LEVELS OF PERFLUORINATED COMPOUNDS IN HUMAN LIVER SAMPLES FROM CATALONIA, SPAIN

Kärrman Anna[1], Nadal Marti[2], García Francisco[2], Domingo José L.[2], van Bavel Bert[1] and Lindström Gunilla[1]

[1]Man-Technology-Environment Research Centre (MTM), Örebro University, SE-701 82 Örebro, Sweden; [2]Laboratory of Toxicology and Environmental Health, "Rovira i Virgili" University, Sant Llorenç 21, 43201 Reus, Catalonia, Spain

Introduction

Perfluorinated compounds (PFCs) form a diverse group of chemicals with surface-active properties, which have been manufactured for over 50 years. In recent years, an increasing number of investigations have reported the ubiquitous distribution of these compounds in humans and wildlife[1, 2]. Among the different classes of PFCs, perfluorooctanesulfonate (PFOS) has received particular attention and has been the most studied. The environmental persistence, the potential for long-term environmental effects, as well as the finding of PFOS in blood of non-occupationally exposed humans, has raised a notable concern. Although the routes of human exposure to PFCs are not well-characterized yet, it has been hypothesized that dietary intake could be one of the major exposure pathways[3, 4].

Although accumulation and trends of PFCs are largely unknown, it is evident that unlike the classical more lipophilic persistent organic pollutants, PFCs do not typically accumulate in lipids. With respect to PFOS, animal studies indicate that it is well absorbed orally and distributes mainly in blood serum and the liver[5]. In recent years, data of PFCs in blood from biomonitoring studies have increased[1, 6, 7]. It has been also found that the liver is the primary target organ of PFOS[5]. Relatively higher hepatic concentrations of PFOS than in other human tissues have been reported[8].

As part of an overall investigation to determine the environmental and biological levels of PFCs, the concentrations of these chemicals are being determined in human tissues of the general population of Tarragona (Catalonia, Spain). The specific goal of the present survey was to measure PFC levels in human liver samples from local residents, as well as to study the influence of other parameters (sex, age and smoking habits) on hepatic PFC.

Materials and methods

Chemicals

Ammonium acetate (>99%, pa for HPLC) was purchased from Fluka (Steinheim, Germany) and methanol (HPLC) was obtained from Labscan (Dublin, Ireland). All water used was laboratory produced ultra pure water. Ammonium hydroxide (25% in water), sodium acetate, and glacial acetic acid (100%) were purchased from E. Merck (Darmstadt, Germany). Supelclean ENVI-Carb (120/400 mesh) was purchased from Supelco (Bellefonte, PA). Perfluorobutanesulfonate (PFBuS) tetrabutylammonium salt (>98%), PFOS potassium salt (>98%), perfluorodecanoic acid (PFDA; >97%), and perfluorohexanoic acid (PFHxA, >97%) were purchased from Fluka. Perfluoroheptanoic acid (PFHpA, 99%), perfluorononaoic acid (PFNA, 97%), perfluorooctanoic acid (PFOA, 96%), perfluoroundecanoic acid (PFUnDA, 95%), and perfluorotetradecanoic acid (PFTDA, 97%) were purchased from Aldrich (Steinheim, Germany, and Milwaukee, WI, USA). *1H,1H,2H,2H*-PFOS (THPFOS, purity unknown), and 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).

Sampling

Twelve human liver samples were collected in 2007 from subjects who had lived in different areas of Tarragona (Catalonia, Spain). Additional data from the individuals, such as age, place of residence and smoking habits, were acquired. Samples from 6 males and 6 females (27-79 years old) were obtained. One gram of liver was

collected during the post-mortem examination, which was done during the first 24 h after the time of death. After extraction, liver samples were stored at -20ºC until analysis.

Analytical determination

PFCs including sulfonates C4, C6, C8 (PFOS), 6:2 tetrahydro PFOS and carboxylates C6, C7, C8 (PFOA), C9, C10, C11, C12, C14 were analyzed using weak anion exchange, solid phase extraction (Waters Oasis® WAX) and ultra-performance chromatography coupled to a tandem mass spectrometer (Acquity UPLC-Quattro Premier XE MS/MS). Liver samples (1 g) were cut into pieces and homogenized using a probe homogenizer. Acetonitrile (10 mL) and internal standards ($^{13}C_4$-PFOA and $^{13}C_4$-PFOS) were added and the mixture was repeatedly vortex mixed and sonicated for 30 min. The supernatant acetonitrile phase was removed after centrifugation (10000xg, 30 min) and the extraction procedure was repeated once. The acetonitrile fractions was combined and reduced in volume to 10 mL after which 25 mL water was added. After mixing and centrifugation the solution was put through a WAX solid phase cartridge (Waters, Milford, MA, USA) previously conditioned with 4 mL methanol followed by 4 mL water. After sample loading the WAX cartridge was washed with 4 mL 25 mM sodium acetate (pH 4) and 4 mL 40v% methanol in water, followed by drying using vacuum suction. A final wash with 8 mL methanol was employed before the perfluorinated compounds were eluted with 2 mL 2% ammonium hydroxide in methanol into a tube with 50 mg ENVI-Carb and 100 µL acetic acid. 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.

Analysis was performed using an Acquity UPLC coupled to an Quattro Premier XE (Waters Corporation, Midford) with an atmospheric electrospray interface operating in negative ion mode (ES-MS/MS). Separation was performed on an Acquity BEH C18 2.1 x 50mm, 1.7 µm kept at 50°C. An extra guard column (Waters prototype) was inserted between the pump and injector to remove any fluorochemicals originating from the HPLC system. Injection volume was 10 µl and the flow rate was set to 400 µL/min. A gradient program delivering mobile phases consisted of 2 mM ammonium acetate in methanol and 2 mM ammonium acetate in water was employed. Multiple reaction monitoring was employed using parent ion $[M]^-$ for sulfonates and $[M\text{-}COOH]^-$ for carboxylates. Qualitative analysis of the PFCs detected in liver samples was performed by measuring a second, and for PFOS and PFOA a third, transition (Table 1). Positive identification of PFCs in the liver samples was set to retention time ± 5% of an identical standard, and relative ion intensity for at least two transitions for each compound identical to ± 50% of an identical standard.

Quantification was performed using the internal standard method with non-extracted standards dissolved in 30 % 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. The mean recoveries for $^{13}C_4$-PFOS and $^{13}C_4$-PFOA internal standards in all samples were 50% and 64%, respectively. The limit of quantification (LOQ) was set to a signal to noise ratio of 10. In the case of blank levels, the mean blank signal of multiple blank extractions was subtracted from the calculated concentrations in the samples. A blank corrected concentration was reported provided that the blank level was equal or less than 50% of the uncorrected concentration.

Data treatment

Data analysis was carried out by means of the statistical package SPSS 15.0. The Levene test was applied to establish whether the data followed a normal distribution. Subsequently, ANOVA or Kruskal Wallis test was used depending on the equality of variances. If the concentration of a specific compound was below their respective limit of detection (LOD), the value was considered as one-half of that LOD (ND = ½ LOD).

Results and discussion

Only six (PFHxS, PFOS, PFOA, PFNA, PFDA, and PFUnDA) of the 12 PFCs analyzed in human liver samples could be detected. The concentrations of these 6 PFCs are summarized in Table 2. PFOS showed the highest mean concentration (26.61 ng/g ww), while PFOA was the compound with the lowest mean level (0.71 ng/g ww). In fact, one-half of the total samples showed PFOA concentrations below its respective LOQ (<0.77 ng/g ww). Because of blank contamination, the LOQs corresponding to PFOA were relatively high. PFHxA, PFHpA,

PFDoDA, PFTDA, PFBuS and THPFOS were not detected in any sample (LOQs: 0.1, 0.06, 2.35, 0.14, 0.03, and 0.01 ng/g ww, respectively).

PFOS were present at relatively higher concentrations in comparison to previously reported levels in human liver. Olsen et al.[7] investigated the concentration of 4 PFCs (PFOS, PFOSA, PFOA and PFHxS) in liver and serum from 30 liver donors from the USA. A mean PFOS concentration of 18.8 ng/g (95% CI 14.1-23.5) was found in liver. However, 50% of the liver samples showed PFOS values below the LOQ. For statistical purposes, the concentration of undetected samples was assumed to be one-half of the LOQ (ND = ½ LOQ). After restricting the results to those 10 individuals whose measured PFOS liver and serum concentrations were always above the LOQ, the mean PFOS concentration in liver was 28.7 ng/g (95% CI 18.7-38.6).

The current PFOS mean level in Tarragona was approximately 2-times higher than those obtained by Maestri et al.[8] in a pooled liver sample corresponding to 7 subjects from northern Italy (13.6 ng/g). In contrast, the PFOA liver concentrations for Italian population seemed to be higher than that reported in Tarragona (3.1 ng/g vs. 0.71 ng/g). However, the comparison is hard to be carried out taking into account that only one pooled sample was analyzed. Olsen et al.[7] also found difficulties to detect PFOA in human liver in USA subjects. Only 2 of 30 samples, with levels of 47 and 2.5 ng/g, presented values above the LOQ, which ranged between <17.9 and <35.9 ng/g. Similarly, PFHxS in the USA was only detected in 3 samples, with levels ranging <3.4-8.2 ng/g. It means a substantially higher concentration than that found for the Spanish population here assessed.

The relationship between hepatic PFOS and PFOA levels was different compared to human blood. In a previous survey[1], the ratio PFOS:PFOA in blood was found to be 4.3:1, while in the present study the ratio PFOS:PFOA in liver was 37.5:1, indicating almost 9-times higher PFOS levels in liver than in blood. As it has been consistently demonstrated in toxicological studies in animals, liver is the primary target organ of PFOS[5]. The information regarding to the relationship of PFOS levels in different biological compartments may be very useful for human risk characterization[7]. In USA, good correlations between liver and serum of 23 paired samples were reported[7], with a PFOS liver to serum ratio of 1.3:1 (95% confidence interval (CI), 0.9:1–1.7:1). In Tarragona, a PFOS mean concentration of 7.64 ng/mL in blood of 48 Tarragona inhabitants was recently found[1]. Although the values cannot be comparable because the serum and liver samples were not extracted from the same subjects, those levels would mean a liver/serum ratio of 3.5:1.

PFOA levels in liver samples from males were significantly higher than those from females (0.96 vs. 0.45 ng/g ww; $p < 0.05$). These results are in agreement with those previously observed in blood of Tarragona population[1]. In that study, males showed significant higher values of PFOA in blood than females (2.02 vs 1.57 ng/l; $p < 0.05$)[1]. With the exception of PFOA, no significant difference in human liver PFC concentrations according to gender could be seen. Similarly, no significant differences were observed when comparing the PFC concentrations from individuals living in an urban area (Tarragona) and those from residents in suburban zones (residential areas and villages). Finally, although non-smokers presented higher concentrations of all the detected PFCs than smokers, the differences did not reach a level of statistical significance ($p > 0.05$). However, it must be taken into account that smokers were much younger than non-smokers (mean age: 33 vs. 62 years old, respectively). Therefore, PFC accumulation could have been due to age rather than smoking habits. In order to establish the influence of age on the PFC concentrations in human liver, a Pearson correlation study was done. With the exception of PFHxS ($p < 0.05$), no correlations were found between PFC levels in liver and age. Regarding to PFOS, no associations had been previously described between measured concentrations and age[7].

In summary, several PFCs could be detected in human liver samples of subjects living in Tarragona. The highest and lowest concentrations corresponded to PFOS and PFOA, respectively. Since labeled internal standards and three transitions were here used, the identity and measured concentration of PFOS and PFOA were considered confident. However, relatively low recoveries were obtained. Because it is not clear whether the amount of sample taken (only 1 g) is representative for the whole organ, further investigations must be focused on establishing the optimally necessary amount of sample.

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Table 1: MS/MS transitions for 12 PFCs analyzed

Compound	Parent > product ion [a]		
PFBuS	298.9 > 79.7		
PFHxS	399> 80	**399> 99**	
PFOS	498.9 > 79.7	**498.9 > 98.7**	498.9 > 129.7
THPFOS	426.9 > 79.9		
PFHxA	313 > 269		
PFHpA	363 > 319		
PFOA	**413 > 369**	413 > 219	413 > 168.7
PFNA	**463 > 419**	463 > 219	
PFDA	**513 > 469**	513 > 269	
PFUnDA	**563 > 519**	563 > 269	
PFDoDA	**613 > 569**	613 > 269	
PFTDA	713 > 669		

[a] Transitions used for quantification are shown in bold

Table 2: Concentrations (ng/g of wet weight) of PFCs detected in human liver samples from Spain.

Sample	PFHxS	PFOS	PFOA	PFNA	PFDA	PFUnDA	Sex	Age	Smoker
1	1.64	52.13	<0.77	1.38	2.06	2.62	F	79	no
2	0.45	15.84	<0.77	0.43	0.66	1.18	F	30	yes
3	0.29	20.73	<0.77	0.56[a]	0.67[a]	0.79	F	70	no
4	0.91	20.80	0.80	0.76	0.86	1.04[a]	F	77	no
5	0.20	23.73	<0.77	0.76	0.73[a]	1.25	F	27	yes
6	0.08	9.67	<0.77	0.42	0.48	1.25	F	56	no
7	0.26	17.00	1.05	0.71	0.54[a]	0.76	M	29	unknown
8	0.34	24.87	0.81	0.66	1.00[a]	3.49	M	35	unknown
9	0.78	46.07	0.91	0.65	0.68[a]	1.49	M	50	no
10	0.25	12.77	0.88	0.77	0.54[a]	0.40	M	43	yes
11	0.20	27.27	<0.77	0.72	0.91	2.34	M	40	no
12	0.62	48.53	1.73	0.91	1.05	0.73	M	63	no
LOQ	0.004	0.04	0.77	0.02	0.06	0.03	-	-	-
Mean[b]	0.50	26.6	0.71	0.73	0.85	1.45	-	50	-
Median[b]	0.31	22.3	0.59	0.72	0.71	1.21	-	47	-
Std dev[b]	0.44	14.4	0.41	0.25	0.42	0.91	-	-	-

[a]Relative ion intensity between first and second transition was 54-86%.[9] [b]Non-detects are replaced with LOQ/2.