Environment International 35 (2009) 712–717

Contents lists available at ScienceDirect

Environment International

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

Relationship between dietary exposure and serum perfluorochemical (PFC) levels—A case study

Anna Kärrman [a,b,c], Kouji H. Harada [a], Kayoko Inoue [a], Takumi Takasuga [b], Etsumasa Ohi [b], Akio Koizumi [a,*]

[a] Department of Health and Environmental Sciences, Kyoto University Graduate School of Medicine, Sakyo Konoe, Kyoto 606-8501, Japan
[b] Shimadzu Techno-Research Inc, Kyoto 604-8436, Japan
[c] MTM Research Centre, Örebro University, 701 82 Örebro, Sweden

ARTICLE INFO

Article history:
Received 2 October 2008
Accepted 23 January 2009
Available online 27 February 2009

Keywords:
Perfluorinated chemicals
Food duplicate
Dietary intake
Japan

ABSTRACT

Daily dietary intake of perfluorinated chemicals (PFCs) in relation to serum levels was assessed by determination of nine PFCs including perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in matched daily diet duplicates and serum samples. Diet and serum were collected in year 2004 from 20 women in Osaka and Miyagi, Japan. Only PFOS and PFOA were detected in the diet samples and no significant difference between cities was seen. After adjusted by water content, diet concentration of PFOA was significantly higher in Osaka. The median daily intake calculated using the measured diet concentrations was 1.47 ng PFOS/kg b.w. and 1.28 ng PFOA/kg b.w. for Osaka, and 1.08 ng PFOS/kg b.w. and 0.72 ng PFOA/kg b.w. for Miyagi. A significant difference between cities was seen for the serum concentrations with median of 31 ng/mL PFOS and PFOA in Osaka, compared to 14 ng/mL PFOS and 4.6 ng/mL PFOA in Miyagi. Carboxylates such as perfluorononanoic acid (PFNA) and perfluoroundecanoic acid (PFUnDA) were also detected in serum at median levels 6.9 ng/mL and 3.2 ng/mL (Osaka), and 2.8 ng/mL and 5.1 ng/mL (Miyagi). Based on one-compartment model under steady state, dietary intake of PFOS and PFOA accounted for only 22.4% and 23.7% of serum levels in Osaka females, and in contrast 92.5% and 110.6% in Miyagi females, respectively.

1. Introduction

Perfluorinated chemicals (PFCs) constitute a large group of substances with special properties resulting in extensive usage for applications like fabric, leather and apparel treatment, protection of food packaging and paper products and as performance chemicals, e.g. fire extinguishing foam and insecticides. An increasing number of studies show that humans are exposed to a large number of chemicals from this group, foremost and in highest concentrations perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) (Calafat et al., 2006; Harada et al., 2007; Kärrman et al., 2006b; Yeung et al., 2006). Several toxicological studies performed mainly on rodents suggest that PFOS and PFOA are potentially hepatotoxic, carcinogenic, endocrine disruptors, immunotoxic, and shows developmental toxicity (Kennedy et al., 2004; Lau et al., 2007; OECD, 2002; Peden-Adams et al., 2008). A limited number of epidemiological studies on PFCs found no consistent effects on humans in terms of cancer and general health problems in adults (Alexander et al., 2003; Gililland and Mandel, 1993; Olsen et al., 2003). Recent epidemiological studies however demonstrated inhibitory effects of PFOS and /or PFOA on human neonatal growth at the background exposure levels in the general population (Apelberg et al., 2007; Fei et al., 2007). Thus there is a growing concern for adverse effects on human neonatal growth in addition to the persistence, biomagnification properties and the global distribution in the ecosystem of PFOS, PFOA and other PFCs (Giesy and Kannan, 2001; Smithwick et al., 2005; Tomy et al., 2004).

Possible pathways of human exposure to PFCs include inhalation of volatile and neutral PFCs for example fluorotelomer alcohols (FTOHs) and perfluorinated sulfonamides, or ingestion of non-volatiles attached to particles, and exposure of contaminated food and water. Dietary intake is regarded to be the most important exposure pathway for lipophilic persistent organic pollutants. Since the chemical and physiological properties as well as bioaccumulation processes for PFCs differs compared to traditional lipophilic POPs, dietary exposure needs to be studied in more detail. Exposure of PFCs through diet has been suggested but the number of reports in the literature is limited. Contaminated fish and other seafood have been reported from the American Great Lakes (Kannan et al., 2005) and China (Gulkowska et al., 2006) and shows that PFOS are present in higher concentrations compared to PFOA. Drinking water from sites with no known contamination source generally contain PFCs in the low ng/L range while contaminated water sources with 51 ng/L PFOS and 10,000 ng/L PFOA have been reported (Emmett et al., 2006; Harada et al., 2003; Skutlarek et al., 2006). In the UK, PFOS was detected in a few food items like potatoes, eggs and canned vegetables while PFOA was only detected in potatoes (FSA, 2006). Fast food and other items with high potential for PFC contamination was chosen in a Canadian study,

* Corresponding author. Tel.: +81 75 753 4456; fax: +81 75 753 4458.
E-mail address: koizumi@pbh.med.kyoto-u.ac.jp (A. Koizumi).

0160-4120/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.envint.2009.01.010

resulting in detection of PFOS and PFOA in 7 and 5 samples, respectively, out of the total 53 samples (Tittlemier et al., 2007). Food from Spain contained PFOS and PFOA in 24 and 2 samples, respectively, out of total 36 food items (Ericson et al., 2008). In the only other duplicate diet study on PFCs found in the literature besides the present study, 214 daily duplicate diet composites from Germany contained PFOS and PFOA resulting in a daily intake median value of 1.4 and 2.9 ng/kg body weight, respectively (Fromme et al., 2007b). It is not clear if cooking changes PFC concentrations, although Del Gobbo and colleagues noticed a decrease in fish concentrations using several different cooking methods (Del Gobbo et al., 2008).

The aim of present study was to assess the daily intake of PFCs through diet and beverage and to evaluate the relationship between the dietary contamination and human serum levels. This case study was performed using human biological and food duplicate samples collected in 2004 and archived in the Kyoto Human Specimen Bank (Koizumi et al., 2005). In the current study, we used daily duplicate samples collected by 20 women in Japan, including all food and beverage that was taken that specific day, together with matched serum samples. It has previously been reported that geographical differences in serum levels exist in Japan (Harada et al., 2007). Two cities were therefore included in the present study, one small town in Miyagi Prefecture, north Honshu, with approximately 8000 inhabitants, and Osaka city with over 2 million inhabitants located in central Honshu.

2. Materials and methods

2.1. Samples

Serum and a one-day composite diet sample were collected in 2004 from 20 women in Japan. Those samples were archived in the Kyoto Human Specimen Bank at −20 °C until analysis (Koizumi et al., 2005). All participants were housewives between 34 and 75 years old (Table 1) and no one were regular blood donors. Ten lived in Osaka city, located at Honshu's south coast in Kansai area, which is next after Tokyo the most densely populated and industrialized area in Japan. The remaining ten women lived in Karakuwa, a small town located in Miyagi prefecture in north Honshu. The duplicate diet composites were prepared in the same manner as by Koizumi et al. (Koizumi et al., 2005). Briefly, the 24 h food and drink of an ordinary day were prepared in duplicate and collected by each participant. The food was collected in the same state as it was eaten, e.g. cooked if eaten cooked. Individual food and beverage items were weighed and recorded before mixed into a one-day composite sample. Weights and water content of the composites can be seen in Table 1. The composite was homogenized and a sub sample of 70–100 g was freeze-dried and pestled. Serum samples were taken after the collection of the duplicate sample was finished. Both serum and fresh diet samples were stored at −20 °C. We obtained approval for this research proposal from the Ethics Committee of the Kyoto University Graduate School of Medicine on 14 November 2003 (E25). Written informed consent was obtained before sample collection.

Table 1
Age of 20 women living in Osaka and Miyagi, Japan, and descriptive data of the one-day duplicate diet sample collected by each woman.

Osaka				
No.	Age	Diet sample (g, wet)	(g, dry)	Water content[a] (weight%)
O1	64	2169	347	84
O2	69	3633	363	90
O3	60	3055	367	88
O4	64	2137	321	85
O5	65	2719	517	81
O6	56	3103	465	85
O7	68	2834	312	89
O8	59	2203	352	84
O9	55	3097	434	86
O10	49	3387	373	89
Miyagi				
	Age	Diet sample (g, wet)	(g, dry)	Water content[a] (weight%)
M1	74	2916	467	84
M2	61	3073	522	83
M3	75	1814	272	85
M4	57	2838	369	87
M5	61	3219	644	80
M6	52	2605	417	84
M7	75	2673	401	85
M8	42	2309	393	83
M9	34	1426	271	81
M10	53	1820	346	81

[a] Water content was determined by loss after freeze-drying.

2.2. Chemicals

Ammonium acetate (>99%, pa for HPLC) was purchased from Aldrich (Steinheim, Germany), methanol (PCB and pesticide grade), acetonitrile (LC–MS grade) and water (distilled LC–MS grade) were from Kanto Chemicals (Tokyo, Japan). Ammonium hydroxide (25% in water) was purchased from Merck (Darmstadt, Germany). Acetic acid was from Nacalai tesque (Kyoto, Japan) and hydrochloric acid (HCl) from Wako (Osaka, Japan). Perfluorobutanesulfonate (PFBuS) tetrabutylammonium salt (>98%), PFOS potassium salt (>98%), perfluorodecanoic acid (PFDA; >97%), and perfluorohexanoic acid (PFHxA, >97%) were purchased from Fluka (Milwaukee, WI). Perfluoroheptanoic acid (PFHpA, 99%), perfluorononaoic acid (PFNA, 97%), perfluorooctanoic acid (PFOA, 96%), perfluoroundecanoic acid (PFUnDA, 95%) were purchased from Aldrich (Steinheim, Germany and Milwaukee, WI). Perfluorohexanesulfonate (PFHxS, 98%) were purchased from Interchim (Montlucon, France). $^{13}C_4$-labeled PFOA, $^{13}C_4$-labeled PFOS and $^{13}C_5$-labeled PFNA were from Wellington Laboratories (Guelph, Ontario, Canada).

2.3. Extraction

The diet samples were extracted using weak anion exchange, solid-phase extraction (Waters Oasis® WAX) based on the method by Taniyasu and colleagues (Taniyasu et al., 2005). Further clean-up was employed using a dispersive carbon method described by Powley et al. (Powley et al., 2005). The latter method was used for the serum samples. Approximately 10 g freeze dried sample and internal standards ($^{13}C_4$-PFOA and $^{13}C_4$-PFOS) were put into a 250 mL polypropylene (PP) centrifugation tube and 10 mL 0.2 M sodium hydroxide was added. After vortex mixing, the sample was left for 20 min. Extraction was performed by vigorously shaking the sample with 40 mL methanol for 30 min followed by addition of 1 mL 1 M HCl in methanol. After centrifugation (11,000 ×g, 30 min) the supernatant was reduced in volume by rotary evaporator to approximately 10 mL, and approximately 15 mL water was added. The solution was put through a WAX solid phase cartridge (6 cc, 150 mg 30 μm, Waters, Milford, MA, USA) previously conditioned with 4 mL methanol and 4 mL water. Subsequent loading of the sample was followed by washing the sorbent using 4 mL 40% (v/v) methanol in water, drying of the cartridges using N_2, and a second wash using 8 mL methanol. The perfluorinated compounds were eluted into a tube with 25 mg ENVI-Carb and 50 μL acetic acid, using 2 mL 2% (v/v) ammonium hydroxide in methanol. The carbon solution was vortex mixed for 30 s and then filtrated through 0.2 μm GHP membrane (Pall, East Hills, NY, USA) and reduced to 200 μL using N_2 after which 300 μL 2 mM ammonium acetate in water and the performance standard $^{13}C_5$-PFNA were added. The serum samples (0.5 mL) together with internal standards ($^{13}C_4$-PFOA and $^{13}C_4$-PFOS) were extracted with 5 mL acetonitrile, followed by centrifugation at 3000 ×g for 30 min. The supernatant was transferred into a tube with 25 mg ENVI-Carb and 50 μL acetic acid,

the solution was vortex mixed for 30 s and centrifuged at 3000 ×g for 15 min. The extract was then reduced in volume and filtrated in the same manner as for the diet samples.

2.4. Analysis

Analysis was performed using a Shimadzu LC 20 system (Tokyo, Japan) coupled to an MDS Sciex API 4000 MS/MS instrument (Applied Biosystems, Toronto, Canada) with an atmospheric electrospray interface operating in negative ion mode (Sciex, Toronto, Canada). Separation was performed on a Discovery HS C18 (50 mm length, 2.1 mm i.d., 3 µm particles, 120 Å pore size) column with a guard column of the same material (20 mm length, 2.1 mm i.d., 3 µm particles, 120 Å pore size, Supelco, Bellefonte PA, USA). Both columns were kept at 40 °C. Injection volume was 10 µL and the flow rate was set to 200 µL/min. A gradient program was employed using two mobile phases consisting of 2 mM ammonium acetate in acetonitrile and 2 mM ammonium acetate in water. A multiple reaction monitoring program divided into four periods with optimized parameters for each analyte was used measuring two product ions for each analyte except for PFOS and PFOA for which three product ions were measured (Table 2).

2.5. Quality assurance

Quantification was performed using the internal standard method with external standards dissolved in 35% methanol in water. $^{13}C_4$-PFOS was used as internal standard for the sulfonates, and $^{13}C_4$-PFOA was used for the carboxylates. $^{13}C_5$-PFNA was used to monitor the recovery of the internal standards. The performance of the method can be seen in Table 2. The recovery for serum samples was between 95 and 111% for all analytes while good recovery was obtained for most analytes in diet composites with somewhat lower recoveries for the longer chain carboxylates. Recoveries were evaluated by three to 11 replicate fortifications to a low-contaminated serum and diet sample. Procedural blank levels were evaluated in duplicates for each ten samples and trace signals were detected for PFOA, PFOS and longer chain carboxylates. In the case of blank levels, the mean blank signal was subtracted from the calculated sample concentration and a value was reported only if the calculated sample concentration was three times the blank concentration. The freeze-dry procedure of the diet composites were checked for contamination by using agar powder as blank matrix. Additionally, possible evaporation or other losses of PFCs and possible degradation of FTOHs or sulfonamides yielding PFCs were tested by adding $^{13}C_4$-PFOS, $^{13}C_4$-PFOA, 8:2 FTOH, perfluorooctanesulfonamidoethanol (FOSE) and perfluorooctanesulfonamidoacid (FOSA) (Wellington Laboratories, Guelph, Ontario, Canada) to diet composite samples prior drying. No contamination or loss of PFOS and PFOA in the freeze-drying process was observed. Neither was increased recoveries observed that could have resulted from degradation of FOSE/FOSA/FTOH to PFOS or PFOA.

Table 2
Validation performance of the methods for analysis of PFCs in human serum and diet composite samples.

Compound	Transition	Recovery and (reproducibility) % (CV%)[a]		Instrument detection limit [b]	Method detection limit	
	Quantification (confirmation)	Serum $n=6$	Diet composite $n=3$	(ng/mL)	Serum (ng/mL)	Diet composite (ng/g d.w.)
PFBuS	298.7>79.9 (298.7>98.9)	95 (6)	83 (24)	0.05	0.05	0.03
PFHxS	398.8>80.0 (398.8>98.8	96 (5)	72 (17)	0.01	0.05	0.03
PFOS	498.9>80.0 (498.9>99.0, 498.9>129.9)	98 (19)	75 (23) [c]	0.05	0.1	0.05
PFHxA	312.9>268.8 (312.9>119.0)	108 (8)	65 (12)	0.1	0.1	0.05
PFHpA	362.9>319.0 (362.9>169.0)	100 (9)	54 (8)	0.05	0.05	0.03
PFOA	412.9>369.0 (412.9>218.9, 412.9>168.9)	98 (14)	67 (15) [c]	0.2	0.5	0.15
PFNA	462.9>419.1 (462.9>219.1)	106 (13)	47 (4)	0.1	0.3	0.1
PFDA	512.8>468.8 (512.8>268.9)	111 (11)	17 (18)	0.05	0.1	0.5
PFUnDA	562.9>518.8 (562.9>268.9)	105 (11)	30 (10)	0.1	0.3	0.5

[a] CV = coefficent of variation.
[b] 10 µl injection.
[c] $n=11$.

2.6. One-compartment kinetic model simulation

A one-compartment pharmacokinetic model was employed to predict serum level of PFOA and PFOS attributed to the measured daily dietary intake. The parameters in the model are taken from Harada et al. (Harada et al., 2005a). In the one-compartment model, the serum concentration at T (days) was expressed as:

$$V\frac{dC(T)}{dT} = E - kC(T) \quad (1)$$

$$k = k_{tot} + k_m \quad (2)$$

where V and $C(T)$ represents the volume distribution (140 mL/kg for PFOA and 220 mL/kg for PFOS (Andersen et al., 2006)) and serum concentration (ng/mL) of PFOS and PFOA at time T days after initiation of exposure, respectively. We incorporated menstrual bleeding as one of the elimination routes. Other parameters represent: E, daily intake (ng/day); k, clearance (mL/day); k_{tot}, total clearance of males (0.092 and 0.077 mL/kg/day for PFOS and PFOA, respectively, calculated from median half-lives reported by Olsen and colleagues (Olsen et al., 2007)); k_m, menstrual serum loss (0.80 mL/kg/month, using a median body weight of the study subjects of 52.8 kg) assumed to start at 10 years and stop at 50 years.

2.7 Statistics

All statistical analyses were carried out using JMP software (SAS Institute Inc). $p<0.05$ was considered to be significant. The differences between means were tested by Student's t test. Correlation was tested by Pearson's product moment correlation coefficient. Analysis of covariance for PFCs levels was used to determine whether sampling site influenced the food levels when adjusted by water content.

3. Results and discussion

A summary of the results for PFOS and PFOA in 20 individual matched diet composite and serum samples is given in Table 3.

Only PFOS and PFOA could be detected in the diet samples with current method detection limits and it should be noted that the found levels of PFOA were relatively close to the method detection limit. PFOS and PFOA were detected in all diet samples and no difference between Osaka (medians 0.027 ng PFOS/g f.w. and 0.023 ng PFOA/g f.w.) and Miyagi (medians 0.028 ng PFOS/g f.w. and 0.018 ng PFOA/g f.w.) was seen. Although PFOS was present at highest concentration in the majority of diet samples the difference between PFOS and PFOA was not statistically significant.

Highest median serum concentration was seen in Osaka with a median concentration of 31 ng/mL for both PFOS and PFOA. Osaka serum levels are significantly higher compared to Miyagi serum levels (median PFOS 14 ng/mL, PFOA 4.6 ng/mL). PFOS was generally found at the highest concentration in serum samples from Miyagi, while for Osaka PFOA was found at the same median concentration (31 ng/mL) as PFOS (Table 3). Two serum samples from Osaka contained exceptional high concentrations of PFOS (161 and 136 ng/mL). Besides PFOS and PFOA up to seven additional PFCs were detected in the serum samples (Table 4). PFNA and PFDA in Osaka samples (medians 6.9

Table 3
Concentrations of PFOS and PFOA in paired serum (ng/mL) and diet samples (ng/g fw) collected in 2004 from females living in Osaka (O) and Miyagi (M).

	Serum (ng/mL)		Diet (ng/g f.w.)		Diet (ng/day)[a]		Diet (ng/kg b.w./day)[b]		Simulated serum (ng/mL)[d]	
No.	PFOS	PFOA	PFOS	PFOA	PFOS	PFOA	PFOS	PFOA	PFOS	PFOA
O 1	27.6	26.1	0.034	0.024	73.8	52.1	1.58	1.12	11.09	5.97
O 2	161	47	0.024	0.019	87.2	69	1.93	1.53	13.17	7.90
O 3	35	34.8	0.019	0.013	58.1	39.7	1.05	0.72	8.57	4.54
O 4	136	45.4	0.015	0.022	32.1	47	0.63	0.92	4.81	5.38
O 5	24.9	22.4	0.029	0.031	78.8	84.3	1.62	1.73	11.85	9.85
O 6	50.4	28.6	0.057	0.04	177	124	2.75	1.93	24.99	13.95
O 7	32.3	25.3	0.019	0.008	53.8	22.7	1.08	0.45	8.11	2.60
O 8	19.9	30.1	0.029	0.037	63.9	81.5	1.13	1.44	9.36	9.30
O 9	27	31.4	0.024	0.017	74.3	52.7	1.36	0.96	10.48	5.92
O 10	30	31.3	0.046	0.033	156	112	2.84	2.04	19.24	10.95
M 1	21.8	7.39	0.054	0.031	157	90.4	2.97	1.71	23.73	10.35
M 2	16.5	12.1	0.087	0.021	267	64.5	5	1.22	39.61	7.38
M 3	13.7	7.08	0.018	0.016	32.7	29	0.62	0.55	4.94	3.32
M 4	8.22	3.22	0.012	0.014	34.1	39.7	0.64	0.75	4.92	4.51
M 5	14.7	4.28	0.037	0.027	119	86.9	2.25	1.64	17.85	9.94
M 6	26.3	7.62	0.009	0.012	23.5	31.3	0.44	0.59	3.12	3.36
M 7	22.4	4.84	0.029	0.016	77.5	42.8	1.46	0.81	11.72	4.90
M 8	3.1	0.61	0.008	0.016	18.5	36.9	0.35	0.7	2.28	3.61
M 9	4.1	0.32	0.026	0.021	37.1	30	0.7	0.57	4.58	2.93
M 10	7.52	1.44	0.062	0.019	112	34.6	2.13	0.65	15.23	3.80
Osaka										
Range	19.9–161	22.4–47.0	0.015–0.057	0.008–0.040	32.1–180	22.7–124	0.63–2.84	0.45–2.0	4.81–24.99	2.60–13.95
Aritm. mean	54.4	32.2	0.03	0.024	85.5	68.5	1.6	1.29	12.2	7.6
Median	31.1	30.7	0.027	0.023	74	60.8	1.47	1.28	10.79	6.94
P25–P75[c]	26.0–93.2	25.7–40.1	0.019–0.040	0.015–0.035	56.0–122	43.4–98.0	1.07–2.34	0.82–1.83	8.46–14.69	5.17–10.13
Miyagi										
Range	3.10–26.3	0.32–12.1	0.008–0.087	0.012–0.031	18.5–267	29.0–90.4	0.35–5.04	0.55–1.71	2.28–39.61	2.93–10.35
Aritm. mean	13.8	4.89	0.034	0.019	88	48.6	1.66	0.92	12.80	5.41
Median	14.2	4.56	0.028	0.018	57.3	38.3	1.08	0.72	8.33	4.16
P25–P75[c]	5.81–22.1	1.03–12.1	0.058–0.022	0.015–0.031	28.1–138	30.6–75.7	0.53–2.61	0.58–1.43	4.22–19.32	3.35–8.02

[a]Calculated using the measured weight of the one-day composite.
[b]Calculated using the actual body weight of the Osaka participants and an average value (53 kg) for Miyagi participants.
[c]Percentile 25%–75%.
[d]Calculated using daily diet intake.

and 2.5 ng/mL) were higher compared to those from Miyagi (2.7 and 0.92 ng/mL). In contrary, PFUnDA was higher in Miyagi (median 3.2 ng/mL) than in Osaka (5.1 ng/mL).

The water content of diet samples from Osaka and Miyagi was compared. Miyagi samples had 83.3% and Osaka 86.1% ($p=0.0234$ by t-test) while dry weights were 409.8 g and 385.1 g for Miyagi and Osaka, respectively ($p=0.55$ by t-test), which implies no difference in calorie intakes. Analysis of covariance for PFOA indicates sampling site was a significant factor ($p=0.03$). Least square mean adjusted by water content were 0.0169 for Miyagi and 0.0267 for Osaka (ng/g f.w.). PFOS was not affected by sampling site ($p=0.92$).

The intake through diet and beverage during one day for 20 women in Japan was between 18.5–267 ng for PFOS, and 22.7–124 ng for PFOA (Table 3). Calculated on a body weight (b.w.) basis gives 0.35–5.04 ng PFOS/kg b.w./day and 0.45–2.0 ng PFOA/kg b.w./day (Table 3). To analyze the variation in food intake, analysis of covariance for intake (ng/day) was performed using two variables, sampling site and dry weight. Dry weight was a significant factor for both PFOS and PFOA ($p=0.014$ and 0.003, respectively). After adjusted by dry weight, sampling site was a significant factor for PFOA ($p=0.02$) and least square mean were 46.2 for Miyagi and 70.9 for Osaka (ng/day).

The median daily intake in present study from the two regions (1.1–1.5 ng PFOS/kg b.w./day and 0.72–1.3 ng PFOA/kg b.w./day) is in the same range as the calculated daily intake in the southern parts of Germany (Fromme et al., 2007b). In the mentioned study a total of 214 daily duplicate diet samples resulted in a median of 1.4 ng PFOS/kg b.w./day and 2.9 ng PFOA kg b.w./day, drinking water not included. The median serum levels in southern Germany was 13.7 ng PFOS/mL and 5.7 ng PFOA/mL, and it was concluded that diet was the major source of exposure for German cases. The German study is the only report in the literature estimate daily intake of PFCs through the duplicate diet approach. Three other studies in the literature estimates daily intake of PFCs but through market food baskets. A 2004 UK Total diet study estimated an upper bound daily intake of 100 ng PFOS/kg b.w. and 70 ng PFOA/kg b.w. (FSA, 2006). The values reported from the UK are higher relative other studies, partly due to inclusion of high LODs in the upper bound calculations. In Canada the dietary intake was estimated in 2004 to be 1.8 and 1.1 ng/kg b.w. for PFOS and PFOA respectively (Tittlemier et al., 2007). In Tarragona County, Spain, a food market study estimated the PFOS daily intake to 1.07 ng/kg b.w. for adult men and the median serum level in the region was estimated to 15.2 ng PFOS/mL (Ericson et al., 2007, 2008).

Based on the daily intake in present study, serum levels of PFOS and PFOA were simulated using one-compartment model for each participant (Table 3). Average serum PFOA concentrations was calculated to 7.6 ng/mL (23.7% of measured concentration) for Osaka females and to 5.4 ng/mL (110.6% of measured concentration) for Miyagi females. Serum PFOS concentrations was calculated to 12.2 ng/mL (22.4% of measured concentration) and 12.8 ng/mL (92.5% of measured concentration) for Osaka and Miyagi females, respectively. This suggests that dietary intake is a major exposure route in Miyagi but other routes of exposure seem to be of greater importance in Osaka. No association between PFOS and PFOA levels in the individual matched serum and diet samples was found as can be seen in the scatter plots in Fig. 1, indicating that diet contamination is not solely responsible for the serum levels. However, this analysis has its limitation as several assumptions have been made. It is assumed that the one-day composite sample is representative for the consumption over several years since the serum levels reflect historical exposure, considering the long half-lives of PFOS and PFOA. Although it has been shown that daily duplicate composites are good predictors of blood levels of compounds with long half-lives (Shimbo et al., 2000) it is currently unkown how PFC levels have changed in the Japanese diet. Moreover, limited number of subjects was studied during one day only. Day-to-day variations are likely to occur and this was shown in a study by Fromme and colleagues, however the authors concluded the variations to be overall not substantial (Fromme et al., 2007b). The one-compartment model used in this study assumes no discernible impact on potential sex differences in total clearance rates as predicted by Harada et al. (Harada et al.,

Table 4
Serum concentrations (ng/mL) of five PFCs for ten women in Osaka and ten women in Miyagi.

	PFHxS	PFHpA	PFNA	PFDA	PFUnDA
Osaka					
Range	1.3–4.1	0.05–0.45	3.1–9.2	1.4–4.2	3.2–9.0
Aritm. mean	2.2	0.26	6.7	2.6	5.8
Median	1.9	0.23	6.9	2.5	3.2
Miyagi					
Range	0.1–3.0	<0.05	0.38–5.4	<0.1–2.9	1.4–12
Aritm. mean	1.2	–	2.8	1.1	5.4
Median	0.93	–	2.7	0.92	5.1

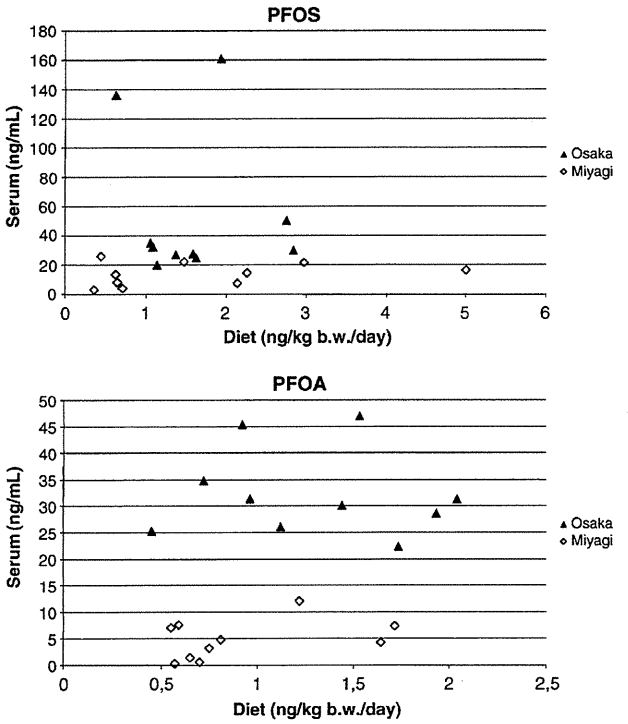


Fig. 1. Scatter plot of PFOS and PFOA concentrations in matched serum and one-day diet composite from ten women in Osaka and ten women in Miyagi, Japan.

2005a). Moreover, the steady-state assumption might not apply, although it has been used in previous studies (Harada et al., 2005a; Trudel et al., 2008). Variations in dietary habits between women, men and younger people might exist and are not covered by the present study since only women were included. Worth mentioning is that the daily intake of PFOS by a breast-feeding infant was calculated to approximately 100 ng/day (Kärrman et al., 2006a) which is in the same range as the female adult intake in the present study. The origin of the contaminants found in the diet could not be addressed in the present study since composites were used. Analysis of individual items in a food market basket approach is more useful for that purpose.

Diet and drinking water have been suggested to be two major PFC exposure sources for humans (Emmett et al., 2006; Fromme et al., 2007b). The number of studies covering this area reported in the literature is however limited. The diet composite samples in present study contained both food and beverage thus summarizing two possible exposure sources. In the present study these two sources are not the reason for elevated Osaka serum levels given the lack of difference in PFOS and PFOA diet levels between Miyagi and Osaka. The exposure source and explanation for the high serum levels of other carboxylates could not be elucidated in the present study. PFNA was found at serum levels around 20% and 50% of the corresponding PFOA levels in Osaka and Miyagi, respectively. Moreover, PFUnDA was higher than PFNA and similar to the PFOA level in Miyagi serum. One explanation for why PFNA and PFUnDA were not detected in any diet sample can be method limitations, since PFOA was detected relatively close to the detection limit and the recovery decreased with increasing chain length of the PFC. The reason for high serum levels of PFCs, and especially carboxylates, in Osaka can be due to a point source but the intake route is not clear. Other possible exposure sources are air and dust (Harada et al., 2005b; Oono et al., 2008; Strynar and Lindstrom, 2008).

The measured median PFOS serum level in Osaka corresponds to reported levels in the US (Calafat et al., 2006, 2007). However, the lower median in Miyagi more resembles reports from European countries, such as Spain, Germany and Sweden (Ericson et al., 2007; Fromme et al., 2007a; Kärrman et al., 2006c). The present study confirms previously reported geographical differences in serum from different regions in Japan (Harada et al., 2004) and levels in Miyagi serum correspond to previous reports from less populated regions in Japan (Harada et al., 2007). The Osaka median in the present study is however higher compared to levels reported in previous studies from Japan. Relatively high concentrations of other carboxylates were also observed, foremost in Osaka serum but from an international perspective also in serum from Miyagi. PFNA was the highest PFC detected after PFOS and PFOA, with a median level of 6.9 ng/mL and 2.7 ng/mL in Osaka and Miyagi serum, respectively (Table 4). PFDA levels (2.5 and 0.92 ng/mL) were lower than PFNA but interestingly PFUnDA levels were higher than PFDA. The median PFUnDA in Osaka serum was 3.2 ng/mL while corresponding value in Miyagi serum was 5.1 ng/mL. The notable high PFUnDA in Miyagi serum was confirmed in the second daughter ion trace. PFNA median levels of 1–2 ng/mL have been reported in serum worldwide but longer carboxylates are rarely detected in serum and if so only at low ng/mL concentrations (Calafat et al., 2007; Ericson et al., 2007; Kärrman et al., 2006b). While PFOA, PFNA and PFUnDA concentrations in Miyagi serum were positively associated to each other, thus indicating a common source, only PFOA and PFNA correlated in Osaka serum (data not shown). This indicates an exposure source for carboxylates in Japan, even in less industrialized areas and profoundly for odd number carboxylates. It has been reported that the fluoropolymer industry in Japan uses mainly PFNA instead of PFOA that is commonly used in Europe and North America, which can be one explanation to the observed pattern in human serum in this study (Prevedouros et al., 2006).

4. Conclusions

Food and beverage are major sources of exposure to PFCs in Japan. However, the importance of the diet for the body burden seems to vary between regions. The lack of association in levels between the matched serum and one-day diet composites is unexplained but can be due to variation in PFC diet levels throughout the last decade. Besides this, other non-dietary sources should be elucidated, especially in the Osaka area. Our results show that focus should also be directed to other carboxylates besides PFOA in order to find the sources for the relatively high serum levels of PFNA, PFDA and PFUnDA.

Acknowledgments

This study was supported by Japan society of promotion of science (JSPS PE7509) and was founded by Special Coordination Funds for Promoting Science and Technology (No. 1300001) sponsored by Japan Science and Technology Agency. Ms. Sayaka Aono and Ms. Sayoko Oono are kindly acknowledged for their valuable contribution to this study.

References

Alexander BH, Olsen GW, Burris JM, Mandel JH, Mandel JS. Mortality of employees of a perfluorooctanesulphonyl fluoride manufacturing facility. Occup Environ Med 2003;60:722–9.

Andersen ME, Clewell HJ, Tan Y-M, Butenhoff JL, Olsen GW. Pharmacokinetic modeling of saturable, renal resorption of perfluoroalkylacids in monkeys—probing the determinants of long plasma half-lives. Toxicology 2006;227:156–64.

Apelberg BJ, Witter FR, Herbstman JB, Calafat AM, Halden RU, Needham LL, et al. Cord serum concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in relation to weight and size at birth. Environ Health Perspect 2007;115:1670–6.

Calafat AM, Kuklenyik Z, Caudill SP, Reidy JA, Needham LL. Perfluorochemicals in pooled serum samples from United States residents in 2001 and 2002. Environ Sci Technol 2006;40:2128–34.

Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Tully JS, Needham LL. Serum concentrations of 11 polyfluoroalkyl compounds in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. Environ Sci Technol 2007;41:2237–42.

Del Gobbo L, Tittlemier SA, Diamond M, Pepper K, Tague B, Yeudall F, et al. Cooking decreases observed perfluorinated compound concentrations in fish. J Agric Food Chem 2008;56:7551–9.

Emmett EA, Shofer FS, Zhang H, Freeman D, Desai C, Shaw LM. Community exposure to perfluorooctanoate: relationships between serum concentrations and exposure sources. J Occup Environ Med 2006;48:759–70.

Ericson I, Gomez M, Nadal M, van Bavel B, Lindström G, Domingo JL. Perfluorinated chemicals in blood of residents in Catalonia (Spain) in relation to age and gender: a pilot study. Environ Int 2007;33:616–23.

Ericson I, Martí-Cid R, Nadal M, van Bavel B, Lindström G, Domingo JL. Human exposure to perfluorinated chemicals through the diet: intake of perfluorinated compounds in foods from the Catalan (Spain) market. J Agric Food Chem 2008;56:1787–94.

Fei CY, McLaughlin JK, Tarone RE, Olsen J. Perfluorinated chemicals and fetal growth: a study within the Danish National Birth Cohort. Environ Health Perspect 2007;115:1677–82.

Fromme H, Midasch O, Twardella D, Angerer J, Boehmer S, Liebl B. Occurence of perfluorinated substances in an adult German population in southern Bavaria. Int Arch Occup Environ Health 2007a;80:313–9.

Fromme H, Schlummer M, Möller A, Gruber L, Wolz G, Ungewiss J, et al. Exposure of an adult population to perfluorinated substances using duplicate diet portions and biomonitoring data. Environ Sci Technol 2007b;41:7928–33.

FSA U. Fluorinated chemicals: UK dietary intakes. London, UK: Food standards agency; 2006.

Giesy JP, Kannan K. Global distribution of perfluorooctane sulfonate in wildlife. Environ Sci Technol 2001;35:1339–42.

Gililland FG, Mandel JS. Mortality among employees of a perfluorooctanoic acid production plant. J Occup Med 1993;35:950–4.

Gulkowska A, Jiang Q, Ka So M, Taniyasu S, Lam PKS, Yamashita N. Persistent perfluorinated acids in seafood collected from two cities of China. Environ Sci Technol 2006;40:3736–41.

Harada K, Saito N, Sasaki S, Inoue K, Koizumi A. Perfluorooctane sulfonate contamination of drinking water in the Tama River, Japan: estimated effects on resident serum levels. Bull Environ Contam Toxicol 2003;71:31–6.
Harada K, Saito N, Inoue K, Yoshinaga T, Watanabe T, Sasaki S, et al. The influence of time, sex and geographic factors on levels of perfluorooctane sulfonate and perfluorooctanoate in human serum over the last 25 years. J Occup Health 2004;46: 141–7.
Harada K, Inoue K, Morikawa A, Yoshinaga T, Saito N, Koizumi A. Renal clearance of perfluorooctane sulfonate and perfluorooctanoate in humans and their species-specific excretion. Environ Res 2005a;99:253–61.
Harada K, Nakanishi S, Saito N, Tsutsui T, Koizumi A. Airborne perfluorooctanoate may be a substantial source contamination in Kyoto area, Japan. Bull Environ Contam Toxicol 2005b;74:64–9.
Harada K, Koizumi A, Saito N, Inoue K, Yoshinaga T, Date C, et al. Historical and geographical aspects of the increasing perfluorooctanoate and perfluorooctane sulfonate contamination in human serum in Japan. Chemosphere 2007;66:293–301.
Kannan K, Tao L, Sinclair E, Pastva SD, Jude DJ, Giesy JP. Perfluorinated compounds in aquatic organisms at various trophic levels in a great lakes food chain. Arch Environ Contam Toxicol 2005;48:559–66.
Kärrman A, Ericson I, van Bavel B, Darnerud PO, Aune M, Lignell S, et al. Exposure of perfluorinated chemicals through lactation — levels in matched human milk and serum and a temporal trend, 1996–2004, in Sweden. Environ Health Perspect 2006a;115:226–30.
Kärrman A, Mueller JF, van Bavel B, Harden F, Toms L-ML, Lindström G. Levels of 12 perfluorinated chemicals in pooled Australian serum collected 2002–2003 in relation to age, gender and region. Environ Sci Technol 2006b;40:3742–8.
Kärrman A, van Bavel B, Järnberg U, Hardell L, Lindström G. Perfluorinated chemicals in relation to other persistent organic pollutants in human blood. Chemosphere 2006c;64:1582–91.
Kennedy GL, Butenhoff JL, Olsen GW, O'Connor JC, Seacat AM, Perkins RG, et al. The toxicology of perfluorooctanoate. Crit Rev Toxicol 2004;34:351–84.
Koizumi A, Yoshinaga T, Harada K, Inoue K, Morikawa A, Muroi J, et al. 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 2005;99:31–9.
Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol Sci 2007;99:366–94.
OECD. Draft assessment of perfluorooctane sulfonate (PFOS) and its salts: complete assessment. Organisation for Economic Co-operation and Development; 2002. p. 362.
Olsen GW, Burris JM, Burlew MM, Mandel JH. Epidemiologic assessment of worker serum perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) concentrations and medical surveillance examinations. J Environ Monitor 2003;45:260–70.
Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL, et al. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ Health Perspect 2007;115:1298–305.
Oono S, Matsubara E, Harada KH, Takagi S, Hamada S, Asakawa A, et al. Survey of airborne polyfluorinated telomers in Keihan Area, Japan. Bull Environ Contam Toxicol 2008;80:102–6.
Peden-Adams MM, Keller JM, EuDaly JG, Berger J, Gilkeson GS, Keil DE. Suppression of humoral immunity in mice following exposure to perfluorooctane sulfonate. Toxicol Sci 2008;104:144–54.
Powley CR, George SW, Ryan TW, Buck RC. Matrix effect-free analytical methods for determination of perfluorinated carboxylic acids in environmental matrices. Anal Chem 2005;77:6353–8.
Prevedouros K, Cousins IT, Buck RC, Korzeniowski SH. Sources, fate and transport of perfluorocarboxylates. Environ Sci Technol 2006;40:32–44.
Shimbo S, Zhang Z-W, Moon C-S, Watanabe T, Nakatsuka H, Matsuda-Inoguchi N, et al. Correlation between urine and blood concentrations, and dietary intake of cadmium and lead among women in the general population of Japan. Int Arch Occup Environ Health 2000;73:163–70.
Skutlarek D, Exner M, Färber H. Perfluorinated surfactants in surface and drinking waters. Environ Sci Pollut Res Int 2006;13:299–307.
Smithwick M, Mabury SA, Solomon KR, Sonne C, Martin JW, Born EW, et al. Circumpolar study of perfluoroalkyl contaminants in polar bears (*Ursus maritimus*). Environ Sci Technol 2005;39:5517–23.
Strynar MJ, Lindstrom AB. Perfluorinated compounds in house dust from Ohio and North Carolina, USA. Environ Sci Technol 2008;42:3751–6.
Taniyasu S, Kannan K, Ka So M, Gulkowska A, Sinclair E, Okazawa T, et al. Analysis of fluorotelomer alcohols, fluortelomer acids and short- and long-chain perfluorinated acids in water and biota. J Chromatogr A 2005;1093:89–97.
Tittlemier SA, Pepper K, Seymour C, Moisey J, Bronson R, Cao XL, et al. Dietary exposure of Canadians to perfluorinated carboxylates and perfluorooctane sulfonate via consumption of meat, fish, fast food, and food items prepared in their packing. J Agric Food Chem 2007;55:3203–10.
Tomy GT, Budakowski WR, Halldorson T, Helm PA, Stern GA, Friesen K, et al. Fluorinated organic compounds in an eastern Arctic marine food web. Environ Sci Technol 2004;38:6475–81.
Trudel D, Horowitz L, Wormuth M, Scheringer M, Cousins IT, Hungerbuhler K. Estimating consumer exposure to PFOS and PFOA. Risk Anal 2008;28:1-19.
Yeung LW, So MK, Jiang G, Taniyasu S, Yamashita N, Song M, et al. Perfluorooctanesulfonate and related fluorochemicals in human blood samples from China. Environ Sci Technol 2006;40:715–20.

Doi: 10.1111/j.1742-7843.2009.00409.x
Journal compilation © 2009 Nordic Pharmacological Society. *Basic & Clinical Pharmacology & Toxicology*, **105**, 136–138

Short Communication

Human Organic Anion Transporter hOAT4 is a Transporter of Perfluorooctanoic Acid

Hatsuki Nakagawa[1,2], Tomohiro Terada[2], Kouji H. Harada[1], Toshiaki Hitomi[1], Kayoko Inoue[1], Ken-ichi Inui[2] and Akio Koizumi[1]

[1]Department of Health and Environmental Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan, and
[2]Department of Pharmacy, Kyoto University Hospital, Kyoto, Japan

(Received 24 October 2008; Accepted 4 January 2009)

Perfluorooctanoic acid is a class of synthetic fluorochemicals used in a variety of applications such as processing aids for fluoropolymer production, surfactants and water-repellent coatings [1,2]. It poses special public health concerns due to its long-term persistence and bioaccumulation in the environment, including humans [3–5]. There have been a number of reports regarding the health effects of perfluorooctanoic acid. It was found to be a carcinogen for rodents [6] while it is not genotoxic in *umu* test [7]. Furthermore, a developmental toxicity study of perfluorooctanoic acid in mice revealed early total loss and delays in general growth and development [8].

Species and sex differences have been reported for the toxicokinetics of perfluorooctanoic acid. Human serum elimination half-life of perfluorooctanoic acid is reported to be 3.8 years [9]. However, its serum elimination half-lives are much shorter in male Wistar rats (5.7 days) [10] suggesting large differences in perfluorooctanoic acid elimination kinetics among species. In clear contrast to rats, renal clearance of perfluorooctanoic acid in humans is negligible [11]. Although several organic anion transporters (OATs) are found to mediate transport of perfluorooctanoic acid in rats and humans, there were no kinetically significant difference in their activities [12,13]. Assuming that perfluorooctanoic acid transport from serum to tubular epithelial cells would be expected to occur in similar manners in both species, it is reasonable to predict a critical role for the re-absorption process as a determinant for the large species differences observed in the renal excretion of perfluorooctanoic acid. OAT4 (*SLC22A11*), a transporter that is only expressed in humans, is an apical type isoform in proximal tubules and mediates the re-absorption of organic anions [14]. Therefore, it may contribute to the long half-life of perfluorooctanoic acid in humans. In the present study, we aimed to characterize the perfluorooctanoic acid transport activity of human OAT4.

Author for correspondence: Akio Koizumi, Department of Health and Environmental Sciences, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan (fax + 81 75 753 4458, e-mail koizumi@pbh.med.kyoto-u.ac.jp).

Materials and Methods

Materials. *p*-[Glycyl-1-^{14}C]aminohippurate (PAH, 1.9 GBq/mmol) and [6,7-^{3}H(*N*)]estrone sulphate, ammonium salt (ES, 2.1 TBq/mmol) were from Perkin-Elmer Life Sciences Inc. (Boston, MA). We also commissioned Perkin Elmer Life Sciences to synthesize [1-^{14}C]perfluorooctanoic acid (34 mCi/mmol). Foetal bovine serum and trypsin were purchased from Invitrogen (Carlsbad, CA). All other compounds used were of the highest purity available.

Cell culture and transfection. HEK 293 cells (American Type Culture Collection CRL-1573), a transformed cell line derived from human embryonic kidney, were cultured in complete medium consisting of Medium 199 (Invitrogen, Carlsbad, CA) with 10% foetal bovine serum (Thermo. Electron Co., Waltham, MA) in an atmosphere of 5% CO_2, 95% air at 37°. Full-length cDNAs of human (h) OAT1 (*SLC22A6*) [15], hOAT3 (*SLC22A8*) [15] and hOAT4 [16] were subcloned into pBK-CMV plasmid vector (Stratagene, La Jolla, CA). HEK 293 cells were transfected with 0.8 μg hOAT1 cDNA, hOAT3 cDNA, hOAT4 or empty vector using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. G418 (Nacalai Tesque, Kyoto, Japan) (0.5 mg/ml) – resistant cells were removed. Cells expressing hOAT1 (HEK-hOAT1) were selected by measuring *p*-[^{14}C]aminohippurate uptake, and cells expressing hOAT3 (HEK-hOAT3) and hOAT4 (HEK-hOAT4) were selected by measuring [^{3}H]estrone sulphate uptake. Expression of *hOAT4* mRNA was analysed by reverse transcription-PCR (forward primer, 5′-TAAGCCAGTCCATCTTCATGTCCG-3′; reverse primer, 5′-TTGCCCTTAATAATCAGCCACCGG-3′), as previously reported [17]. Cells transfected with empty

vector (HEK-pBK) were used as control cells. These transfectants were maintained in complete medium with G418 (0.5 mg/ml).

Uptake experiments. Uptake experiments were performed as described previously [18] with some modifications. HEK cells were seeded on poly-D-lysine-coated 24-well plates at a density of 2×10^5 cells/well for the uptake of [1-^{14}C]perfluorooctanoic acid. At 48 hrs after seeding, the uptake of these compounds by HEK cells was examined. The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose and 5 HEPES (pH 7.4). The cells were pre-incubated with 0.2 ml of the incubation medium for 10 min. at 37°. After the pre-incubation, the medium was replaced with 0.2 ml of incubation medium containing [1-^{14}C]perfluorooctanoic acid. At the end of the incubation period of 15 min., the medium was aspirated and then cells were washed two times with 1 ml of ice-cold incubation medium. The cells were lysed in 0.25 ml of 0.5 N NaOH solution and the radioactivity in aliquots was determined in 3 ml of ACSII (Amersham International, Buckingham shire, UK). The protein contents of the solubilized cells were determined by the method of Bradford [19] using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA) with bovine γ-globulin as a standard.

Statistical analysis. Each uptake experiment was performed in triplicate. Data are expressed as means and standard errors of the mean. Statistical differences were determined using Student's unpaired t-test. Differences were considered significant at $P < 0.01$.

Results

Uptake of perfluorooctanoic acid by OATs transfected into HEK293 cells. Firstly, we confirmed expression of hOAT4 mRNA (fig. 1). Strong expression of hOAT4 mRNA was observed in pBK-hOAT4-transfected HEK 293 cells while weak expression was detected in mock-transfected cells. Secondly, we examined the [^{3}H]ES uptake rate into HEK293 cells transfected with hOAT4 (fig. 2A). We confirmed the functional expression of hOAT4 using its optimal substrate. Thirdly, we examined [^{14}C]perfluorooctanoic acid uptake by hOATs expressed in HEK293 cells. The uptake for 15 min. was stimulated in cells transfected with human OAT1, OAT3, and OAT4 (fig. 2B). The uptake of [^{14}C]perfluorooctanoic acid by HEK-hOATs was 1.6–2.7-fold higher than that by control cells ($P < 0.01$ for each cells). Moreover, the accumulation of perfluorooctanoic acid was significantly greater in HEK-hOAT4 than in HEK-hOAT1 ($P < 0.01$). To investigate the contribution of possible non-specific binding of

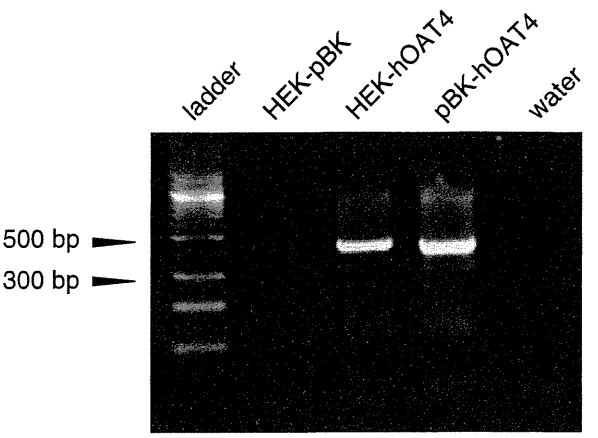


Fig. 1. Expression of hOAT4 mRNA in *hOAT4* cDNA-transfected HEK293 cells. RT-PCR products were electrophoresed in 1.5% agarose gel. HEK-pBK: cells transfected with empty vector; HEK-hOAT4: cells transfected with hOAT4 cDNA; pBK-hOAT4: PCR products from plasmid vector.

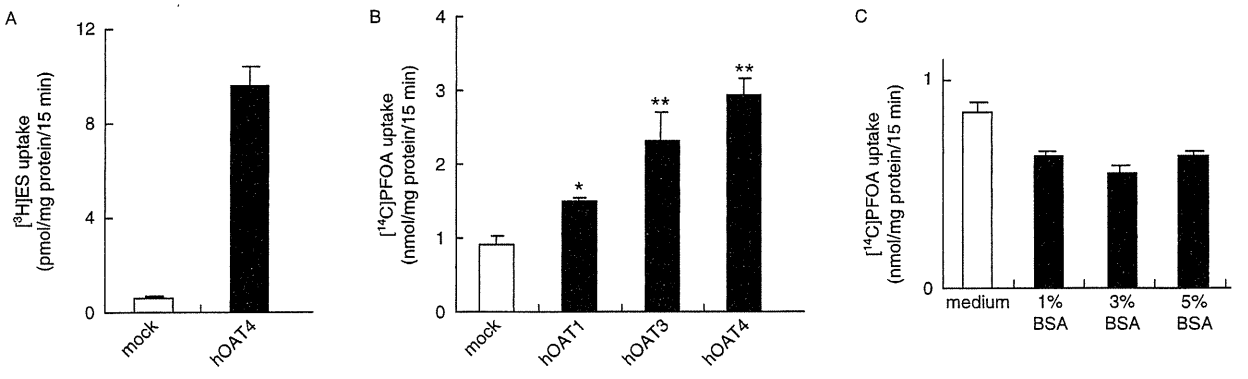


Fig. 2. Transport activity of hOAT4 expressed in HEK293 cells. (A) HEK293 cells transfected with the pBK vector alone (mock, white bar) and hOAT4 (black bar) were incubated in a solution containing 20 nM [^{3}H]ES at 37° for 15 min. (B) [^{14}C]perfluorooctanoic acid uptake by hOAT1, hOAT3, and hOAT4 expressed in HEK293 cells. HEK293 cells transfected with pBK were incubated in a solution containing 10 μM [^{14}C]perfluorooctanoic acid at 37° for 15 min. (C) Effect of BSA washing on [^{14}C]perfluorooctanoic acid uptake by HEK293 cells. HEK293 cells were incubated in a solution containing 10 μM [^{14}C]perfluorooctanoic acid at 37° for 15 min., and then washed with incubation medium containing 1%, 3%, and 5% of bovine serum albumin. Each value represents the mean ± S.E.M. of three determinations. *$P < 0.01$, **$P < 0.001$ versus mock.

perfluorooctanoic acid on cell membrane, HEK293 cells were washed with bovine serum albumin after incubation with perfluorooctanoic acid (fig. 2C). BSA washing reduced perfluorooctanoic acid uptake by 30% at most, suggesting that perfluorooctanoic acid uptake in transfected cells was mediated by transporters.

Discussion

In the present study, we found that human OAT4 transported perfluorooctanoic acid. The uptake by hOAT4 was greater than that by hOAT1.

Previously, we have found that the transport activities of hOAT1 and hOAT3 for perfluorooctanoic acid were nearly equal to those for PAH which is a good substrate for both OAT1 and OAT3 [12]. Therefore, hOAT1 and hOAT3 could involve in perfluorooctanoic acid transport from serum to tubular epithelial through the basolateral membrane, while only a small amount of perfluorooctanoic acid is detected in urine in humans [20]. Perfluorooctanoic acid transport activity by hOAT4 suggests that re-absorption of perfluorooctanoic acid from tubule might cause poor excretion into urine. Further studies are required to evaluate the contributions of OAT4 to species-difference in the toxicokinetics of perfluorooctanoic acid.

In addition, hOAT4 mRNA is abundantly expressed in the placenta as well as in the kidney [21]. hOAT4 is expressed in basal (foetal facing) membrane and could be involved in elimination of toxic substances from foetuses. Moderate partition of perfluorooctanoic acid from maternal blood to foetus was observed in humans [22]. The contribution of OAT4 to distribution of perfluorooctanoic acid warrants further investigation.

In conclusion, we have demonstrated that OAT4 have perfluorooctanoic acid transport.

Acknowledgement

This study was mainly supported by a grant (H15-Chemistry-004) from the Ministry of Health, Labor and Welfare of Japan.

References

1 Kissa E. Fluorinated Surfactants and Repellents, 2nd ed. Marcel Dekker, New York. 2001.
2 Hogue C. Perfluorinated pollutant puzzle. Chem Eng News 2004;**82**:17–9.
3 Kannan K, Choi JW, Iseki N, Senthilkumar K, Kim DH, Masunaga S *et al.* Concentrations of perfluorinated acids in livers of birds from Japan and Korea. Chemosphere 2002;**49**:225–31.
4 Harada K, Koizumi A, Saito N, Inoue K, Yoshinaga T, Date C *et al.* Historical and geographical aspects of the increasing perfluorooctanoate and perfluorooctane sulfonate contamination in human serum in Japan. Chemosphere 2007;**66**:293–301.
5 Nakayama S, Harada K, Inoue K, Sasaki K, Seery B, Saito N *et al.* Distributions of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) in Japan and their toxicities. Environ Sci 2005;**12**:293–313.
6 Biegel LB, Hurtt ME, Frame SR, O'Connor JC, Cook JC. Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. Toxicol Sci 2001;**60**:44–55.
7 Oda Y, Nakayama S, Harada KH, Koizumi A. Negative results of *umu* mutagenecity test of fluorotelomer alcohols and perfluorinated alkyl acids. Environ Health Prev Med 2007;**12**:217–9.
8 Lau C, Thibodeaux JR, Hanson RG, Narotsky MG, Rogers JM, Lindstrom AB *et al.* Effects of perfluorooctanoic acid exposure during pregnancy in the mouse. Toxicol Sci 2006;**90**:510–8.
9 Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL *et al.* Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ Health Perspect 2007;**115**:1298–305.
10 Kudo N, Katakura M, Sato Y, Kawashima Y. Sex hormone-regulated renal transport of perfluorooctanoic acid. Chem Biol Interact 2002;**139**:301–16.
11 Harada K, Inoue K, Morikawa A, Yoshinaga T, Saito N, Koizumi A. Renal clearance of perfluorooctane sulfonate and perfluorooctanoate in humans and their species-specific excretion. Environ Res 2005;**99**:253–61.
12 Nakagawa H, Hirata T, Terada T, Jutabha P, Miura D, Harada KH *et al.* Roles of organic anion transporters in the renal excretion of perfluorooctanoic acid. Basic Clin Pharmacol Toxicol 2008;**103**:1–8.
13 Katakura M, Kudo N, Tsuda T, Hibino Y, Mitsumoto A, Kawashima Y. Rat organic anion transporter 3 and organic anion transporting polypeptide 1 mediate perfluorooctanoic acid transport. J Health Sci 2007;**53**:77–83.
14 Ekaratanawong S, Anzai N, Jutabha P, Miyazaki H, Noshiro R, Takeda M *et al.* Human organic anion transporter 4 is a renal apical organic anion/dicarboxylate exchanger in the proximal tubules. J Pharmacol Sci 2004;**94**:297–304.
15 Takeda M, Hosoyamada M, Cha SH, Sekine T, Endou H. Hydrogen peroxide downregulates human organic anion transporters in the basolateral membrane of the proximal tubule. Life Sci 2000;**68**:679–87.
16 Kusuhara H, Sekine T, Utsunomiya-Tate N, Tsuda M, Kojima R, Cha SH *et al.* Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. J Biol Chem 1999;**274**:13675–80.
17 Islam R, Anzai N, Ekaratanawong S, Kundu-Srivastava S, Ji Jin C, Noshiro-Kofuji R *et al.* Characterization of human organic anion transporter 4 (hOAT4) as a low affinity p-aminohippurate (PAH) transporter. J Kyorin Med Soc 2007;**38**:61–8.
18 Urakami Y, Akazawa M, Saito H, Okuda M, Inui KI. cDNA cloning, functional characterization, and tissue distribution of an alternatively spliced variant of organic cation transporter hOCT2 predominantly expressed in the human kidney. J Am Soc Nephrol 2002;**13**:1703–10.
19 Bradford MM. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 1976;**72**:248–54.
20 Harada K, Inoue K, Morikawa A, Yoshinaga T, Saito N, Koizumi A. Renal clearance of perfluorooctane sulfonate and perfluorooctanoate in humans and their species-specific excretion. Environ Res 2005;**99**:253–61.
21 Cha SH, Sekine T, Kusuhara H, Yu E, Kim JY, Kim DK *et al.* Molecular cloning and characterization of multispecific organic anion transporter 4 expressed in the placenta. J Biol Chem 2000;**275**:4507–12.
22 Midasch O, Drexler H, Hart N, Beckmann MW, Angerer J. Transplacental exposure of neonates to perfluorooctanesulfonate and perfluorooctanoate: a pilot study. Int Arch Occup Environ Health 2007;**80**:643–8.

Chemosphere 77 (2009) 1333–1337

Contents lists available at ScienceDirect

Chemosphere

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

Contamination levels of mercury in the muscle of female and male spiny dogfishes (*Squalus acanthias*) caught off the coast of Japan

Tetsuya Endo [a,*], Yohsuke Hisamichi [a], Osamu Kimura [a], Yuichi Kotaki [b], Yoshihisa Kato [c], Chiho Ohta [d], Nobuyuki Koga [d], Koichi Haraguchi [e]

[a] *Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, 1757 Ishikari-Tobetsu, Hokkaido 061-0293, Japan*
[b] *School of Marine Biosciences, Kitasato University, Sanriku, Ofunato 022-0101, Japan*
[c] *Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, 1314-1 Shido, Sanuki, Kagawa 769-2193, Japan*
[d] *Faculty of Nutritional Sciences, Nakamura Gakuen University, 5-7-1 Befu, Johnan-Ku, Fukuoka 814-0198, Japan*
[e] *Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-Cho, Minami-Ku, Fukuoka 815-8511, Japan*

ARTICLE INFO

Article history:
Received 11 July 2009
Received in revised form 17 September 2009
Accepted 18 September 2009
Available online 27 October 2009

Keywords:
Spiny dogfish (*Squalus acanthias*)
Mercury
Stable isotope (^{13}C and ^{15}N)
Gender difference

ABSTRACT

We analyzed the total mercury (T-Hg) and stable isotopes of ^{13}C and ^{15}N in the muscle of spiny dogfish (*Squalus acanthias*) caught off the coast of Japan. The average body length of the female spiny dogfish sampled (94.9 ± 20.2 cm, 50.5–131.0 cm, $n = 40$) was significantly larger than that of the males sampled (77.8 ± 10.8 cm, 55.5–94.0 cm, $n = 35$), although the ages of the samples were unknown. The T-Hg concentration in the muscle samples rapidly increased after maturity in the females (larger than about 120 cm) and males (larger than about 90 cm), followed by a continued gradual increase. Contamination level of T-Hg in female muscle samples (0.387 ± 0.378 μg (wet g)$^{-1}$, $n = 40$) was slightly higher than that in male muscle samples (0.316 ± 0.202 μg (wet g)$^{-1}$, $n = 35$), probably due to the greater longevity of females. In contrast, the contamination level of T-Hg in females smaller than 94.0 cm in length (0.204 ± 0.098 μg (wet g)$^{-1}$, $n = 20$) was slightly lower than that in the males, probably due to the faster growth rate of females. Although the $\partial^{13}C$ and $\partial^{15}N$ values in the muscle samples increased with an increase in body length, there were no significant differences between the females (−17.2 ± 0.4‰ and 12.4 ± 0.9‰, respectively) and males (−17.3 ± 0.4‰ and 12.4 ± 0.8‰, respectively). A positive correlation was found between $\partial^{13}C$ and $\partial^{15}N$ values, suggesting trophic enrichment due to the growth.

1. Introduction

The spiny dogfish (*Squalus acanthias*) is a small schooling shark. Reproduction is ovoviviparous and the gestation period is the longest known for any vertebrate at an estimated 24 months (Ferrari and Ferrari, 2000). They eat small fishes and invertebrates such as krill, crabs, squid and octopus. Ketchen (1975) determined the age of spiny dogfish from markings on the second dorsal spine. Although the growth rate of this shark varies with habitat, the body length and life span of the female are both greater than those of the male (Forrester et al., 1972; Ketchen, 1975). The spiny dogfish is fished for food in Europe, US, Canada, Australia, New Zealand and other countries. The dogfish is also used as a source of liver oil, and in fertilizer and pet food. Small-scale of commercial fishing is undertaken in Aomori and Hokkaido Prefectures, the northern area of Japan (Fig. 1), and the fish taken are used for food, the production of liver oil and in dietary supplements.

Mercury (Hg) is typically accumulated in predatory fishes via the marine food web. Contamination levels of Hg in the spiny dogfish have been investigated in Canada (Forrester et al., 1972; Ketchen, 1975), US (Greig et al., 1977), New Zealand (van den Broek et al., 1981) and Australia (Walker, 1988). Like other predatory fishes, the contamination level of Hg in the dogfish muscle increases with body length (age), and varies by habitat. For instance, the contamination level of total mercury (T-Hg) in the muscle of dogfish caught off Australia was 0.86 ± 0.43 μg (wet g)$^{-1}$ (0.28–2.3 μg (wet g)$^{-1}$, $n = 77$) (Walker, 1988), whereas that of dogfish caught off New Zealand was 0.29 ± 0.26 μg (wet g)$^{-1}$ (0.01–1.13 μg (wet g)$^{-1}$, $n = 61$) (van den Broek et al., 1981), although the average body lengths of the fish in both surveys were almost the same (77.0 cm vs 78.9 cm). The T-Hg content in the muscle of fish caught off the Strait of Georgia, Canada, increased with increases in body length and reached levels of 0.5 μg (wet g)$^{-1}$ at a body length of 72 cm and 77 cm for males and females, respectively; the relation between Hg contamination and body length differed markedly between males and females, probably due to slower growth rate of males (Forrester et al., 1972; Ketchen, 1975). However, little information on the contamination level of Hg in the spiny dogfish caught off the coast of Japan is available.

Levels of Hg in predatory fishes and the health risk due to their consumption is of great interest to consumers. Due to concerns

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

0045-6535/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2009.09.041

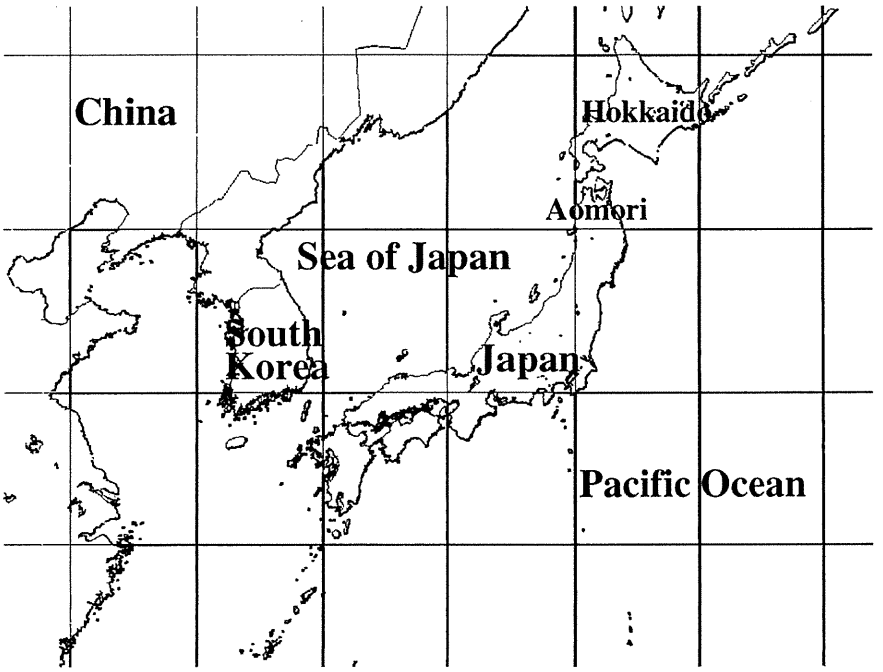


Fig. 1. Map of Japan showing Aomori and Hokkaido Prefectures.

over the impact of methylmercury (M-Hg) on the developing fetus, the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) lowered its guideline value for the provisional tolerable weekly intake (PTWI) of M-Hg from 3.3 μg (kg-bw)$^{-1}$ to 1.6 μg (kg-bw)$^{-1}$ (JECFA, 2003). The authorities in many countries have also advised pregnant women to limit their consumption of predatory fish such as tuna, shark and swordfish.

Stable isotopes of ^{13}C and ^{15}N are useful for the delineation and understanding of the trophic relationships and origins of marine prey as well as in terrestrial food webs. Recently, stable isotope ratios of $\partial^{13}C$ and $\partial^{15}N$ have been used to elucidate differences in the mechanism of Hg bioaccumulation between two or more species or in the same species from different areas (Yoshinaga et al., 1992; Al-Reasi et al., 2007; Bank et al., 2007; Rigét et al., 2007; Loseto et al., 2008). To our knowledge, however, stable isotope ratios have not yet been compared between male and female sharks, which are known to have different growth rates.

The purposes of present study are to compare the contamination levels of T-Hg and $\partial^{13}C$ and $\partial^{15}N$ values between male and female spiny dogfishes caught off the coast of Japan, and investigate growth-related changes in T-Hg, $\partial^{15}N$ and $\partial^{13}C$.

2. Materials and methods

2.1. Sampling

Seventy-five spiny dogfishes caught off the north Pacific coast of Japan were purchased at random from a storehouse of a fish company in Aomori Prefecture, Japan (Fig. 1), in August 2008, and extracted muscle and liver samples were stored at −20 °C until analysis.

2.2. Chemical analyses

Total mercury (T-Hg) in the subsamples of muscle and liver was analyzed using a flameless atomic absorption spectrophotometer (Hiranuma Sangyo, HG-1, Ibaragi, Japan) after digestion by a mixture of HNO_3, $HClO_4$ and H_2SO_4 (Endo et al., 2002). As reported previously (Endo et al., 2002, 2004, 2007), DOLT-2 (National Research Council of Canada) was used as an analytical quality control sample for the determination of T-Hg. The T-Hg concentrations shown in Table 1 were the mean of duplicate or triplicate analyses.

Dried subsamples of spiny dogfish muscle were analyzed for stable isotopes (^{15}N and ^{13}C) after the removal of lipids using chloroform/methanol extraction (Logan and Lutcavage, 2008). $\partial^{15}N$ and $\partial^{13}C$ 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, Kyoto University (Kyoto, Japan). Precision of the on-line procedure was better than ±0.2‰. The natural abundances of ^{15}N and ^{13}C are expressed by per mil (‰) deviation from international standards; ^{15}N or $^{13}C = (R_{sample}/R_{standard} - 1) \times 1000(‰)$, where R in $\partial^{15}N$ or $\partial^{13}C$ is $^{15}N/^{14}N$ or $^{13}C/^{12}C$, respectively. Atmospheric nitrogen and Pee Dee Belemnite (PDB) are the international standards for nitrogen and carbon, respectively. Nitrogen and carbon concentrations were determined using a thermal conductivity detector (TCD) attached to an elemental analyzer (Tayasu et al., 2002).

2.3. Statistical analyses

The data were analyzed by Student's t-test using the statcell 12 program, and the level of significance was set at $p < 0.05$. All data were expressed as the mean ± standard deviation (SD).

3. Results and discussion

3.1. Body size of analyzed spiny dogfish

Table 1 summarizes the body size of spiny dogfish purchased at random from a fish company. The average body length of females (94.9 ± 20.2 cm, $n = 40$) was significantly larger than that of males (77.8 ± 10.8 cm, $n = 35$); the maximal body lengths of female and

Table 1
Analytical data for male and female spiny dogfish (mean ± SD with range).

	Body length (cm)	Hg in muscle μg (wet g)$^{-1}$	Hg in liver μg (wet g)$^{-1}$	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	Estimated age (yr)
Male	77.8 ± 10.8	0.316 ± 0.202	0.023 ± 0.012	−17.3 ± 0.4	12.4 ± 0.8	27 ± 7
(n = 35)	(55.5–94.0)	(0.112–0.859)	(0.010–0.071)	(−18.0 to −16.6)	(11.4–12.8)	(16–41)
Female[a]	94.9 ± 20.2*	0.387 ± 0.378	0.031 ± 0.022	−17.2 ± 0.4	12.4 ± 0.9	36 ± 11*
(n = 40)	(50.5–131.0)	(0.110–1.871)	(0.011–0.110)	(−18.0 to −16.5)	(10.7–14.4)	(18–61)

[a] Body length, Hg concentration in muscle and estimated age of females smaller than 94 cm (n = 21) were 78.8 ± 11.3 cm, 0.202 ± 0.095 μg (wet g)$^{-1}$ and 27 ± 4 yr, respectively.
* Significantly different from male ($p < 0.05$).

male samples were 131.0 cm and 94.0 cm, respectively. Most of the females larger than 88 cm had gametes, and some females larger than 108 cm had gametes and fetuses. According to information from the fish company that sold these shark samples, the maximal body lengths of female and male spiny dogfishes caught off the northern coast of Japan are about 130 cm and 95 cm, respectively, and the body lengths of female and male dogfishes analyzed in this study may represent the natural population; however, little information is available regarding age. The larger body length of females was also reported for the spiny dogfish caught off British Columbia Water, Canada (Forrester et al., 1972; Ketchen, 1975). Although the growth rate and maturity of spiny dogfishes varies according to habitat, a female with a body length of 90 cm and aged 23 yr and a male with a body length of 70 cm and aged 14 yr caught off the Washington coast of BC, Canada, were reported to have reached maturity (Ketchen, 1975). Similarly, a larger body length in females has also been reported for dogfish (*Squalus mitsukuri*) (Taguchi et al., 1979) and other shark species (Lyle, 1984; de Pinho et al., 2002).

The age of spiny dogfish analyzed in this study was estimated from the relationship between age and body length (Table 1). Among three equations postulated by Ketchen (1975), we chose the equation for spiny dogfish caught off the Washington coast because the maximal body lengths of our female and male samples did not fit the equations derived from other areas. The estimated age of the females sampled (36 ± 11 yr, 18–61 yr, n = 40) was slightly greater than that of the males sampled (27 ± 7 yr, 16–41 yr, n = 35), which is in agreement with longevity data reported for the female spiny dogfish caught off the Washington coast (Ketchen, 1975). However, the estimated age of our samples is expected to include some variance, as the growth rate of spiny dogfish varies markedly with habitat. The determination of exact age by markings on the dorsal spine (Ketchen, 1975) is necessary.

3.2. Relationship between mercury concentration and body length

The T-Hg concentration in the muscle of individual fish was one order of magnitude higher than that in the liver (Table 1). Similarly, Greig et al. (1977) reported a higher accumulation of T-Hg in the muscle than in other organs, such as the liver and kidney of spiny dogfish. Available data for other shark species indicate higher concentrations of T-Hg in the muscle than in the liver (Taguchi et al., 1979; Branco et al., 2007) and similar levels of T-Hg in the muscle and liver (Marcovecchi et al., 1991). Recently, we reported a higher T-Hg concentration in the muscle than in the liver of immature tiger sharks, whereas that in the muscle of mature tiger sharks was lower than that in the liver (Endo et al., 2008a). The reason for these differences in Hg distribution remains open.

The average T-Hg concentrations in both the muscle and liver of females sampled were slightly higher than those of the males. The longevity of females may be one cause (Ketchen, 1975). T-Hg concentrations in gametes and fetuses were trace (below 0.05 μg (wet g)$^{-1}$, data not shown), suggesting little transfer from mother to gametes. Likewise, trace levels of T-Hg in gametes and fetuses have been reported in dogfish (Taguchi et al., 1979).

The relationships between T-Hg concentrations in the muscle and liver samples and body length of the spiny dogfish was also investigated (Fig. 2). The trends showing the relation between T-Hg concentration in the muscle and body length differed markedly between males and females: the T-Hg concentrations in the muscle samples from females and males rapidly increased from a body length of about 120 cm and 90 cm, respectively, although the increases had been gradual up to those body lengths. The average T-Hg concentration in the muscle of females smaller than 94 cm (0.202 ± 0.095 μg (wet g)$^{-1}$, n = 21) was lower than that of males (0.316 ± 0.202 μg (wet g)$^{-1}$, n = 35). Gender differences in the rapid increases in T-Hg in the muscle have been previously reported in spiny dogfish (Forrester et al., 1972) and dogfish (Taguchi et al., 1979), and explained by the continuous intake of Hg via food, the cessation of growth at maturity and the slower growth rate of males. In contrast, the T-Hg concentration in the liver samples from females and males remained at trace levels and increased little with increases in body length. We previously reported rapid increases in hepatic T-Hg concentration after maturation in tiger sharks (Endo et al., 2008a) and melon-headed whales (Endo et al., 2008b). These increases can be explained not only by the cessation of growth at maturity but also the formation of HgSe in the liver (Endo et al., 2002, 2005, 2006; Branco et al., 2007). The low level of HgSe formation may be one reason for the trace level of T-Hg observed in the spiny dogfish liver (Fig. 2), although the formation of HgSe is supposed to preferentially occur in the liver (not in muscle) after the demethylation of M-Hg in this organ (Endo et al., 2002, 2005, 2006). Domi et al. (2005) reported the absence of any correlation between T-Hg and Se concentrations in the mus-

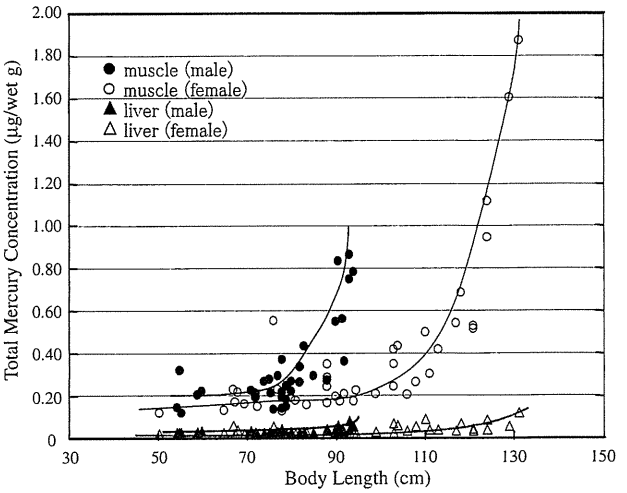


Fig. 2. Relationships between total mercury concentrations in the muscle and liver and body length of female and male spiny dogfishes.

cle of spiny dogfish, suggesting no formation of HgSe in the dogfish muscle. The commercially available powder from spiny dogfish muscle (DORM-2) and liver (DOLT-2), prepared by mincing, defatting and drying, are used in trace metal analyses as certificated standards. The percentages of M-Hg to T-Hg in DORM-2 and DOLT-2 are 96% and 33%, respectively. These suggest that most T-Hg present in the muscle samples existed as M-Hg, not as HgSe. Further analysis of M-Hg and Se concentrations in the muscle and liver of spiny dogfish is now in progress.

As the T-Hg concentration in the muscle of large females (larger than 125 cm) exceeded 1.0 μg (wet g)$^{-1}$ (Fig. 2), the M-Hg concentration in the muscle of larger females may exceed 1.0 μg (wet g)$^{-1}$, which is the US FDA action level and Cordex Alimentarius guideline level of M-Hg in predatory fish. The T-Hg concentrations in the muscle of spiny dogfish caught off the coast of Japan were slightly lower than those in fish of the same body length caught off Canada (Forrester et al., 1972) and Australia (Walker, 1988), and similar to those in fish caught off New Zealand (van den Broek et al., 1981).

3.3. Relationship among stable isotopes, mercury concentration and body length

No differences were found in the average ∂^{15}N and ∂^{13}C values between female and male samples (Table 1). The similarity between the values determined for the female and male samples suggest the same trophic position of females and males, in spite of marked differences in their body lengths. To our knowledge, ∂^{13}C and ∂^{15}N values have not yet been compared between males and females in shark species in which body sizes markedly differ between sexes. Although the sample size was limited ($n = 6$) and the gender was not mentioned, Domi et al. (2005) analyzed T-Hg, ∂^{13}C and ∂^{15}N in the muscle of spiny dogfish caught in the Celtic sea. The present results for body length, T-Hg concentration and ∂^{15}N (Table 1) were greater than those reported by Domi et al. (2005), whereas that for ∂^{13}C was similar.

Values of ∂^{13}C and ∂^{15}N in the muscle of samples from males and females increased with increases in body length (Fig. 3). However, the slopes of the regression lines generated by body length and ∂^{13}C or ∂^{15}N were slightly higher in the males than in the females ($p > 0.05$). The slower growth rate of the males may be a reason for the observed differences in the slopes, and might relate to the higher T-Hg concentration in male muscle than in the muscle from females of a similar size (Fig. 1).

The increase in ∂^{13}C was significantly correlated with the increase in ∂^{15}N in the combined muscle samples from males and females (Fig. 4; $\gamma = 0.735$, $p < 0.05$). It is noteworthy that no particular differences between the samples from males and females were observed in the map of ∂^{13}C–∂^{15}N, despite the significant differences in body length between males and females. The positive correlation found between ∂^{13}C and ∂^{15}N suggests a trophic enrichment due to the growth (Al-Reasi et al., 2007; Bank et al., 2007; Rigét et al., 2007; Loseto et al., 2008).

Fig. 5 shows the relation between ∂^{15}N and the T-Hg concentration in muscle samples. The T-Hg concentration in samples from females rapidly increased when ∂^{15}N exceeded 13, while the increase in T-Hg concentration due to increases in ∂^{15}N was not so noticeable in the male samples. The plots of the male samples overlapped with those of the female samples, which was in contrast to the plots of body length and T-Hg concentration (Fig. 2). The ∂^{15}N value of 13.0 corresponded to a body length of about 120 cm in females and 90 cm in males, respectively (Fig. 3), which coincided with the body lengths of maturation in the female and male. The ∂^{15}N value may be a common indicator of the maturity of female and male spiny dogfishes and the rapid increase found in female samples may be related to the longevity-related higher T-Hg.

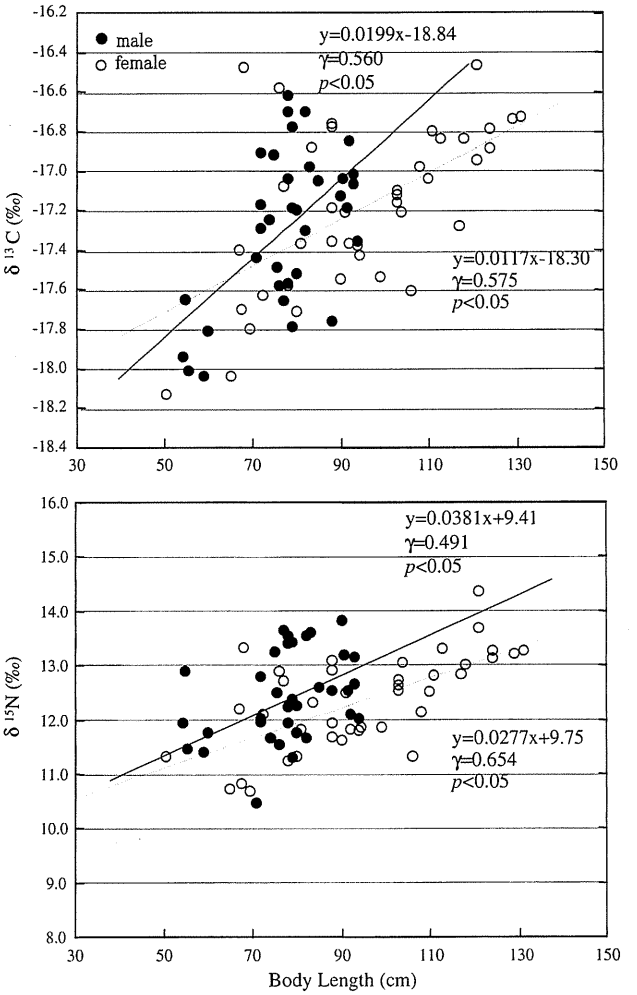


Fig. 3. Correlation between body length and ∂^{13}C or ∂^{15}N in the muscle of female and male spiny dogfishes.

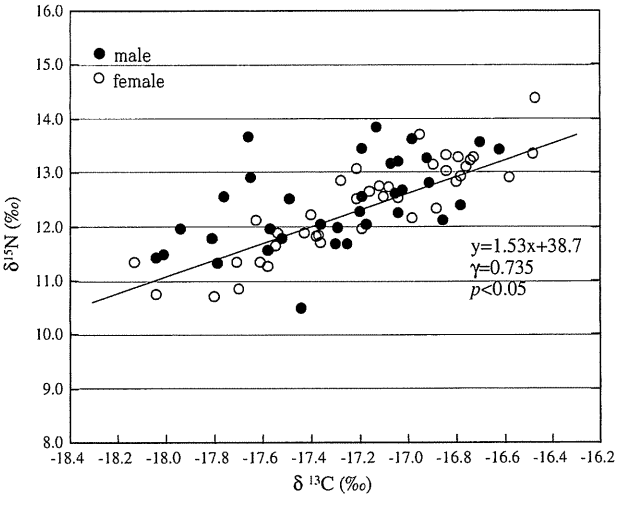


Fig. 4. Correlation between ∂^{13}C and ∂^{15}N in the muscle of female and male spiny dogfishes.

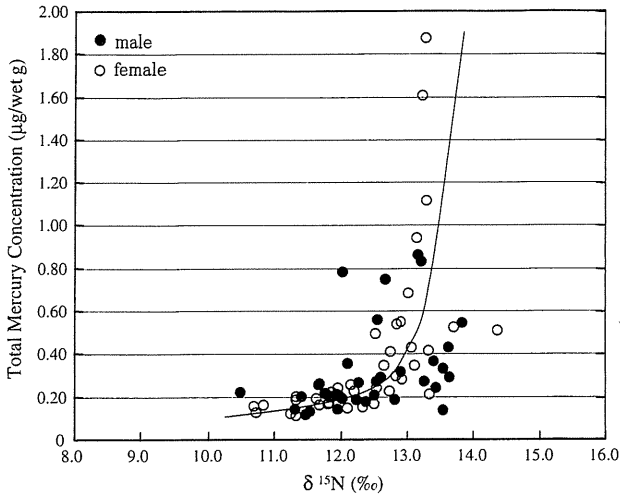


Fig. 5. Relationships between total mercury concentration and $\partial^{15}N$ in the muscle of female and male spiny dogfishes.

4. Conclusion

The body length of female spiny dogfish caught off the coast of Japan was significantly larger than that of males. The T-Hg concentration in the muscle was significantly higher than that in the liver, irrespective of gender. The average concentration of T-Hg in the muscle from females was slightly higher than that in the muscle from males, probably due to the greater longevity of females. The average concentration of T-Hg in the muscle from smaller female was slightly lower than that in the muscle from males of a similar size, probably due to the faster growth rate of females. Contamination of T-Hg rapidly increased after maturation in females (about 120 cm in body length) and male (about 90 cm in body length). The average values of $\partial^{13}C$ and $\partial^{15}N$ in the muscle were the same in samples from males and females, in spite of marked differences in body length.

Acknowledgements

We deeply appreciate the assistance provided by Drs. I. Tayasu, and R. Hirasawa of Kyoto University, Japan, in analyzing the stable isotopes.

This work was supported by Grant-in-Aid from the Japan Society of for the Promotion of Science (B20404006 and C21590135).

References

Al-Reasi, H.A., Ababneh, F.A., Lean, D.R., 2007. Evaluating mercury biomagnification in fish from a tropical marine environment using stable isotopes ($\partial^{13}C$ and $\partial^{15}N$). Environ. Toxicol. Chem. 26, 1572–1581.

Bank, M.S., Chesney, E., Shine, J.P., Maage, A., Senn, D.B., 2007. Mercury bioaccumulation and trophic transfer in sympatric snapper species from the gulf of Mexico. Ecol. Appl. 17, 2100–2110.

Branco, V., Vale, C., Canário, J., dos Santos, M.N., 2007. Mercury and selenium in blue shark (*Prionace glauca*, K. 1758) and swordfish (*Xiphias gladius*, L. 1758) from two areas of the Atlantic Ocean. Environ. Pollut. 150, 373–380.

de Pinho, A.P., Guimarães, J.R.D., Martins, A.S., Costa, P.A.S., Olavo, G., Valentin, J., 2002. Total mercury in muscle tissue of five shark species from Brazilian offshore waters: effects of feeding habit, sex, and length. Environ. Res. 89, 250–258.

Domi, N., Bouquegneau, J.M., Das, K., 2005. Feeding ecology of five commercial shark species of the Celtic sea through stable isotope and trace metal analysis. Mar. Environ. Res. 60, 551–569.

Endo, T., Haraguchi, K., Sakata, M., 2002. Mercury and selenium concentrations in the internal organs of toothed whales and dolphins marketed for human consumption in Japan. Sci. Total. Environ. 300, 15–22.

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. 3, 5703–5708.

Endo, T., Kimura, O., Hisamichi, Y., Minoshima, Y., Haraguchi, K., Kakumoto, C., Kobayashi, M., 2006. Distribution of total mercury, methyl mercury and selenium in pod of killer whales (*Orcinus orca*) stranded in the northern area of Japan: comparison of mature females with calves. Environ. Pollut. 144, 145–150.

Endo, T., Kimura, O., Hisamichi, Y., Minoshima, Y., Haraguchi, K., 2007. Age-dependent accumulation of heavy metals in a pod of killer whale (*Orcinus orca*) stranded in the northern area of Japan. Chemosphere 67, 51–59.

Endo, T., Hisamichi, Y., Koichi, H., Kato, Y., Ohta, C., Koga, N., 2008a. Hg, Zn and Cu levels in the muscle and liver of tiger sharks (*Galeocerdo cuvier*) from the coast of Ishigaki island, Japan: relationship between metal concentrations and body length. Mar. Pollut. Bull. 56, 1774–1780.

Endo, T., Hisamichi, Y., Kimura, O., Haraguchi, K., Baker, C.S., 2008b. Contamination levels of mercury and cadmium in melon-headed whales (*Peponocephala electra*) from a mass stranding on the Japanese coast. Sci. Total Environ. 401, 73–80.

Ferrari, A., Ferrari, A., 2000. *tuto Squali*/Sharks and Rays of the World, Japanese Translation Edition by Mifune, A. Yamamoto, T. Hankyu Communication Co., Tokyo, Japan.

Forrester, C.R., Ketchen, K.S., Wong, C.C., 1972. Mercury content of spiny dogfish (*Squalus acanthias*) in the strait of Georgia, British Columbia. J. Fish. Res. Board Can. 29, 1487–1490.

Greig, R.A., Wenzloff, D., Shelpuk, C., Adams, A., 1977. Mercury concentrations in three species of fish from north Atlantic offshore waters. Arch. Environ. Contam. Toxicol. 5, 315–323.

JECFA, 2003. Summary and Conclusions of the 61st Meeting of the Joint FAO/WHO Expert Committee of Food Additives (JECFA). JECFA/61/SC, Rome, Italy.

Ketchen, K.S., 1975. Age and growth of dogfish *Squalus acanthias* in British Columbia waters. J. Fish. Res. Board. Can. 32, 43–59.

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.

Loseto, L.L., Stern, G.A., Ferguson, S.H., 2008. Size and biomagnification: how habitat selection explains beluga mercury levels. Environ. Sci. Technol. 42, 3982–3988.

Lyle, J.M., 1984. Mercury concentrations in four carcharhinids and three hammerhead sharks from coastal waters of the northern territory. Aust. J. Mar. Freshwater Res. 35, 441–451.

Marcovecchi, J.E., Moreno, V.J., Pérez, A., 1991. Metal accumulation in tissues of sharks from the Bahia Balanca estuary, Argentina. Mar. Environ. Res. 31, 263–274.

Rigét, F., Moller, P., Dietz, R., Nielsen, T.G., Asmund, G., Strand, J., Larsen, M.M., Hobson, K.A., 2007. Transfer of mercury in the marine food web of west Greenland. J. Environ. Monit. 8, 877–883.

Taguchi, M., Yasuda, K., Toda, S., Shimizu, M., 1979. Study of metal contents of elsmobranch fishes: part 1 – metal concentration in the muscle tissue of dogfish *Squalus mitsukuri*. Mar. Environ. Res. 2, 239–249.

Tayasu, I., Hyodo, F., Abe, T., 2002. Caste-specific N and C isotope ratios in fungus-growing termites with special reference to uric acid preservation and their nutritional interpretation. Ecol. Entomol. 27, 355–361.

van den Broek, W.L.F., Tracey, D.M., Solly, S.R.B., Avrahami, M., 1981. Mercury levels in some New Zealand sea fishes New Zealand. J. Mar. Freshwater Res. 15, 137–146.

Walker, T.I., 1988. Mercury concentrations in edible tissues of elasmobranches, teleosts, crustaceans and mollusks from South-eastern Australian waters. Aust. J. Mar. Freshwater 39, 39–49.

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

0090-9556/09/3710-2095–2102$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 37, No. 10
28621/3512324
Printed in U.S.A.

DMD 37:2095–2102, 2009

4-Hydroxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl-Mediated Decrease in Serum Thyroxine Level in Mice Occurs through Increase in Accumulation of Thyroxine in the Liver

Yoshihisa Kato, Koichi Haraguchi, Makiko Kubota, Yoshiki Seto, Shin-ichi Ikushiro, Toshiyuki Sakaki, Nobuyuki Koga, Shizuo Yamada, and Masakuni Degawa

Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Sanuki, Kagawa, Japan (Y.K.); Daiichi College of Pharmaceutical Sciences, Fukuoka, Japan (K.H.); School of Pharmaceutical Sciences and Global Center of Excellence Program in the 21st Century, University of Shizuoka, Shizuoka, Japan (M.K., Y.S., S.Y., M.D.); Faculty of Engineering, Toyama Prefectural University, Toyama, Japan (S.I., T.S.); and Faculty of Nutritional Sciences, Nakamura Gakuen University, Fukuoka, Japan (N.K.)

Received May 27, 2009; accepted July 6, 2009

ABSTRACT:

4-Hydroxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl (4-OH-CB187) was selected as a major hydroxylated polychlorinated biphenyl metabolite detected from serum of wildlife and humans and was examined for its effect on level of serum thyroid hormone in mice. Four days after treatment of C57BL/6 and DBA/2 mice with 4-OH-CB187 (1.0 mg/kg), the serum total thyroxine (T_4) and free T_4 levels were decreased in both strains of mice. On the other hand, no significant changes in the level and activity of the T_4-UDP-glucuronosyltransferases, including UGT1a and UGT1a1, by the 4-OH-CB187 treatment were observed in either strain of mice. No 4-OH-CB187-mediated change in level of serum thyroid-stimulating hormone was observed in either strain of mice. Binding levels of [^{125}I]T_4 to serum proteins after administration of [^{125}I]T_4 were significantly changed in 4-OH-CB187-pretreated mice: a decrease in the level of serum [^{125}I]T_4-transthyretin (TTR) complex and an increase in the binding level of [^{125}I]T_4 to serum albumin and thyroxine binding protein in both strains of mice. Clearance from serum of T_4 was promoted by 4-OH-CB187 pretreatment in both C57BL/6 and DBA/2 mice, and the levels of T_4 in several tissues, especially the liver, were increased. In addition, 4-OH-CB187-mediated decreases in serum total T_4 and free T_4 levels were observed in wild-type and TTR-heterozygous mice but not in TTR-deficient mice. The present findings show that 4-OH-CB187 shows a definite ability to decrease serum T_4 level and further indicate that the 4-OH-CB187-induced decrease would occur through increase in accumulation of T_4 in the liver.

A large number of hydroxylated polychlorinated biphenyls (OH-PCBs) have been found in the blood of humans, birds, seals, and polar bears (Letcher et al., 2000; Sjödin et al., 2000; Hovander et al., 2002). The concentration of OH-PCBs may exceed 10% of the total amount of polychlorinated biphenyls (PCBs) in human serum (Fängström et al., 2002; Hovander et al., 2002, 2006; Sandau et al., 2002). As the major OH-PCBs from human serum, 4-hydroxy-2,3,3′,4′,5-pentachlorobiphenyl (4-OH-CB107), 3-hydroxy-2,2′,4,4′,5,5′-hexachlorobiphenyl (3-OH-CB153), 4-hydroxy-2,2′,3,4′,5,5′-hexachlorobiphenyl (4-OH-CB146), 3′-hydroxy-2,2′,3,4,4′,5′-hexachlorobiphenyl (3′-OH-CB138), and 4-hydroxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl (4-OH-CB187) are identified. These OH-PCBs, especially 4-OH-CB187 (Fig. 1), show a high binding affinity for serum transthyretin (TTR) (Lans et al., 1993; Brouwer et al., 1998; Ucán-Marín et al., 2009). The binding affinity of 4-OH-CB187 is 5.3-fold higher than that of endogenous ligand thyroxine (T_4) (Malmberg, 2004), strongly suggesting that 4-OH-CB187 would modify the metabolic fate and action of serum thyroid hormone.

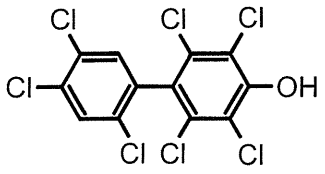
Most PCB congeners are known to decrease the levels of serum thyroid hormone in rats (Van Birgelen et al., 1995; Craft et al., 2002). As possible mechanisms for the PCB-mediated decrease, the induction of hepatic UDP-glucuronosyltransferases (UDPGTs), especially UGT1As, responsible for thyroid hormone metabolism and the competitive inhibition on the thyroid hormone-TTR complex formation are considered (Barter et al., 1994; Brouwer et al., 1998). However, we have recently shown that a consecutive treatment with Kanechlor-500 resulted in significant decrease in level of serum total T_4 not only in Wistar but also in Gunn rats (UGT1A-deficient Wistar rats) and further indicated that the Kanechlor-500-induced decrease would oc-

This work was supported in part by grants-in-aid for Scientific Research (C) [Grant 20510070] and for Scientific Research (B) [Grant 19310042] from Japan Society for the Promotion of Science.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.109.028621.

ABBREVIATIONS: OH-PCB, hydroxylated polychlorinated biphenyl; PCB, polychlorinated biphenyl; 4-OH-CB107, 4-hydroxy-2,3,3′,4′,5-pentachlorobiphenyl; 3-OH-CB153, 3-hydroxy-2,2′,4,4′,5,5′-hexachlorobiphenyl; 4-OH-CB146, 4-hydroxy-2,2′,3,4′,5,5′-hexachlorobiphenyl; 3′-OH-CB138, 3′-hydroxy-2,2′,3,4,4′,5′-hexachlorobiphenyl; 4-OH-CB187, 4-hydroxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl; TTR, transthyretin; T_4, thyroxine; UDPGT, UDP-glucuronosyltransferase; TSH, thyroid-stimulating hormone; HPLC, high-performance liquid chromatography; TBG, thyroxine binding globulin.



4-OH-CB187

FIG. 1. Chemical structure of 4-OH-CB187.

cur through increase in accumulation (transportation from serum to tissues) of T_4 in several tissues, especially the liver, rather than increase in hepatic T_4-UDPGT activity (Kato et al., 2007).

In the present study, we selected 4-OH-CB187 as a major hydroxylated PCB detected from serum of wildlife and humans and examined its effect on the level of serum thyroid hormone in mice. The present results revealed that 4-OH-CB187 showed a definite ability to decrease serum T_4 level and strongly suggested that its decrease occurred mainly through an increase in accumulation of T_4 in the liver.

Materials and Methods

Chemicals. Panacete 810 (medium-chain triglycerides) was purchased from Nippon Oils and Fats Co. Ltd. (Tokyo, Japan). The [^{125}I]T_4, radiolabeled at the 5′-position of the outer ring, was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). 4-OH-CB187 was synthesized by the Cadogan (1962) coupling reaction of 2,4,5-trichloroaniline with 2,3,5,6-tetrachloroanisole and subsequent demethylation of the resulting 4-methoxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl using boron tribromide. The purity of the compound was >99% when analyzed by gas chromatography.

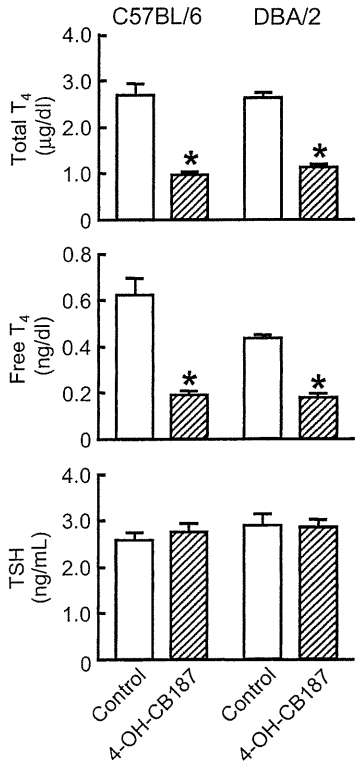


FIG. 2. Effects of 4-OH-CB187 on levels of serum total T_4, free T_4, and TSH in mice. Animals were killed 4 days after the administration of 4-OH-CB187 (1.0 mg/kg), and levels of serum thyroid hormones were measured as described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for four to six animals. *, $P < 0.01$, significantly different from each control.

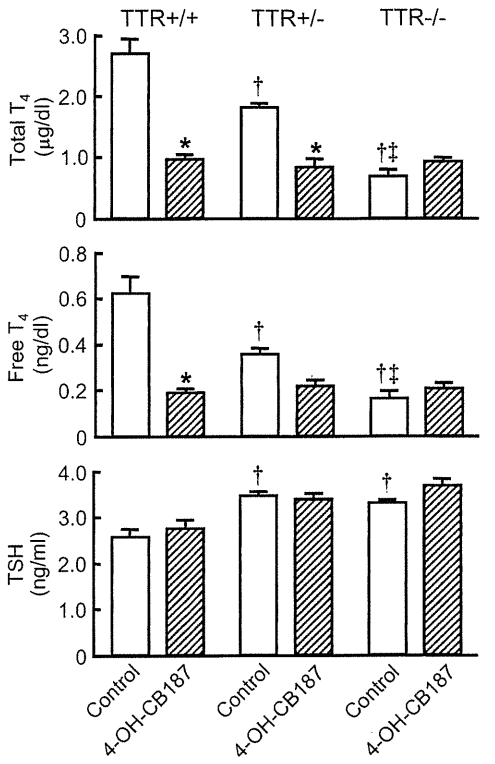


FIG. 3. Effects of 4-OH-CB187 on levels of serum total T_4, free T_4, and TSH in TTR-heterozygous and TTR-deficient mice. Animals were killed 4 days after the administration of 4-OH-CB187 (1.0 mg/kg), and levels of serum thyroid hormones were measured as described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for three to six animals. *, $P < 0.01$, significantly different from corresponding control. †, $P < 0.05$, significantly different from control of TTR(+/+) mice. ‡, $P < 0.05$, significantly different from control of TTR(+/−) mice.

Animal Treatments. Male C57BL/6 mice (18–31 g) and the DBA/2 mice (18–28 g) were obtained from Japan SLC, Inc. (Shizuoka, Japan). TTR-deficient [TTR(−/−)] mice (15–24 g) were generated by using 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 C57BL/6, DBA/2, TTR(+/−), and TTR(−/−) 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–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). Mice received an intraperitoneal injection of 4-OH-CB187 (1.0 mg/kg) dissolved in Panacete 810 (5 ml/kg). Control animals were treated with vehicle alone (5 ml/kg).

In Vivo Study. Mice were killed by decapitation 4 days after the administration of 4-OH-CB187. The liver was removed, and 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, free T_4, and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay using a Total T4 and Free T4 kit (Diagnostic Products, Los Angeles, CA) and the rTSH [^{125}I] Biotrak assay system (GE Healthcare, Little Chalfont, Buckinghamshire, UK), respectively.

Hepatic microsomal T_4-UDPGT activity. The amount of hepatic microsomal protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The activity of microsomal UDPGT toward T_4

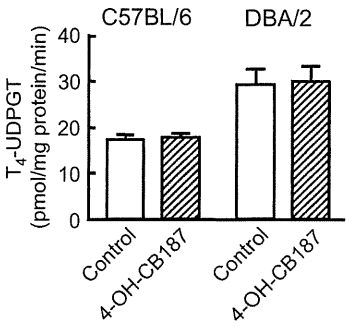


FIG. 4. Effect of 4-OH-CB187 on the activity of hepatic microsomal T_4-UDPGT in mice. Hepatic microsomes from individual animals were used for T_4-UDPGT enzyme assay, as described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for four to seven animals. *, $P < 0.05$, significantly different from each control.

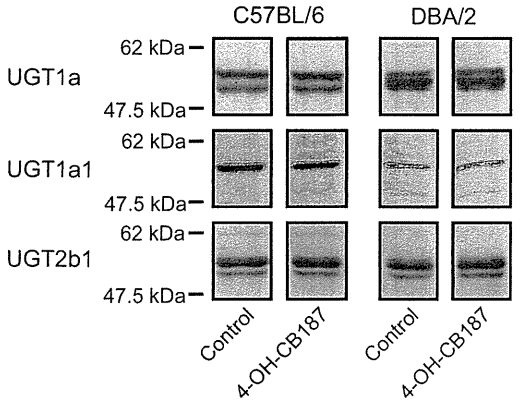


FIG. 5. Representative Western blot profiles for hepatic microsomal UGT isoforms in the 4-OH-CB187-treated mice. Hepatic microsomes from individual animals were used for Western blot analysis, as described under *Materials and Methods*.

(T_4-UDPGT activity) was determined by the methods of Barter and Klaassen (1992).

Western blot analysis. The polyclonal antipeptide antibodies against the common region of rat UGT1A isoforms and specific antibodies against rat UGT1A1 and UGT2B1, which were established by Ikushiro et al. (1995, 1997), were used. Western blot analyses for microsomal UGT isoforms were performed by the method of Luquita et al. (2001). The bands of mouse UGT1a1 and UGT2b1, which correspond to rat UGT1A1 and UGT2B1, respectively, were detected using chemical luminescence (ECL detection kit; GE Healthcare), and the level of each protein band was determined densitometrically with LAS-1000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Ex Vivo Study. Four days after treatment with 4-OH-CB187 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 the animal's body was warmed to 37°C. Fifteen minutes later, the mice were given intravenously 0.1 ml of [^{125}I]T_4 (15 μCi /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. Two aliquots (15 μl each) of each serum were used for determination of [^{125}I]T_4 level by a gamma counter (COBRA II AUTO-GAMMA 5002; PerkinElmer Life and Analytical Sciences).

Biliary excretion of [^{125}I]T_4 glucuronide. Amount of biliary [^{125}I]T_4 glucuronide was determined with high-performance liquid chromatography (HPLC) as described by Vansell and Klaassen (2001). In brief, after the administration of [^{125}I]T_4, bile was collected on ice for 2 h at 30-min intervals. Bile volume was determined gravimetrically. Two aliquots (10 μl each) were taken from each bile sample for determination of [^{125}I]T_4 level by a gamma counter (COBRA II AUTO-GAMMA 5002; PerkinElmer Life and Analytical Sciences). A portion (10 μl) of bile was added to 2 volumes of methanol and kept at −20°C for 1 h to precipitate protein. After the mixture was centrifuged at 12,000*g* (4°C) for 10 min, the resultant supernatant was collected for HPLC analysis. The HPLC analysis was performed using a ChromSpher C18 column (10 × 0.3 cm) (Chrompack, Inc., Raritan, NJ) in combination with both a ChromSep reverse-phase guard column (10 × 2 mm) (Chrompack, Inc.) and an Adsorbosphere C18 reverse-phase guard column (7.5 × 4.6 mm) (Alltech Associates, Deerfield, IL). Then, 0.02 mM ammonium acetate, pH 4.0, containing 16 to 45% of acetonitrile solution was used for elution of [^{125}I]T_4 glucuronide; 16% of acetonitrile was used as a initial solution for 6 min, and then the elution solution was changed by a linear increase to 27% over 12 min, held for 4 min, followed by a linear increase to 45% over 5 min and held for 11 min. The levels of biliary [^{125}I]T_4 glucuronide were determined by Radioisotope Detector 171 (Beckman Coulter, Fullerton, CA).

To identify [^{125}I]T_4 glucuronides, 100 μl of bile was incubated for 4 h at 37°C with β-glucuronidase (250 units) in 100 mM phosphate buffer (100 μl, pH 6.8), and the reaction was stopped by addition of 50 μl of methanol and cooling on ice. After the reaction mixture was centrifuged at 12,000*g* (4°C) for 10 min, the resultant supernatant was collected for HPLC analysis. [^{125}I]T_4 glucuronide in bile was confirmed by the disseemance of a peak responsible for [^{125}I]T_4 glucuronides by treatment with β-glucuronidase.

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 with 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 to 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 incubated with [^{125}I]T_4 were also applied on a 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.). The levels of [^{125}I]T_4-TBG, [^{125}I]T_4-albumin, and [^{125}I]T_4-TTR in serum were deter-

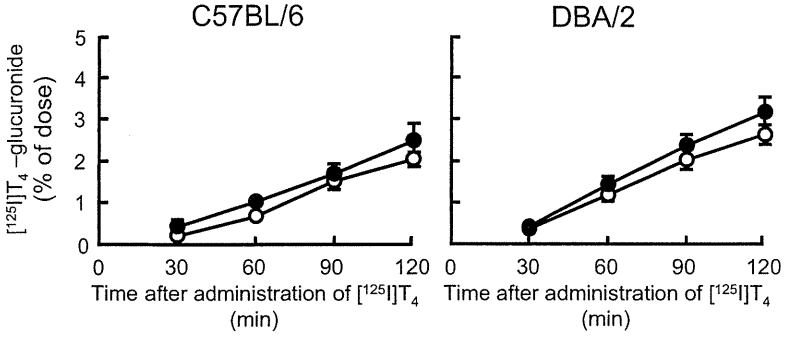


FIG. 6. Effect of 4-OH-CB187 on amount of the biliary [^{125}I]T_4-glucuronide in mice. The level of [^{125}I]T_4-glucuronide excreted was measured in bile collected at 30-min intervals after the intravenous administration of [^{125}I]T_4. Each point represents the mean ± S.E. (vertical bars) for five to eight mice. *, $P < 0.01$, significantly different from each control. —○—, control; —●—, 4-OH-CB187.

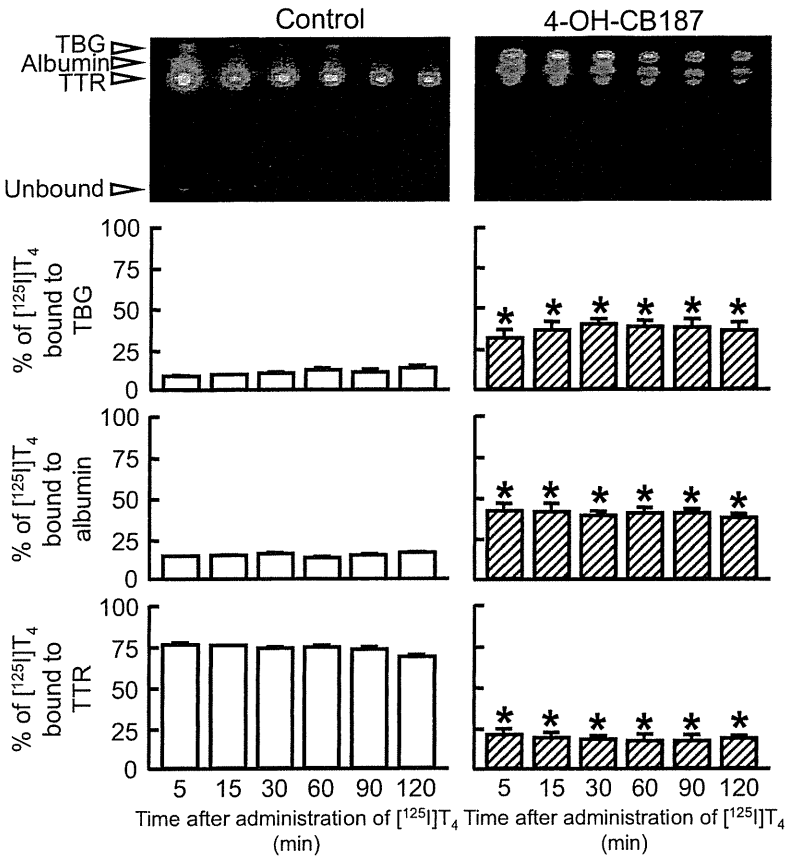


FIG. 7. Effect of 4-OH-CB187 on the binding of $[^{125}I]T_4$ to serum proteins in C57BL/6 mice. The amounts of $[^{125}I]T_4$ bound to the serum proteins 5 min after $[^{125}I]T_4$ administration were assessed by the method as described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for four to five animals. *, $P < 0.001$, significantly different from each control.

mined by counting the corresponding gel fractions identified with Bio Imaging Analyzer (BAS-2000II IP Reader; Fuji Photo Film Co., Ltd.).

Tissue distribution of $[^{125}I]T_4$. Tissue distribution of $[^{125}I]T_4$ was assessed according to the modified method of Oppenheimer et al. (1968). In brief, 5 min after administration of $[^{125}I]T_4$ to 4-OH-CB187-pretreated mice, blood was sampled from the abdominal aorta. Then, 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, cecum, 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; PerkinElmer Life and Analytical Sciences), and amounts of $[^{125}I]T_4$ in the tissues were shown as ratios to the amount in serum.

Statistics. The data obtained were statistically analyzed according to Student's *t* test or Dunnett's test after analysis of variance. In addition, clearance of $[^{125}I]T_4$ from serum, amount of biliary $[^{125}I]T_4$ glucuronide, and the binding level of $[^{125}I]T_4$ 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. The effect of 4-OH-CB187 on the level of serum thyroid hormone was examined in dioxin-sensitive C57BL/6 mice and dioxin-resistant DBA/2 mice (Fig. 2). In both C57BL/6 and DBA/2 mice, 4-OH-CB187 treatment resulted in significant decreases in the levels of serum total T_4 and free T_4. On the other hand, no significant change in TSH level was observed in either strain of mice.

To further clarify whether the 4-OH-CB187-mediated decrease in serum total T_4 level is dependent on the inhibition of a thyroid hormone-TTR complex formation, we examined effects of 4-OH-CB187 on the level of serum thyroid hormone in TTR-heterozygous and TTR-deficient mice (Fig. 3). 4-OH-CB187-mediated decreases in serum total T_4 and free T_4 levels were observed in the wild-type and TTR-heterozygous mice but not in TTR-deficient mice. In addition, constitutive levels of serum total T_4 and free T_4 were 33 to 43% lower in TTR-heterozygous [TTR(+/−)] mice and 74 to 75% lower in TTR-deficient [TTR(−/−)] mice compared with that in wild-type [TTR(+/+)] mice.

Hepatic T_4-UDPGT Enzymes. The effect of 4-OH-CB187 on hepatic microsomal activity of T_4-UDPGTs was examined in C57BL/6 and DBA/2 mice. Treatment with 4-OH-CB187 resulted in no significant change in hepatic T_4-UDPGT activity in either strain of mice (Fig. 4).

Levels of the proteins responsible for T_4-UDPGT enzymes, such as UGT1a, UGT1a1, and UGT2b1, were determined by Western blot analysis. No significant changes in the protein level of hepatic UGT1a, UGT1a1, and UGT2b1 after 4-OH-CB187 treatment were observed in either C57BL/6 or DBA/2 mice (Fig. 5).

Biliary Excretion of $[^{125}I]T_4$ Glucuronide. Effect of 4-OH-CB187 on the biliary excretion of $[^{125}I]T_4$-glucuronide was examined in C57BL/6 and DBA/2 mice. No significant change in the amount of biliary $[^{125}I]T_4$-glucuronide after 4-OH-CB187 pretreatment was seen in either strain of mice (Fig. 6).

Serum Proteins Bound to $[^{125}I]T_4$. Effects of 4-OH-CB187 on the binding of $[^{125}I]T_4$ to serum proteins, such as TTR, albumin, and TBG, were examined in C57BL/6 and DBA/2 mice (Figs. 7 and 8). In both strains of mice, pretreatment with 4-OH-CB187 resulted in a significant decrease in the level of $[^{125}I]T_4$-TTR complex. On the contrary, the pretreatment led to significant increases in the levels of $[^{125}I]T_4$ bound to TBG and albumin.

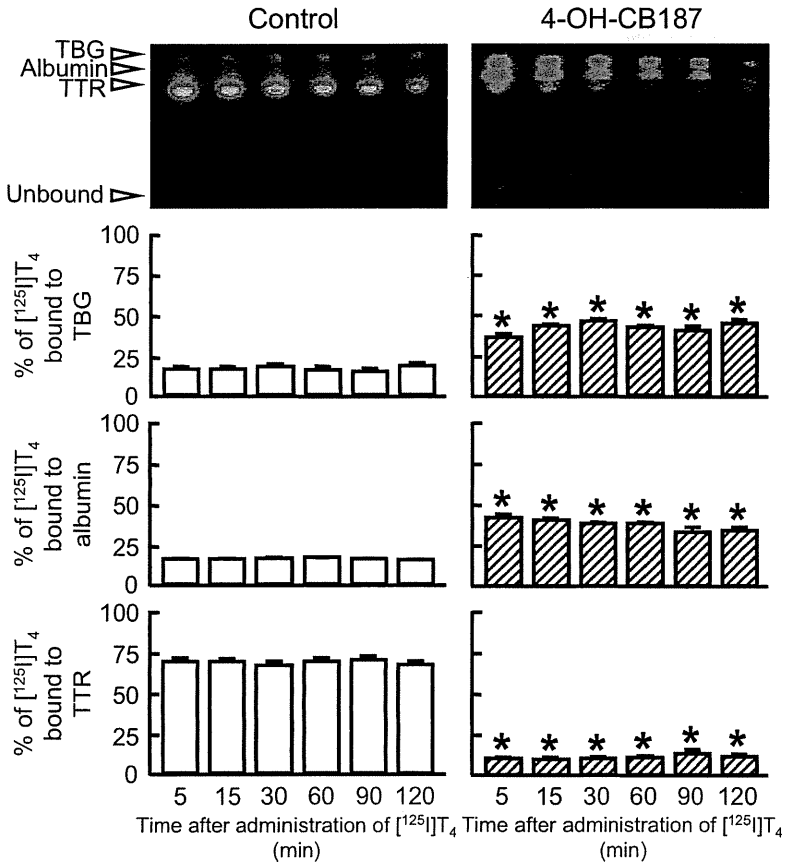


FIG. 8. Effect of 4-OH-CB187 on the binding of $[^{125}I]T_4$ to serum proteins in DBA/2 mice. Experimental protocols were the same as those described in the legend of Fig. 7. Each column represents the mean ± S.E. (vertical bars) for five to seven animals. *, $P < 0.001$, significantly different from each control.

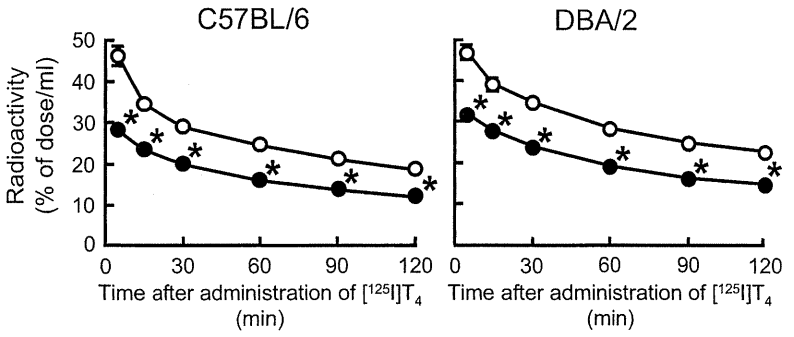


FIG. 9. Effects of 4-OH-CB187 on the clearance of $[^{125}I]T_4$ from serum in mice. The amount of serum $[^{125}I]T_4$ was measured at the indicated times after the intravenous administration of $[^{125}I]T_4$. Each point represents the mean ± S.E. (vertical bars) for five to eight mice. *, $P < 0.05$, significantly different from each control. —○—, control; —●—, 4-OH-CB187.

Clearance of $[^{125}I]T_4$ from Serum. After an intravenous administration of $[^{125}I]T_4$ to the 4-OH-CB187-pretreated C57BL/6 and DBA/2 mice, concentrations of $[^{125}I]T_4$ in the serum were measured at the indicated times (Fig. 9). In both C57BL/6 and DBA/2 mice, pretreatment with 4-OH-CB187 promoted the clearance of $[^{125}I]T_4$ from serum. Their serum $[^{125}I]T_4$ levels were decreased by approximately 35% of the corresponding control levels within 5 min, and the decreases remained up to 120 min later. The serum pharmacokinetic parameters of the $[^{125}I]T_4$ estimated from these data (Fig. 9) were summarized in Table 1. The mean total body clearances (Cl_{tb}) of $[^{125}I]T_4$ in the 4-OH-CB187-pretreated C57BL/6 and DBA/2 mice increased approximately 1.5 times compared with the corresponding control mice. The steady-state volumes of distribution in the 4-OH-CB187-pretreated mice also increased 1.5 times, compared with the corresponding control mice.

Tissue Distribution of $[^{125}I]T_4$. Effects of 4-OH-CB187 pretreatment on the tissue-to-serum concentration ratio (K_p value) and distribution level of $[^{125}I]T_4$ in tissue after the administration of $[^{125}I]T_4$ were examined using C57BL/6 and DBA/2 mice. The K_p values in the thyroid gland and liver were the greatest in either strain of control (4-OH-CB187-untreated) mice (Fig. 10). Pretreatment with 4-OH-CB187 resulted in a significant increase in the K_p values in the tissues, including thyroid gland and liver in either strain of mice (Fig. 10).

Accumulation level of $[^{125}I]T_4$ was the highest in the liver among the tissues examined in either strain of mice (Fig. 11). In both strains of mice, pretreatment with 4-OH-CB187 resulted in a significant increase in the accumulation in tissues, especially the liver, and the level in the liver achieved more than 40% of the $[^{125}I]T_4$ dosed (Fig. 11). In addition, the accumulation level per gram of liver was also increased in 4-OH-CB187-pretreated mice (Table 2). On the other

TABLE 1

Pharmacokinetic parameters for [^{125}I]T_4 after the administration of [^{125}I]T_4 to the 4-OH-CB187-pretreated mice

The data shown were calculated from the data in Fig. 9. The values shown are expressed as the mean ± S.E. for five to eight mice.

Animal	Pretreatment	Mean Total Body Clearance × 100	Distribution Volume
		ml/min	*ml*
C57BL/6	None (control)	1.48 ± 0.08	2.90 ± 0.17
	4-OH-CB187	2.23 ± 0.15*	4.48 ± 0.24*
DBA/2	None (control)	1.24 ± 0.02	2.47 ± 0.06
	4-OH-CB187	2.01 ± 0.12*	3.76 ± 0.24*

* $P < 0.05$, significantly different from each control.

hand, no significant change in the liver weight after 4-OH-CB187 pretreatment was observed in either strain of mice (Table 3).

Discussion

In the present study, we first show that the treatment with 4-OH-CB187 (a single intraperitoneal administration at a dose of 1.0 mg/kg) promoted accumulation of T_4 in several tissues, especially the liver, and resulted in a drastic decrease in the levels of serum total T_4 and free T_4 in both C57BL/6 and DBA/2 mice.

As a possible explanation for a chemical-induced decrease in serum thyroid hormones, a hepatic T_4-UDPGT-dependent mechanism is generally considered because T_4-UDPGT inducers, including PCB, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, phenobarbital, 3-methylcholanthrene, pregnenolone-16α-carbonitrile, and clobazam, show strong activities for decreasing level of serum total thyroid hormones, including T_4 and T_3 (Barter and Klaassen, 1994; Van Birgelen et al., 1995; Miyawaki et al., 2003). However, among experimental animals treated with T_4-UDPGT inducers, difference in magnitude of decrease in the level of serum total T_4 is not necessarily correlated with that of increase in hepatic T_4-UDPGT activity (Craft et al., 2002; Hood et al., 2003; Kato et al., 2003). Furthermore, our previous studies (Kato et al., 2004, 2005, 2007) using Wistar and Gunn rats supported a hypothesis that decreases in the level of serum total thyroid hormones by PCB and phenobarbital occur primarily in a hepatic T_4-UDPGT-independent pathway.

In the present study, we showed that the level and activity of hepatic T_4-UDPGTs, especially the UGT1a and UGT1a1 responsible for glucuronidation of T_4, were little changed by 4-OH-CB187 treatment in both C57BL/6 and DBA/2 mice, although serum total T_4 level was markedly decreased in both strains of mice by the treatment. This result indicates that 4-OH-CB187-induced decrease in serum T_4 level occurs in a T_4-UDPGT-independent manner.

Furthermore, 4-OH-CB187 treatment led to no significant change in the level of serum TSH in either strain of mice, although serum TSH is considered one of the factors regulating the level of serum total T_4. No significant change in the level of serum TSH in PCB-treated rats has also been reported (Liu et al., 1995; Hood et al., 1999; Hallgren et al., 2001; Kato et al., 2004).

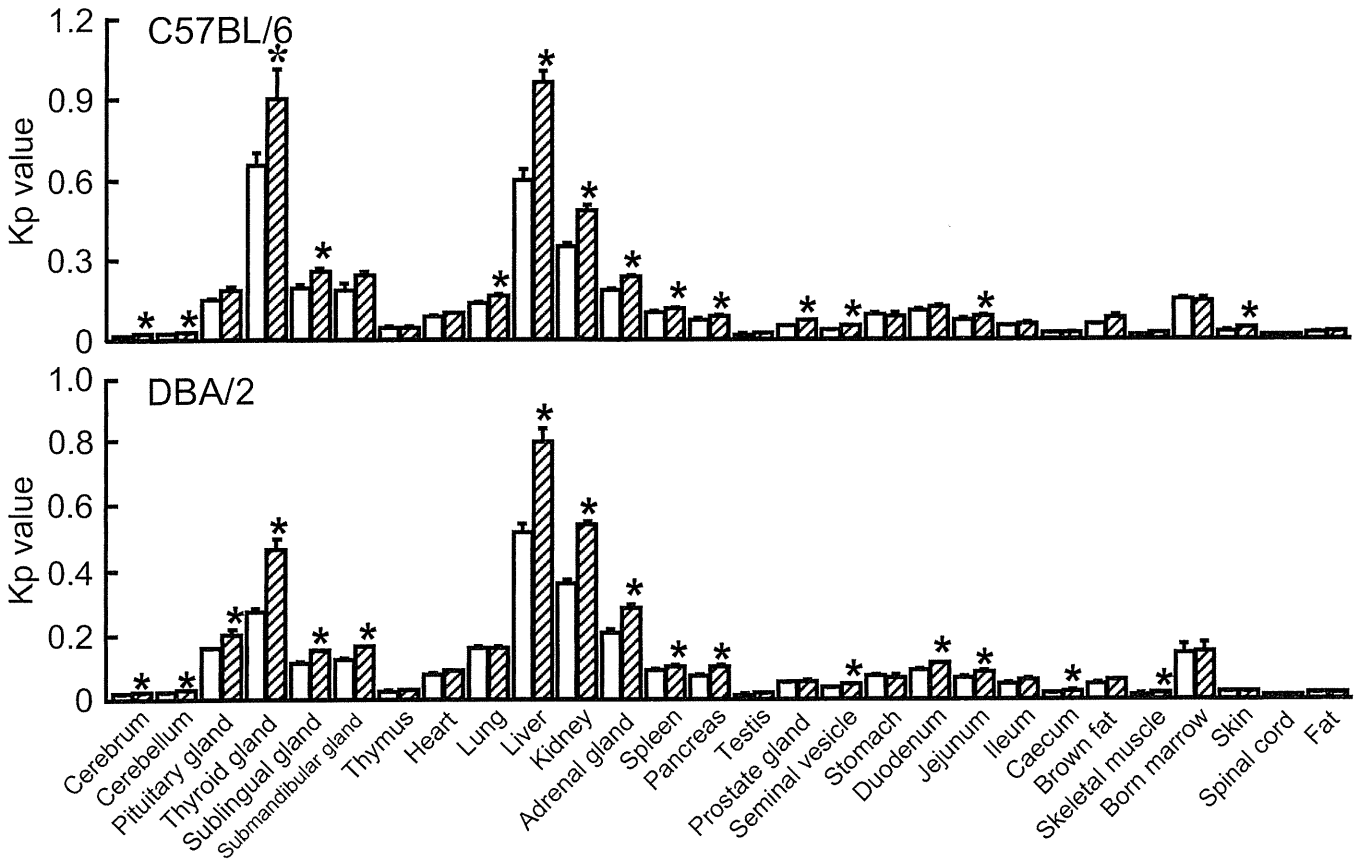


FIG. 10. Tissue-to-serum concentration ratio (K_p value) of [^{125}I]T_4 in various tissues after administration of [^{125}I]T_4 to 4-OH-CB187-pretreated mice. 4-OH-CB187 (1.0 mg/kg) was given to mice, and 96 h after the 4-OH-CB187-treatment, [^{125}I]T_4 was further administered to the mice. At 5 min after the [^{125}I]T_4 administration, the radioactivity in each tissue was measured, as described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for five to seven animals. *, $P < 0.05$, significantly different from each control. Open bars, control; hatched bars, 4-OH-CB187.