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Determination of perfluorinated carboxylic acids in biological samples by high-performance liquid chromatography

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Abstract

This paper describes a method for the quantitative determination of perfluorinated carboxylic acids (PFCAs), perfluorohexanoic acid (C6-PFCA), perfluoroheptanoic acid (C7-PFCA), perfluorooctanoic acid (C8-PFCA), perfluorononanoic acid (C9-PFCA) and perfluorodecanoic acid (C10-PFCA), in biological samples. PFCA in liver homogenates was extracted as an ion pair with tetrabutylammonium (TBA) ion into organic solvent, then the PFCA was derivatized with 3-bromoacetyl-7-methoxycoumarin (BrAMC) and quantified by HPLC with fluorescence detection. This method is applicable for the studies on tissue accumulation and elimination of PFCAs in animals after the administration. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Perfluorinated carboxylic acids (PFCAs) are analogs of fatty acids with straight chains in which all the aliphatic hydrocarbons have been replaced by fluorine. PFCAs have been used widely as a wetting agents, lubricants, corrosion inhibitors and foam fire extinguishants. Owing to superior surfactant properties and chemical and thermal stability, industrial production and applications of PFCAs have recently increased. Although PFCAs had been considered to be metabolically inert and therefore to be non-toxic, these compounds were revealed to be biologically active and toxic. Namely, PFCAs were shown to induce a proliferation of peroxisomes [1,2] and to cause alterations of lipid metabolism [3–5] in livers of rodents. Recently, it has been demonstrated that perfluorooctanoic acid (C8-PFCA) altered a function of Leydig cells [6] and may be a cancer promoter [7]. C8-PFCA has been reported to be the principal organic fluorine which was found in the serum of all human populations studies [8]. Moreover, PFCAs are thought to have long biological half-lives. Accordingly, information about the accumulation of PFCAs in human and experimental animals is required for the assessment of toxicity of PFCAs.

The methods to analyze PFCAs in biological samples by gas chromatography (GC) with electron-capture detection (ECD) [9] and flame ionization detection [10,11] were developed. In a previous study [12], we reported the improved method of analysis of PFCAs by GC–ECD.

In the present study, we developed a method for a

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quantitative determination of perfluorohexanoic acid (C6-PFCA), perfluoroheptanoic acid (C7-PFCA), C8-PFCA, perfluorononanoic acid (C9-PFCA) and perfluorodecanoic acid (C10-PFCA) in biological samples by high-performance liquid chromatography (HPLC) with fluorescence detection using 3-bromoacetyl-7-methoxycoumarin (BrAMC) as a fluorescence derivatization reagent. The method described here is more sensitive than the GC methods reported previously.

2. Experimental

2.1. Chemicals and reagents

C6-PFCA was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). C7-PFCA, C8-PFCA and C10-PFCA were purchased from Aldrich (Milwaukee, WI, USA). C9-PFCA was obtained from Lancaster Synthesis (Morecambe, UK). Tetrabutylammonium (TBA) hydroxide solution (0.5 *M*) was prepared by dissolving TBA hydrogen sulphate (Wako Pure Chemical Industries, Osaka, Japan) in water; the pH of the solution was adjusted to 10 with 2 *M* sodium hydroxide. Stock solutions of PFCAs were prepared by dissolving them in methanol. BrAMC was prepared according to the method of Takadate et al. [13]. All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. Animals

Male Wistar rats were purchased from SLC (Hamamatsu, Japan). After one week acclimatization, rats were decapitated and livers were isolated. Some rats were injected intraperitoneally with C10-PFCA as a solution of propylene glycol–water (1:1, v/v) at a dose of 20 mg/kg body mass. Twenty-four hours after the injection, livers were isolated.

2.3. Sample preparation

The livers were perfused with ice-cold 0.9 % NaCl and then homogenized in nine volumes of 0.25 *M* sucrose–1 m*M* EDTA–10 m*M* Tris–HCl, pH 7.4.

2.4. Apparatus and HPLC conditions

High-resolution (HR) mass spectra were measured on a DX-300 mass spectrometer (JEOL, Tokyo, Japan). The HPLC analyses were performed on two Shimadzu (Kyoto, Japan) LC 10A pumps, a SCL-10A system controller, a RF-10A fluorescence detector and a C-R3A chromatopac integrator. The analytical column was a Wakosil-II 3C18 HG ODS column, 50×4.6 mm I.D., supplied by Wako Pure Chemical Industries. The column was kept at 30°C. Isocratic separation was achieved using a mobile phase consisting of acetonitrile–water (3:1, v/v), which was maintained at a flow-rate of 0.5 ml/min. After the fluorescence derivatives of PFCAs (AMC derivatives of PFCAs) were eluted, the column was washed with isopropanol at a flow-rate of 1 ml/min for 10 min to remove the contaminants which were retained on the column. A fluorescence detector was set at an excitation wavelength of 366 nm with the emission wavelength at 419 nm to detect the analytes.

2.5. Extraction and derivatization procedures

Liver homogenate (50 mg tissue) was placed in a screw-cap glass tube. To the tube were added an internal standard, 1 ml of water, 1 ml of 2 *M* sodium carbonate buffer (pH 10) and 1 ml of 0.5 *M* TBA solution. After mixing, the tube was sonicated in a water bath for 10 min. The internal standards used were as follows; C7-PFCA for C6-PFCA determination, C8-PFCA for C7-PFCA determination, C7-PFCA for C8-PFCA determination, C8-PFCA for C9-PFCA determination, C9-PFCA for C10-PFCA determination. The ion pair of PFCA with TBA was extracted with 3×4 ml of ethyl acetate–hexane (1:1, v/v) by shaking the tube in a mechanical shaker for 15 min. The extracts were combined and then taken to dryness under a stream of nitrogen. To the resulting residue was added 2 ml of 0.2% (w/v) BrAMC in acetone. The tube was capped, heated at 70°C for 25 min, cooled on ice and kept at −30°C for more than 2 h to precipitate the excess BrAMC. This sample can be stored for at least 48 h at −30°C. After the mixture was filtered through a glass-wool filter, 10 μl of the filtrate was injected into the HPLC system.

2.6. HR mass spectra of AMC derivatives of PFCAs

One hundred microliters of 50 m*M* PFCA methanol solution was added to 1 ml of water in a screw-cap glass tube. To the tube were added 1 ml of 2 *M* sodium carbonate buffer (pH 10) and 1 ml of 0.5 *M* TBA solution. The mixture was extracted with 3×4 ml of ethyl acetate–hexane (1:1, v/v). The extracts were combined and taken to dryness under a stream of nitrogen. To the resulting residue were added 6 mg of BrAMC and 2 ml of acetone. The tube was capped, heated at 70°C for 25 min, cooled on ice and kept at −30°C for 2 h. After the mixture was filtered, 10 μl of the filtrate was injected into the HPLC system then a fraction of the peak corresponding to the AMC derivative of PFCA was collected. The eluate collected was evaporated to dryness in vacuo and the residue was subjected to HR mass spectrometry (MS).

3. Results and discussion

3.1. Conditions for extraction and derivatization

We have sought a fluorescent labeling reagent for the determination of PFCA by using HPLC. Among a number of fluorescent labeling reagents for carboxylic acids, BrAMC was found to have a reactivity with PFCAs.

The extraction of PFCAs into organic solvent as an ion pair with a lipophilic counterion such as TBA ion is a useful procedure, since the free PFCAs are known to be lost during the extraction and the derivatization due to their volatility and absorption on the glass [9]. Ylinen et al. [10] demonstrated that an ion pair of PFCA with TBA was quantitatively extracted into organic solvent. The extraction conditions employed in the present study were based on those developed by Ylinen et al. [10]. Although they used ethyl acetate as an extraction solvent, ethyl acetate–hexane (1:1, v/v) was used in this study to reduce the extraction of contaminants. The solvent gave the same extraction yield as did ethyl acetate.

The derivatization with BrAMC of fatty acids having a hydrocarbon chain is performed in the presence of basic anion-exchange resin as a catalyst, however, the reaction of PFCA–TBA ion pair with BrAMC was found to proceed without the catalyst. The reaction scheme is illustrated in Fig. 1.

Although the dervatization reaction of fatty acids with BrAMC proceeded at room temperature, no reaction was brought about in the case of PFCAs at that temperature. Heating was required to derivatize PFCAs (Fig. 2). In the reaction at 70°C, the fluorescence intensity reached a maximum within 20 min. When heated at 60°C, prolonged heating was required. The reaction was completed within 15 min at

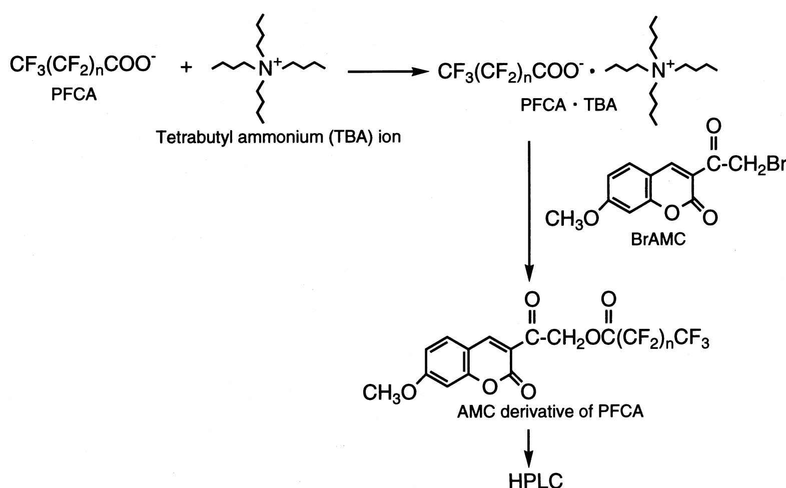

Fig. 1. Scheme of derivatization reaction.

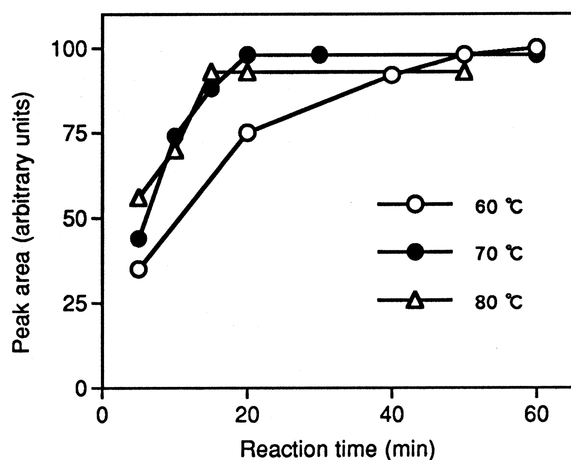

Fig. 2. Effects of reaction time and temperature on the derivatization of PFCAs. Water (0.5 ml) spiked with 5 nmol of C8-PFCA was treated according to the standard procedure, except for temperature.

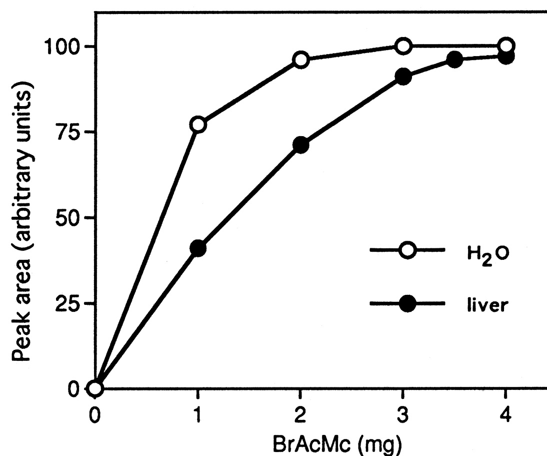

Fig. 3. Effect of the amount of BrAMC on the derivatization of PFCAs. Water (0.5 ml) and rat liver homogenates (50 mg liver) spiked with 5 nmol of C8-PFCA were treated according to the standard procedure, except for the amount of BrAMC.

80°C, but the maximum fluorescence peak area was slightly smaller compared with those produced by the reaction at 70°C or 60°C.

To optimize the amount of BrAMC used, the derivatization was examined using water and liver homogenates from the non-treated control rats, spiked with 5 nmol of C8-PFCA. In the derivatization reaction of the water sample, the fluorescence peak area reached a plateau at 2 mg of BrAMC, while in the case of the homogenate sample, the peak area reached a maximum at 3.5 mg of BrAMC probably due to the contaminants which consumed BrAMC (Fig. 3). Based on these results, the procedure for the derivatization was decided as described in Section 2.5.

3.2. Fluorescence properties

The excitation and emission spectra of the AMC derivative of C8-PFCA were obtained by using the fraction of fluorescence peak collected from HPLC. The excitation maximum at 366 nm and the emission maximum at 419 nm were almost the same as those of AMC derivatives of fatty acids having aliphatic hydrocarbon chains [13].

3.3. Identification of AMC derivatives of PFCAs

To verify that the fluorescence peaks of the HPLC chromatogram were AMC derivatives of PFCAs, the fractions of these peaks were collected and subjected to HR-MS analyses. The data obtained by HR-MS revealed that the compounds corresponding to each peak were identical with the AMC derivatives of PFCAs (Table 1).

3.4. Chromatography

Fig. 4 shows the HPLC chromatogram of AMC derivatives of PFCAs. A good separation of the

Table 1
HR-MS analyses of derivatized products of PFCAs by BrAMC[a]

PFCA	Formula of product	M^+ (m/z)	
		Found	Calculated
C6-PFCA	$C_{18}H_9O_6F_{11}$	530.0223	530.0217
C7-PFCA	$C_{19}H_9O_6F_{13}$	580.0192	580.0195
C8-PFCA	$C_{20}H_9O_6F_{15}$	630.0159	630.0142
C9-PFCA	$C_{21}H_9O_6F_{17}$	680.0127	680.0118
C10-PFCA	$C_{22}H_9O_6F_{19}$	730.0096	730.0095

[a] AMC derivatives of each PFCA isolated by using HPLC were subjected to HR-MS analysis.

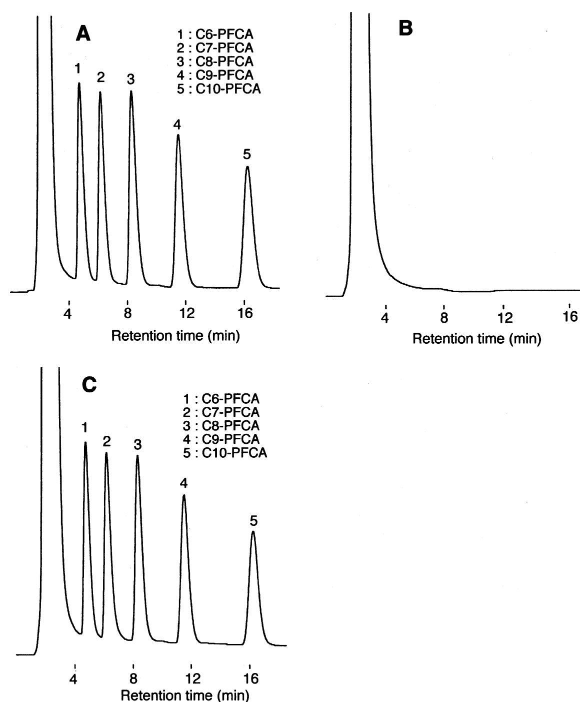

Fig. 4. Chromatograms obtained for standard PFCAs (5 nmol of each PFCA was spiked) (A), blank liver sample (B) and liver sample spiked with 5 nmol of each PFCA (C).

derivatives was achieved on a Wakosil-II 3C18 HG column with an isocratic elution using acetonitrile–water (3:1, v/v) (Fig. 4A). Typical chromatograms obtained for blank liver homogenates (50 mg liver) and for liver homogenates (50 mg liver) spiked with PFCAs are also shown in Fig. 4B and C, respectively. No endogenous compounds were found to interfere with the assay under the present experimental conditions.

3.5. Calibration curves and recovery

The calibration curves ($y=ax+b$) were prepared by using blank liver homogenates (50 mg liver) to which PFCA had been added in the range of 0.1–10 nmol and a fixed amount of internal standard (1 nmol). The internal standards used were described in Section 2.5. A high correlation was found between the amount of PFCA (x ; nmol/50 mg liver) and the peak area ratio (y) (Table 2). The detection limits

Table 2
Calibration line for the HPLC assay of PFCAs[a]

PFCA	Linear regression equation ($y=ax+b$)			n
	Slope (a)	Intercept (b)	Correlation (r)	
C6-PFCA	1.06	−0.126	1.000	7
C7-PFCA	0.883	−0.040	0.999	7
C8-PFCA	1.03	−0.003	0.999	7
C9-PFCA	0.886	−0.031	0.999	7
C10-PFCA	0.882	−0.008	0.999	7

[a] PFCA (0.1–10 nmol) and internal standard were added to liver homogenates (50 mg liver). The determination was made according to the standard procedure.

were 50 pmol/50 mg liver for the PFCAs tested in this study at a signal-to-noise ratio of 3.

Recoveries determined from the liver homogenates (50 mg liver) spiked with 1 nmol PFCA and 10 nmol PFCA are summarized in Table 3. High recoveries (>90 %) were obtained for all PFCAs tested.

3.6. Accuracy and precision

The intra-day and inter-day accuracies of the measurements were determined by adding 1 nmol of PFCA and 1 nmol of an internal standard to liver homogenates (50 mg liver, $n=4$). Accuracy was calculated as the percentage of the measured versus the known concentrations. Precision was determined as the coefficient of variation (C.V.). The intra-day accuracies and precisions for the assay of PFCAs were 99.2% and 7.4% for C6-PFCA; 103.8% and 5.5% for C7-PFCA; 96.5% and 5.2% for C8-PFCA;

Table 3
Recoveries of PFCAs from rat liver homogenate[a]

PFCA	Amount of PFCA spiked	
	1 nmol/50 mg liver (recovery, %)	10 nmol/50 mg liver (recovery, %)
C6-PFCA	97.9±2.5 ($n=5$)	93.1±1.4 ($n=5$)
C7-PFCA	98.9±2.3 ($n=5$)	91.8±1.2 ($n=5$)
C8-PFCA	94.0±4.4 ($n=5$)	93.6±1.0 ($n=5$)
C9-PFCA	92.8±3.6 ($n=5$)	97.1±2.2 ($n=5$)
C10-PFCA	91.6±1.6 ($n=5$)	95.3±1.2 ($n=5$)

[a] PFCA (1 nmol or 10 nmol) was added to liver homogenates (50 mg liver). The determination was made according to the standard procedure.

100.1% and 3.0% for C9-PFCA; and 102.8% and 5.3% for C10-PFCA, respectively. The inter-day accuracies and precisions were 102.3% and 0.9% for C6-PFCA; 103.7 and 6.3% for C7-PFCA; 93.4% and 4.8% for C8-PFCA; 98.8% and 4.1% for C9-PFCA; and 94.7% and 6.4% for C10-PFCA, respectively.

3.7. Applications

The present method was applied to determine the concentrations of C10-PFCA in livers of rats ($n=3$) which were sacrificed 24 h after a single intraperitoneal administration of C10-PFCA (20 mg/kg). A typical HPLC chromatogram is shown in Fig. 5. In addition, the same samples were analyzed using the GC method developed previously [12]. The close data were obtained from the present method and the GC method (129.90±3.31 μg/g liver and 116.75±12.09 μg/g liver, respectively).

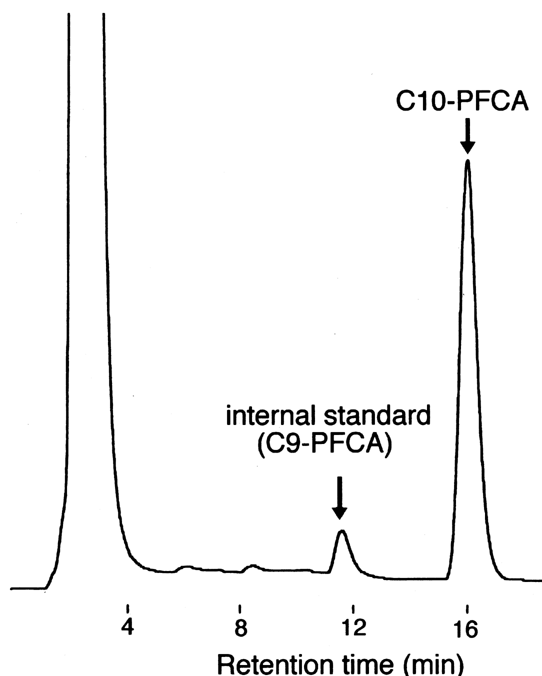

Fig. 5. Chromatogram obtained for liver sample of the rat which was sacrificed 24 h after a single intraperitoneal administration of C10-PFCA (20 mg/kg). Liver homogenates (50 mg tissue), which was spiked with 1 nmol of C9-PFCA as an internal standard, was assayed according to the standard procedure.

3.8. Concluding discussion

PFCAs have been analyzed in biological samples by GC after derivatization to their methyl [9] or benzyl [10,11] esters. Belisle and Hagen [9] reported an analysis of C8-PFCA by using GC–ECD after conversion to its methyl ester. However, this method had a difficulty for a precise determination due to interfering peaks in chromatogram. We have reported the method for the precise determination of C8-PFCA, C9-PFCA and C10-PFCA by using GC–ECD [12]. However, C6-PFCA and C7-PFCA could not be determined by this method because these PFCAs were lost during purification from biological samples due to their high volatility. The simple and precise method for the determination of C6-, C7-, C8-, C9- and C10-PFCAs was required for a study on an accumulation and elimination of their PFCAs in animal tissues.

Although the analytical methods using HPLC are common and useful, no HPLC method for the determination of PFCAs has been developed because of its weak absorption in the UV–Vis region and no or low reactivity with labeling reagents such as bromomethyl coumarin-type reagents which are widely used for the fluorescence derivatization of carboxylic acids. Among the fluorescence labeling reagents for carboxylic acids, BrAMC was found to be reactive with PFCAs and applicable to the determination of PFCAs by using HPLC. The method developed in the present study was composed of three steps; first, extraction of PFCAs as an ion pair with TBA into organic solvent; second, derivatization reaction with BrAMC; and third, determination of PFCAs by using HPLC. In conclusion, this method was simple and successfully used even in the determination of volatile PFCAs such as C6-PFCA and C7-PFCA.

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