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MICROANALYSIS OF FREE FATTY ACIDS IN PLASMA OF EXPERIMENTAL ANIMALS AND HUMANS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) method was developed for microanalysis of thirteen free fatty acids using 200 μl of plasma. Fatty acids were derivatized with 9-anthryldiazomethane for HPLC analysis. Use of an ODS minicolumn for pretreatment of plasma gave a more accurate determination of free fatty acids in plasma than by chloroform extraction. Using this method, thirteen free fatty acids in the plasma of normal human, dog, rabbit, guinea pig and rat were determined.

INTRODUCTION

Investigations in cardiology have been directed towards phospholipids from prostanoids, because prostanoids are derived from arachidonic acid, which is released from phospholipids. Determination of free fatty acids, including arachidonic acid, therefore seems to afford information on cardiovascular disease. In order to accomplish this, trace amounts of free fatty acids need to be analysed accurately.

Analysis of fatty acids has previously been carried out by gas chromatography (GC) [1—3]. However, fatty acids derivatized with various fluorescent reagents have been determined recently by high-performance liquid chromatography (HPLC) [4—8]. Ikeda et al. [6] reported that HPLC determination showed a smaller distribution in the results of quadruplicate measurements compared with GC determination of fatty acids. Therefore, HPLC seems to be a useful method for the measurement of fatty acids.

In a previous paper [10], we reported the HPLC analysis of prostaglandins;

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the present paper describes the HPLC analysis of fatty acids. This paper also describes the comparison of two clean-up methods (ODS minicolumn method and conventional chloroform extraction method). We determined free fatty acids in the plasma of normal humans and experimental animals that are derivatized with 9-anthryldiazomethane (ADAM). From this experiment, with phosphatidyl inositol as a model phospholipid, we found that the chloroform extraction method is uncertain.

EXPERIMENTAL

Reagents

Arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5) were purchased from Nakarai Chemicals (Kyoto, Japan). ADAM, phosphatidyl inositol sodium salt from soybean, and other authentic fatty acids such as myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), 11,14-eicosadienoic acid (C20:2), 8,11,14-eicosatrienoic acid (C20:3) and docosahexaenoic acid (C22:6), were purchased from Funakoshi Pharmaceutical (Tokyo, Japan). Acetonitrile, methanol, 1,4-dioxane, ethyl acetate and chloroform of HPLC-reagent grade were used in this work. Other chemicals were ultrafine or guaranteed reagent grade.

Plasma samples

Plasma samples were obtained from normal humans, Wistar male rats, Hortley male guinea pigs, Japanese white male rabbits and male mongrel dogs. Blood of rat and guinea pig were obtained by cardiac puncture. Blood of rabbit was obtained from its ear vein and blood of human and dog were obtained from their brachium veins. To compare the two methods of pretreatment, rabbits (operated rabbits) in which the carotid and the femoral artery were exposed were used, and blood was obtained from their femoral arteries.

Purification of ADAM

We used purified ADAM for maintenance of the analytical column and to prevent overloading the detector. An HLC-803A high-pressure pump (Toyo Soda) was used to deliver ethyl acetate with a flow-rate of 1.0 ml/min. Two columns (300 × 7.8 mm I.D.) packed with TSK gel G1000HXL were used in series. A UV-8 Model II spectrophotometer (Toyo Soda) was used to monitor the absorbance at 254 nm. A 95-μl aliquot of 1% (w/v) ADAM solution was injected and a fraction between 28 min 15 s and 29 min 45 s was collected. This fraction was evaporated and dissolved again in 500 μl of ethyl acetate. A 50-μl aliquot of this pure ADAM solution was used for labelling the free fatty acids.

Separation of free fatty acids from plasma

ODS minicolumn. The separation method of Po[illegible]ell [9] was used, with some modifications. Quantities of 100 mg of Toyopak ODS were packed in three strainless-steel columns (10 × 4 mm I.D.) placed in series. These columns were supplied with eluent by an HLC-803A high-pressure pump.

Before application of a plasma sample, these columns were washed successively with methanol, water, 1 *M* acetic acid and water. A 200-μl aliquot of plasma, to which 20 μl of 1 *M* hydrochloric acid and 500 μl of water were added, was applied onto these columns using a high-pressure pump equipped with a 1-ml sample loop. The columns were eluted successively with 30 ml of water, 30 ml of ethanol—water (15:85) and 35 ml of light petroleum (b.p. 30–60°C). The light petroleum fraction containing fatty acids was concentrated in vacuo and transferred into a 2-ml brown test-tube.

Extraction with chloroform. Fatty acids were extracted from plasma according to the method reported by Shimomura et al. [8]. A 0.2-ml aliquot of plasma was mixed with 1 ml of 1/30 *M* phosphate buffer (pH 6.4) and 6 ml of chloroform. The mixture was shaken vigorously for 5 min, and 4 ml of the chloroform layer were taken after centrifugation (900 *g*, 5 min). The chloroform extract containing the fatty acids was evaporated under a stream of nitrogen. It was also tested to make sure that fatty acids were released from phospholipid during this extraction procedure.

Phosphatidyl inositol (1.2 mg) in 6 ml of chloroform was added to 1 ml of 1/30 *M* phosphate buffer (pH 6.4) and the extraction was carried out by the same procedure as described above.

Esterification with ADAM

Authentic sample or fatty acid sample solutions obtained by two separation methods were dried under nitrogen gas in a 2-ml brown test-tube and esterified with pure ADAM solution (50 μl). The tube was stoppered tightly, using silicone rubber, and warmed at 40°C for 30 min. The mixture was then dried under nitrogen gas and dissolved again in 5 μl of ethyl acetate and 0.5 ml of methanol. A suitable volume (e.g. 1, 5 or 10 μl) of the resulting mixture was analysed by HPLC.

High-performance liquid chromatography

An HLC-803D high-pressure pump (Toyo Soda) was used. A column (250 × 4.6 mm I.D.) packed with TSK gel ODS-80TM (5 μm particle size) was maintained at 40°C during chromatography. FS-950 Fluoromat (Toyo Soda) was used as a detector at 0.1 range, the fluorescence being measured at 418 nm with excitation at 365 nm. Chromatograms were recorded on a 3390 A integrator (Hewlett-Packard) at peak-height mode. The mobile phase used for elution 42 min after injection was methanol–acetonitrile—1,4-dioxane—water (16:1:1:2), and thereafter methanol—acetonitrile—1,4-dioxane—water (17:1:1:1), with a flow-rate of 1.5 ml/min.

RESULTS AND DISCUSSION

As fluorescent reagents for fatty acids, 1-naphthylamine [5], 9-aminophenanthrene [6], 4-bromomethyl-7-acetoxycoumarin [7] and ADAM [4, 8] have been developed. ADAM, with which we have already esterified prostaglandins [10], is easier for derivatizing carboxyl groups than other reagents. We intend to analyse prostaglandins and fatty acids in the same sample by HPLC in the future. For these reasons, ADAM was used in this work.

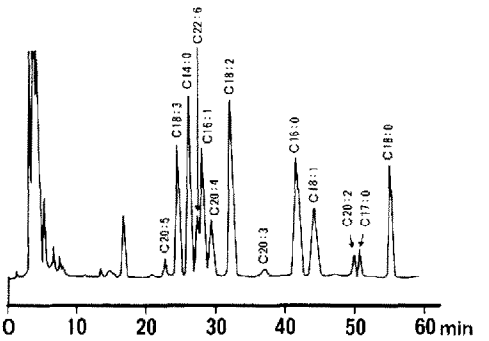

Fig. 1. High-performance liquid chromatogram of ADAM derivatives of authentic fatty acids. Fatty acids and amounts injected: C20:5 = eicosapentaenoic acid, 280 pg; C18:3 = linolenic acid, 1.4 ng; C14:0 = myristic acid, 1.8 ng; C22:6 = docosahexaenoic acid, 1.0 ng; C16:1 = palmitoleic acid, 1.4 ng; C20:4 = arachidonic acid, 1.1 ng; C18:2 = linoleic acid, 2.9 ng; C20:3 = eicosatrienoic acid, 270 pg; C16:0 = palmitic acid, 2.3 ng; C18:1 = oleic acid, 1.6 ng; C20:2 = eicosadienoic acid, 320 pg; C17:0 = heptadecanoic acid, 280 pg; C18:0 = stearic acid, 1.4 ng.

ADAM derivatives of thirteen fatty acids were separated by HPLC (Fig. 1).

To make calibration curves, thirteen authentic fatty acids (0.2—1 μg) were worked up, and the resultant solution was diluted and applied to analysis. We confirmed that the following amounts of fatty acids were detectable with the present method: > 120 pg (injected amount) of C17:0, C20:2, C20:3 and C20:5; 0.4 ng of C20:4 and C22:6; 0.6 ng of C14:0, C16:1, C18:0, C18:1 and C18:3; 0.9 ng of C16:0; and 1.2 ng of C18:2. ADAM derivatives of C20:3

TABLE I

COMPARISON OF TWO METHODS FOR SEPARATION OF FREE FATTY ACIDS FROM OPERATED RABBIT PLASMA

Amounts of fatty acids are expressed in nmol/ml. Fatty acids are expressed as the number of carbon atoms and double bonds. N.D. = Not detectable.

Fatty acid	ODS minicolumn (A)	Chloroform extraction (B)	B/A
C14:0	4.90	5.30	1.1
C16:0	92.0	108.0	1.2
C16:1	4.78	5.72	1.2
C17:0	1.52	2.24	1.5
C18:0	11.6	37.4	3.2
C18:1	48.1	57.7	1.2
C18:2	38.0	38.9	1.0
C18:3	10.9	12.1	1.1
C20:2	0.24	0.40	1.7
C20:3	0.23	0.20	0.9
C20:4	1.28	1.17	0.9
C20:5	0.21	0.23	1.1
C22:6	N.D.	N.D.	
Total	213.76	269.36	1.3

were eluted near the retention time, with small impurities contained in the purified ADAM (im-ADAM). However, both could be distinguished from each other, because there was a slight difference in retention time between C20:3 and im-ADAM. Taking note of this difference, calculation for determination was carried out by peak-height mode.

Two clean-up methods for free fatty acids from plasma are compared, as shown in Table I. Nine fatty acids (C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:3, C20:2 and C20:5), especially C18:0, were detected in larger amounts using the liquid–liquid extraction method. If the data for C18:0 are neglected,

TABLE II

FATTY ACIDS RELEASED FROM PHOSPHATIDYL INOSITOL BY CHLOROFORM EXTRACTION

R · PI indicates that fatty acids were clearly released from phosphatidyl inositol by chloroform extraction. *PI is a control experiment in which phosphatidyl inositol was not treated. Fatty acids of R · PI and *PI are expressed as peak heights when 5 μl of preparative sample solution were injected into the liquid chromatograph. Amounts of five fatty acids calculated are shown in parentheses. N.D. = Not detectable

Fatty acid	R · PI peak height (amount: nmol/ml)	*PI peak height
C14:0	8171 (1.26)	1285
C16:0	9520 (2.85)	1730
C16:1	546	N.D.
C17:0	602	N.D.
C18:0	5551 (0.71)	1001
C18:1	4435 (1.43)	487
C18:2	2729	806
C18:3	603	N.D.
C20:2	N.D.	N.D.
C20:3	510 (0.16)	N.D.
C20:4	456	N.D.
C20:5	N.D.	N.D.
C22:6	N.D.	N.D.

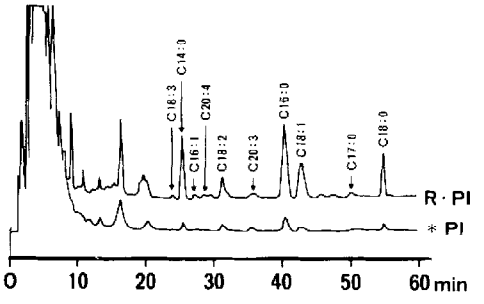

Fig. 2. High-performance liquid chromatograms of ADAM derivatives of fatty acids released from phosphatidyl inositol. R · PI indicates that fatty acids were clearly released from phosphatidyl inositol by chloroform extraction. *PI is a control experiment in which phosphatidyl inositol was not treated.

TABLE III

FREE FATTY ACIDS IN PLASMA OF NORMAL HUMANS AND EXPERIMENTAL ANIMALS

Results are expressed as nmol/ml; +, detection limit; —, no detection.

Subject		C14:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:2	C20:3	C20:4	C20:5	C22:6	Total
Human	M	4.58	11.8	1.11	0.21	3.45	8.78	5.56	0.70	—	—	0.82	0.55	1.16	38.7
	M	3.00	6.80	3.93	0.06	0.72	8.66	12.9	1.94	—	—	0.75	0.34	0.78	39.9
	M	3.33	8.07	4.09	0.09	1.01	7.35	7.15	1.02	—	0.25	0.76	0.34	0.61	34.1
	F	9.79	57.3	2.20	0.72	8.17	40.2	11.3	1.41	—	0.53	1.55	1.77	6.58	142
Dog		4.97	59.7	3.09	1.15	11.7	53.3	47.4	1.69	+	0.45	2.04	0.22	—	186
		4.64	57.1	17.7	0.39	3.87	83.9	47.4	2.17	0.50	0.55	3.38	0.59	—	222
		4.82	67.3	9.79	0.99	16.4	11.3	47.1	1.02	0.83	0.71	2.40	0.14	—	163
Rabbit		0.73	2.32	0.31	0.04	0.46	1.22	2.03	0.91	—	—	0.26	—	—	8.28
		0.76	1.62	0.30	0.04	0.51	1.38	1.14	0.48	—	—	0.31	0.03	—	6.57
		0.47	1.61	0.21	0.03	0.28	0.74	1.57	0.61	—	—	0.24	0.11	—	5.87
Guinea pig		1.74	16.4	1.00	0.46	2.90	15.6	20.1	7.22	0.21	0.76	0.69	0.21	—	67.3
		2.16	19.9	1.19	0.69	4.73	19.6	21.8	9.41	0.17	0.36	0.72	0.22	+	81.0
		4.53	73.1	4.21	2.55	18.8	61.8	59.0	24.6	0.61	0.42	1.28	0.45	—	251
Rat		4.95	8.83	6.64	+	0.43	4.16	16.8	2.39	+	0.19	2.66	1.67	1.55	50.3
		2.45	4.41	2.34	+	0.28	2.09	8.13	1.42	+	—	1.81	0.84	0.90	24.7
		2.23	4.62	2.12	+	0.46	2.16	7.86	1.05	+	—	2.09	0.77	1.20	24.6

the phenomenon may be explained merely as a lower recovery of the ODS minicolumn clean-up method. However, it is difficult to consider that only C18:0 has a different character from the other twelve fatty acids. Fatty acids may be released from phospholipids in plasma during extraction. Phospholipids such as phosphatidyl inositol (PI), phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl glycerol and cardiolipin possess esterified fatty acids and their fatty acid compositions differ from each other and from what they were originally. In this study, PI sodium salt (1.2 mg) (from soybean) was treated according to the chloroform extraction method. The results are shown in Table II and the chromatograms are shown in Fig. 2. The amount of PI in the sample solution was decided by refering to the phospholipids contents in human serum [11]. C14:0, C16:0, C18:0 and C18:1 were remarkably released from PI by chloroform extraction but from non-treated PI, fatty acids were hardly detected. It is, therefore, easy to think of fatty acids as being released from phospholipids in the plasma during the process of chloroform extraction. Consequently, in the case of determination of free fatty acids in plasma, use of an ODS minicolumn seems to be more accurate than use of chloroform extraction.

Free fatty acids in the plasma of normal humans, dogs, rabbits, guinea pigs and rats were determined by the present method, involving ODS minicolumn pretreatment; the results are shown in Table III and the chromatograms are shown in Figs. 3—7. C17:0 is normally used as the internal standard because it has not been reported to be present in human plasma. However, a substance was detected at virtually the same retention time as authentic C17:0 derivatized with ADAM in all the different plasmas used in this study (Figs. 3—7). Also, C20:2, C20:3 and C22:6 are not suitable as internal standards because a small quantity of these fatty acids was detected in certain kinds of plasma. No internal standard, therefore, was added to the plasma sample in this study.

Detection of C17:0 in plasma samples may be attributed to a higher sensitivity of the present method. Thus, C17:0 in plasma should be identified

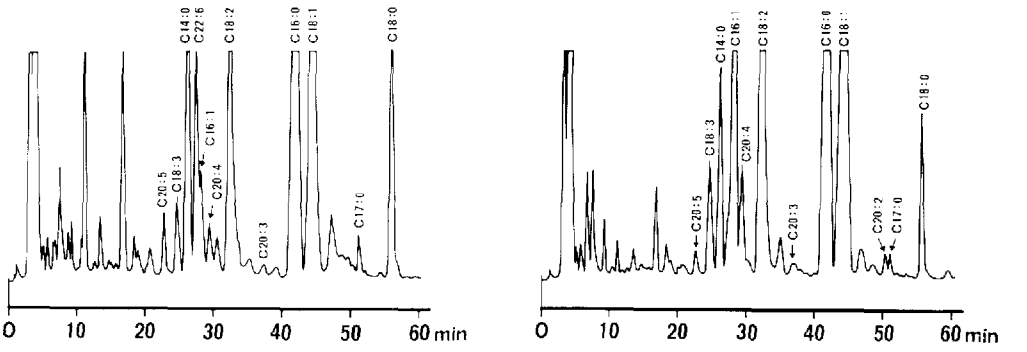

Fig. 3. High-performance liquid chromatogram of ADAM derivatives of free fatty acids in human (female) plasma. A 5-μl aliquot of pretreatment solution was injected into the chromatograph.

Fig. 4. High-performance liquid chromatogram of ADAM derivatives of free fatty acids in dog plasma. A 5-μl aliquot of pretreatment solution was injected into the chromatograph.

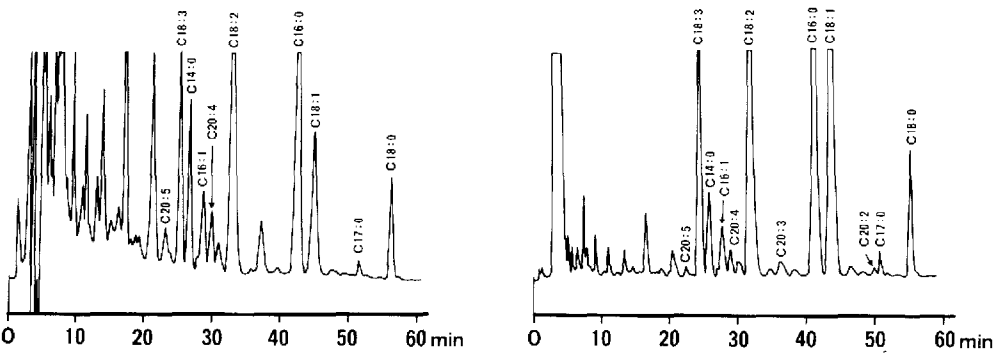

Fig. 5. High-performance liquid chromatogram of ADAM derivatives of free fatty acids in rabbit plasma. A 40-μl aliquot of pretreatment solution was injected into the chromatograph.

Fig. 6. High-performance liquid chromatogram of ADAM derivatives of free fatty acids in guinea pig plasma. A 5-μl aliquot of pretreatment solution was injected into the chromatograph.

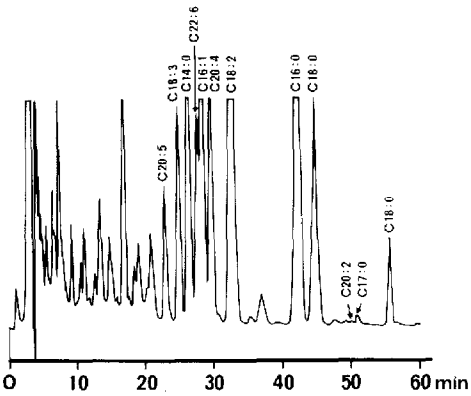

Fig. 7. High-performance liquid chromatogram of ADAM derivatives of free fatty acids in rat plasma. A 20-μl aliquot of pretreatment solution was injected into the chromatograph.

by GC—mass spectrometry (MS) and we are now taking GC—MS data. In the present study, the substance corresponding to C17:0 is expressed tentatively as C17:0 in order to show its amount in the plasma, because it was detected at virtually the same retention time as authentic C17:0 with several kinds of eluent system.

The amounts of free fatty acids in human plasma obtained in this work were lower than those obtained in other works [1, 2, 5—8]. Our data do not include fatty acids that were released from phospholipids during pretreatment. Each amount of free fatty acid in operated rabbit plasma was greater than in normal rabbit plasma (Tables I and III). This fact indicates that the amounts of free fatty acid increased, depending on incise. The present microanalysis method, which involves ODS minicolumn pretreatment of plasma, ADAM derivatization and subsequent HPLC, seems to be useful for quantitative determination of such variable free fatty acids in plasma.

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