of the most important areas for commercial fishing in Japan, where Pacific cod is consumed by Japanese as one of major dietary fishery products. Furthermore, PFCA contamination of Pacific cods was compared among the Pacific Ocean, the Sea of Okhotsk and the Sea of Japan to evaluate current and historical release of PFCA. The present study also examined correlations between the concentration of PFCAs and other persistent organic pollutants (POPs), such as dichlorodiphenyltrichloroethane (DDT) metabolites, hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs), *trans*-nonachlor (*trans*-NC) and *beta*-hexachlorocyclohexane (β-HCH).

2. Material and methods

2.1. Sample collection

Seawaters around Hokkaido are a major distribution area for Pacific cod (*Gadus macrocephalus*), which commonly live in the coastal to continental shelf region (Mishima, 1989). The biggest catch is usually made by offshore trawl fishery in the Sea of Okhotsk and/or coastal fishery both in the Pacific Ocean and the Sea of Japan. The annual catch of Pacific cod in fishery was estimated at 25000–30000 tons in 2012, of which more than half was from the coastal waters of the Pacific Ocean, Hokkaido (Chimura and Tanaka, 2014). We purchased fillets and whole Pacific cod from retail outlets in Hokkaido, Japan between October 2012 and January 2013. These collected Pacific cods were originated from three seawaters (Fig. 1.). We collected 22 cod samples in total: 12 samples were from the Pacific Ocean, Hokkaido, 5 samples were from the Sea of Okhotsk, and 5 samples were from the Sea of Japan, Hokkaido (Fig. 1). A summary of the fish sample information is provided in Table 1. Neither fat content nor body weight of the fish samples showed any statistical significance between three seas (ANOVA, $p < 0.05$). The fish samples were stored at −20 °C until analyses and edible muscle parts were used for the evaluation.

Table 1
Characteristics of Pacific cod samples obtained from Hokkaido, Japan.

Water area	*n*	Weight (kg)		Fat content (%)	
Pacific Ocean	12	2.6 ± 1.3[a]	n.s.[b]	5.2 ± 1.0	n.s.[b]
Sea of Japan	5	1.6 ± 0.7	n.s.[b]	5.2 ± 1.3	n.s.[b]
Sea of Okhotsk	5	3.1 ± 1.6	n.s.[b]	5.0 ± 0.8	n.s.[b]

Data are presented as the mean ± standard deviation.
[a] Body weight of one fish sample is a blind figure.
[b] n.s.:not significant ($p > 0.05$, ANOVA).

2.2. Chemicals

Methanol (LC-MS grade) was obtained from Merck KGaA (Darmstadt, Germany). Methyl tert-butyl ether (MTBE) (HPLC grade) was obtained from Kanto Chemicals Co., Ltd. (Tokyo, Japan). Benzyl bromide was obtained from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tetrabutylammonium hydrogen sulfate, sodium carbonate, sodium hydrogen carbonate and 11H-perfluoroundecanoic acid (11H-PFUnDA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). A mixture of $^{13}C_4$-labeled PFOA, $^{13}C_5$-labeled perfluorononanoic acid (PFNA or C9), $^{13}C_2$-labeled perfluorodecanoic acid (PFDA or C10), $^{13}C_2$-labeled perfluoroundecanoic acid (PFUnDA or C11) and $^{13}C_2$-labeled perfluorododecanoic acid (PFDoDA or C12) were purchased from Wellington Laboratories Inc. (MPFAX-MXA; Guelph, Canada). For POPs analysis, selected organohalogen pesticides (pesticide mix 1037) and PCBs (BPMS) were purchased from Kanto Chemical Co.

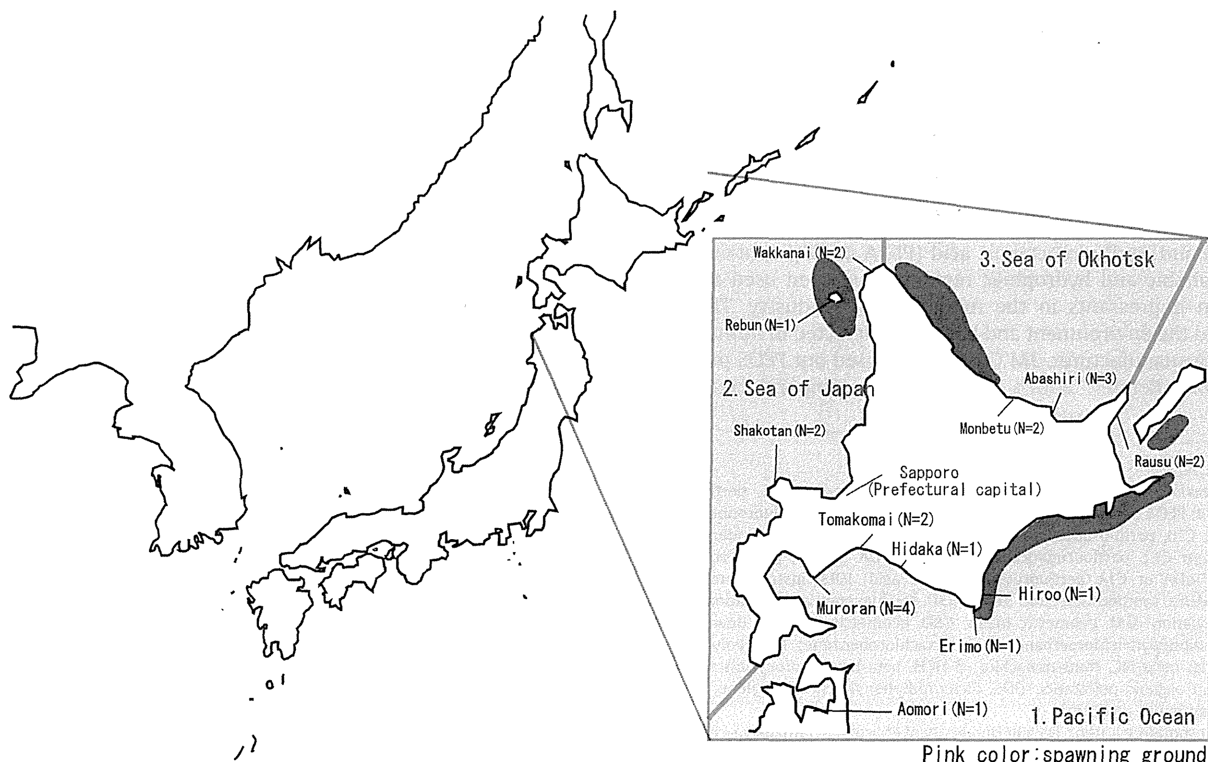

Fig. 1. Map showing the sampling locations for Pacific cod (N = 22). The migration area of each fish group is localized around the spawning ground (Chimura and Tanaka et al., 2014; Hattori, 1994).

Ltd (Tokyo Japan) and Wellington Laboratories Inc (Guelph, Canada), respectively.

2.3. Determination of PFCAs and POPs concentrations in fish samples

2.3.1. Clean-up procedure

PFOA, PFNA, PFDA, PFUnDA, PFDoDA, perfluorotridecanoic acid (PFTrDA or C13) and perfluorotetradecanoic acid (PFTeDA or C14) and five selected POPs (dichlorodiphenyldichloroethylene (DDE), HCB, PCB153, *trans*-NC and β-HCH) were analyzed by gas chromatography-mass spectrometry (GCMS, Agilent 6890GC/5973inertMSD). PFCAs were measured as described elsewhere (Fujii et al., 2012a, 2013). Briefly, 1–2 g of fish samples and 10 mL of methanol were placed in a 50 mL centrifuge tube and homogenized. Part of the homogenate (1 mL) was transferred into a 15 mL polypropylene tube. A recovery surrogate mixture (1 ng each of, $^{13}C_4$-labeled PFOA, $^{13}C_5$-labeled PFNA, $^{13}C_2$-labeled PFDA, $^{13}C_2$-labeled PFUnDA, and $^{13}C_2$-labeled PFDoDA) was added to the tube. Next, 2 mL of 0.5 mol L^{-1} tetrabutylammonium hydrogen sulfate/0.25 mol L^{-1} sodium carbonate buffer (pH adjusted to 10 using $NaHCO_3$) and 2 mL of MTBE were added to the samples and the tubes were vortex mixed for 60 s. The samples were then centrifuged at 870 × *g* for 10 min and the organic layer was removed. This step was repeated and the organic layers were combined in a clean tube and then dried. The residue was redissolved in 100 μL of a 0.1 mol L^{-1} benzyl bromide/MTBE solution containing 10 ng of 11H-PFUnDA as external calibration standard. The solution was then derivatized to benzyl esters at 60 °C for 1 h. No further clean-up was conducted. Organochlorine pollutants (DDE, PCB153, HCB, *trans*-NC, and β-HCH) were measured as described elsewhere (Haraguchi et al., 2009). Briefly, 10 g of fish materials were extracted with 0.1% formic acid/ethanol, diethylether and hexane. A gel permeation column and silica gel were used to purify the fatty materials for GC–MS.

2.3.2. Instruments and quantification

Derivatized PFCAs were analyzed by GC–MS with electron-capture negative ionization (GC/ECNI/MS) in selected ion monitoring mode. PFCA benzyl esters were separated on an HP-5MS column (30 m × 0.25 mm i.d., 0.25-μm-thick film) with helium carrier gas (99.999% purity). Splitless injections (1 μL) were performed with an injector temperature of 220 °C, and the split vent was opened after 1.5 min. The initial oven temperature was 70 °C for 1.5 min, after which it was increased to 230 °C at 20 °C min^{-1}, then to 280 °C at 4 °C min^{-1}, and then held at 280 °C for 5 min. Methane (99.99% purity) was used as the reagent gas (2 mL min^{-1}). 4,4′-DDE, HCB, PCB153, *trans*-NC and β-HCH were selected and determined for POP analysis in Pacific cods. The GC–MS conditions for POPs are the same as those for PFCAs, except for using the electron impact ionization mode. The target ions for determination of PFCAs are summarized in Supplemental Table 1. The detection limit was defined as the mass of the analyte producing a peak with a signal-to-noise ratio of 3 for PFCAs and 10 for the other POPs.

2.4. Quality assurance

We used Milli-Q water as the procedural blank control. The average blank values ($n = 6$) are listed in Supplemental Table 1. In the case of blank levels, the mean blank signal was subtracted from the calculated sample concentration and the method detection limits (MDLs) were defined by the following equation; $MDL = \alpha + 3\beta$, where α is the mean of the blank signals and β is the standard deviation of the blank signals. The calibration was linear and characterized by good correlation coefficients (>0.99) for all of the studied compounds. The quality of the method was verified by standard reference materials (fish tissue standard reference material from the National Institute of Standards and Technology, 1946) for PCBs and PFCAs (Reiner et al., 2012). The recoveries of the PFCAs were examined by spiking each standard compound into the Pacific cod samples. The mean recoveries were between 85 and 97%.

Table 2
Concentrations of PFCAs in Pacific cod samples.

		pg g-wet weight^{-1}							
		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA	Total
		(C8)	(C9)	(C10)	(C11)	(C12)	(C13)	(C14)	∑C8 to C14
Pacific Ocean ($n = 12$)	n > MDL (%)	7(58)	12(100)	12(100)	12(100)	12(100)	12(100)	12(100)	12(100)
	Median (Range)	35(<26–136)	97(43–178)	137(65–269)	865(453–1472)	182(38–349)	467(165–916)	105(42–182)	2107(819–3310)
	Mean ± SD	51 ± 45 n.s.[a]	104 ± 45 n.s.[a]	143 ± 66 n.s.[a]	917 ± 373 A[a]	190 ± 95 n.s.[a]	550 ± 268 n.s.[a]	110 ± 50 n.s.[a]	2066 ± 835 n.s.[a]
	GM (GSD)	34(2.6)	94(1.6)	130(1.6)	847(1.5)	163(1.9)	486(1.7)	99(1.6)	1899(1.6)
Sea of Japan ($n = 5$)	n > MDL (%)	4(80)	4(80)	4(80)	5(100)	4(80)	5(100)	5(100)	5(100)
	Median (Range)	39(<26–58)	81(<21–124)	102(<23–171)	890(147–1278)	237(<37–325)	808(99–1296)	152(42–188)	2308(377–3362)
	Mean ± SD	38 ± 17 n.s.[a]	72 ± 41 n.s.[a]	109 ± 63 n.s.[a]	882 ± 456 AB[a]	210 ± 124 n.s.[a]	701 ± 451 n.s.[a]	131 ± 57 n.s.[a]	2144 ± 1160 n.s.[a]
	GM (GSD)	34(1.8)	56(2.6)	80(3.0)	708(2.5)	148(3.3)	524(2.7)	117(1.8)	1718(2.4)
Sea of Okhotsk ($n = 5$)	n > MDL (%)	5(100)	5(100)	5(100)	5(100)	5(100)	5(100)	5(100)	5(100)
	Median (Range)	88(53–404)	108(80–183)	75(49–86)	421(312–513)	85(38–122)	277(127–325)	62(24–96)	1184(784–1401)
	Mean ± SD	140 ± 149 n.s.[a]	122 ± 45 n.s.[a]	70 ± 17 n.s.[a]	420 ± 84 B[a]	84 ± 30 n.s.[a]	259 ± 76 n.s.[a]	60 ± 29 n.s.[a]	1155 ± 228 n.s.[a]
	GM (GSD)	101(2.3)	116(1.4)	68(1.3)	413(1.2)	79(1.5)	247(1.5)	54(1.7)	1135(1.2)
Total	n > MDL (%)	16(73)	21(95)	21(95)	22(100)	21(95)	22(100)	22(100)	22(100)
	Median (Range)	51(<26–404)	93(<21–183)	99(<23–269)	746(147–1472)	146(<37–349)	404(99–1296)	93(24–188)	1728(377–3362)
	Mean ± SD	68 ± 83	101 ± 46	119 ± 63	796 ± 397	171 ± 101	518 ± 319	104 ± 52	1877 ± 891
	GM (GSD)	44(2.6)	88(1.9)	100(1.9)	691(1.8)	135(2.2)	424(2.0)	90(1.8)	1651(1.7)

MDL: method detection limit; SD: standard deviation; GM: geometric mean; GSD: geometric standard deviation.
Concentrations lower than the detection limits were considered to be equal to half of the detection limit for statistical analyses.
[a] Values with different letters differ significantly ($p < 0.05$, Tukey–Kramer HSD test). For example, the letters A and B indicate that the corresponding values differ. n.s.:no significant differences in ANOVA.

2.5. Statistical analysis

All statistical analyses were conducted using the JMP® 10 (SAS Institute Inc., Cary, NC, USA) and the SPSS software Version 22 (SPSS Inc., IL, USA). Values of $p < 0.05$ were considered to indicate statistical significance. Concentrations lower than the MDLs were considered to be equal to half of the MDLs for statistical analyses. Differences between mean values among the three seas were tested by Tukey–Kramer's honestly significant difference (HSD) test when statistical tests by ANOVA were significant. Differences between mean values among carbon chain length were tested by paired t-test with Bonferroni adjustment. Correlations were tested by Pearson correlation after logarithmic transformation of the PFCA concentrations. A principal component analysis (PCA) was used to reveal the potential difference of target compounds between PFCAs and POPs. The analyses were conducted via a correlation matrix. When the eigenvalues were >1, eigenvectors were employed for the analysis. Varimax rotation was applied to these eigenvectors.

3. Results and discussion

3.1. PFCA and classical POP concentrations in Pacific cods

In the present study, we first demonstrated the presence of PFCAs in edible fish muscle from Japanese coastal waters. Concentrations of PFCAs in Pacific cods are shown in Table 2. All samples contained long-chain PFCAs (C9≤), while PFOA (C8) was detected in 73% of all samples. PFCA levels (C8–C14, median) were 51 pg g^{-1} wet weight for C8, 93 for C9, 99 for C10, 746 for C11, 146 for C12, 404 for C13, and 93 for C14. The PFCAs with longer chains (C9–C14) than C8 represented 96% of the total PFCAs averaged over the three seas, suggesting that the Japanese population have been exposed to long-chain PFCAs through sea fish consumption. Among these detected long-chain PFCAs, odd-numbered PFCAs (C11 and C13) were detected at higher concentrations than neighboring, even-numbered PFCAs (C10, C12 and C14) (<0.0001, by paired t-test). Interestingly, long-chain PFCA (C10–C14) concentrations in samples from the Pacific Ocean and the Sea of Japan were about two to three times higher than those in samples from the Sea of Okhotsk, although a significant difference was only observed for the C11 concentration ($p < 0.05$, HSD test). The lack of significance could be due to the small sample size (The sea of Japan and the sea of Okhotsk, both $n = 5$). Previous studies have revealed that some consumer products such as sunscreen and cosmetics contain relatively high levels of long-chain PFCAs (Fujii et al., 2013). In

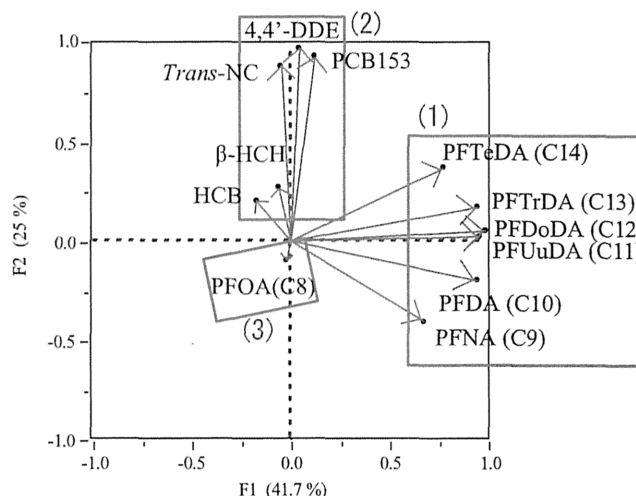

Fig. 2. Principal component analysis between PFCAs (C8 to C14) and classical POPs. When the eigenvalues were >1, eigenvectors were employed for the analysis. Varimax rotation was applied to these eigenvectors. (1) long-chain PFCAs (C9–C14), (2) classical lipophilic POPs, and (3) PFOA (C8).

Hokkaido, the coast of the Sea of Japan is inhabited by the largest human population of the three seas, followed by the Pacific Ocean and then the Sea of Okhotsk. This indicates that difference in PFCA concentrations could be related to consumption activity, depending on the size of the population. In addition, long-chain PFCAs can be transported by ocean flows from distant areas such as the main island of Japan and nearby countries.

For classical POPs, the concentrations of 4,4′-DDE and HCB were one order of magnitude higher than that of PCB153, *trans*-NC and β-HCH across the three Seas (Supplemental Table 2). The levels of 4,4′-DDE and PCB153 are higher in the Sea of Japan, followed by the Sea of Okhotsk and then the Pacific Ocean. However, HCB, *trans*-NC and β-HCH levels did not differ across the three Seas. This result indicates that there may be current or historical emission sources for 4,4′-DDE and PCB153 from the coast of the Sea of Japan and/or nearby countries.

3.2. Correlations between PFCAs and classical POPs

The correlation coefficients between PFCAs (C8–C14) and five classical POPs (4,4′-DDE, HCB, PCB153, *trans*-NC, β-HCH) ($n = 22$)

Table 3
Correlation among different chain length PFCAs and classical POPs ($n = 22$).

		PFCAs							Classical POPs			
		PFOA(C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)	4,4′-DDE	HCB	PCB153	*trans*-NC
PFCAs	PFOA (C8)	1										
	PFNA (C9)	**0.431**[a]	1									
	PFDA (C10)	0.011	**0.748**[b]	1								
	PFUnDA (C11)	−0.228	**0.523**[a]	**0.920**[b]	1							
	PFDoDA (C12)	−0.135	**0.560**[b]	**0.886**[b]	**0.944**[b]	1						
	PFTrDA (C13)	−0.151	**0.435**[a]	**0.828**[b]	**0.917**[b]	**0.965**[b]	1					
	PFTeDA (C14)	−0.235	.189	**0.569**[b]	**0.703**[b]	**0.825**[b]	**0.824**[b]	1				
Classical POPs	4,4′-DDE	0.057	−0.268	−0.134	0.045	0.061	0.176	0.316	1			
	HCB	0.187	0.011	−0.157	−0.196	−0.210	−0.131	−0.341	0.264	1		
	PCB153	0.038	−0.170	−0.074	0.079	0.149	0.228	0.390	**0.936**[b]	0.167	1	
	trans-NC	0.093	−0.242	−0.163	−0.010	−0.044	0.033	0.165	**0.899**[b]	0.202	**0.781**[b]	1
	β-HCH	0.340	−0.090	−0.154	−0.159	−0.084	0.033	−0.010	0.278	**0.555**[b]	0.208	0.137

Bold font: |r|>0.4.
[a] Correlation is significant at the 0.05 level (2-tailed).
[b] Correlation is significant at the 0.01 level (2-tailed).

Table 4
Comparison of PFCA concentration of sea and river fish with reported data.

Sampling country	Concentrations (pg g-wet weight^{-1})											
	Water area	Sampling year	Fish type		PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)	Reference
Sweden	Baltic sea	2001	edible fish muscle	Median	<100–200	100–260	<80–170	<80–300	<80–90	190–230	<0.15	Berger et al., 2009
Japan	Pacific Ocean	2012	edible fish muscle	Mean	100	129	176	955	230	563	110	This study
	Japan Sea	2012	edible fish muscle	Mean	87	99	143	920	248	714	131	This study
	Sea of Okhotsk	2012	edible fish muscle	Mean	191	148	103	457	123	272	60	This study
	River in Tokyo[a]	2007	carp muscle	Range	<50	100–900	200–1400	900–9800	200–2100	300–4200	100–1000	Murakami et al., 2011.
China (6 costal province)	East China Sea	–	fatty fish	Mean	16–70	n.d.–69	39–353	–	–	–	–	Wu et al., 2012.
China	South China Sea	2009	edible fish muscle	Mean	<LOD-1000	<LOD-860	<LOD-600	<LOD-710	<LOD	<LOD-750	<LOD	Zhao et al., 2011.
Czech	6 rivers	2010	fish muscle	Mean	200	400	1960	2470	3850	2720	3590	Hloušková et al., 2013.

[a] Drainage canal near a sewage-treatment plant outfall.

are listed in Table 3. Among the PFCAs, C10, C11, C12, C13 and C14 showed a strong association ($r = 0.569$ to 0.965, $p < 0.01$), but were not associated with the five classical POPs. Based on this result, we conducted a PCA to reveal the potential difference of studied compounds. The PCA revealed the 1st factor (F1), 2nd factor (F2) and 3rd factor (F3), accounted for 40.0%, 26.1%, and 14.9% of the total variance (with eigenvalues of >1), respectively (Supplemental Table 3). In the PCA, F1 indicated higher eigenvectors for long-chain PFCAs (C9–C14) while F2 had positive eigenvectors for classical POPs (4,4′-DDE, PCB153, and *trans*-NC). F3 had a moderate positive eigenvector for the other compounds (mainly, C8) (Supplemental Table 3). In summary, as shown in Fig. 2, the PCA divided the target compounds into three groups; (1) long-chain PFCAs (C9–C14), (2) classical lipophilic POPs, and (3) the others (mainly, C8). This indicates that the long-chain PFCAs (C9–C14) have a similar emission source, which differs from that of classical POPs and PFOA (C8). In human serum, the levels of classical POPs and PFOA (C8) have decreased over a decade, but long-chain PFCA levels are still increasing (Fujii et al., 2014; Harada et al., 2011), which may simply reflect the differences in emission trends of those compounds over time. Moreover, the result of the PCA may also reflect the different bioaccumulation mechanisms of the studied compounds. PFOA (C8) has both hydrophobic and hydrophilic regions and tends to accumulate in serum due to its binding affinity to serum proteins (Fujii et al., 2015; Jones et al., 2003). On the other hand, the long-chain PFCAs are known lipophilic contaminants, since the lipophilicity increases with increasing chain lengths and are anticipated to have a similar distribution pattern to the lipophilic POPs, which accumulate in lipid-rich tissues. However, in this study the long-chain PFCAs were independent of the POPs (Table 3 and Supplemental Table 2, Fig. 2), suggesting different bioaccumulation mechanisms. This result may be explained by previous in vivo and in vitro studies, showing that long-chain PFCAs may accumulate preferentially in liver over lipid-rich tissues due to a high affinity for fatty acid binding proteins in the liver (Fujii et al., 2015; Zhang et al., 2013).

3.3. Comparison of PFCA concentrations with reported data

Although data concerning the PFCA levels in edible sea fish samples are not as abundant as those in freshwater fish, there are several reports from Greenland, Norway and China, and the relevant data are summarized in Table 4. The levels of PFCAs in sea fish tend to be lower than those in river fish (Hloušková et al., 2013; Murakami et al., 2011). PFCA levels in sea fish in Japan were comparable to previous studies from China and Greenland, while slightly lower than in Norway (Berger et al., 2009; Wu et al., 2012; Zhao et al., 2011). Odd-numbered PFCAs are slightly predominant in all previous studies in sea fish, which matches the data from the Japanese fish samples. However, the levels of PFCAs in freshwater

Table 5
Daily intake estimations and risk assessment for the Japanese population in Hokkaido.

		ng day^{-1}						
		PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)
(1) Estimated intake of PFCAs via fish consumption[a]	ng/day	2.9	4.3	5.1	34.0	7.3	22.1	4.4
(2) Reported total dietaly intake of PFCAs[b]	ng/day	<18.1	7.8	<3.6	20.6	4.9	14.5	<3.6
Ratio of (1) to (2)	(1)/(2)	0.3[c]	0.6	2.8[c]	1.7	1.5	1.5	2.4[c]
% of TDI[d]	%	0.004	–	–	–	–	–	–

[a] Average raw fish consumption in Japan was assumed as 42.7 g day^{-1}(National Health and Nutrition Survey, reported by the Ministry of Health, Labour and Welfare of Japan (2012)).
[b] Dietary intake of PFCAs from composite food samples in Hokkaido area (all food and drink items that they consumed over a 24 h period) (Fujii et al., 2012a).
[c] Concentrations lower than the MDLs were considered to be equal to half of the MDLs.
[d] Provisional tolerable daily intake (PTDI) is 1500 ng kg-body weight^{-1} day^{-1}) for C8 given by the Scientific Panel on Contaminants in the Food Chain requested by the European Food Safety Authority in 2008. Body weight is set to 50 kg.

fish in the Czech Republic did not show such odd-number predominance (Table 4) (Hloušková et al., 2013). These differences may reflect the contamination pattern in that particular area of water.

3.4. Daily intake and toxicological impact of PFCAs

3.4.1. Fish as a source of PFCAs exposure to humans

We estimated the PFCAs intake through fish consumption to assess the contribution of sea fish consumption to PFCA exposure of the Japanese population in Hokkaido. Average unprocessed fish consumption of the Japanese population has been reported to be 42.7 g day^{-1} by the National Health and Nutrition Survey (the Ministry of Health, Labor and Welfare of Japan. 2012). In this study, the mean PFCA intake through fish was estimated to be 2.9, 4.3, 5.1, 34.0, 7.3, 22.1 and 4.4 ng day^{-1} for C8, C9, C10, C11, C12, C13, and C14, respectively (Table 5), assuming that all consumed fish species in the survey contain similar levels as Pacific cods. Recently we reported the dietary PFCAs exposure based on food-duplicate containing all food and drink items that humans consumed over a 24 h period in the Hokkaido area (Fujii et al., 2012a) As shown in Table 5, the relative ratio between the estimated PFCAs intake through fish consumption and the reported total dietary exposure were lower than 1 for C8 and C9 (0.3 for C8, and 0.6 for C9), but were higher than 1 for the long-chain PFCA (2.8 for C10, 1.7 for C11, 1.5 for C12, 1.5 for C13, and 2.4 for C14). This result strongly suggests that fish consumption is a significant source of dietary exposure to long-chain PFCA (C10–C14). The results also showed that odd-numbered PFCAs predominated in the cod fish samples. This is consistent with a previous biomonitoring study of human serum (Harada et al., 2011; Okada et al., 2013), for which the intake of PFCAs through ingestion of fish may be an important route of exposure. In the present study, C8 and C9 made up less than 0.6 of total dietary intake, indicating that C8 and C9 have a different exposure source such as drinking water.

3.4.2. Risk assessment of dietary PFCAs intakes

The tolerable daily intake (TDI) for C8 was set to be 1500 ng kg body weight^{-1} d^{-1} by the Scientific Panel on Contaminants in the Food Chain created by the European Food Safety Authority in 2008 (Fujii et al., 2012b). As of 2014, there is no established TDI for PFCAs that are longer than C8. The dietary intakes of C8 through fish consumption were much lower than the TDI in this study (less than 0.004% of TDI) (Table 5). These observations may indicate that the health risks of C8 for dietary exposure through fish consumption are limited.

Bioaccumulation factors of PFCAs in biota have been reported to be increased with increasing perfluoroalkyl chain length (generally, >C8) (Martin et al., 2003). This is in agreement with our results showing that the concentrations of the longer chain PFCAs (C9 to C14) comprised 96% of the total studied PFCAs (C8 to C14) in fish samples. Those facts strongly suggest that the long chain PFCAs should be major targets of PFCAs risk assessment.

Toxicological implication for human exposed to long-chain PFCAs via diet is not known. The potential toxicities of PFCs based on laboratory studies of animals (monkeys and rats) are reduced body weight, increased liver weight, hepatoxicity, alteration of hepatic lipid metabolism and peroxisome, reduction of serum cholesterol and thyroid hormones (Lau et al., 2004). A recent research revealed antibody productions by immunization of tetanus and diphtheria in children can be suppressed depending on serum PFCA concentrations in the general human population (Grandjean et al., 2012). Moreover, in vivo study, long chain PFCAs have caused higher biological responses than C8 for immunotoxicity (Corsini et al., 2012). Considering that edible fish is a major human dietary source of long chain PFCAs, it is important to investigate the marine environmental fate of long chain PFCAs and their risk assessment of human exposure.

4. Conclusions

We analyzed the PFOA (C8) and PFCAs (C9–C14) from 22 cod samples collected in coastal areas of Hokkaido, northern Japan. The long-chain PFCAs (C9–C14) reached 96% of the total PFCAs averaged across the three seas. The results indicate that long-chain PFCAs (C10–C14) in sea fish is a significant source of dietary exposure to PFCA intake. Furthermore, the levels of long-chain PFCAs were strongly correlated to each other, but not to C8 and the other classical POPs, indicating that long-chain PFCAs have a different emission source and/or bioaccumulation mechanism than C8 and the POPs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.01.007.

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Toxicokinetics of perfluoroalkyl carboxylic acids with different carbon chain lengths in mice and humans

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Abstract: Toxicokinetics of perfluoroalkyl carboxylic acids with different carbon chain lengths in mice and humans: Yukiko FUJII, *et al.* Department of Health and Environmental Sciences, Kyoto University Graduate School of Medicine—Objectives: Perfluoroalkyl carboxylic acids (PFCAs) consist of analogs with various carbon chain lengths. Their toxicokinetics have remained unexplored except in the case of perfluorooctanoic acid (8 carbon chemicals). This study aimed to investigate the toxicokinetics of PFCAs with six to fourteen carbon atoms (C6 to C14) in mice and humans. **Methods:** We applied a two-compartment model to mice administered PFCAs intravenously or by gavage. The time courses of the serum concentration and tissue distribution and elimination were evaluated for 24 hours after treatment. For human samples, urine from healthy volunteers, bile from patients who underwent biliary drainage, and cerebral spinal fluid (CSF) from brain drainage were collected. **Results:** The mouse experiment showed that short-chained PFCAs (C6 and C7) were rapidly eliminated in the urine, whereas long-chain PFCAs (C8 to C14) accumulated in the liver and were excreted slowly in feces. Urinary clearance of PFCAs in humans also decreased with increasing alkyl chain lengths, while biliary clearances increased. C9 to C10 had the smallest total clearance for both mice and humans. However, disparities existed in the magnitude of the total clearance between mice and humans. A slightly higher partition ratio (brain/serum) was observed for long-chained PFCAs in mice, but this was not observed in the corresponding partition ratio in humans (CSF/serum). **Conclusions:** The large sequestration volumes of PFCAs in the liver seem to be attributable to the liver's large binding capacity in both species. This will be useful in evaluating PFCA bioaccumulation in other species.
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Key words: Human, Mice, Perfluoroalkyl carboxylates, Perfluorooctanoic acid, Toxicokinetics

Perfluorochemicals, such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid [PFOA, chemicals with eight carbon atoms (C8)], have been detected in the environment, and their toxicokinetics have been examined extensively. Their biological half-lives are significantly longer in humans than in other laboratory animal models[1,2]. The reason for the longer biological half-lives in humans remains unknown.

C8 PFOA has been found to cause hepatotoxicity, developmental toxicity, immunotoxicity and endocrine disruption[3]. Consequently, perfluoroalkyl carboxylic acids (PFCAs) other than C8 PFOA with shorter chain lengths, such as perfluorobutanoic acid and perfluorohexanoic acid (C4 to C6), have been used for commercial applications[4]. These short-chained PFCAs seemed to be less toxic than C8 PFOA[5,6], possibly stemming from their relatively short half-lives compared to the C8 PFOA[7,8]. In contrast, long-chained PFCAs, such as perfluorononanoic acid (PFNA, C9) and perfluorodecanoic acid (PFDA, C10), showed relatively longer half-lives than PFOA in rodents[1,9,10]. It is well known that straight-chain PFCAs are not metabolized biologically[11]. Furthermore, several *in vitro* studies have found that biological activities are dependent on the alkyl chain length of the parent

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compounds[12–14]. Nevertheless, increasing levels of long-chained PFCAs have been found in the human serum[15, 16] and daily diet[17] in recent decades.

The present study aimed to investigate the toxicokinetic differences of C6 to C14 PFCAs in mice and humans. Serum concentration and tissue distribution and elimination were evaluated for 24 hours after intravenous (IV) and gavage PFCA administration in mice. Urinary clearance, biliary clearance and cerebral spinal fluid (CSF) partitions of PFCAs in humans were examined for comparison. No such comparison has ever been reported, despite its toxicological importance.

Material and Methods

Animal experiments

1) Animals. All experiments were performed with mice aged 8–10 weeks (body weight 20–30 g). FVB/NJcl mice were purchased from CLEA Japan, Inc. (Tokyo, Japan), and housed in the Kyoto University Institute of Laboratory Animals. A standard commercial lab chow diet (F-2, 3.73 kcal/g, Funahashi Farm Corp., Chiba, Japan) was used. All animals were maintained at an ambient temperature of 24 ± 2°C and 50 ± 10% humidity with a 12-h light/dark cycle (lights on at 7:00 a.m.). Mice were individually placed in metabolic cages and were provided with free access tap water and food.

2) Sample collection. Each PFCA was administered by IV or gavage. PFCAs were dissolved in ethanol/water/dimethyl sulfoxide (5:4:1) and prepared with Milli-Q water. In this study, both IV and gavage administration were applied to evaluate the absorbed ratios of PFCAs. Single doses of PFCAs were administered through the tail vein (IV dose 0.31 μmol/kg, injection volume 10 m*l*/kg) or orally (gavage dose 3.13 μmol/kg, injection volume 10 m*l*/kg). Each group contained 18 mice: 9 males and 9 females.

To observe the time course of the serum PFCA concentrations, whole blood samples (10 μl) were collected from the tail veins at 0, 1, 3, 6, 12 and 24 hours after IV or gavage administration. An additional collection was made at 0.5 hours for IV administration. The study protocol is summarized in Table S1.

After 24 hours, urine and feces were collected in metabolic cages. Mice were then placed under sevoflurane anesthesia and euthanized by cervical dislocation. A portion of the whole blood was collected and centrifuged (370 g) to isolate the serum. Liver, kidney and brain tissues were collected and weighed. Adipose tissue was collected from the abdominal mesenteric fat. The total serum in the mice was estimated to be 56 m*l*/kg mouse body weight for male mice and 65 m*l*/kg mouse body weight for female mice[18]. The total adipose tissue was assumed to be 2.3% of the total body weight of mice[18]. All experimental procedures were approved by the Kyoto University Animal Research Committee (MedKyo11067).

Paired human samples: urine, bile and CSF serum pairs

All paired human samples (bile-serum, CSF-serum and urine-serum) were obtained from the archived samples in the Kyoto University Human Specimen Bank[19, 20]. The characteristics of the participants are summarized in Table S2. Bile samples were taken by nasobiliary drainage, percutaneous transhepatic biliary drainage or percutaneous transhepatic gallbladder drainage for 24 hours. Paired 5-m*l* blood samples were collected from the cubital vein into polypropylene tubes on the same day. CSF samples were taken by cerebral drainage, spinal drainage, ventriculoperitoneal shunt or duraplasty. Ten milliliters of blood was also donated from the donor on the same day. Healthy volunteers were requested to collect 24-h pooled urine samples and to donate 10 m*l* of blood at the end of urine collection. The research protocol was reviewed and approved by the ethics committee of Kyoto University (E25). Written informed consent was obtained from all participants before sample collection.

Determination of PFCA concentration in biological samples

1) Sample homogenization and preparation. Mouse tissue and feces were weighed and diluted with Milli-Q water/methanol (1:1) at a ratio of 15 m*l* water/methanol per gram of mouse tissue. The sample was homogenized using a homogenizer. Part of the homogenate (0.1–1 m*l*, depending on the concentration) was transferred into a 15-m*l* polypropylene tube. For whole blood, serum and urine samples, approximately 10–100 μl of each sample and 1 m*l* of methanol were placed in a 1.5 m*l* microcentrifuge tube and mixed for 3 hours. Part of the resulting solution (0.1–1 m*l*, depending on the concentration) was then transferred into a 15 m*l* polypropylene tube. For the human samples, approximately 0.5–30 m*l* of each sample was directly transferred into 15 or 50 m*l* polypropylene tubes.

2) Determination of PFCAs. Determination of PFCA concentrations in all samples was performed using a method previously reported[17]. Target chemicals included perfluorohexanoic acid (PFHxA, C6), perfluoroheptanoic acid (PFHpA, C7), PFOA (C8), PFNA (C9), PFDA (C10), perfluoroundecanoic acid (PFUnDA, C11), perfluorododecanoic acid (PFDoDA, C12), perfluorotridecanoic acid (PFTrDA, C13) and perfluo-

rotetradecanoic acid (PFTeDA, C14). Procedural blank controls were analyzed after every 10 samples. The method detection limit (MDL) was defined as the concentration that produced a signal three times that of the blank (Table S3). Total recoveries are shown in Table S4.

Toxicokinetic analysis of PFCAs

The ratio of PFCAs between whole blood and serum at 24 hours was used to convert PFCA concentrations in whole blood samples into serum PFCA concentrations. Serum concentration data were analyzed using a two-compartmental model described by the following equation:

$$C(t)=C_1 exp(-\lambda_1 * t)+C_2 exp(-\lambda_2 * t). \quad Eq\ (1)$$

To obtain C_1, C_2, λ_1 and λ_2, PFCA levels in the serum were fitted into a two-compartment toxicokinetic model by nonlinear optimization with a least-square approach[21]. The volume distribution in the IV injection study was defined as follows:

$$Volume\ distribution = Dose\ /\ C(0). \quad Eq\ (2)$$

PFCA clearance in mouse and human samples

Mouse urinary clearance ($CL_{u\text{-}mice}$) was determined by dividing the total amount excreted in the urine during a 24-h period with the area under the curve (AUC) of the serum concentration of each PFCA between 0 to 24 hours. Mouse fecal clearance ($CL_{f\text{-}mice}$) was determined by dividing the total amount excreted in the feces during a 24-h period with the AUC of the serum concentration of each PFCA between 0 to 24 hours.

Human urinary ($CL_{u\text{-}humans}$) and biliary clearance ($CL_{b\text{-}humans}$) of each PFCA was determined by dividing the cumulative urine or bile excretion in a 24-h period with the serum concentration of each PFCA.

Statistical analysis

Concentrations lower than the detection limits were given a value half that of the detection limit for statistical analyses. Differences between mean values of each PFCA in human CSF were tested using the Student's *t*-test. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

Toxicokinetic analyses in mice after IV administration

The ratios of whole blood to the serum concentrations of each PFCA (mean ± SD) were 0.60 ± 0.1 for C8, 0.43 ± 0.1 for C9, 0.50 ± 0.1 for C10, 0.53 ± 0.1 for C11, 0.70 ± 0.2 for C12, 0.88 ± 0.2 for C13, and 1.05 ± 0.2 for C14. The mean ratio of each chemical was multiplied by the whole blood concentrations to calculate the corresponding serum concentration.

The time course and fitted curves for PFCAs in logarithmic scale are shown in Fig. 1. As C6 was not detected in the serum at even 1 hour after administration, its serum kinetics was not analyzed. For the other PFCAs (C7 to C14), the serum levels were above the MDLs. As shown in Fig. 1, C7 disappeared from the serum in a time-dependent manner. The other compounds (C8 to C14) demonstrated very unique kinetic profiles characterized by slow elimination from the serum (Table 1). The two-compartment model successfully described the kinetics of PFCAs in mice. The parameters obtained from the serum PFCA concentrations are depicted in Table 1.

The volume distributions of the PFCAs (C7 to C14) exhibited no differences between sexes, with the volume increasing as a function of length in both males and females (Fig. S1). The distributions corresponded roughly to the total volume of blood with C7, extracellular water with C8 and C9 and body water with C11 and C12. Tissue binding was suggested for C13 and C14. These results indicated that chain length was a determining factor for volume distribution (Table 1). The AUCs reached their maximums at C8 and decreased with increasing chain length (Table 1).

Table S5 shows the tissue distribution of PFCAs 24 hours after administration. Total recoveries for all C6 to C14 were greater than 76% in males and somewhat lower in females (greater than 58%). For C6 and C7 PFCAs, almost all of the administered doses were recovered in the urine after 24 hours (101 and 99% for males, 66 and 79% for females), with only a small portion excreted in the feces (5 and 3% for males, 16 and 13% for females). In contrast, only a small portion of C8 was excreted in the urine (6% for males, 7% for females), and even less was excreted in the feces (<1% for both sexes); the majority was retained in the serum and liver (80% for males, 62% for females), with a discernible amount retained in the kidney (1% for both sexes). For C9 to C14, the distribution pattern was similar to that of C8. However, C9 to C14 excretion in the urine and feces for both males and females was much lower than that of C8; most were retained in liver (64–80% for males, 46–55% for females).

Toxicokinetics of PFCAs in mice after gavage administration

After gavage administration, C6 was not detected in the serum at any sampling points. Thus, a two compartment analysis was not conducted for C6. As shown in Fig. 2, the time courses for C7 to C14 were well simulated by the two-compartment toxicokinetic models with no differences in sex (Table 1). The

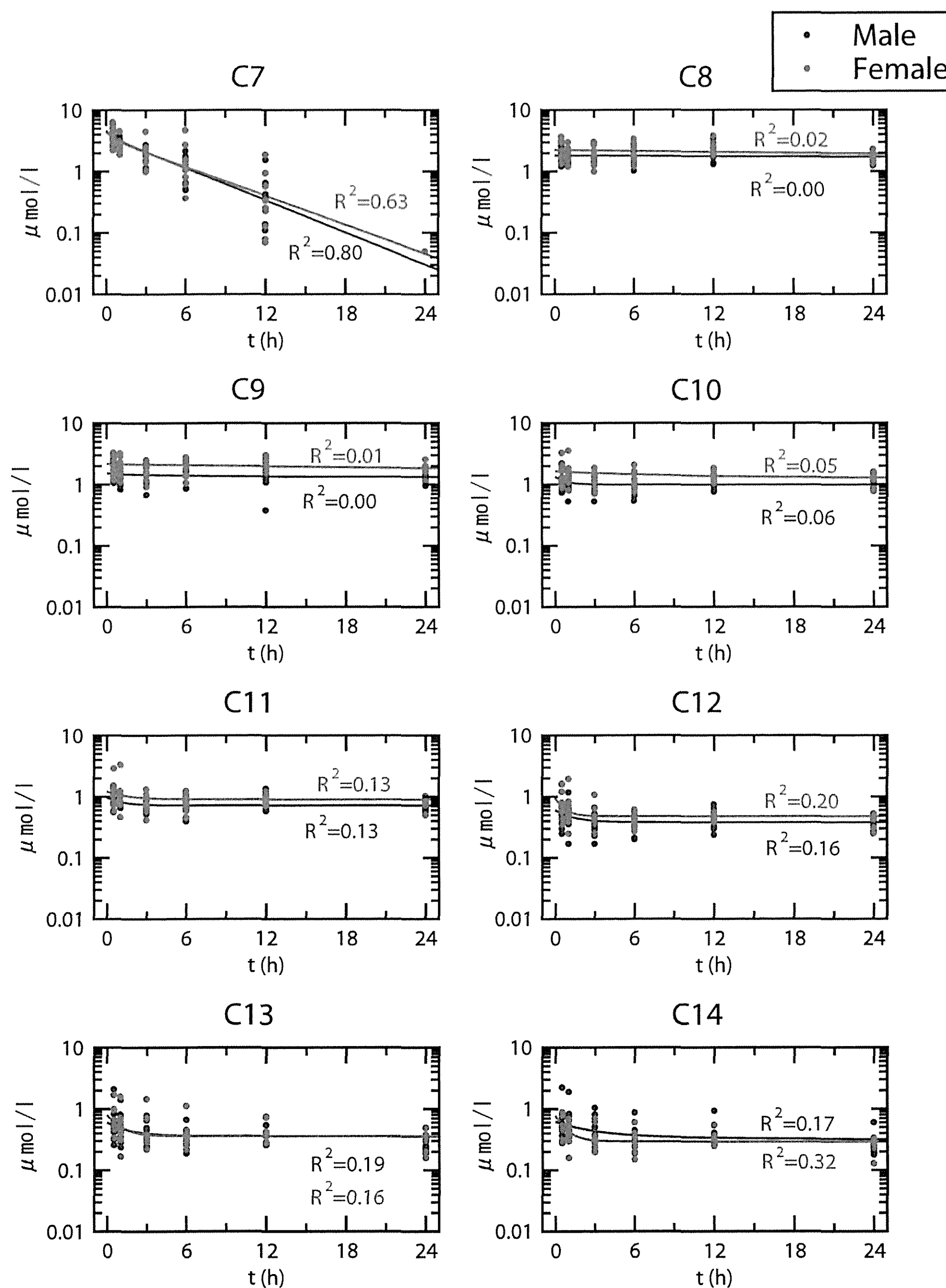


Fig. 1. Simulated serum concentrations in mice after IV administration (0 to 24 hours).
Values derived from Table 1.
Dots indicate the observed values with IV administration.

AUCs increased with decreasing numbers of carbon, with C8 having the largest AUC. The adjusted AUC ratios of gavage to IV administration were close to 1 for C7 to C13 and less than 1 for C14 (Table 1).

Mass balance studies indicated lower total recoveries for C6, C7 and C13 to C14 with gavage administration than those with IV administration (Table S5 and Table S6). The exact reason for those lower recoveries is not well-known. The administrated PFCAs may be distributed in some part other than the collected samples (e.g., stomach and gut wall). The overall distribution profiles of gavage administration are close to those of IV administration: most of the C8 to C14 were recovered in the liver and serum, while C6 and C7 were recovered in the urine. Only small volumes of PFCAs were excreted in feces,

Table 1. Elimination of PFCAs determined by the two-compartment model in mice after IV or gavage administration

$$C(t)=C_1*\exp(-\lambda_1*t) + C_2*\exp(-\lambda_2*t)$$

a. Intravenous injection with a target dose of 0.313 μmol/kg

		PFHpA (C7)	PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)
	Male								
C_1	$\mu mol\ l^{-1}$	0.8 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
λ_1	h^{-1}	1.59 ± 0.50	0.03 ± 0.02	0.18 ± 0.11	1.20 ± 0.37	0.82 ± 0.25	0.75 ± 0.33	0.62 ± 0.32	0.38 ± 0.22
C_2	$\mu mol\ l^{-1}$	3.8 ± 0.9	1.6 ± 0.4	1.3 ± 0.4	1.0 ± 0.2	0.7 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
λ_2	h^{-1}	0.20 ± 0.1	0.00014 ± 0.0001	0.000046 ± 0.00004	0.00013 ± 0.0001	0.000052 ± 0.00004	0.00035 ± 0.0002	0.00058 ± 0.0003	0.0043 ± 0.002
AUC of 24 hours	$\mu mol\ l^{-1}$ hour (0 to 24 hours)	22.2 ± 8.4	42.2 ± 9.9	33.2 ± 10.3	24.2 ± 6.0	17.6 ± 3.7	9.5 ± 3.1	9.2 ± 3.1	9.0 ± 3.2
Volume distribution[a]	$l\ kg^{-1}$	0.07 ± 0.01	0.18 ± 0.04	0.22 ± 0.06	0.25 ± 0.06	0.33 ± 0.06	0.57 ± 0.21	0.58 ± 0.20	0.55 ± 0.18
	Female								
C_1	$\mu mol\ l^{-1}$	0.9 ± 0.2	0.5 ± 0.3	0.6 ± 0.3	0.4 ± 0.2	0.3 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.5 ± 0.2
λ_1	h^{-1}	1.40 ± 0.63	0.03 ± 0.02	0.03 ± 0.02	0.11 ± 0.07	0.61 ± 0.16	1.50 ± 0.70	0.98 ± 0.51	1.24 ± 0.62
C_2	$\mu mol\ l^{-1}$	3.5 ± 1.1	1.7 ± 0.5	1.6 ± 0.4	1.3 ± 0.3	0.9 ± 0.3	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
λ_2	h^{-1}	0.18 ± 0.08	0.00021 ± 0.0001	0.00042 ± 0.0003	0.00046 ± 0.0003	0.00043 ± 0.0003	0.00023 ± 0.0002	0.00027 ± 0.0002	0.00079 ± 0.0005
AUC of 24 hours	$\mu mol\ l^{-1}$ hour (0 to 24 hours)	23.6 ± 14.2	49.5 ± 11.9	47.4 ± 11.0	33.4 ± 8.4	22.2 ± 6.9	11.9 ± 3.3	9.3 ± 2.6	7.5 ± 1.7
Volume distribution[a]	$l\ kg^{-1}$	0.08 ± 0.02	0.15 ± 0.04	0.15 ± 0.04	0.20 ± 0.05	0.28 ± 0.08	0.35 ± 0.10	0.43 ± 0.14	0.43 ± 0.13

b. Gavage administration with a target dose of 3.13 μmol/kg

		PFHpA (C7)	PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)
	Male								
C_1	$\mu mol\ l^{-1}$	−19 ± 2	−20 ± 2	−19 ± 3	−18 ± 4	−15 ± 4	−11 ± 4	−9 ± 3	−6 ± 3
λ_1	h^{-1}	0.8 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
C_2	$\mu mol\ l^{-1}$	29 ± 6	23 ± 4	20 ± 4	19 ± 4	16 ± 4	11 ± 4	9 ± 3	6 ± 2
λ_2	h^{-1}	0.18 ± 0.06	0.025 ± 0.004	0.014 ± 0.004	0.021 ± 0.01	0.033 ± 0.01	0.041 ± 0.01	0.042 ± 0.01	0.040 ± 0.01
AUC of 24 hours	$\mu mol\ l^{-1}$ hour (0 to 24 hours)	141 ± 51	348 ± 76	335 ± 63	277 ± 44	170 ± 30	90 ± 21	69 ± 21	44 ± 17
	Female								
C_1	$\mu mol\ l^{-1}$	−15 ± 1	−17 ± 5	−16 ± 5	−14 ± 4	−8 ± 3	−4 ± 1	−4 ± 0	−3 ± 1
λ_1	h^{-1}	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
C_2	$\mu mol\ l^{-1}$	38 ± 6	30 ± 3	27 ± 3	22 ± 3	14 ± 4	6 ± 1	4 ± 1	3 ± 1
λ_2	h^{-1}	0.14 ± 0.05	0.021 ± 0.0004	0.0022 ± 0.001	0.0070 ± 0.002	0.0081 ± 0.003	0.0058 ± 0.002	0.0021 ± 0.0002	0.0048 ± 0.001
AUC of 24 hours	$\mu mol\ l^{-1}$ hour (0 to 24 hours)	215 ± 156	495 ± 64	535 ± 63	414 ± 61	248 ± 78	117 ± 27	84 ± 23	51 ± 12

c. Ratio of dose-adjusted AUC (gavage average AUC / IV average AUC ratio, both adjusted with the administrated dose)

	PFHpA (C7)	PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)
Male								
	0.6	0.8	1.0	1.1	1.0	0.9	0.7	0.5
Female								
	0.9	1.0	1.1	1.2	1.1	1.0	0.9	0.7

Values are means ± SD. a) See Eq (2).

PFCA: perfluoroalkyl carboxylic acid.

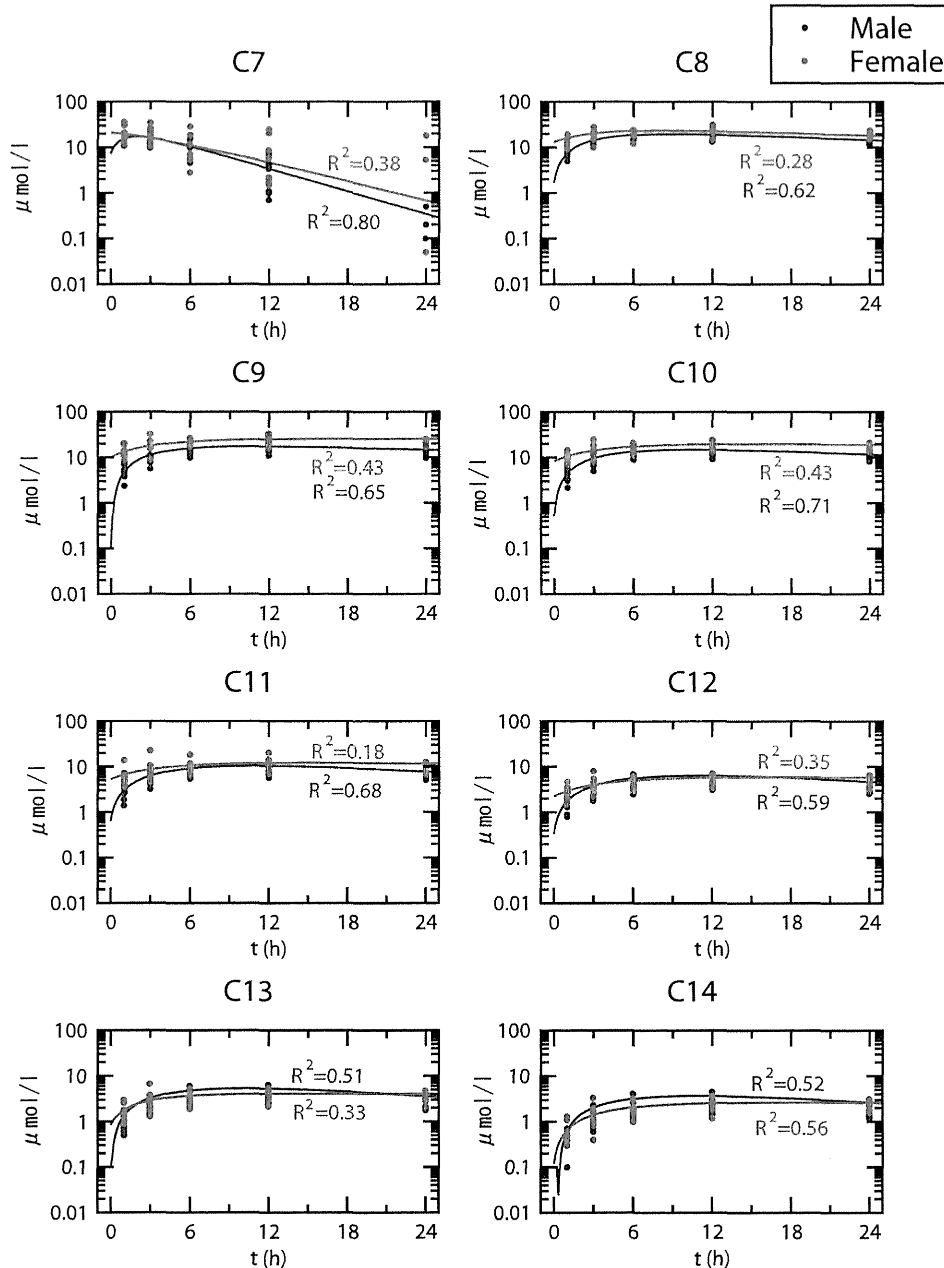


Fig. 2. Simulated serum concentrations in mice after gavage administration (0 to 24 hours).
Values derived from Table 1.
Dots indicate the observed values with gavage administration.

confirming efficient absorption and enterohepatic circulation from the gut.

Urinary and fecal clearance of PFCAs in mice

Urinary and fecal clearance of PFCAs for both IV and gavage administration in mice are shown in Table S7. The urinary clearance of IV-administered C8 (males, 13.1 m*l*/day/kg; females, 9.8 m*l*/day/kg) was significantly smaller when compared with that of C7 (males, 336.7 m*l*/day/kg; females, 216.3 m*l*/day/kg) (Table S7). C7 had the highest fecal clearance, although the level was still smaller than the level of C7 urinary clearance, and C9 had the lowest level of fecal clearance. C7 had the greatest total clearance (males, 347.4 m*l*/day/kg; females, 265.7 m*l*/day/kg), and C10 had the lowest (males, 2.2 m*l*/day/

kg; females, 2.8 ml/day/kg). There were no marked differences between sexes.

Gavage-administered PFCAs had similar clearance patterns as IV-administrated PFCAs. C8 had a significantly lower urinary clearance (males, 9.2 ml/day/kg; females, 6.6 ml/day/kg) than C7 (males, 248.8 ml/day/kg; females, 166.7 ml/day/kg) (Table S7). C7 had the highest fecal clearance, although it was still smaller than the urinary clearance, and C9 had the lowest fecal clearance. C7 had the highest total clearance (males, 292.5 ml/day/kg; females, 190.2 ml/day/kg), and C10 had the lowest (males, 3.9 ml/day/kg; females, 2.2 ml/day/kg).

When the fecal clearances of gavage- and IV-administered PFCAs were compared, disparities existed in the long-chain PFCAs (C13 and C14) (Table S7). The feces after 24 hours of gavage administration contained both PFCAs eliminated in the bile and unabsorbed PFCAs that passed through the gut. The actual fecal clearances of PFCAs were represented by the fecal clearances of IV-administrated PFCAs. To evaluate the absorbed ratios of PFCAs, we calculated the theoretically absorbed portion using the following equation:

$$\text{Theoretical absorbed portion (\%)} = 100 - \text{Recovery in feces by gavage (\%)} \times \frac{\text{Fecal CL by gavage} - \text{Fecal CL by IV}}{\text{Fecal CL by gavage}} . \qquad Eq\ (3)$$

The results are recorded in Table S7. The theoretical absorbed portions for IV and gavage administration ranged from 94 to 104% for both males and females, suggesting that the PFCAs were efficiently absorbed in the gut.

Toxicokinetic model evaluation

The physiologically based pharmacokinetic model of PFOA in rats and monkeys was previously developed using chemical parameters obtained from several animal studies[22, 23]. For this study, we developed a simple two-compartment model based on the PFCA concentration in mouse serum. This model described the time courses of serum concentrations containing single 3.13 μmol (1.3 mg for PFOA)/kg doses via gavage administration. To evaluate this model, we applied it to the toxicokinetics of serum concentrations with repeated gavage doses of 20 mg/kg[24]. The dose model, shown in Figure S2, using a gavage dose of 20 mg/kg was selected because single gavage doses of 40 mg/kg or more were necessary to observe any nonlinear pharmacokinetics for PFOA in mice[24]. PFOA serum concentrations reached a steady state by about 8 days after the first dose, and the minimum and maximum serum levels were approximately 260 and 185 μg/ml, respectively, for males and 300 and 400 μg/ml, respectively, for females. A previous study showed that daily gavage doses of 20 mg/kg yielded serum PFOA concentrations of 181 μg/ml for males and 178 μg/ml for females after 7 days and 199 μg/ml for males and 171 μg/ml for females after 17 days[25]. The simulated serum concentrations in this study revealed that males showed similar results, while females showed slightly higher results (Fig. S2). These results confirmed that repeated doses could be simulated using our simple two-compartment toxicokinetic model with only single doses of PFOA. Moreover, if the model could be applied to other PFCAs, it would be enable us to predict PFCA clearances in repeated gavage administration by using only single gavages. Second, the doses could also be scaled up from 1.3 to 20 mg/kg. Taken together, this model might be able to represent the toxicokinetics in both chronic and trace-level PFCA exposure. However, the experimental proof for this was not demonstrated in this study. Thus, our discussion at the current conditions waits further confirmation.

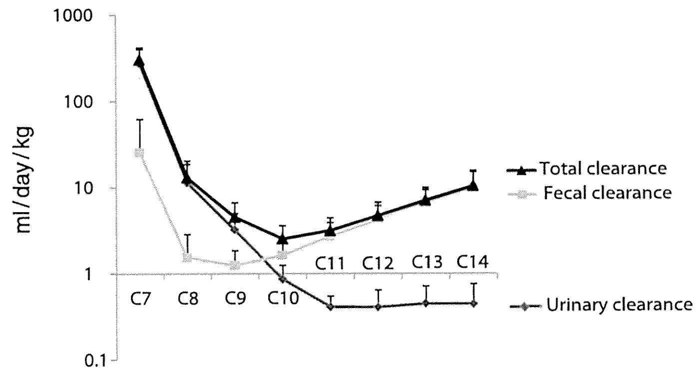
Table 1 presents the numerical results of the model. The λ_2 values of the PFCAs ($\geq$C8) were much smaller than the λ_1 values for both IV and gavage administration, indicating that the PFCAs were distributed rapidly into the body tissue and might have equilibrated between the blood and tissues in their early phases. These result suggested that the first exponential equation would be negligible for long-term observations and that a one-compartment toxicokinetic model would be sufficient for predicting the toxicokinetics of PFCAs ($\geq$C8) in human serum[26].

Urinary and biliary clearance in humans

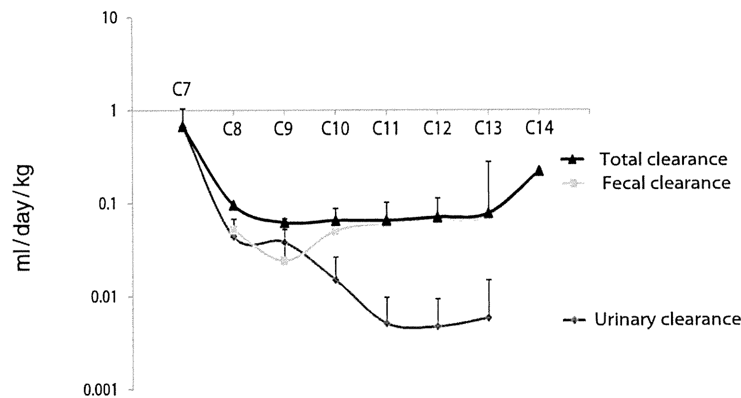
The human urinary and biliary clearances of PFCAs are shown in Table 2. The concentrations of PFCAs in human serum, bile and urine are shown in Table S8. C6 was not analyzed for clearances, as it was not detected in the human serum. Human urinary clearances for PFCAs were more than 200 times smaller than those in mice and decreased with respect to chain length (Fig. 3). Biliary clearance was lowest for C9 and increased as a function of chain length for C9 to C14 PFCAs (Table 2).

To calculate fecal excretion, we estimated the reabsorption rate of PFCAs excreted in the bile, as PFCAs are known to circulate enterohepatically and to be reabsorbed into the bile. Assuming a volume distribution of 200 ml/kg (based on previous reported mouse experiments[26, 27]), a serum half-life of 3.8 years[2], and that C8 could only be excreted into the urine and feces via the bile, the reabsorption rate of bile excreting C8 was calculated as 0.98. We assumed that this reabsorption rate was applicable to the other PFCAs.

a. Mice PFCA clearances (IV administration)



b. Human PFCA clearances



c. Comparison of PFCA clearances in mice and humans

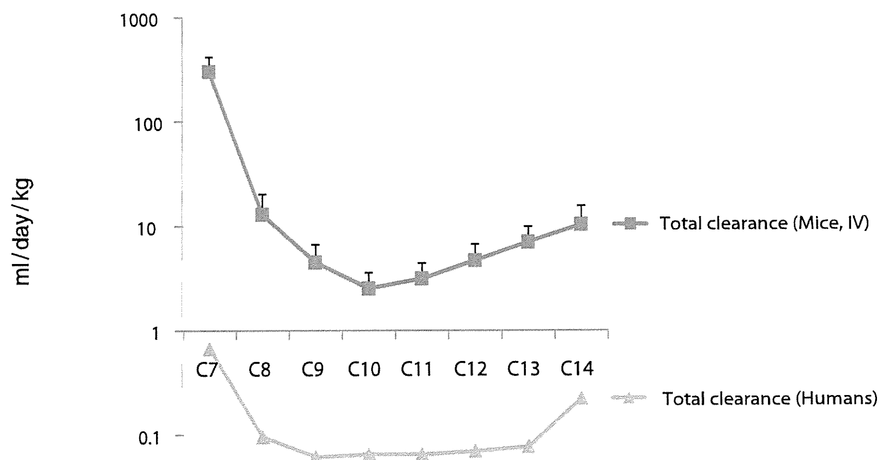


Fig. 3. PFCA (perfluoroalkyl carboxylic acid) clearances in mice and humans (values are means ± SD).

Table 2 shows the fecal clearances for PFCAs estimated from biliary clearances. If the mouse PFCA clearances by single gavage administration are comparable to those in chronic and trace level exposure (Section "Toxicokinetics model evaluation"), the estimated fecal clearances would be similarly two times smaller in humans than in mice. The total clearances (urinary plus fecal clearances) were similar between humans and mice: total clearance in humans decreased as a function of chain length for C7 to C14, and C9

Table 2. Urinary, biliary and fecal clearances of PFCAs in humans

		PFHpA (C7)	PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)
Urinary clearance (Male, N=5; Female, N=5)	ml/day/kg[b]	0.674 ± 0.374	0.044 ± 0.01	0.038 ± 0.01	0.015 ± 0.01	0.005 ± 0.00	0.005 ± 0.00	0.006 ± 0.01	<MDL[c]
Biliary clearance (Male, N=3; Female, N=2)	ml/day/kg[b]	<MDL[c]	2.62 ± 3.6	1.20 ± 1.2	2.51 ± 2.1	3.02 ± 3.0	3.27 ± 3.2	3.57 ± 3.3	11.22 ± 4.4
Estimated fecal clearance[a]	ml/day/kg[b]	—	0.052 ± 0.05	0.024 ± 0.02	0.050 ± 0.04	0.060 ± 0.04	0.065 ± 0.04	0.071 ± 0.05	0.224 ± 0.20
Total clearance (Urinary clearance + Estimated fecal clearance)	ml/day/kg[b]	0.674	0.096	0.062	0.066	0.065	0.070	0.077	0.224

Values are means ± SD. a) See Section "Urinary and biliary clearance in humans". b) Calculated by assuming a human body weight of 50 kg. c) Method delection limit. PFCA: perfluoroalkyl carboxylic acid.

showed the lowest total clearance (0.062 m*l*/day/kg) (Fig. 3). Nevertheless, the total clearances in humans were 50–100 times smaller than those in mice.

PFCAs in mouse and human central nervous systems

PFOS and C8 PFOA are known to alter cell membrane potentials, which in turn affects channel gating properties[28–30]. This suggests that PFCAs may cause neurological toxicities. We have previously reported large concentration gradients of PFOS and C8 PFOA between the CSF and serum[27], suggesting that PFOS and C8 PFOA cannot enter the central nervous system through the blood-brain barrier. These reasons led us to evaluate the concentration gradients of PFCAs between the brain and serum in mice (Table 3). The gradients generally increased with respect to chain length and were large for C8, C9 and C10 but small for C11 to C14. These results suggested that PFCAs might not pass freely through the human blood-brain barrier.

In humans, the PFCA concentrations in CSF were smaller than those in the serum by two orders of magnitude (Table 3). The mean PFCA concentration in hydrocephalus patients ranged from 0.38 pg/m*l* to 37 pg/m*l*, whereas the mean concentrations in patients with cerebral hemorrhage and liquorrhea ranged from 1.3 pg/m*l* to 70 pg/m*l*. The ratio of PFCAs to serum was smaller for hydrocephalus patients than for patients with cerebral hemorrhage and liquorrhea. It is of interest that significantly higher PFCAs (C11 and C13) in the CSF were detected in patients with cerebral hemorrhage and liquorrhea ($p<0.05$, Student's *t*-test). This phenomenon may be associated with the direct infusion of serum into the central nervous system[31]. However, generalization based on these patients only require further confirmations, as the number of patients in this study was relatively small.

Implications of PFCA bioaccumulation

This study clearly demonstrated that the toxicokinetics of PFCAs was dichotomous: C6 and C7 PFCAs were excreted rapidly from the body into the urine, while PFCAs with alkyl chains longer than or equal to C8 were deposited mainly in the liver. Elimination by urine was more rapid than by the liver. Such toxicokinetic characteristics could predict whether PFCAs accumulated in the body. The total clearances of C10 to C14 PFCAs increased with respect to chain length, implying a link with the lipophilicity of PFCAs and they were mainly cleared into the feces via the bile. Thus, C9 to C11 PFCAs accumulated the most in mice. C6 and C7 PFCAs, which were efficiently eliminated through the urine, had significantly shorter half-lives than the other longer-chain PFCAs.

The biochemical mechanisms that cause bioaccumulation as a function of chain length are not well understood. Our study observed that the volume distribution of PFCAs increased with increasing chain length, which may be linked with the lipophilicity of PFCAs. In addition to this, our study indicated that longer-chain PFCAs had an increasing affinity for serum and liver fatty acid binding proteins. Our interpretation was supported by previous studies showing short-chain PFCAs binding modestly to avian serum proteins and binding increasing with longer chains[32]. These results suggest that unbound C6 and C7 PFCAs were excreted by glomerular filtration in the kidney, while the ≥C8 PFCAs were mainly eliminated by bile in the liver. At the same time, long alkyl-chain PFCAs (≥C9) may accumulate preferentially in the liver because of their high affinity for liver fatty acid binding proteins[33]. It is already a known fact that the binding affinity of PFCAs increases with longer alkyl chains[33]. Further studies are required to understand the large PFCA (≥C8) depositions in the liver.

Implications for species difference

This study reports the toxicokinetic profile of PFCAs with 9 different carbon chain lengths in humans and mice. We found that the total clearances (urinary plus fecal clearances) were similar between the two species despite large magnitude differences.

Table 3. Concentration gradients of PFCAs between the serum and brain in mice and the serum and CSF in humans

a. Whole brain tissue in mice (male, N=9; female, N=9)

		Compound (carbon atoms)							
		PFHpA (C7)	PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)
Mouse experiment samples (male, N=9; female, N=9)	Brain tissue concentration (pmol/g)[a]	4 ± 3	25 ± 7	66 ± 23	66 ± 23	93 ± 29	82 ± 23	104 ± 28	97 ± 27
	Brain /serum ratio	<MDL (Serum)	0.015 ± 0.01	0.059 ± 0.02	0.059 ± 0.02	0.125 ± 0.03	0.211 ± 0.06	0.360 ± 0.12	0.413 ± 0.15

b. Cerebral spinal fluids in Human (Male N=6, Female N=1)

	Disease		PFHpA (C7)	PFOA (C8)	PFNA (C9)	PFDA (C10)	PFUnDA (C11)	PFDoDA (C12)	PFTrDA (C13)	PFTeDA (C14)
Human samples (male, N=6; female, N=1)	Total	CSF concentration (pg/ml)	<5.6	50.8 ± 19.1	18.2 ± 8.1	9.1 ± 6.7	19.4 ± 25.7	2.2 ± 2.3	3.3 ± 3.9	0.8 ± 0.7
		CSF/serum ratio	n.d.	0.021 ± 0.013	0.015 ± 0.012	0.015 ± 0.016	0.010 ± 0.012	0.016 ± 0.022	0.011 ± 0.011	0.025 ± 0.024
	Hydrocephalus (male, N=4)	CSF concentration (pg/ml)	<5.6	36.7 ± 8.8	13.8 ± 7.4	5.6 ± 3.9	5.8 ± 2.7	0.5 ± 0.0	1.0 ± 1.0	0.4 ± 0.2
		CSF/serum ratio	n.d. n.s.[b]	0.013 ± 0.005 n.s.[b]	0.007 ± 0.003 n.s.[b]	0.005 ± 0.002 n.s.[b]	0.003 ± 0.003 *[b]	0.003 ± 0.001 n.s.[b]	0.003 ± 0.003 *[b]	0.011 ± 0.005 n.s.[b]
	cerebral hemorrhage and liquorrhoea (male, N=2; female, N=1)	CSF concentration (pg/ml)	<5.6	69.6 ± 7.0	24.1 ± 5.0	13.8 ± 7.4	37.4 ± 33.5	4.4 ± 1.6	6.5 ± 4.1	1.3 ± 0.7
		CSF/serum ratio	n.d. n.s.[b]	0.031 ± 0.015 n.s.[b]	0.025 ± 0.012 n.s.[b]	0.029 ± 0.015 n.s.[b]	0.020 ± 0.012 *[b]	0.035 ± 0.024 n.s.[b]	0.021 ± 0.008 *[b]	0.045 ± 0.025 n.s.[b]

Values are means ± SD. a) Brain tissues were collected 24 hours after IV injection (IV dose: 0.313 μmol/kg). b) *Significant difference between the CSF/serum ratios ($p<0.05$, the Student's t-test). n.s.: not significant ($p>0.05$). PFCA: perfluoroalkyl carboxylic acid.

Differences in the PFCA elimination rates between species have remained unclear. An epidemiological study of retired workers from a C8 PFOA production plant operated by 3M revealed that the serum elimination half-life was 3.8 years[2]. Another study found that the serum elimination half-life of C8 PFOA was much shorter in mice (15–20 days), rats (<1–15 days) and Cynomolgus monkeys (20–35 days)[3].

The reason for the extremely long half-life of PFCAs in humans is still not well understood. PFCAs are not metabolized biologically[11], thus these differences in half-lives are likely due to the difference in clearances. In the current study, the long half-lives of the PFCAs in humans were attributed to poor elimination from the kidney[26, 34]. Renal clearance of PFOA was reduced by probenecid, which decreases organic acid excretion in the urine, suggesting that organic anion transporters may have some important role for PFCA excretion[35]. Other previous experiments studying transporters in rats and mice *in vivo* and in humans *in vitro* have also suggested that organic anion transporters in the proximal kidney tubules might be responsible[36–38]. Indeed, the urinary clearances of C7 and C8 in mice in this study were 500 and 300 times larger than those in humans, respectively. Thus, the species differences are mainly due to the difference in urinary clearances. However, the fecal clearances also differed by one order of magnitude, indicating the other membrane transporters in the liver may also be involved. Furthermore, a slightly higher partition ratio was observed for long-chain PFCAs in mice, but this was not observed in the corresponding partition ratio in humans (CSF/serum). PFCAs in the human CSF ranged from 1.0 to 2.5% of those in serum. This result indicated a species difference in the brain concentrations of PFCAs between human and mice. A previous study revealed that the blood-brain barrier in both humans and mice is maintained by several organic anion transporters, such as organic anion transporter 3, which may actively transport PFCAs from the CSF into the serum[38]. However, there are some species differences in substance selectivity between mouse and human organic anion transporter 3[40]. The species difference in this study may be explained by a difference in substrate selectivity of the transporters between humans and mice.

Limitations of this study

This study had several limitations. First, the PFCA toxicokinetic model was for short-term observations. Nevertheless, our model could simulate both single and repeated doses and scale up C8 doses (Section "Toxicokinetic model evoluation"). It is unknown whether the model is applicable to other PFCAs and in accordance with actual experimental data from

chronic and trace-level administration. This warrants further investigation. Second, several parameters, such as the rate of PFCA reabsorption into the human enterohepatic circulation and the serum to CSF ratio in mice, were estimated, and this therefore increases the uncertainty in the current study. Third, the number of human samples was relatively small, which may prevent generalization based on the current results.

Conclusion

The present study was a comprehensive toxicokinetics study of PFCAs in mice and humans. The highlight of this study was evaluation of various PFCAs of different alkyl chain lengths in mice and humans based on biological residual potency. The large deposition of PFCAs (≥C8) may suggest that fatty acid binding proteins in the liver have an important role for PFCA bioaccumulation. In addition, a simple two-compartment toxicokinetic model for PFOA was shown to simulate serum concentrations by both single or repeated dosing and small or large doses. This information will be useful for evaluating bioaccumulation of PFCAs in a variety of species.

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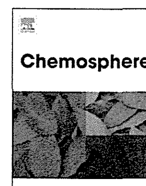

Pentafluorobenzyl esterification of haloacetic acids in tap water for simple and sensitive analysis by gas chromatography/mass spectrometry with negative chemical ionization

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HIGHLIGHTS

- Haloacetic acids (HAAs) levels in tap water are determined.
- Pentafluorobenzyl esterification is used for pretreatment.
- Gas chromatography/mass spectrometry with negative chemical ionization is used.
- Method has low detection limits (8–94 ng L^{-1}) and good recovery rates (89–99%).
- The HAAs concentrations are in range 0.54–7.83*f* Kg L^{-1} in Japanese tap water.

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ABSTRACT

Chlorine is the most widely used disinfectant for control of waterborne diseases in drinking water treatment. It can react with natural organic matter in water and form haloacetic acids (HAAs). For analysis of HAA levels, derivatization with diazomethane is commonly recommended as the standard methodology in Japan. However, diazomethane is a carcinogenic alkylating agent. Therefore, in this study, a safe, simple, and sensitive quantification method was developed to monitor HAAs in drinking water. Pentafluorobenzyl esterification was used for pretreatment. The pentafluorobenzyl-ester derivative was detected by gas chromatography–negative ion chemical ionization-mass spectrometry analysis with very high sensitivity for HAAs analysis. The method has low detection limits (8–94 ng L^{-1}) and good recovery rates (89–99%) for HAAs. The method was applied to 30 tap water samples from 15 cities in the Kansai region of Japan. The levels of HAAs detected were in the range 0.54–7.83 μg L^{-1}. Dichloroacetic acid, trichloroacetic acid, and bromochloroacetic acid were the major HAAs detected in most of the tap water, and accounted for 29%, 20% and 19% of the total HAAs, respectively. This method could be used for routine monitoring of HAAs in drinking water without exposure of workers to occupational hazards.

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1. Introduction

In drinking water treatment, chlorine is widely used for control of waterborne diseases such as cholera, typhoid, hepatitis and gastro-intestinal illness (Morris and Levin, 1995). However, it can react with natural organic matter in water to form a variety of disinfection by-products, including trihalomethanes and haloacetic acids (HAAs) (Badawy, 1992).

There are nine HAAs commonly found in drinking water, including monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), monobromoacetic acid (MBAA), dibromoacetic acid (DBAA), tribromoacetic acid (TBAA), bromochloroacetic acid (BCAA), dibromochloroacetic acid (DBCAA), and

Abbreviations: HAAs, haloacetic acids; MCAA, monochloroacetic acid; DCAA, dichloroacetic acid; TCAA, trichloroacetic acid; MBAA, monobromoacetic acid; DBAA, dibromoacetic acid; TBAA, tribromoacetic acid; BCAA, bromochloroacetic acid; DBCAA, dibromochloroacetic acid; BDCAA, bromodichloroacetic acid; PFBBr, 2,3,4,5,6-Pentafluorobenzyl bromide; MTBE, methyl *tert*-butyl ether; GC–NCI-MS, gas chromatography–negative ion chemical ionization-mass spectrometry; MCL, maximum contaminant level; IDL, instrumental detection limit; MDL, method detection limit; MQL, method quantification limit.

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bromodichloroacetic acid (BDCAA) (Magnuson and Kelty, 2000). The International Agency for Research on Cancer has categorized TCAA as Group 3 (not classifiable as to its carcinogenicity in humans) despite animal tests showing that TCAA and DCAA are hepatotoxic and DCAA and DBAA adversely affecting male reproductive function (Linder et al., 1994). Both TCAA and DCAA caused substantial systematic organ toxicity, including liver and kidney damage, in rats in a 13-week subchronic study (Mather et al., 1990). In epidemiological studies, HAA exposure was significantly correlated with an increased risk of bladder (Villanueva et al., 2004) and colorectal cancers (Rahman et al., 2010).

Because of the suspected adverse health effects of HAAs, the United States Environmental Protection Agency has regulated five HAA species (HAA_5 = MCAA, DCAA, TCAA, MBAA, DBAA) to a maximum contaminant level (MCL) of 60 $\mu g L^{-1}$ based on a running annual average (USEPA, 1999, 2001). The World Health Organization (WHO) provides guidelines for the regulation of MCAA, DCAA and TCAA of 20, 50, and 200 $\mu g L^{-1}$, respectively (World Health Organization, 1993).

In Japan, sodium hypochlorite (NaOCl) is the most common primary disinfectant used in tap water treatment. Consequently, it is expected that HAAs are ubiquitous in drinking water in Japan. The Japanese Drinking Water Quality Standards for MCAA, DCAA, and TCAA are 20, 40 and 200 $\mu g L^{-1}$, respectively (Wakayama, 2003). However, guidelines have not been established for other HAAs and monitoring data are not available.

For chemical analysis of HAAs in tap water, derivatization with diazomethane is commonly recommended as the standard methodology in Japan. However, diazomethane is a carcinogenic alkylating agent and its use presents hazards to laboratory workers (International Programme on Chemical Safety, 1995). Acidic methanol esterification was introduced (Munch et al., 1995; Xie et al., 1998; Nikolaou et al., 2002), and widely used in Asia, Europe and North America. Even though methylester derivatives of TBAA, DBCAA and BDCAA are prone to thermal decomposition and hydrolysis in the GC injection port (Munch et al., 1995; Xie, 2001), the method has been modified to improve methylation efficiency (Domino et al., 2004; Xie et al., 2002, 1998).

Above method employs gas-chromatography/electron capture detector (GC–ECD). Mass-spectrometry (MS) technique is another method to detect target analytes qualitatively and quantitatively with fragment ions (Xie, 2001). Derivatization of HAAs to the 2,4-difluoroanilide with carbodiimide and GC–MS analysis has been described (Alaee and Scott, 1998) and adopted for the determination of HAAs in water sample. However, with this derivatization, recoveries tend to decrease with increasing concentrations of the HAAs. Reports in the literature suggest that pentafluorobenzyl bromide (PFBBr) could increase the electron capture capability of the analyte (Galdiga and Greibrokk, 1998), and enhance both the separation and detection of organic acids (Knapp, 1979). In addition, negative chemical ionization (NCI)-MS analysis for pentafluorobenzyl (PFB)-ester derivatives can provide very high sensitivities for HAAs analysis in biological samples (Jia et al., 2003).

The aim of the present study was to develop a safe, simple, and sensitive quantification method, as an alternative to the diazomethane method to monitor HAAs in drinking water. Thus, in this study we optimized PFBBr derivatization of HAAs and analyzed them using NCI-MS. In parallel with the newly developed method, HAAs levels were monitored in 30 tap water samples obtained from 15 cities in the Kansai region of Japan.

2. Materials and methods

2.1. Materials and reagents

Potassium carbonate and sodium chloride were obtained from Nacalai Tesque (Kyoto, Japan). 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr), 2,3-dibromopropionic acid (recovery surrogate), methyl *tert*-butyl ether 5000 (MTBE, HPLC grade), 11H-eicosafluoroundecanoic acid (internal standard), and 1,4,7,10,13,16-hexaoxacyclooctadecane were purchased from Wako Pure Chemical Industries (Osaka, Japan). Halogenated Acetic Acid Standard Stock Solution II was obtained from Kanto Chemicals (Tokyo, Japan). A derivatization reagent (3% PFBBr) was prepared by dissolving 300 µL of PFBBr and 100 mg of 1,4,7,10,13,16-hexaoxacyclooctadecane in 10 mL of MTBE.

All glassware was washed with detergent, rinsed with tap water and then Milli-Q (MQ, Millipore, Billerica, MA) water, and oven dried before use.

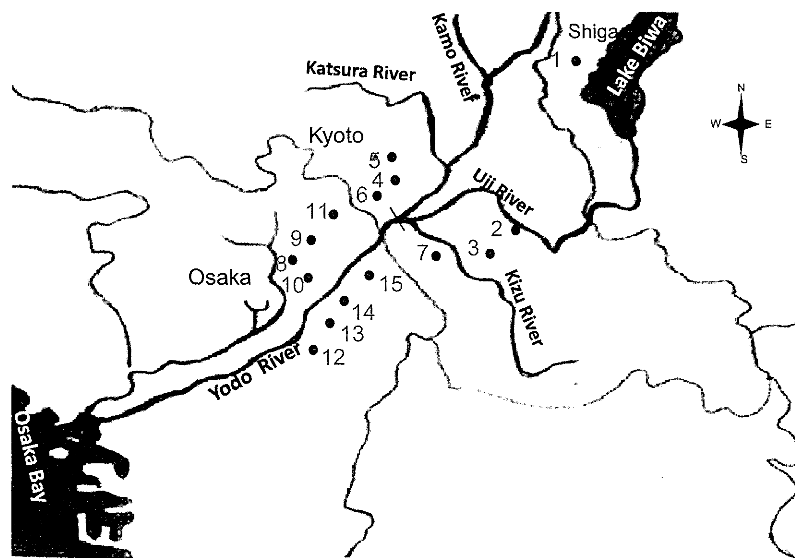

Fig. 1. Locations of water sampling sites in the Kansai region of Japan.