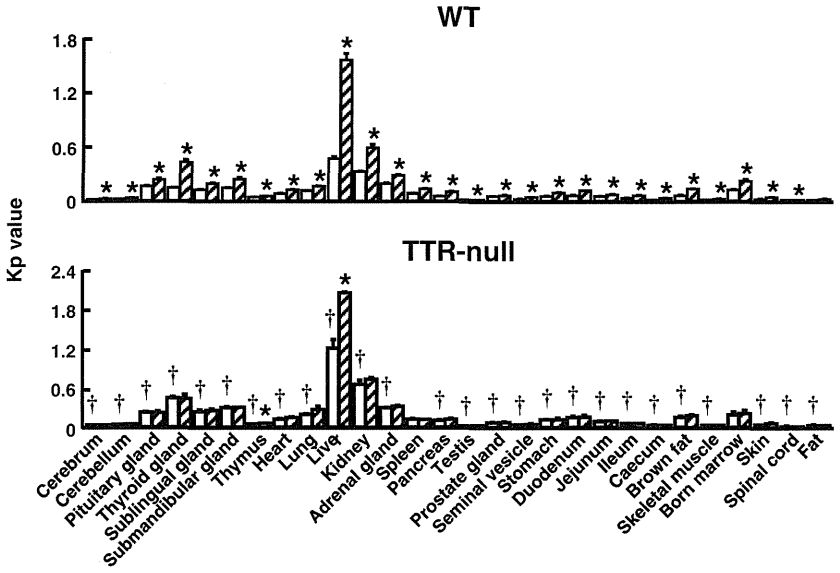

Fig. 5. Effects of PentaCB pretreatment on the tissue-to-serum concentration ratios (Kp values) of [^{125}I]T_4. A portion of [^{125}I]T_4 (15 μCi/ml) was intravenously administered to the mice pretreated with PentaCB or vehicle alone (control), and at 5 min after the [^{125}I]T_4 administration, the radioactivity in each tissue was measured. Each column represents the mean ± S.E. (vertical bars) for four to six animals. *P<0.05, significantly different from each control. †P<0.05, significantly different from control WT mice. □, control; ▨, PentaCB-pretreatment.

3′,4′-$(OH)_2$-PentaCB, was detected in WT mice, but not in TTR-null mice (Table 4). The amounts of the total hydroxylated metabolites of PentaCB in WT and TTR-null mice were almost the same.

Discussion

In the present study, we found that PentaCB-treatment resulted in a drastic decrease in the levels of serum total T_4 in WT mice, but only a slight decrease in TTR-null mice. On the other hand, PentaCB-treatment was demonstrated to significantly enhance hepatic accumulation of T_4 in both strains of mice.

As a cause for the PentaCB-mediated decrease in the level of serum total T_4 in WT mice, the competitive inhibition of a T_4–TTR complex formation by the hydroxylated PentaCB metabolites is considered, because the hydroxylated metabolites of PCBs are reported to show high affinities to TTR (Brouwer et al., 1998; Kato et al., 2004; Lans et al., 1993; Meerts et al., 2002). Incidentally, in the present study, the production of the mono- and/or di-hydroxylated PentaCB metabolites was demonstrated in PentaCB-treated mice. Since di-hydroxylated PCBs show much higher affinity for TTR than mono-hydroxylated PCBs (Lans et al., 1993), PentaCB-mediated inhibition of a T_4–TTR complex formation in WT mice might be mainly dependent on the formation of 3,4-dihydroxylated

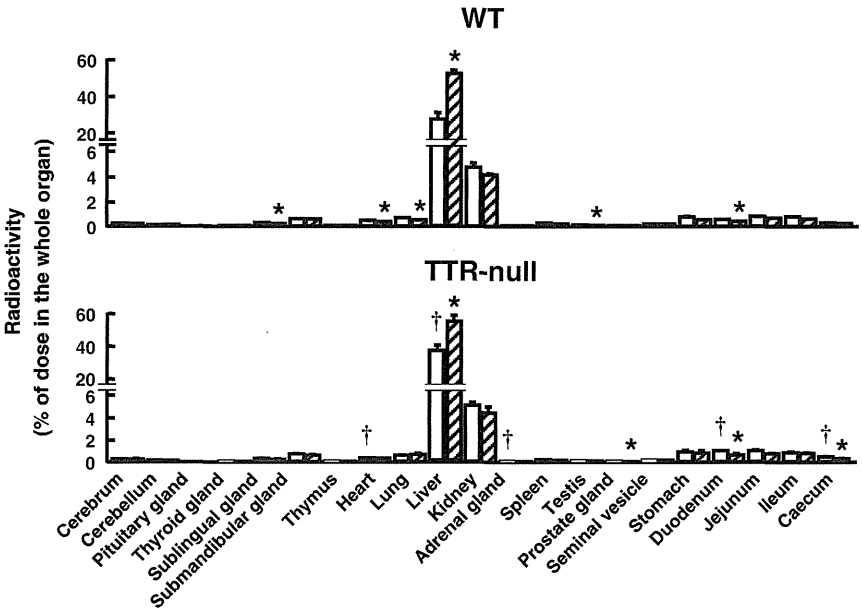

Fig. 6. Tissue distribution of [^{125}I]T_4 after the administration of [^{125}I]T_4 to PentaCB-pretreated WT and TTR-null mice. Four days after treatment with PentaCB (112 mg/kg), [^{125}I]T_4 was administered to the mice, and after 5 min of the [^{125}I]T_4 administration, the radioactivity in each tissue was measured, as described in Materials and methods. Each column represents the mean ± S.E. (vertical bar) for four to six animals. *P<0.05, significantly different from each control. †P<0.05, significantly different from control WT mice. □, control; ▨, PentaCB-pretreatment.

Table 2
Accumulation of [^{125}I]T_4 in the liver of PentaCB-pretreated mice.

Pretreatment	[^{125}I]T_4 (% of dose/g liver)	
	WT	TTR-null
Control	22.33 ± 0.38	33.01 ± 0.54†
PentaCB	36.47 ± 1.89*	43.07 ± 2.55*

The radioactivity in the liver was measured at 5 min after the [^{125}I]T_4-administration, as described in Materials and methods. The values shown are expressed as the mean ± S.E. for four to six animals.
* Significant differences from the strain-matched control: $P<0.05$.
† Significant difference from the corresponding WT mice: $P<0.001$.

Table 4
Serum concentrations of PentaCB and its hydroxylated metabolites in the PentaCB-treated WT and TTR-null mice.

Animal	Concentration (μg/ml serum)			
	PentaCB	3-OH-PentaCB	4-OH-PentaCB	3′,4′-$(OH)_2$-PentaCB
WT	0.258 ± 0.015	0.150 ± 0.019	0.022 ± 0.006	0.156 ± 0.019
TTR-null	0.366 ± 0.021*	0.265 ± 0.036*	0.007 ± 0.004	ND

Animals were killed 4 days after administration of PentaCB (112 mg/kg), and serum concentrations of PentaCB and its hydroxylated metabolites were measured, as described in Materials and methods. The values shown are expressed as the mean ± S.E. for four to six mice.
* $P<0.05$, significant differences from the corresponding WT mice. ND: not detected.

PentaCB. In addition, an affinity of PentaCB itself to TTR is reported to be lower than natural T_4 (Chauhan et al., 2000). Furthermore, since the hydroxylated metabolites of PCBs have been detected in the PCB-exposed human serum (Hovander et al., 2006; Park et al., 2007), the results obtained in this study strongly suggest that disruption of serum total T_4 level in PCB-exposed humans occurs through the hydroxylated metabolite-mediated inhibition of a formation of T_4–TTR complex in the serum.

The constitutive level of serum total T_4 was herein demonstrated to be lower in TTR-null mice than in WT mice, confirming that serum TTR plays an important role in the maintenance of serum T_4 level through forming a T_4–TTR complex (Episkopou et al., 1993). Treatment of WT mice with PentaCB led to not only a remarked decrease in the level of a serum T_4–TTR complex but also significant increases in levels of the serum T_4–albumin and TBG complexes, whereas no such PentaCB-mediated changes were observed in TTR-null mice. Considering the PentaCB-mediated decrease in the level of serum total T_4 and increase in the accumulation level of hepatic T_4 in both WT and TTR-null mice, free T_4 and the T_4 bound to albumin and/or TBG seem to be efficiently incorporated into the liver as compared with the T_4 bound to TTR.

It has been proposed that PCB-mediated decrease in the level of serum T_4 occurs, at least in part, in a hepatic T_4-UGT-dependent fashion (Barter and Klaassen, 1994; Schuur et al., 1997; Van Birgelen et al., 1995). However, we have previously demonstrated that the PentaCB- or KC500-mediated decreases in the level of serum total T_4 occurred in both Wistar and Gunn (UGT1A-deficient Wistar) rats (Kato et al., 2004, 2007). Furthermore, in the present study, PentaCB-mediated increase in the accumulation of T_4 in the liver was observed in either WT or TTR-null mice, despite the level of hepatic Ugt1a1, one of the representative T_4-UGT enzymes (Visser, 1996), was increased by PentaCB. Accordingly, the PentaCB-mediated increase in hepatic accumulation of T_4 is thought to occur mainly through the inhibition of efflux of T_4 and/or the promotion of influx of T_4 into hepatic cells.

Distribution analyses of [^{125}I]T_4 in the serum and several tissues at 5 min after the [^{125}I]T_4 administration to PentaCB-pretreated mice indicated that a mean total body clearance of [^{125}I]T_4 was clearly promoted by PentaCB-pretreatment in WT mice, but slightly in TTR-null mice. The clearance pattern of serum [^{125}I]T_4 after 5–120 min in the PentaCB-pretreated WT mice was almost same as that in PentaCB-untreated (control) TTR-null mice. On the other hand, a tissue-to-serum concentration ratio (Kp value) on the distribution of [^{125}I]T_4 was increased in several tissues, especially the liver, by PentaCB-pretreatment in both WT and TTR-null mice. Consequently, more than 52%–55% of the [^{125}I]T_4 dose was accumulated in the liver of the PentaCB-pretreated WT and TTR-null mice, although no PentaCB-mediated development of liver hypertrophy occurred in either strain of mice. In addition, PentaCB-treatment led to no significant change in the level of serum TSH, one of the factors regulating the level of serum total T_4 (Capen 1997; McClain 1989; Saito et al., 1991), in either strain of mice. These results are identified with those in previous reports concerning the effects of PCB on the level of serum TSH in rats and mice (Hallgren et al., 2001; Hood et al., 1999; Kato et al., 2004, 2007, 2010a; Liu et al., 1995).

In conclusion, the present findings indicate that PentaCB-mediated decrease in serum T_4 level in mice is mainly dependent on the increase in hepatic accumulation (promotion of the transportation from serum to liver and/or inhibition of the efflux from hepatic cells) of T_4 and strongly suggest that in WT mice, PentaCB-mediated decrease in serum T_4 occurs, at least in part, through the inhibition of a T_4–TTR complex formation by the hydroxylated metabolites of PentaCB. The present findings also indicate that the PentaCB-mediated decrease primarily occurs in a T_4-UGT-independent manner. In addition, the clearance profile of serum [^{125}I]T_4 in the PentaCB-pretreated WT mice was almost the same as that in control TTR-null mice, conforming that serum TTR plays an important role in the maintenance of serum T_4 level. Furthermore, the serum level of total hydroxylated PCB metabolites herein found in the PentaCB-treated mice was about 400-fold higher than a previously reported level in Yusho patients (Masuda, 2009). Accordingly, contribution of the hydroxylated PCB metabolites to disruption of serum T_4 level in Yusho patients is not necessarily clear. However, the previous report (Feldt-Rasmussen and Rasmussen, 2007) and the present findings that constitutive level of serum T_4 in humans, as well as TTR-null mice, primarily maintains a T_4–TBG complex (75% of total T_4-serum protein complexes in humans), but not a T_4–TTR complex, suggest that the decreased level of serum T_4 in PCB-exposed humans (Koopman-Esseboom et al., 1994; Persky et al., 2001) is, at least in part, attributed to the PCB/its hydroxylated metabolite-mediated promotion of the accumulation of T_4 in several tissues, especially the liver.

Table 3
Changes in liver weight after administration of PentaCB to mice.

Pretreatment	Relative liver weight (% of body weight)	
Control	5.23 ± 0.38	5.09 ± 0.10
PentaCB	5.88 ± 0.18	5.53 ± 0.31

Mice were killed 4 days after administration of PentaCB (112 mg/kg). The body and liver were weighted, and the relative liver weight (% of body weight) was calculated. The values shown are expressed as the mean ± S.E. for four to six mice.

Funding

This work was supported in part by the Grant-in-Aid for Scientific Research (C) (no. 23510083, Y.K.) from Japan Society for the Promotion of Science.

Conflict of interest statement

The authors do not have any conflict of interest.

Acknowledgments

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

References

Barter, R.A., Klaassen, C.D., 1994. Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. Toxicol. Appl. Pharmacol. 128, 9–17.

Brouwer, A., Morse, D.C., Lans, M.C., Schuur, A.G., Murk, A.J., Klasson-Wehler, E., Bergman, Å., Visser, T.J., 1998. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. Toxicol. Ind. Health 14, 59–84.

Brouwer, A., Longnecker, M.P., Birnbaum, L.S., Cogliano, J., Kostyniak, P., Moore, J., Schantz, S., Winneke, G., 1999. Characterization of potential endocrine-related health effects at low-dose levels of exposure to PCBs. Environ. Health Perspect. 107, 639–649.

Cadogan, J.I.G., 1962. A convenient new method of aromatic arylation. J. Chem. Soc. 4257–4258.

Capen, C.C., 1997. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol. Pathol. 25, 39–48.

Chauhan, K.R., Kodavanti, P.R.S., McKinney, J.D., 2000. Assessing the role of *ortho*-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. Toxicol. Appl. Pharmacol. 162, 10–21.

Craft, E.S., DeVito, M.J., Crofton, K.M., 2002. Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. Toxicol. Sci. 68, 372–380.

Davis, P.J., Spaulding, S.W., Gregerman, R.I., 1970. The three thyroxine-binding proteins in rat serum: binding capacities and effects of binding inhibitors. Endocrinology 87, 978–986.

Duignan, D.B., Sipes, I.G., Leonard, T.B., Halpert, J.R., 1987. Purification and characterization of the dog hepatic cytochrome P450 isozyme responsible for the metabolism of 2,2′,4,4′,5,5′-hexachlorobiphenyl. Arch. Biochem. Biophys. 255, 290–303.

Duignan, D.B., Sipes, I.G., Ciaccio, P.J., Halpert, J.R., 1988. The metabolism of xenobiotics and endogenous compounds by the constitutive dog liver cytochrome P450 PBD-2. Arch. Biochem. Biophys. 267, 294–304.

Episkopou, V., Maeda, S., Nishiguchi, S., Shimada, K., Gaitanaris, G.A., Gottesman, M.E., Robertson, E.J., 1993. Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. Proc. Natl. Acad. Sci. U. S. A. 90, 2375–2379.

Feldt-Rasmussen, U., Rasmussen, Å.K., 2007. Diseases of the thyroid in childhood and adolescence. In: Krassas, G.E., Rivkees, S.A., Kiess, W. (Eds.), Thyroid Hormone Transport and Actions. : Pediatr Adolesc Med, vol. 11. Karger, Basel, pp. 80–103.

Hallgren, S., Sinjari, T., Håkansson, H., Darnerud, P.O., 2001. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. Arch. Toxicol. 75, 200–208.

Haraguchi, K., Kato, Y., Kimura, R., Masuda, Y., 1998. Hydroxylation and methylthiolation of mono-*ortho*-substituted polychlorinated biphenyls in rats: identification of metabolites with tissue affinity. Chem. Res. Toxicol. 11, 1508–1515.

Haraguchi, K., Koga, N., Kato, Y., 2005. Comparative metabolism of polychlorinated biphenyls and tissue distribution of persistent metabolites in rats, hamsters, and guinea pigs. Drug Metab. Dispos. 33, 373–380.

Hood, A., Hashmi, R., Klaassen, C.D., 1999. Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. Toxicol. Appl. Pharmacol. 160, 163–170.

Hovander, L., Linderholm, L., Athanasiadou, M., Athanassiadis, I., Bignert, A., Fängström, B., Kocan, A., Petrik, J., Trnovec, T., Bergman, Å., 2006. Levels of PCBs and their metabolites in the serum of residents of a highly contaminated area in Eastern Slovakia. Environ. Sci. Technol. 40, 3696–3703.

Ikushiro, S., Emi, Y., Iyanagi, T., 1995. Identification and analysis of drug-responsive expression of UDP-glucuronosyltransferase family 1 (UGT1) isozyme in rat hepatic microsomes using anti-peptide antibodies. Arch. Biochem. Biophys. 324, 267–272.

Ikushiro, S., Emi, Y., Iyanagi, T., 1997. Protein–protein interactions between UDP-glucuronosyltransferase isozymes in rat hepatic microsomes. Biochemistry 36, 7154–7161.

Kato, Y., Haraguchi, K., Kawashima, M., Yamada, S., Masuda, Y., Kimura, R., 1995. Induction of hepatic microsomal drug-metabolizing enzymes by methylsulphonyl metabolites of polychlorinated biphenyl congeners in rats. Chem. Biol. Interact. 95, 257–268.

Kato, Y., Yamazaki, T., Haraguchi, K., Ito, Y., Nemoto, K., Masuda, Y., Degawa, M., Kimura, R., 2001. Effects of 2,2′,4,5,5′-pentachlorobiphenyl and 2,2′,3,3′,4,6′-hexachlorobiphenyl on serum hormone levels in rats and mice. Organohalogen Compd. 53, 44–46.

Kato, Y., Ikushiro, S., Haraguchi, K., Yamazaki, T., Ito, Y., Suzuki, H., Kimura, R., Yamada, S., Inoue, T., Degawa, M., 2004. A possible mechanism for decrease in serum thyroxine level by polychlorinated biphenyls in Wistar and Gunn rats. Toxicol. Sci. 81, 309–315.

Kato, Y., Haraguchi, K., Yamazaki, T., Kimura, R., Koga, N., Yamada, S., Degawa, M., 2005. The decrease in level of serum thyroxine by 2,2′,4,5,5′-pentachlorobiphenyl in rats and mice: no correlation with formation of methylsulfonyl metabolites. Drug Metab. Dispos. 33, 1661–1665.

Kato, Y., Ikushiro, S., Takiguchi, R., Haraguchi, K., Koga, N., Uchida, S., Sakaki, T., Yamada, S., Kanno, J., Degawa, M., 2007. A novel mechanism for polychlorinated biphenyl-induced decrease in serum thyroxine level in rats. Drug Metab. Dispos. 35, 1949–1955.

Kato, Y., Haraguchi, K., Kubota, M., Seto, Y., Okura, T., Ikushiro, S., Koga, N., Yamada, S., Degawa, M., 2010a. A possible mechanism for the decrease in serum thyroxine level by a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like polychlorinated biphenyl congener, 3,3′,4,4′,5-pentachlorobiphenyl in mice. Drug Metab. Dispos. 38, 150–156.

Kato, Y., Haraguchi, K., Ito, Y., Fujii, A., Yamazaki, T., Endo, T., Koga, N., Yamada, S., Degawa, M., 2010b. Polychlorinated biphenyl-mediated decrease in serum thyroxine level in rodents. Drug Metab. Dispos. 38, 697–704.

Koopman-Esseboom, C., Morse, D.C., Weisglas-Kuperus, N., Lutkeschipholt, I.J., van der Paauw, C.G., Tuinstra, L.G., Brouwer, A., Sauer, P.J., 1994. Effects of dioxins and polychlorinated biphenyls on thyroid hormone status of pregnant women and their infants. Pediatr. Res. 36, 468–473.

Lans, M.C., Klasson-Wehler, E., Willemsen, M., Meussen, E., Safe, S., Brouwer, A., 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-*p*-dioxins and -dibenzofurans with human transthyretin. Chem. Biol. Interact. 88, 7–21.

Liu, J., Liu, Y., Barter, R.A., Klaassen, C.D., 1995. Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. J. Pharmacol. Exp. Ther. 273, 977–985.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Luquita, M.G., Catania, V.A., Pozzi, E.J.S., Veggi, L.M., Hoffman, T., Pellegrino, J.M., Ikushiro, S., Emi, Y., Iyanagi, T., Vore, M., Mottino, A.D., 2001. Molecular basis of perinatal changes in UDP-glucuronosyltransferase activity in maternal rat liver. J. Pharmacol. Exp. Ther. 298, 49–56.

Masuda, Y., 2009. Toxic effects of PCB/PCDF to human observed in Yusho and other poisonings. Fukuoka Acta Medica. 100, 141–155.

McClain, R.M., 1989. The significance of hepatic microsomal enzyme induction and altered thyroid function in rats: implications for thyroid gland neoplasia. Toxicol. Pathol. 17, 294–306.

McConnell, E.E., 1989. Acute and chronic toxicity and carcinogenesis in animals, Chapter 6, In: Kimbrough, R.D., Jensen, A.A. (Eds.), Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products, 2nd ed. Elsevier Science Publishers, Amsterdam, pp. 161–193.

Meerts, I.A.T.M., Assink, Y., Cenijn, P.H., van den Berg, J.H.J., Weijers, B.M., Bergman, Å., Koeman, J.H., Brouwer, A., 2002. Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat. Toxicol. Sci. 68, 361–371.

Mimura, K., Tamura, M., Haraguchi, K., Masuda, Y., 1999. Analysis of 209 PCB congeners by high separation gas chromatography/low resolution mass spectrometer. Fukuoka Acta Med. 90, 192–201.

Ness, D.K., Schantz, S.L., Moshtaghian, J., Hansen, L.G., 1993. Effects of perinatal exposure to specific PCB congeners on thyroid hormone concentrations and thyroid histology in the rat. Toxicol. Lett. 68, 311–323.

Oppenheimer, J.H., Bernstein, G., Surks, M.I., 1968. Increased thyroxine turnover and thyroidal function after stimulation of hepatocellular binding of thyroxine by phenobarbital. J. Clin. Invest. 47, 1399–1406.

Park, J.S., Linderholm, L., Charles, M.J., Athanasiadou, M., Petrik, J., Kocan, A., Drobna, B., Trnovec, T., Bergman, Å., Hertz-Picciotto, I., 2007. Polychlorinated biphenyls and their hydroxylated metabolites (OH-PCBS) in pregnant women from eastern Slovakia. Environ. Health Perspect. 115, 20–27.

Persky, V., Turyk, M., Anderson, H.A., Hanrahan, L.P., Falk, C., Steenport, D.N., Chatterton Jr., R., Freels, S., Great Lakes Consortium, 2001. The effects of PCB exposure and fish consumption on endogenous hormones. Environ. Health Perspect. 109, 1275–1283.

Safe, S.H., 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit. Rev. Toxicol. 21, 51–88.

Safe, S.H., 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. Crit. Rev. Toxicol. 24, 87–149.

Saito, K., Kaneko, H., Sato, K., Yoshitake, A., Yamada, H., 1991. Hepatic UDP-glucuronyltransferase(s) activity toward thyroid hormones in rats: induction and effects on serum thyroid hormone levels following treatment with various enzyme inducers. Toxicol. Appl. Pharmacol. 111, 99–106.

Schuur, A.G., Boekhorst, F.M., Brouwer, A., Visser, T.J., 1997. Extrathyroidal effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on thyroid hormone turnover in male Sprague–Dawley rats. Endocrinology 138, 3727–3734.

Tabata, K., Yamaoka, K., Kaibara, A., Suzuki, S., Terakawa, M., Hata, T., 1999. Moment analysis program available on Microsoft Excel®. Xenobio. Metabol. Dispos. 14, 286–293.

Van Birgelen, A.P.J.M., Smit, E.A., Kampen, I.M., Groeneveld, C.N., Fase, K.M., van der Kolk, J., Poiger, H., van den Berg, M., Koeman, J.H., Brouwer, A., 1995. Subchronic effects of 2,3,7,8-TCDD or PCBs on thyroid hormone metabolism: use in risk assessment. Eur. J. Pharmacol. 293, 77–85.

Visser, T.J., 1996. Pathways of thyroid hormone metabolism. Acta Med. Austriaca 23, 10–16.

Ecotoxicology and Environmental Safety 79 (2012) 35–41

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

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

Highlighted Article

Stable isotope ratios and mercury levels in red meat products from baleen whales sold in Japanese markets

Tetsuya Endo [a,*], Yohei Hotta [a], Yohsuke Hisamichi [a], Osamu Kimura [a], Rie Sato [b], Koichi Haraguchi [c], Naoko Funahashi [d], C. Scott Baker [e]

[a] Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, 1757, Ishikari-Tobetsu, Hokkaido 061-0293, Japan
[b] SI Science Co. Ltd., 473-3 Hongou, Sugito-machi, Kitakatsushika, Saitama 345-0023, Japan
[c] Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-Cho, Minami-Ku, Fukuoka 815-8511, Japan
[d] International Fund for Animal Welfare, NishiShinjuku Well BLDG 6F, 5-24-16 NishiShinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
[e] Marine Mammal Program and Department of Fisheries and Wildlife, Oregon State University, Newport, Oregon 97365, USA

ARTICLE INFO

Article history:
Received 7 October 2011
Received in revised form
25 January 2012
Accepted 29 January 2012
Available online 23 February 2012

Keywords:
Stable isotope ratio
Mercury
Minke whale
Bryde's whale
Sei whale
Fin whale

ABSTRACT

We analyzed the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values and Hg concentration in red meat products originating from the predominant types sold in Japan for human consumption: two populations of common minke (J- and O-types), Bryde's and sei whales in the western North Pacific Ocean, and fin and Antarctic minke whales in the Southern Ocean. The order of the trophic positions, evaluated by $\delta^{15}N$ values and Hg concentrations, coincided with their known feeding habits: common minke (J-type)=common minke (O-type) > Bryde's ≥ sei ≥ Antarctic minke ≥ fin. The Hg concentrations in the combined samples from the six samples were significantly correlated with their $\delta^{15}N$ values ($\gamma=0.455$, $n=66$, $p<0.05$), reflecting overall differences in the trophic level. This correlation was not significant for within-species comparison for the common minke (J- and O-types) or the Bryde's whale, probably reflecting the higher $\delta^{15}N$ value and lower Hg concentration in the North Pacific Ocean around Japan. Determination of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ could be used to discriminate between the red meat products originating from the whale species in the North Pacific and Southern Oceans. However, the four whale species or populations in the Pacific Ocean could not be discriminated on basis of these values, nor could the two species in the Southern Ocean. Positive correlations between the $\delta^{13}C$ and $\delta^{15}N$ values and negative correlations between the $\delta^{15}N$ and $\delta^{18}O$ values and the $\delta^{13}C$ and $\delta^{18}O$ values, probably reflecting migration patterns, were found in some whale species in the North Pacific and Southern Oceans.

1. Introduction

Products from whales, dolphins and porpoises (Suborder Cetacea) are sold in Japan for human consumption. Currently, most whale products for human consumption are supplied from the scientific whaling of baleen whales, small-type coastal whaling of toothed whales, and the drive and hand-harpoon fishing of small whales, dolphins and porpoises as well as incidental catch by set nets (Endo et al., 2003). Red meat (muscle) products are the most popular whale products sold in Japan, and most Japanese consumers prefer the red meat originating from mysticetes (baleen whales) to that from odontocetes (toothed whales, dolphins and porpoises). Most red meats from mysticetes sold in Japan originate from the Antarctic minke whale (*Balaenoptera bonaerensis*) and fin whale (*Balaenoptera physalus*) taken in the Southern Ocean and the common minke whale (*Balaenoptera acutorostrata*), Bryde's whale (*Balaenoptera edeni*) and sei whale (*Balaenoptera borealis*) taken in the western North Pacific Ocean. Common minke whales can be categorized into at least two types: the "O type", found primarily in the offshore Pacific Ocean, and the "J type", found primarily in the Sea of Japan and nearshore waters along Japan's Pacific coast (Wade et al., 2010). O-type minke whales are the primary target of Japanese scientific whaling in both coastal and offshore waters of the Pacific, while J-type minke whales are primarily taken as bycatch in coastal set nets around the entire Japanese coastline. Although most baleen whales are assumed to migrate annually between feeding habitat in high latitudes and breeding habitat in low latitudes, the pattern of migration is poorly known for some of the species sampled here.

As odontocetes are long-lived and occupy the top levels of the marine food web, feeding mainly on fish and squid, they biomagnify marine pollutants such as heavy metals and organochlorine compounds (Haraguchi et al., 2000). Among these pollutants,

* Corresponding author. Fax: +81 133 23 3902.
E-mail address: endotty@hoku-iryo-u.ac.jp (T. Endo).

0147-6513/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2012.01.020

contamination with mercury (Hg) is prominent (Endo et al., 2003, 2004, 2005). The contamination levels of pollutants in mysticetes are lower than those in odontocetes, reflecting their preference for plankton and small fish species (i.e., their lower trophic positions). Among the baleen whale species sold in Japan, common minke whales are opportunistic and omnivorous feeders that change their prey temporally and regionally. Compared with common minke whales, Bryde's and sei whales are only moderately omnivorous feeders (Mitani and Bando, 2008; Yasunaga and Fujise, 2009a, b), and Antarctic minke and fin whales are generally zooplankton feeders. In our previous survey of Hg levels (Endo et al., 2003), only one of the 62 red meat products originating from mysticetes showed a Hg concentration exceeding the Japanese permitted level for fish and shellfish (0.4 μg/wet g), whereas all red meat products originating from odontocetes (n=137) exceeded the permitted level.

Stable isotope analysis has been used as a tool to obtain information on the feeding ecology of marine species. The $\delta^{15}N$ value shows a stepwise increase in the trophic level of a food chain (Kelly, 2000), and a positive correlation between the $\delta^{15}N$ value and the Hg concentration in biota has been reported (Yoshinaga et al., 1992; Kidd et al., 1995). On the other hand, the $\delta^{13}C$ value is used to indicate the relative contribution to the diet of potential primary sources, and can demonstrate differences between species taking coastal and offshore prey or between those taking pelagic and benthic prey (Kelly, 2000). A significant increase in $\delta^{15}N$ of 3.4 ± 1.1‰ has been shown to occur between consumer and prey (Minagawa and Wada, 1984), whereas only a small enrichment of about 1‰ is found in the $\delta^{13}C$ value (DeNiro and Epstein, 1981). We recently reported that the $\delta^{15}N$ and $\delta^{13}C$ values in odontocetes caught off or stranded on the coast of northern Japan were higher and lower, respectively, than those in whales in the southern area, probably reflecting the variations in marine environment around Japan (Endo et al., 2010). Mitani et al. (2006) analyzed the $\delta^{13}C$ and $\delta^{15}N$ values in the baleen plates of common minke whales caught during scientific research whaling, and tried to elucidate the migration pattern in relation to dietary shift. However, little information is available about the $\delta^{13}C$ and $\delta^{15}N$ values in the muscle of baleen whales, including common minke, Bryde's and sei whales, caught in the western North Pacific Ocean and Antarctic minke and fin whales caught in the Southern Ocean.

Recently, the $\delta^{18}O$ value, in addition to the $\delta^{13}C$ and $\delta^{15}N$ values, has been used to discriminate, verify and identify the habitat of plants and animals, as the $\delta^{18}O$ value reflects the water environment, temperature and humidity of their habitats. For instance, the $\delta^{18}O$ values in beef oil (Heaton et al., 2008), underground water (Mizota and Kusakabe, 1994) and cultured rice (Suzuki et al., 2009) all tend to decrease with latitude (temperature). To our knowledge, however, the $\delta^{18}O$ values in cetacean species have not yet been reported. According to the above latitude-dependent changes, we speculated that the $\delta^{18}O$ value would be lower in cetaceans caught off the northern areas than off the southern areas of Japan, and that $\delta^{13}C$ and $\delta^{15}N$ values would be negatively correlated with the $\delta^{18}O$ value in the whale products sold in Japan.

The purpose of the present study was to analyze the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ and the Hg concentration in red meat products originating from the common minke (J- and O- types), Bryde's and sei whales caught in the western North Pacific Ocean and Antarctic minke and fin whales caught in the Southern Ocean. We discuss the correlations between the trophic level, as evaluated by $\delta^{15}N$ value, and the Hg contamination and among the $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$ values, and the possibility of verifying the species origins of red meat products sold in Japan using these stable isotope ratios.

2. Materials and methods

2.1. Sampling of red meat products and genetic analysis for species origin

Red meat products originating from common minke whale (J- and O-types), Bryde's and sei whales caught in the Northwest Pacific Ocean and Antarctic minke and fin whales caught in the Antarctic Ocean were purchased from retail outlets in Japan between 2000 and 2006, as described previously (Endo et al., 2003, 2005). Samples were stored at −20 °C until analysis.

As reported elsewhere (Baker et al., 1996, 2006), the species origin of cetacean products was identified by mitochondrial DNA sequences (control region and cytochrome b) amplified from the products via PCR. The population origin of the common minke whale products (i.e., J- or O-type) was inferred from sequence variation in the mtDNA control region, as described in Baker et al. (2000).

2.2. Chemical analyses

The total mercury (Hg) concentration in the red meat products was determined by a Mercury Analyzer SP-2 (Nippon Instruments Corporation, Tokyo, Japan), as reported previously (Endo et al., 2007). DOLT-2 (National Research Council of Canada) was used as an analytical quality control for Hg. The recovery of Hg was 94 ± 3% (n=5). The Hg concentration in the red meat products was expressed on a wet weight basis.

Dried subsamples of red meat products were analyzed for stable isotopes (^{13}C, ^{15}N and ^{18}O) after the removal of lipids by chloroform/methanol extraction (Logan and Lutcavage, 2008). The $\delta^{13}C$ and $\delta^{15}N$ analyses were performed using a mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany) coupled with an elemental analyzer (EA1108, Fisons, Rodano, Milan, Italy) held in the Center for Ecological Research (CER), Kyoto University (Kyoto, Japan), as reported previously (Endo et al., 2009, 2010). The $\delta^{18}O$ analysis was performed using a mass spectrometer (Delta V PLUS, Thermo Fisher Scientific, Tokyo, Japan) coupled with an elemental analyzer (TC/EA, Thermo Fisher Scientific, Tokyo, Japan) held in the SI Science Co. Ltd. (Saitama, Japan). The natural abundances of ^{13}C, ^{15}N and ^{18}O are expressed as per mil (‰) deviation from the standards as defined by the following equation:

$$\delta^{13}C, \delta^{15}N \text{ or } \delta^{18}O = (R_{sample}/R_{standard} - 1) \times 1000(‰),$$

where $R = {}^{13}C/{}^{12}C$, $^{15}N/^{14}N$ or $^{18}O/^{16}O$. CERKU-1, 2 and 5, certified by CER, were used as $\delta^{13}C$ and $\delta^{15}N$ reference materials (Tayasu et al., 2011), and benzoic acid (A and B), certified by Indiana University (IN, USA), was used as the $\delta^{18}O$ reference material.

2.3. Statistical analyses

The data are shown as mean ± S.D., and were analyzed by Turkey–Kramer multiple comparison test and Pearson's correlation coefficient test, using the Statcell program. The level of significance was set at $p < 0.05$.

3. Results and discussion

The stable isotope ratios of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ and the Hg concentration in sixty-six red meat product samples from baleen whale species and populations were analyzed (Table 1), and the analytical results are summarized in Table 2. Fig. 1 shows the relationship between the $\delta^{15}N$ value and the Hg concentration in the combined products from the six samples (n=66), and Fig. 2 shows the relationships among the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values. Table 3 shows a summary of relationships among the Hg concentration, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values for each baleen whale species or population.

In agreement with previously published results (Endo et al., 2003), the contamination levels of Hg in the red meat products were in the following order: common minke whales (J-type)= common minke whales (O-type) > Bryde's whale=sei whale=fin whale ≥ Antarctic minke whale (Table 2). A similar order was found in the $\delta^{15}N$ values for these species (Table 2). The Hg concentrations in the combined products of the six samples were significantly correlated with their $\delta^{15}N$ values (Fig. 1, r=0.455, n=66, $p < 0.05$). As data not shown in Figure, significant correlations were found between the $\delta^{15}N$ values and the Hg concentrations in the combined samples from the North Pacific Ocean (r=0.418, n=46, $p < 0.05$) and from the Southern Ocean (r=0.541, n=20, $p < 0.05$). These correlations between the Hg

Table 1
Analytical results of mercury and stable isotope ratios in red meat products originating from baleen whales sold in Japanese markets.

Species origin	Sample code	Hg (μg/wet g)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{18}O$ (‰)
Common minke whale, J-type	1	0.050	−18.4	11.5	11.9
	2	0.125	−19.3	11.6	10.6
	3	0.074	−17.2	15.8	9.7
	4	0.070	−19.1	11.4	11.6
	5	0.136	−18.0	15.0	10.5
	6	0.239	−17.5	12.6	11.9
	7	0.180	−18.6	10.3	13.3
	8	0.119	−18.7	11.0	12.8
	9	0.031	−19.1	12.1	12.2
	10	0.027	−18.6	12.7	11.8
	11	0.041	−18.3	11.5	12.9
	12	0.061	−17.5	9.6	13.9
	13	0.029	−19.0	11.2	13.4
Common minke whale, O-type	1	0.053	−19.0	12.1	13.0
	2	0.053	−18.3	12.1	13.9
	3	0.056	−18.5	11.6	13.8
	4	0.044	−19.2	11.5	13.1
	5	0.121	−18.0	11.5	13.6
	6	0.053	−20.3	11.4	12.0
	7	0.160	−17.9	12.0	13.8
	8	0.014	−19.1	9.7	13.7
	9	0.174	−17.6	11.1	12.3
	10	0.176	−17.6	12.0	12.2
	11	0.254	−18.9	10.2	12.9
	12	0.027	−18.9	10.9	12.2
Bryde's whale	1	0.037	−17.2	8.6	15.5
	2	0.090	−17.5	9.3	15.5
	3	0.063	−15.9	11.9	13.7
	4	0.053	−17.6	10.1	16.0
	5	0.070	−16.3	11.6	13.9
	6	0.027	−17.2	9.7	16.7
	7	0.055	−15.9	11.2	14.0
	8	0.056	−16.9	9.5	16.1
	9	0.045	−17.2	9.5	15.0
	10	0.055	−17.1	9.8	14.6
	11	0.067	−16.9	8.4	14.9
Sei whale	1	0.026	−23.1	6.3	15.3
	2	0.082	−18.3	9.6	15.8
	3	0.079	−19.1	8.5	13.7
	4	0.028	−21.7	7.1	16.5
	5	0.045	−19.4	7.6	15.6
	6	0.090	−18.9	8.7	15.4
	7	0.046	−18.7	7.6	14.6
	8	0.054	−18.6	10.3	13.1
	9	0.033	−18.1	8.1	14.9
	10	0.061	−18.7	9.5	15.0
Fin whale	1	0.047	−23.0	5.7	15.2
	2	0.050	−21.2	6.1	15.6
	3	0.026	−23.9	6.0	16.5
	4	0.042	−20.8	6.2	15.4
	5	0.031	−23.0	5.7	15.4
	6	0.052	−22.8	5.9	15.6
	7	0.041	−21.9	5.6	15.3
	8	0.090	−21.9	6.3	13.5
	9	0.026	−23.6	5.4	14.4
	10	0.031	−23.1	4.9	14.4
Antarctic minke whale	1	0.027	−24.3	6.1	14.9
	2	0.051	−24.8	6.0	14.2
	3	0.013	−24.2	5.7	13.8
	4	0.013	−25.1	5.9	14.9
	5	0.077	−24.7	6.0	15.2
	6	0.018	−23.9	6.1	13.2
	7	0.014	−24.7	6.3	14.5
	8	0.027	−25.1	6.4	15.1
	9	0.013	−24.7	6.7	15.5
	10	0.014	−24.7	7.2	14.3

level and the trophic level, as evaluated by $\delta^{15}N$ value, were firstly reported in the food products from Papuan New Guinea (Yoshinaga et al., 1992) and from the freshwater biota in Ontario, Canada (Kidd et al., 1995). Although there was an overall correlation in the combined sample from the six whale species or populations, only the sei whale had a significant within-species correlation between the Hg concentration and the $\delta^{15}N$ value ($\gamma=0.651$, $n=10$, $p<0.05$) (Table 3). This probably reflects the marine environment around Japan (higher $\delta^{15}N$ value and lower Hg concentration in the northern area of Japan; Endo et al., 2010). The fin whale in the Antarctic Ocean had high but non-significant correlation between the Hg concentration and the $\delta^{15}N$ value ($\gamma=0.618$, $n=10$, $p>0.05$), while the Antarctic minke whale had negative correlation. The reason for this negative correlation remains unknown.

According to latest reports (Mitani et al., 2006; Yasunaga and Fujise, 2009a, b), O-type common minke whales may be categorized into coastal and offshore whales. The Hg concentration is lower in the coastal whales (about 0.22 ± 0.07 μg/wet g) than in the offshore whales (about 0.3 μg/wet g) as the coastal whales feed on zooplankton, saury and anchovies (the Hg concentrations in these species were below 0.05 μg/wet g) while the offshore whales feed on these three species as well as on pomfret (0.232 ± 0.027 μg/wet g) (Yasunaga and Fujise, 2009a, b). The present Hg value in the O-type whales (0.099 ± 0.076 μg/wet g) is closer to the Hg value in the coastal whales than to that in the offshore O-type whales. The determination of $\delta^{13}C$ and $\delta^{15}N$ may also allow for the discrimination between the coastal and offshore species (Kelly, 2000). However, there has not yet been any comparison of these values between the coastal and offshore populations of common minke whales. We previously analyzed the Hg levels in cetacean products sold in South Korean markets (Endo et al., 2007), and the Hg concentration in the common minke whale (most of the whales were speculated to be J-type from coastal waters) was 0.22 ± 0.11 μg/wet g (0.03–0.43 μg/wet g, $n=30$), which is higher than the present data for the J-type whale (0.091 ± 0.065 μg/wet g). The difference in Hg concentrations in the common minke whale between the previous and present studies may be due to differences in their diet and habitat.

In the present study (Table 2), no differences were found in the results for Hg concentration, or $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values between the J- and O-types of common minke whales. Compared with the J- and O-types of common minke whales (0.091 ± 0.065 and 0.099 ± 0.076 μg/wet g, respectively), Bryde's and sei whales are only moderately omnivorous feeders and their Hg concentrations were lower (0.056 ± 0.017 and 0.054 ± 0.023 μg/wet g, respectively); Yasunaga and Fujise (2009a) reported similar Hg concentrations in the muscle of Bryde's whales (0.046 ± 0.008 μg/wet g) and sei whales (0.052 ± 0.009 μg/wet g) caught in the western North Pacific Ocean. The Antarctic minke and fin whales in the Southern Ocean are plankton feeders and their Hg levels were slightly lower than those of Bryde's and sei whales in the western North Pacific Ocean (Table 2).

Gendron et al. (2001) analyzed the $\delta^{13}C$ and $\delta^{15}N$ values in skin samples from Bryde's, fin and blue (*Balaenoptera musculus*, a plankton-feeder) whales in the Gulf of California, Mexico. The mean values of $\delta^{13}C$ and $\delta^{15}N$ in the skin samples of the Bryde's, fin and blue whales were −18.1 and 15.8‰ ($n=2$), −16.0 and 15.4‰ ($n=2$), and −18.2 and 12.9‰ ($n=2$), respectively. This order of $\delta^{15}N$ values is consistent with our knowledge of the feeding habits of those whale species, although the $\delta^{15}N$ values in the Bryde's and fin whales are higher than those in the present study (10.0 ± 1.2‰ and 5.8 ± 0.4‰, respectively, Table 2). The variation in $\delta^{15}N$ at the base of the food web is considered to be an important factor in the $\delta^{15}N$ values observed in the upper trophic levels. The $\delta^{15}N$ value in euphausiids (krill) along the west coast of the Gulf of California was 11.0 ± 1.2‰ (Gendron et al., 2001),

Table 2
Summary of analytical results for mercury and stable isotope ratios in red meat products originating from baleen whales sold in Japanese markets.

	Hg (μg/wet g)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{18}O$ (‰)
Common minke whale (J-type), $n=13$	0.091 ± 0.065^{a}	-18.4 ± 0.7^{a}	12.0 ± 1.7^{a}	12.0 ± 1.2^{a}
Common minke whale (O-type), $n=12$	0.099 ± 0.076^{a}	-18.6 ± 0.8^{a}	11.4 ± 0.7^{a}	13.0 ± 0.7^{a}
Bryde's whale, $n=11$	0.056 ± 0.017^{ab}	-16.9 ± 0.6^{b}	10.0 ± 1.2^{b}	15.1 ± 1.0^{b}
Sei whale, $n=10$	0.054 ± 0.023^{ab}	-19.5 ± 1.6^{c}	8.3 ± 1.3^{c}	15.0 ± 1.0^{b}
Fin whale, $n=10$	0.044 ± 0.019^{ab}	-22.5 ± 1.0^{d}	5.8 ± 0.4^{d}	15.1 ± 0.8^{b}
Antarctic minke whale, $n=10$	0.027 ± 0.021^{b}	-24.6 ± 0.4^{e}	6.2 ± 0.4^{d}	14.6 ± 0.7^{b}

See Table 1.
Different superscripts indicate significant differences ($p < 0.05$).

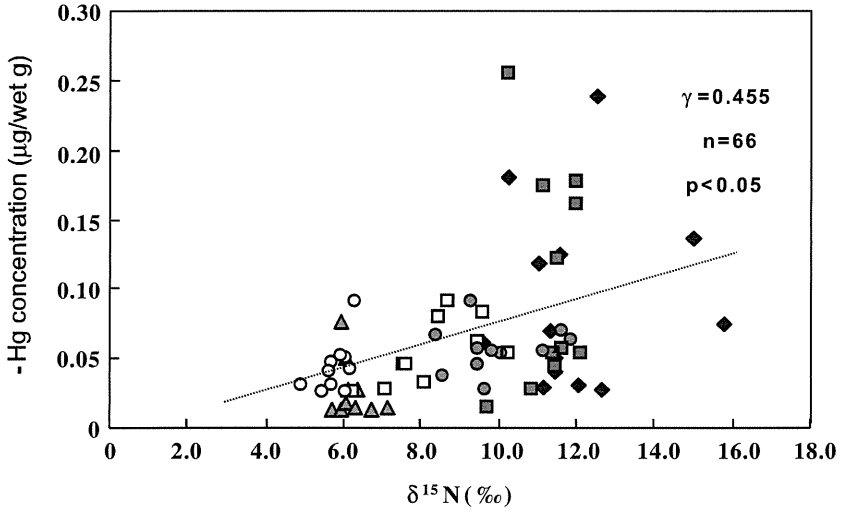

Fig. 1. Relationship between the $\delta^{15}N$ value and the Hg concentration in red meat products originating from baleen whale species or population. See Table 1. J-type common minke whale (◆), O-type common minke whale (■), Bryde's whale (●), sei whale (□), Antractic minke whale (▲), fin whale (○). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

while that in krill found in the stomach of common minke whales caught in the western North Pacific Ocean was 7.2 ± 0.5‰ (Mitani and Bando, 2008). Thus, the trophic positions of Bryde's and fin whales in the western North Pacific Ocean appear to be similar to those in the Gulf of California, respectively.

The $\delta^{13}C$ values in common minke (J- and O-types), Bryde's and sei whales caught in the western North Pacific Ocean were significantly different from those in fin and Antarctic minke whales caught in the Southern Ocean (Table 2), probably reflecting differences in their habitats. Krahn et al. (2008) reported the $\delta^{13}C$ and $\delta^{15}N$ values in the biota of Antarctica: the $\delta^{13}C$ and $\delta^{15}N$ values in the skin of an Antarctic minke whale ($n=1$) were −24.3 and 7.6‰, respectively, and those in the serum of crabeater seals (krill feeders) and in krill were -26.5 ± 1.0 and 8.4 ± 1.6‰ ($n=30$), and -29.8 ± 0.6 and 3.6 ± 0.2‰ ($n=12$), respectively. These $\delta^{13}C$ and $\delta^{15}N$ values in the Antarctic minke whale are in agreement with the present values from the muscle (red meat product) of Antarctic minke and fin whales caught in the Southern Ocean (Table 2). The $\delta^{15}N$ value in krill in the Antarctic Ocean (3.6 ± 0.2‰) was markedly lower than that in the stomach of common minke whales caught in Pacific Ocean (7.2 ± 0.5‰; Mitani and Bando, 2008). Lower $\delta^{15}N$ values in Antarctic minke and fin whales than common minke whale (Table 2) may reflect lower trophic levels of Antarctic minke and fin whales as well as lower $\delta^{15}N$ at the base of Southern food web.

The $\delta^{18}O$ values in common minke whales (J- and O-types) were significantly lower than those in the other whale species (Table 2), whereas the $\delta^{18}O$ values in Bryde's and sei whales caught in the western North Pacific Ocean and those in fin and Antarctic minke whales caught in the Southern Ocean were similar. As far as we know, no information on $\delta^{18}O$ values in cetaceans is available. As the temperature of the Antarctic feeding habitat is lower than that of the temperate North Pacific Ocean habitat, we expected to observe lower $\delta^{18}O$ values in whales in the Antarctic. However, the $\delta^{18}O$ values in the fin and Antarctic minke whales caught in the Antarctic were similar to those of Bryde's and sei whales caught in the North Pacific Ocean. Further study is necessary to explain these unexpected data.

We previously reported the $\delta^{13}C$ and $\delta^{15}N$ values and the Hg concentration in the toothed whale species hunted or stranded along the coast of Japan. The $\delta^{15}N$ values and the Hg concentrations in the toothed whale species (Endo et al., 2005) were markedly higher than those in the baleen whale species in this study (Table 2), reflecting their higher trophic positions. Further determination of $\delta^{18}O$ in the toothed whales from a broad latitudinal range is needed to elucidate whether $\delta^{18}O$ is higher in the toothed whales inhabiting the northern area and whether ^{18}O is bioaccumulated via the food web.

Significant positive correlations ($p < 0.05$) were found between the $\delta^{13}C$ and $\delta^{15}N$ values for Bryde's and sei whales (Table 3), and non-significant positive correlations ($p > 0.05$) were found in the other species caught in the western North Pacific Ocean and the Antarctic Ocean. We previously reported a positive correlation between $\delta^{13}C$ and $\delta^{15}N$ values in wild bluefin tuna taken from different areas around Japan (both values were lower in fish from the northern area), probably reflecting the change in diet due to the wide ranging annual migration from the southern to the northern areas (Hisamichi et al., 2010). Baleen whales, such as the

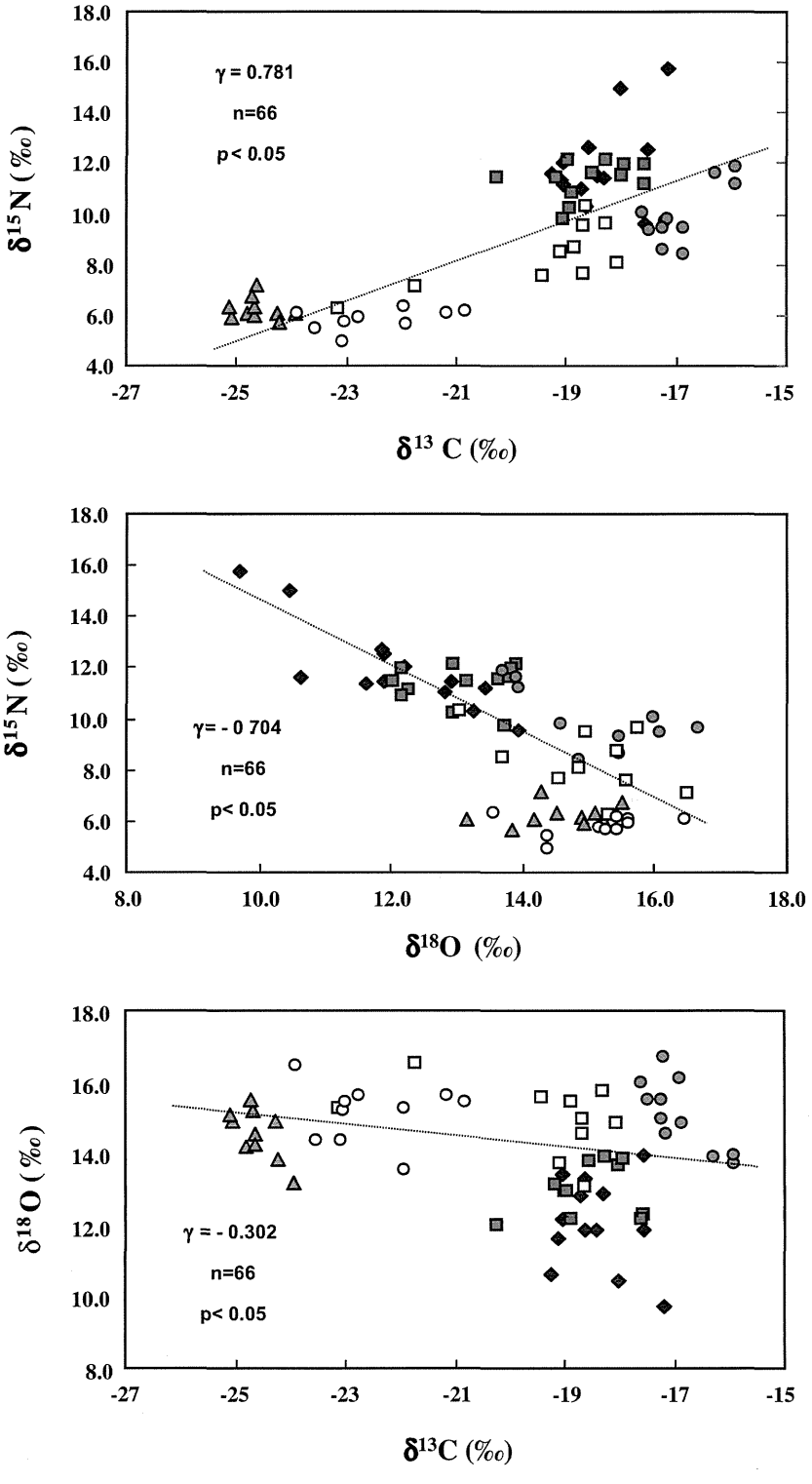

Fig. 2. Relationship among values of $\delta^{13}C$ and the $\delta^{18}O$ in red meat products originating from baleen whale species or population. See Table 1. J-type common minke whale (◆), O-type common minke whale (■), Bryde's whale (●), sei whale (□), Antractic minke whale (▲), fin whale (○). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

common minke (J- and O-types) and Antarctic minke whales, migrate over wide ranges in the North Pacific Ocean and the Southern Ocean, respectively (Kasamatsu et al., 1995; Wade et al., 2010). The positive correlations between the $\delta^{13}C$ and $\delta^{15}N$ values found in the baleen whale species could reflect their migration.

Unfortunately, we do not have any information on whale products with regard to location or date that each whale was killed or the age of the whale. Consequently, it is unclear whether the higher $\delta^{13}C$ and $\delta^{15}N$ values found in the red meat product samples come from whales in the southern or northern areas off

Table 3
Correlation coefficients (γ) of mercury and natural isotopes for within-species samples and overall samples for species or populations of baleen whales.

	Hg vs. $\delta^{15}N$	$\delta^{13}C$ vs. $\delta^{15}N$	$\delta^{15}N$ vs. $\delta^{18}O$	$\delta^{13}C$ vs. $\delta^{18}O$
Common minke whale (J-type), $n=13$	0.074	0.435	−0.852*	−0.185
Common minke whale (O-type), $n=12$	−0.077	0.315	0.199	0.182
Bryde's whale, $n=11$	0.188	0.740*	−0.640*	−0.774*
Sei whale, $n=10$	0.651*	0.751*	−0.484	−0.398
Fin whale, $n=10$	0.618	0.527	0.190	−0.250
Antarctic minke whale, $n=10$	−0.303	0.229	0.229	−0.672*
Overall, $n=66$	0.455*	0.781*	−0.704*	−0.302*

See Table 1.

* $p<0.05$.

Japan. Based on the negative correlation between the $\delta^{13}C$ and $\delta^{18}O$ values and the $\delta^{15}N$ and $\delta^{18}O$ values (Table 3), it is assumed that the lower $\delta^{18}O$ values in whales in the northern areas result in the higher $\delta^{13}C$ and $\delta^{15}N$ values in whales in the northern area of Japan. However, this hypothesis is not supported by the lower $\delta^{13}C$ and $\delta^{15}N$ values found in wild bluefin tuna in the northern area of Japan (Hisamichi et al., 2010). Further study is necessary to confirm our assumption of spatial variations in $\delta^{18}O$, $\delta^{15}N$ and $\delta^{13}C$ values.

The $\delta^{13}C$–$\delta^{15}N$ plots and the $\delta^{13}C$–$\delta^{18}O$ plots can be discriminated into two groups (Table 2 and Fig. 2): the red meat products originating from the western North Pacific Ocean (J- and O-type common minke whales, Bryde's and sei whales) and the Antarctic Ocean (fin and Antarctic minke whales). We previously analyzed organohalogen compounds such as PCBs and DDTs and reported that the levels were markedly lower in the red meat products originating from the Southern Ocean than in products from the western North Pacific Ocean (Haraguchi et al., 2000). Thus, discrimination between the red meat products originating from the western North Pacific Ocean and the Antarctic Ocean could be achieved by the chemical analysis of stable isotope ratios and the pollutants without the need for genetic analysis. However, Antarctic minke and fin whales, J- and O-type common minke whales and Bryde's and sei whales could not be discriminated on the basis of chemical analysis. On the other hand, the $\delta^{13}C$ and $\delta^{15}N$ values in the red meat products originating from baleen whales sold in Japan were markedly different from those in products originating from toothed whales (Endo et al., 2010). Furthermore, contamination levels of Hg as well as organohalogens found in the baleen whales were markedly lower than those in toothed whales. Thus, the red meat originating from mysticetes and odonotocetes sold in Japan can be discriminated through chemical analysis.

In conclusion, we analyzed the Hg concentration and the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values in red meat products originating from common minke (J- and O-types), Bryde's and sei whales in the western North Pacific Ocean and fin and Antarctic minke whales in the Southern Ocean. The range of Hg concentrations and the $\delta^{15}N$ values in the baleen species and populations were in agreement with the known feeding habits of those. The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values could be used to discriminate between the red meat products originating from the mysticetes in the western North Pacific Ocean and those from the Southern Ocean. However, the four mysticetes in the western North Pacific Ocean and the two mysticetes in the Southern Ocean could not be identified on the basis of these data alone. A positive correlation between the $\delta^{13}C$ and $\delta^{15}N$ values and negative correlations between the $\delta^{13}C$ and $\delta^{18}O$ values and the $\delta^{15}N$ and $\delta^{18}O$ values, probably reflecting migration, were found in some species in the western North Pacific Ocean and the Southern Ocean.

Acknowledgments

This work was supported by Grants-in-Aid from Japan Society for the Promotion of Science (C21590135) and International Fund for Animal Welfare (IFAW). Determinations of $\delta^{13}C$ and $\delta^{15}N$ were conducted using Joint Usage/Research Grant of Center for Ecological Research, Kyoto University. We deeply appreciate the help provided by Mr. Akira Hanawa, a representative director of the Isotope Research Institute Co. Ltd., (Yokohama, Japan), in cross-checking the stable isotope determinations.

References

Baker, C.S., Cipriano, F., Palumbi, S.R., 1996. Molecular genetic identification of whale and dolphin products from commercial markets in Korea and Japan. Mol. Ecol. 5, 671–685.

Baker, C.S., Lento, G.L., Cipriano, F., Palumbi, S.R., 2000. Predicted decline of protected whales based on molecular genetic monitoring of Japanese and Korean markets. Proc. R. Soc. London Ser. B 267, 1191–1199.

Baker, C.S., Lukoschek, V., Lavery, S., Dalebout, M.L., Yong-Un, M., Endo, T., Funahashi, N., 2006. Incomplete reporting of whale, dolphin and porpoise 'bycatch' revealed by molecular monitoring of Korean markets. Anim. Conserv. 9, 474–482.

DeNiro, M.J., Epstein, S., 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochim. Cosmochim. Acta 45, 341–351.

Endo, T., Hotta, Y., Haraguchi, K., Sakata, M., 2003. Mercury contamination in the red meat of whales and dolphins marketed for human consumption in Japan. Environ. Sci. Technol. 37, 2681–2685.

Endo, T., Haraguchi, K., Cipriano, F., Simmonds, M.P., Hotta, Y., Sakata, M., 2004. Contamination by mercury and cadmium in the cetacean products from Japanese market. Chemosphere 54, 1653–1662.

Endo, T., Haraguchi, K., Hotta, Y., Hisamichi, Y., Lavery, S., Dalebout, M.L., Baker, C.S., 2005. Total mercury, methyl mercury, and selenium levels in the red meat of small cetaceans sold for human consumption in Japan. Environ. Sci. Technol. 39, 5703–5708.

Endo, T., Yong-Um, M., Baker, C.S., Funahashi, N., Lavery, S., Dalebout, M.L., Lukoschek, V., Haraguchi, K., 2007. Contamination level of mercury in red meat products from cetaceans available from South Korea markets. Mar. Pollut. Bull. 54, 669–677.

Endo, T., Hisamichi, Y., Kimura, O., Kotaki, Y., Kato, Y., Ohta, C., Koga, N., Haraguchi, K., 2009. Contamination levels of mercury in the muscle of female and male of spiny dogfish (*squalus acanthias*) caught off the coast of Japan. Chemosphere 77, 1333–1337.

Endo, T., Hisamichi, Y., Kimura, O., Haraguchi, K., Lavery, S., Dalebout, M.L., Funahashi, N., Baker, C.S., 2010. Stable isotope ratios of carbon and nitrogen and mercury concentration in 13 toothed whale species from the western Pacific Ocean off Japan. Environ. Sci. Technol. 44, 2675–2681.

Gendron, D., Aguiniga, S., Carriquiry, J.D., 2001. $\delta^{15}N$ and $\delta^{13}C$ in skin biopsy samples: a note on their applicability for examining the relative trophic level in three rorqual species. J. Cetacean Res. Manage. 3, 41–44.

Haraguchi, K., Endo, T., Sakata, M., Masuda, Y., 2000. Contamination survey of heavy metals and organochlorine compounds in cetacean products purchased in Japan. J. Food Hyg. Soc. Jpn. 41, 249–287.

Heaton, K., Kelly, S.D., Hoogewerff, J., Woolfe, M., 2008. Verifying the geographical origin of beef: the application of multi-element isotope and trace element analysis. Food Chem. 107, 506–515.

Hisamichi, Y., Haraguchi, K., Endo, T., 2010. Contamination levels of mercury and organochlorine compounds, and stable isotope ratios in three tuna species taken from different regions of Japan. Environ. Sci. Technol. 44, 5971–5978.

Kasamatsu, F., Nishiwaki, S., Ishikawa, H., 1995. Breeding areas and southbound migrations of southern minke whales *Balaenoptera acuturostrata*. Mar. Ecol. Prog. Ser. 119, 1–10.

Kelly, J.F., 2000. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. Can. J. Zool. 78, 1–27.

Kidd, K.A., Hesslein, R.H., Fudge, R.J.P., Hallard, K.A., 1995. The influence of trophic level as measured by $\delta^{15}N$ on mercury concentrations in freshwater organisms. Water Air Soil Pollut. 80, 1011–1015.

Krahn, M.M., Pitman, R.L., Burrows, D.G., 2008. Use of chemical tracers to assess diet and persistent organic pollutions in Antarctic Type C killer whales. Mar. Mamm. Sci. 24, 643–663.

Logan, J.M., Lutcavage, M.,.E., 2008. A comparison of carbon and nitrogen stable isotope ratios of fish tissues following lipid extractions with non-polar and traditional chloroform/methanol solvent systems. Rapid Commun. Mass Spectrom. 22, 1081–1086.

Mitani, Y., Bando, T., 2008. Feeding migration of the common minke whale *Balaenoptera acuturostrata* in the western North Pacific. In: Tominaga, O., Takai, N. (Eds.), Discoveries in Aquatic Animal Ecology Presented by Stable Isotope—Bivalve to Whale—Koseisya Co. Ltd, Tokyo, Japan, pp. 153–161.

Mitani, Y., Bando, T., Takai, N., Sakamoto, W., 2006. Patterns of stable carbon and nitrogen isotopes in the baleen of common minke whale *Balaenoptera acutorostrata* from the western North Pacific. Fish Sci. 72, 69–76.

Minagawa, M., Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains further evidence and relation between ^{15}N and animal age. Geochim. Cosmochim. Acta 48, 1135–1140.

Mizota, C., Kusakabe, M., 1994. Spatial distribution of δD–$\delta^{18}O$ values of surface and shallow groundwaters from Japan, South Korea and East China. Geochem. J. 28, 387–410.

Suzuki, Y., Nakashita, R., Akamatsu, F., Korenaga, T., 2009. Multiple stable isotope analyses for verifying geographical origin and agricultural practice of Japanese rice samples. Bunseki Kagaku 58, 1053–1058.

Tayasu, I., Hirasawa, R., Ogawa, N.O., Ohkouchi, N., Yamada, K., 2011. New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. Limnology 12, 261–266.

Wade, P.R., Brownell, Jr. R.L., Kasuya, T., 2010. A Review of the Biology of Western North Pacific Minke Whales Relevant to Stock. Report to the Scientific Committee of the International Whaling Commission. SC62/NPM13 Rev. 〈http://iwcoffice.org/_documents/sci_com/SC62docs/SC-62-NPM13rev.pdf〉.

Yasunaga, G., Fujise, Y., 2009a. Temporal Trends and Factors Affecting Mercury Levels in Common Minke, Bryde's and Sei Whales and their Prey Species in the Western North Pacific. Report to the Scientific Committee of the International Whaling Commission. SC/J09/JR23. 〈http://iwcoffice.org/_documents/sci_com/workshops/SC-J09-JRdoc/SC-J09-JR23.pdf〉.

Yasunaga, G., Fujise, Y., 2009b. Additional Analyses of Temporal Trends and Factors Affecting Mercury Levels in Common Minke, Bryde's and Sei Whales in the Western North Pacific. Report to the Scientific Committee of the International Whaling Commission. SC/61/JR3(additional analysis—SC/J09/JR23). 〈http://iwcoffice.org/_documents/sci_com/sc61docs/SC-61-JR3.pdf〉.

Yoshinaga, J., Suzuki, T., Hongo, T., Minagawa, M., Ohtsuka, R., Kawabe, T., Inaoka, T., Akimichi, T., 1992. Mercury concentration correlates with the nitrogen stable isotopes ratio in the animal food of Papuans. Ecotoxicol. Environ. Saf. 24, 37–45.

Anal Bioanal Chem (2012) 404:197–206
DOI 10.1007/s00216-012-6132-2

ORIGINAL PAPER

Selective determination of mono- and dihydroxylated analogs of polybrominated diphenyl ethers in marine sponges by liquid-chromatography tandem mass spectrometry

Yoshihisa Kato · Syohei Okada · Kazutaka Atobe · Tetsuya Endo · Koichi Haraguchi

Received: 24 March 2012 / Revised: 17 May 2012 / Accepted: 20 May 2012 / Published online: 8 June 2012

Abstract A number of bioactive brominated secondary metabolites, including hydroxylated polybrominated diphenyl ethers, have been isolated from algae, sponges, and bacteria. In the present study, a screening method using liquid-chromatography tandem mass spectrometry was developed for the identification and selective determination of dihydroxy (diOH), hydroxy-methoxy (OH-MeO), and dimethoxy (diMeO) analogs of tetra- to hexa-BDEs in marine biota. In negative atmospheric pressure chemical ionization (APCI) mode, diOH and OH-MeO analogs provided intense $[M-H]^-$ ions, whereas diMeO analogs provided characteristic $[M-Br+O]^-$ and $[M-CH_3]^-$ ions. This enabled the diOH-, OH-MeO-, and diMeO-PBDEs to be distinguished using selected reaction monitoring transitions in the APCI source. Recoveries of 2′-OH-6-MeO-2,3′,4,5′-tetra-BDE in spiked marine samples were 84±5 %, with a limit of quantification at 9.1 ng mL^{-1} (signal-to-noise ratio=10). The developed method was used to analyze two sponge species collected from Palau, Micronesia; the concentration ratio of diOH-tetra-BDE:OH-MeO-tetra-BDE was 10:1 for the *Lamellodysidea* sp., whereas it was 1:30 for the *Callyspongia* sp.

Keywords LC/MS/MS · APCI · Dihydroxy-PBDE · Hydroxy-methoxy-PBDE · Dimethoxy-PBDE · Marine sponge

Y. Kato · S. Okada · K. Atobe
Kagawa School of Pharmaceutical Sciences,
Tokushima Bunri University,
1314-1 Sanuki,
Kagawa 769-2193, Japan

T. Endo
Faculty of Pharmaceutical Sciences,
Health Sciences University of Hokkaido,
1757 Ishikari-Tobetsu,
Hokkaido 061-0293, Japan

K. Haraguchi (✉)
Daiichi College of Pharmaceutical Sciences,
22-1 Tamagawa-cho, Minami-ku,
Fukuoka 815-8511, Japan
e-mail: k-haraguti@daiichi-cps.ac.jp

Introduction

The marine environment is a rich source of halogenated compounds produced by marine plants, animals, and bacteria [1]. Brominated secondary metabolites are produced by cyanobacterial symbionts [2–4] or by bacterial *Vibrio* spp. [5] in sponge tissues, although the profiles of these metabolites differ as a result of the diversity of species involved. The family Dysideidae, in particular, yields a number of hydroxylated polybrominated diphenyl ethers (OH-PBDEs), including dihydroxy (diOH), hydroxy-methoxy (OH-MeO), and dimethoxy (diMeO) analogs of tri- to hexa-BDEs [6,7]. These phenolic PBDE analogs exhibit a variety of bioactivities, such as antibacterial and antifungal properties [8], cytotoxicity, and enzyme inhibition [9]. Structure–activity relationships indicate that these activities may depend on the numbers and positions of hydroxyl groups [7]. In addition to PBDE homologs found in marine sponges, OH-PBDEs (e.g., 6-OH-BDE47) and the corresponding MeO-PBDEs (e.g., 2′-MeO-BDE68) have been reported to be present in marine algae [10], mussels [11], fish blood [12], as well as the marine food web [13–18]. Although there have as yet been no reports of diOH-PBDEs in higher trophic organisms, *O*-methylated PBDEs (e.g., 2′,6-diMeO-BDE68) have been found to accumulate in shark liver [19] and whale blubber [20].

The extraction, chromatographic separation, and detection of phenolic PBDE analogs have recently been reviewed [21]. In general, OH-PBDEs are routinely quantified by gas chromatography (GC), using either electron-capture detection, electron-ionization high-resolution mass spectrometry, electron-capture negative ionization mass spectrometry, or tandem mass spectrometry (MS/MS), after derivatization of OH-PBDEs separated from neutral PBDEs and/or MeO-PBDEs [22]. GC/MS methods for OH-PBDEs provide the greatest selectivity in the discrimination of PBDEs and MeO-PBDEs from other halogenated organics. However, diazomethane derivatization of OH-MeO-PBDEs for GC/MS analysis can be duplicated by that of diOH-PBDEs. Other derivatizations (e.g., acetylation with acetic anhydride) of diOH-PBDE in the presence of OH-MeO-PBDEs result in several derivatized products and would make the identification difficult.

For the direct determination of OH-PBDEs, several ionization techniques using liquid-chromatography tandem mass spectrometry (LC/MS/MS) have been developed. Hua et al. [23] have studied high-performance liquid chromatography (HPLC) in negative electrospray ionization (ESI) mode for the quantitative analysis of OH-tri-BDE. Mas et al. [24] have further developed negative ion spray-LC/MS/MS and determined eight OH-PBDEs in an environmental setting. Chang et al. [25] have developed the quantification of phenolic PBDEs, including bisphenol A and bromophenols, in ESI mode. Lupton et al. [26] have reported LC/atmospheric pressure chemical ionization (APCI)-MS/MS for OH-PBDEs, ranging from tri- to hexa-brominated, in negative APCI mode. Lai et al. [27] have developed a robust ultra-performance LC/MS/MS for rapid determination of nine OH-PBDEs to study the pharmacokinetics of 6-OH-BDE47 in ESI mode. We have also developed the simultaneous determination of OH- and MeO-PBDEs in APCI mode using both ($[M-H]^- \rightarrow Br^-$) and ($[M-Br+O]^- \rightarrow Br^-$) transitions [28]. However, these methods were validated for monohydroxylated PBDEs but not for the mixed dioxygenated (diOH- and OH-MeO-PBDEs) congeners by using different MRM transitions. An alternative LC/MS/MS method is therefore worth considering for the analysis of dihydroxylated analogs in the environment and for investigating the metabolic processes of diOH-PBDEs.

The aim of this study was to develop an LC/MS/MS method in negative APCI mode for direct determination of diOH, OH-MeO, and diMeO analogs for tetra- to hexa-BDEs, as well as mono substituted OH- and MeO-tetra-BDEs. The LC/MS/MS method was validated using 2′-OH-BDE68, 2′,6-diOH-BDE68 and their *O*-methylated analogs. During our survey of brominated products in marine sponges, we found a set of dihydroxylated PBDE analogs that were dominant in *Callyspongia* sp. and *Lamellodysidea* sp. collected in Palau, Micronesia. In this paper, we describe the overall LC/MS/MS profiles and concentrations of these oxygenated PBDEs in two sponge species and compare the total profiles of the derivatized compounds using GC/MS in EI mode.

Experimental

Chemicals

Standards of 2′-hydroxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-OH-BDE68) and 2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE68), were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). The standard 2′,6-dimethoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′,6-diMeO-BDE68) was a gift from Dr. G. Marsh (Stockholm University, Sweden). Standards of 2′,6-dihydroxy-2,3′,4,5′-tetrabromodiphenyl ether (2′,6-diOH-BDE68) and 2′-hydroxy-6-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-OH-6-MeO-BDE68) were synthesized by demethylation of 2′,6-diMeO-BDE68 in the presence of boron tribromide (2 M) in dichloromethane. The demethylated products (OH-MeO-tetra-BDEs) were identified by GC-MS in EI mode on the basis of its fragmentation pattern with M^+ (*m/z* 526) and $[M-CH_3Br]^+$ ion (*m/z* 434) [29]. The $[M-CH_3Br]^+$ ion was characteristic for 2′-MeO-6-OH-BDE68 but not for the isomeric 2′-OH-6-MeO-BDE68. Thus, both compounds can be distinguished by the abundance of this ion. The purities of

Table 1 Characterization of reference compounds and MRM parameters used for quantitative determination

Compound	LC t_R (min)	LOQ (ng mL^{-1})	Recovery (%)	RSD (%)	MRM transition (*m/z*)	DP (V)	CE (V)
Mono-substituent							
2′-OH-BDE68	7.2	1.9	80–89	15	500.3→78.6 $[M-H]^- \rightarrow Br^-$	−15	−44
2′-MeO-BDE68	13.6	9.5	87–92	16	450.7→78.6 $[M-Br+O]^- \rightarrow Br^-$	−40	−58
Di-substituent							
2′,6-diOH-BDE68	5.1	9.1	76–92	20	516.3→78.9 $[M-H]^- \rightarrow Br^-$	−35	−64
2′-OH-6-MeO-BDE68	6.6	9.1	77–92	22	530.7→78.9 $[M-H]^- \rightarrow Br^-$	−30	−40
2′,6-diMeO-BDE68	11.5	15	88–93	15	480.8→78.8 $[M-Br+O]^- \rightarrow Br^-$	−40	−66