Tissue Distribution and Hepatic Subcellular Distribution of Perfluoroctanoic Acid at Low Dose Are Different from Those at High Dose in Rats

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Fate of perfluoroctanoic acid (PFOA) after an intravenous injection to male rats at the dose of 0.041 mg/kg body weight was compared with that at the dose of 16.56 mg/kg body weight. In the liver, 52% and 27% of PFOA dosed was recovered 2 h after an intravenous injection at the low and the high doses, respectively. By contrast, larger proportion of PFOA dosed was distributed to serum, other tissues and carcass at the high dose compared with the low dose. Subcellular distribution of PFOA was determined in the liver. At the dose of 0.041 mg/kg, 45%, 34%, 18% and 3% were distributed to 8000 g pellet, 18000 g pellet, 105000 g pellet and 105000 g supernatant fraction, respectively; 28%, 17%, 13% and 43% of PFOA were distributed to these fractions, respectively, at the dose of 16.56 mg/kg. The higher the concentration of hepatic PFOA was, the more the PFOA was distributed to 105000 g supernatant fraction. Biliary excretion index increased as PFOA concentration raised in the liver. These results suggest that PFOA is preferentially taken-up by the liver, and distributed to membrane fractions, especially 18000 g pellet, and hardly excreted into bile when exposed at very low dose.

Key words perfluoroctanoic acid; low dose; subcellular distribution; renal clearance; biliary clearance

Perfluoroalkyl chemicals have been used since the 1950s in a wide variety of industrial and consumer products. Among them, perfluorooctanoic acid (PFOA) was used primarily in an ammonium salt form as an emulsifier in the production of fluoropolymers, such as poly(tetrafluoroethylene) and poly(vinylidine fluoride).1 These polymers have been used in various consumers and industrial products, such as water-repellants for leather paper and textiles. The toxicity of PFOA has been characterized in numerous studies with various species.2 Early studies have shown that organic fluorine accumulated in the serum of occupationally exposed people.3 Recent studies have revealed that PFOA and perfluorooctanesulfonic acid have been found in water,4–6 sediments,7 wildlife8–10 and human.11–15 These findings suggest that general population is exposed to such perfluorochemicals which have globally spread at very low levels.

PFOA is thought to remain in humans for long time by the study that has estimated PFOA half-life for 9 retirees from chemical plant to be 4.37 years on the average.16 On the other hand, several studies have been carried out on the fate of PFOA in experimental animals including rats have shown that biological half-life of PFOA in male rats was calculated to be 105 h after an intraperitoneal administration at the dose of 50 mg/kg,17 9 d after an intraperitoneal administration at a dose of 4 mg/kg,18 and 6.8 d after an intravenous administration at a dose of 20 mg/kg,19 respectively. In the studies using experimental animals, PFOA has been shown to be mainly distributed to the liver and serum/plasma, and easily excreted into urine.18–20 The reason for such species difference in half-life of PFOA between humans and experimental animals may be due to the differences in the proteins responsible for distribution, binding and transport of PFOA. Alternative explanation is that the concentrations of PFOA used for the calculation of half-life of PFOA were quite different between humans and the experimental animals. In fact, serum concentrations of PFOA were shown be 11.7 µg/ml 24 h after an intraperitoneal administration at a dose of 4 mg/kg18 and 61.5 µg/ml 24 h after an intravenous injection at a dose of 20 mg,19 respectively, while serum samples of human that have been used for the calculation of half-life contained PFOA at the concentrations of 0.06–1.84 µg/ml.16 To date, however, toxicokinetic study has not been performed at the serum concentrations of PFOA corresponding to the levels in serum of humans.

In the view of toxicological aspects, it is important to know the toxicokinetic data of PFOA at very low serum concentrations. In the present study, we demonstrated that tissue distribution of PFOA at very low dose is markedly different from those at high doses in experimental animals.

MATERIALS AND METHODS

Materials PFOA was purchased from Sigma Aldrich Japan (Tokyo, Japan). [1-14C]PFOA (2.04 GBq/nmol) was purchased from BlyChem Ltd. (Bellingham, U.K.). p-Bromophenacyl-8TM reagent was purchased from Pierce Biotechnology, Inc. (Rockford, IL, U.S.A.). 3-Bromoacetyl-7-methoxy-coumarin was prepared as previously described.21 All other chemicals were of analytical grade.

Purification of [1-14C]PFOA In our preliminary study, [1-14C]PFOA purchased contains a few % of impurities at a few %, mainly perfluorohexanoic acid and perfluorohexanoic acid. Toxicokinetics of perfluorocarboxylic acids with different carbon chain length are known to be quite different; the shorter the perfluoroalkyl chain is, the faster perfluorocarboxylic acid is excreted.19 Therefore, the purchased [1-14C]PFOA was further purified as follows: [1-14C]PFOA was dissolved in methanol, diluted with water and extracted with ethyl acetate: hexane (1:1, v/v) as an ion pair with tetrabutylammonium in the presence of 0.29 M NaCO3 buffer (pH

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10%). The extract was taken to dryness, and to the residue was added p-bromophenacyl-\textsuperscript{8}TM reagent to obtain p-bromophenacyl derivative of PFOA. The reaction mixture was then applied to high-performance liquid chromatography using a reverse-phase column (YMC-Pack pro, 4.6×50 mm, YMC, Kyoto, Japan). The fraction containing p-bromophenacyl derivative of [1\textsuperscript{-14}C]PFOA was collected and was taken to dryness by flushing nitrogen, and p-bromophenacyl derivative of [1\textsuperscript{-14}C]PFOA was hydrolyzed with 10% (w/v) KOH/90% methanol (v/v) at 80 °C for 60 min.

frozen and kept at -30 °C until the determination of radioactivity was completed. Tissue distribution of PFOA at different doses and subcellular distribution of PFOA To [1\textsuperscript{-14}C]PFOA was added 1M NaOH at equimolar, and sodium salt of [1\textsuperscript{-14}C]PFOA was dissolved in rat serum obtained from male rats at the concentrations of 0.041—16.56 mg/ml. Rats were intravenously administered with [1\textsuperscript{-14}C]PFOA (approximately 0.2 MBq) via jugular vein at the doses of 0.041, 0.12, 0.41, 1.23, 4.14 and 16.56 mg/kg body weight. Two hours after the injection, blood samples were collected from vena cava under light ether anesthesia and then the rats were killed. Tissues were quickly removed and rinsed with ice-cold 0.9% (w/w) NaCl.

Animals Male Wistar rats were purchased from SLC (Hamamatsu, Japan) and acclimatized in a humidity- and temperature-controlled environment with a 12h-light/dark cycle for at least 1 week before use, and subjected to the experiments at the age of 9 weeks (280—300 g body weight). Animals were free access to water and a laboratory chow (CE-2, Clea Japan, Tokyo). All animal studies complied with institutional board for animal study, Josai University.

Determination of Tissue Distribution and Subcellular Distribution of PFOA To [1\textsuperscript{-14}C]PFOA was added 1M NaOH at equimolar, and sodium salt of [1\textsuperscript{-14}C]PFOA was dissolved in rat serum obtained from male rats at the concentrations of 0.041—16.56 mg/ml. Rats were intravenously administered with [1\textsuperscript{-14}C]PFOA (approximately 0.2 MBq) via jugular vein at the doses of 0.041, 0.12, 0.41, 1.23, 4.14 and 16.56 mg/kg body weight. Two hours after the injection, blood samples were collected from vena cava under light ether anesthesia and then the rats were killed. Tissues were quickly removed and rinsed with ice-cold 0.9% (w/w) NaCl. The tissues other than livers and the remaining carcass were frozen and kept at -30 °C. Serum samples were obtained from the blood by centrifugation. The tissues were homogenized with 4 volumes of 0.25 M sucrose/0.1 mM EDTA/10 mM Tris–HCl (pH 7.4) using a Polytron homogenizer. An aliquot of the homogenates was transferred to a glass tube and PFOA was extracted with ethyl acetate : hexane (1 : 1, v/v) as an ion pair with tetrabutylammonium in the presence of 0.29 M NaCO\textsubscript{3} buffer (pH 10.0). The extract was taken to dryness by flushing nitrogen. After the addition of 6 M HCl to the residue, [1\textsuperscript{-14}C]PFOA was extracted with n-hexane. The extract was used as a purified [1\textsuperscript{-14}C]PFOA. Radiochemical purity of [1\textsuperscript{-14}C]PFOA was analyzed by high-performance liquid chromatography after derivatization with 3-bromoacetyl-7-methoxy-coumarin as described previously\textsuperscript{21} where more than 99% of the radioactivity was recovered in PFOA fraction, and less than 0.1% was found in the fractions of perfluorooctanoic acid and perfluorohexanoic acid.

Determination of Renal Clearance (\textit{CL}_{R}) and Bile Clearance (\textit{CL}_{B}) of PFOA The \textit{CL}_{R} of [1\textsuperscript{-14}C]PFOA was determined with at least four animals in each group. Rats were anesthetized with urethane at a dose of 0.9 g/kg of body weight, and underwent surgical catheter implantation \textit{via} femoral vein, bladder (SP-45) and bile duct (SP-10). One hour after the surgery finished, these rats were placed in a restrainer and injected with PFOA \textit{via} femoral vein at the doses of 0.041, 0.41, 2.07, 4.14 and 12.42 and 16.56 mg/kg of body weight. Blood samples were withdrawn at several time points between 0 and 300 min after the injection. Urine and bile samples were collected into polyethylene tubes. The tubes were collected at several time points between 0 and 300 min.

**RESULTS**

### Tissue Distribution of PFOA at Different Doses

Tissue distribution of PFOA 2 h after an intravenous injection was determined at the doses of 0.041 and 16.56 mg/kg body weight (Fig. 1). The dose of 16.56 mg/kg corresponds to the dose that has been used for toxicokinetic studies in other studies.\textsuperscript{18–20} The dose of 0.41 mg/kg was used as the lowest dose because the amounts of PFOA in some samples were close to the detection limit in the animals received PFOA at this dose in the present study. We selected the time point of 2 h after dosing because the two-compartment model analysis of time-course study after an intravenous injection have revealed that the first 2 h is thought to be distribution phase (Fig. 2). Most of PFOA dosed was found in the liver, blood and carcass at the both doses (Fig. 1). Distribution of PFOA to the liver represented 52% at the dose of 0.041 mg/kg body weight while it was 27% at the dose of 16.56 mg/kg body weight. By contrast, more PFOA was distributed to extrahepatic tissues including blood, carcass, intestine, lung, stomach, epididymal fat and heart at the dose of 16.56 mg/kg body weight. The proportion of PFOA distributed to the liver decreased as hepatic concentration of PFOA increased within a range of dosing at 0.041 to 16.56 mg/kg body weight (Fig.
Concentrations of PFOA in various tissues were shown in Table 1. PFOA concentrations in various tissues at the dose of 16.56 mg/kg were higher than those at the dose of 0.041 mg/kg, respectively, however, the fold-increase were different between tissues. PFOA concentrations in the liver and the kidney were higher compared with that of serum when rats were administered with PFOA at the dose of 0.041 mg/kg body weight. The ratio of hepatic PFOA concentration to serum of PFOA concentration at the dose of 0.041 mg/kg body weight was calculated to be 2.22, which was 2.7 times larger than that at 16.56 mg/kg body weight. Based on an assumption that volumes of blood and serum are 5.8 and 3.1% of body weight, respectively, almost all PFOA in blood exist in serum at both doses.

Subcellular Distribution of PFOA in the Liver at Different Doses

Subcellular distribution of PFOA was determined in the liver because this tissue was a primary target to which PFOA was distributed (Fig. 4). To avoid the formation of salt of PFOA, any cationic reagent was not included to 0.25 M sucrose solution. At the dose of 0.041 mg/kg body weight, PFOA distributed to 8000 g pellets (cell debris, nu-
clei and mitochondria), 18000 g pellets (lysosomes and peroxisomes), 105000 g pellets (microsomes) and 105000 g supernatant (cytosol) in order. By contrast, PFOA was the most abundant in cytosolic fraction where 43% of PFOA was distributed at the dose of 16.56 mg/kg body weight. The higher the hepatic PFOA concentration became, the more the PFOA was distributed to cytosolic fraction (Fig. 5).

Effects of Dose on Biliary and Renal Clearance of PFOA

Effects of PFOA doses to $\text{CL}_{\text{B}}$ of PFOA was studied. Biliary clearance value was calculated to be 0.07 ml/h/kg body weight at the dose of 0.041 mg/kg body weight and increased in a dose-dependent manner, although the differences among the doses were not statistically significant (Fig. 6A). The ratio of hepatic PFOA concentration to plasma PFOA concentration was 60% at the dose of 0.041 mg/kg body weight. The higher the hepatic PFOA concentration became, the more the PFOA was distributed to cytosolic fraction (Fig. 5).

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Next, $\text{CL}_{\text{R}}$ was estimated at the difference doses because urine is a primary route for PFOA elimination. $\text{CL}_{\text{R}}$ was not significantly different among the doses of 0.041, 0.41, 2.07, 4.14, 12.42, and 16.56 mg/kg body weight (Fig. 7A). Different from the liver, the ratio of renal PFOA concentration to plasma PFOA concentration was not altered (Fig. 7B). The ratio of the amount of PFOA excreted into urine to the amount of renal PFOA (urinary excretion index) was not significantly different at the different doses (Fig. 7C).

**DISCUSSION**

The present study showed that tissue distribution of PFOA was quite different when rats were received this chemical at different doses. In our present study, serum PFOA concentration was 0.25 µg/ml at 2 h after an intravenous administration with 0.041 mg/kg PFOA. The value was far lower than those reported in the previous studies; that is, 23.5 µg/ml in blood...
less than 4 μg/g liver, almost PFOA was found in membrane fractions, and only 3% was found in cytosolic fraction (Fig. 5). The proportion of PFOA in cytosolic fraction increased when hepatic concentration increased, suggesting that PFOA preferentially binds to some constituents in membrane fractions, especially 18000 g pellet fraction. The information about the molecules that PFOA binds to in membrane fraction is very limited. It may be possible that PFOA covalently binds to some constituents such as proteins or lipids. Intracellular distribution of PFOA has been shown in the study by Han et al., where 25% of PFOA was distributed to cytosolic fraction of the liver. Although the utilized dose of PFOA was 25 mg/kg body weight that was higher than those in our present study, the proportion was less than that of our present study (40% at the dose of 16.56 mg/kg body weight). Instead, sum of 6000 g and 600 g pellets were accounted to over 50%. The reason for the discrepancy has not been clarified.

$CL_R$ values were not significantly different at different doses, because the proportion of PFOA in the liver decreased as the dose increased (Fig. 6). However, the ratios of the amount of PFOA excreted into bile to the amount of PFOA in the liver were significantly different at the different doses. Interestingly, the ratio increased at the hepatic concentrations of PFOA over 20 μg/g liver. Thus, the higher the concentration of PFOA in the liver was, the higher the ratio of plasma concentration to liver concentration was, and the larger the proportion distributed to cytosolic fraction became, and the higher the ratio of the amount of PFOA excreted into bile to the amount of PFOA in the liver became. These facts strongly suggest that cytosolic PFOA is susceptible transported to bile or back to plasma and that the intracellular distribution of PFOA strongly affects tissue distribution. By contrast, neither the ratio of plasma PFOA concentration to renal PFOA concentration nor the ratio of the PFOA amounts excreted into urine to those in the kidney altered at any concentrations of renal PFOA studied.

It has been shown that half-life of PFOA in humans is expected to be 4.37 years that is much longer than those observed in experimental animals. The discrepancy may be due to species difference based on the difference in the molecules responsible for binding, transport, storage of PFOA although the molecules responsible for PFOA toxicokinetics have not been fully elucidated. It is also possible that even in the same species, biological half-life is different if the amount of PFOA exposed to animals is quite different. In the present study, we demonstrated that tissue distribution and biliary excretion rate of PFOA at the very low dose is quite different from those at the high doses that have been used in the previous studies on toxicokinetics of PFOA, even in the same species. These facts provide a novel and important information for studying toxicokinetics in humans. The level of PFOA to which human is exposed in the environment is thought to be very low. The present study demonstrated the preferential accumulation of PFOA in the liver at low dose. Therefore, it is plausible that hepatic concentration of PFOA in human and wildlife is higher than plasma or serum. To date, however, little information is available about the relationship between plasma and hepatic PFOA concentrations in wildlife. In humans, liver/serum ratio of PFOA concentrations was reported to be ranging from 0.3 to 3.7.
As for perfluorooctanesulfonic acid, hepatic concentration was higher than plasma concentration in various fishes in Japan. Therefore, further studies are required to elucidate distribution of PFOA in human and other animals that is exposed to PFOA at low dose in the environment. The present information that the distribution of PFOA when administered at very low dose is markedly different from that administered at high dose may be of great use to consider toxicokinetics of PFOA exposed at low level.

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