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FLUOROMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 9-AMINOPHENANTHRENE-DERIVATIZED FREE FATTY ACIDS

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SUMMARY

The application of 9-aminophenanthrene (9-AP), a fluorescence-labeling reagent for free fatty acids (FFA), was examined. 9-AP dissolved in benzene was added to a benzene solution of FFA chlorides derived from FFA and oxalyl chloride. The mixture was allowed to react for 45 min at 70°C. By the method, 9-AP-tagged FFA with a strong fluorescence was formed. The materials thus obtained have a λ_{max} at around 303 nm for excitation and 376 nm for emission. By using this derivatization method, recoveries were measured for seven kinds of FFA added to 0.5 ml of healthy human serum. Significant recoveries ranging from 96 to 107% (coefficient of variation 1.4—5.0%) were obtained for each FFA The proposed method was clinically applied to the determination of FFA in 0.5 ml of healthy human serum, and almost satisfactory results were obtained. Detection limits of FFA by this derivatization method were 10 pmol for $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$, and 15 pmol for $C_{18:0}$ and $C_{20:4}$. As a quantitative measurement of FFA, gas chromatography and high-performance liquid chromatography with fluorescence detection, which have been routinely used, were chosen for comparison with the present method.

INTRODUCTION

In biological tissues and fluids, the amount of free fatty acids (FFA) increases or decreases according to the physiological and pathological changes in the living body. Therefore, the quantitative determination of FFA has been widely used in the search for the causes of various kinds of diseases [1—3].

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High-performance liquid chromatography (HPLC) has recently been used for the determination of FFA. Accordingly, the development of derivatization reagents with high sensitivity for FFA detection has been desired. To date several kinds of derivatization reagents have been reported. Most of the reagents are ultraviolet (UV) tagging agents for the analytes, and provide almost identical detection sensitivity in gas—liquid chromatography, with which several nanograms of FFA can be detected. Recently, fluorescence-labeling reagents for FFA have been developed, and 4-bromomethyl-7-methoxycoumarin, the first fluorescence derivatization reagent for FFA, was reported by Dünges [4]. The development of this reagent was followed by that of 9,10-diaminophenanthrene [5], 9-anthryldiazomethane (ADAM) [6, 7] and 1-bromoacetylpyrene [8]. These fluorescence-labeling reagents provided the highly sensitive detection required to permit the measurement of picomole levels of FFA.

Most of the derivatization reagents for FFA which have already been reported are newly synthesized ones [9—11] with high reactivity for FFA, and much time has been spent in their synthesis.

In previous reports [12, 13] we described the induction of 1-naphthylamine (NA) to acid chloride of FFA as a new derivatization method for FFA. In this derivatization method the presence of a primary amine makes the induction of reagents with fluorescent and UV absorption to FFA easy, with high reactivity of acid chloride and amine. From the present study we may conclude that 9-aminophenanthrene (9-AP) is useful for fluorescence-labeling of FFA.

EXPERIMENTAL

Reagents

Myristic acid ($C_{14:0}$, ML), palmitic acid ($C_{16:0}$, PT), palmitoleic acid ($C_{16:1}$, PL), stearic acid ($C_{18:0}$, ST), linoleic acid ($C_{18:2}$, Ll), arachidonic acid ($C_{20:4}$, AR) were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Oleic acid ($C_{18:1}$, OL) and palmitoyl chloride (PT · Cl) were purchased from Sigma (St. Louis, MO, U.S.A.). Margaric acid ($C_{17:0}$) was from Nakarai Chemicals (Kyoto, Japan). Linoleoyl chloride (Ll · Cl) and triethylamine (TEA) were from Tokyo Kasei Kogyo (Tokyo, Japan). Oxalyl chloride, $(COCl)_2$, was from Wako Pure Chemical (Osaka, Japan). 9-Aminophenanthrene (9-AP) was from Aldrich (Milwaukee, WI, U.S.A.). 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi Chemical (Tokyo, Japan). The ethereal diazomethane solution was prepared by the established method [14].

Apparatus

A Hitachi high-performance liquid chromatograph Model 635A equipped with a JASCO FP-110 spectrofluorometer and a Hitachi multiwavelength UV monitor was used. For measuring the infrared (IR) spectra, a JASCO A-3 IR spectrometer was used. ^{1}H-NMR spectra were determined on a JEOL Fx-200 nuclear magnetic resonance (NMR) spectrometer with tetramethylsilane (TMS) as an internal standard. UV spectra and mass spectra were measured with a Shimadzu UV-210A and a Hitachi RMU-7MG spectrometer, respectively. Gas chromatographic (GC) analysis was performed on a Shimadzu GC-3BF

equipped with a flame ionization detector. Fluorescence spectra were recorded with a Hitachi fluorescence spectrometer 204-S.

HPLC conditions

Column: μBondapak C_{18} (30 × 0.4 cm I.D., particle size 8–10 μm). Fluorescence detector: the excitation wavelength was set at 303 nm and the emission wavelength at 376 nm. Mobile phase: methanol–acetonitrile–water (53:27:20). Flow-rate: 2.0 ml/min. Column temperature: 40°C.

Purification of 9-AP

9-AP was purified by the method of Altiparmakian et al. [15]. In 30 ml of ethanol 50 mg of crude 9-AP were dissolved and filtered. Hydrochloric acid-saturated ether was added to the filtrate until the white precipitation no longer appeared. The precipitates were filtered, washed with ether, and dried in a vacuum desiccator. The dried crystals were dissolved in hot water, and the solution was made basic by the addition of ammonia to yield white crystals (m.p. 134°C).

Preparation of N-palmitoyl-9-aminophenanthrene (PT · AP) and N-linoleoyl-9-aminophenanthrene (Ll · AP)

PT · AP (0.25 mmol), 9-AP (0.25 mmol) and TEA (0.25 mmol) were dissolved in 20 ml of benzene in a reaction vial, and the solution was allowed to react by mixing for 30 min in an oil bath at 70°C. The solvent was removed at reduced pressure and the residue was recrystallized from ethanol to give PT · AP: m.p. 130.5°C. IR ν_{max}^{KBr} cm^{-1}: 3280 (NH), 1660 (CO). NMR (C^2HCl_3) δ: 0.88 (3H, t, J = 6.4 Hz, 16-H), 1.26 (26H, brs), 1.81 (1H, br, NH), 2.48 (2H, brs, 2-H), 7.38–8.79 (9H, m, aromatic H). Mass spectrum m/z: 431 (M^+).

In the same way as the preparation of PT · AP, Ll · AP was obtained by recrystallization with ethanol after a 30-min reaction at 70°C in benzene solution containing Ll · Cl (0.25 mmol), 9-AP (0.25 mmol), and TEA (0.25 mmol): m.p. 95.5°C. IR ν_{max}^{KBr} cm^{-1}: 3250 (NH), 1640 (CO). NMR (C^2HCl_3) δ: 0.88 (3H, t, J = 6.6 Hz, 18-H), 1.29 and 1.36 (16H, each brs, 3-H – 7-H + 15-H – 17-H), 1.85 (1H, br, NH), 2.03 (4H, 8-H + 14-H), 2.55 (2H, brs, 2-H), 2.77 (2H, t, J = 5.3 Hz, 11-H), 5.36 (4H, m, 9-H, 10-H, 12-H, 13-H), 7.28–8.70 (9H, m, aromatic H). Mass spectrum m/z: 455 (M^+).

Preparation of a 9-AP solution

9-AP, 77.6 μmol, was dissolved in benzene to give a total volume of 10 ml. The solution, thus prepared, was kept shielded from light.

Preparation of a 0.1% TEA solution

TEA, 720 μmol, was dissolved in benzene to give a total volume of 10 ml.

Preparation of a 2% oxalyl chloride solution

Oxalyl chloride, 586 μmol, was dissolved in benzene to give a total volume of 10 ml.

Derivatization procedures

According to the reaction conditions which we had examined previously [13], FFA was converted to the acid chloride. FFA (1—700 nmol) dissolved in benzene (0.2—0.4 ml) was mixed with the same volume of a 2% oxalyl chloride solution, shaken sufficiently and the mixture allowed to react for 30 min in an oil bath at 70°C. After reaction, the excess oxalyl chloride and solvent were removed under a stream of nitrogen gas; 0.1 ml of a 9-AP solution and 0.1 ml of a 0.1% TEA solution were added to the preparation. The mixture was allowed to react at 70°C for 45 min, and the 9-AP derivative of FFA was obtained.

FFA extraction from serum

To 0.5 ml of serum were added 0.1 ml of a methanol solution containing 1 μg of margaric acid and 1.4 ml of *M*/15 phosphate buffer (pH 7.0); this mixture was shaken sufficiently and poured into a modified Extrelut column [16]. After adsorbtion for 20 min, FFA was eluted out with 10 ml of chloroform.

Recovery of the FFA added to human serum

To 0.5 ml of human serum were added 0.1 ml of methanol solution containing the internal standard, 5 μg of margaric acid, and the following composition of FFA : ML 2 μg, PT 20 μg, PL 4 μg, ST 4 μg, OL 40 μg, Ll 10 μg, AR 4 μg. Separation and determination of FFA were performed according to the methods described above.

Method A (HPLC of FFA using ADAM reagent)

FFA was allowed to react with ADAM reagent (which has been developed by Nimura and Kinoshita [6]) in methanol at room temperature. An ADAM-derivatized FFA solution was injected onto a μBondapak C_{18} column, and eluted with acetonitrile—water (90:10) at a flow-rate of 1.5 ml/min. FFA derivative was detected by a fluorescence detector (excitation 365 nm, emission 412 nm).

Method B (determination of FFA by GC)

FFA was methylated with the ethereal diazomethane solution according to the method of Shlenk and Gellerman [17]. The excess diazomethane—ether solution was evaporated under a stream of nitrogen. Methylated FFA was dissolved in acetone and applied to GC. GC was carried out using a glass column (200 × 0.3 cm I.D.) packed with 15% diethylene glycol succinate (DEGS) on 80—100 mesh Chromosorb W AW DMCS. The injection temperature was maintained at 225°C and the column oven at 195°C. The flow-rate of nitrogen gas was 60 ml/min. The amount of each FFA was calculated from the calibration curves which were previously drawn on the basis of the peak height ratio of FFA to that of the internal standard ($C_{17\cdot 0}$).

RESULTS AND DISCUSSION

From among the 9-AP-derivatized FFA, PT · AP and Ll · AP were selected, and their fluorescence and UV spectra were measured. Both PT · AP and

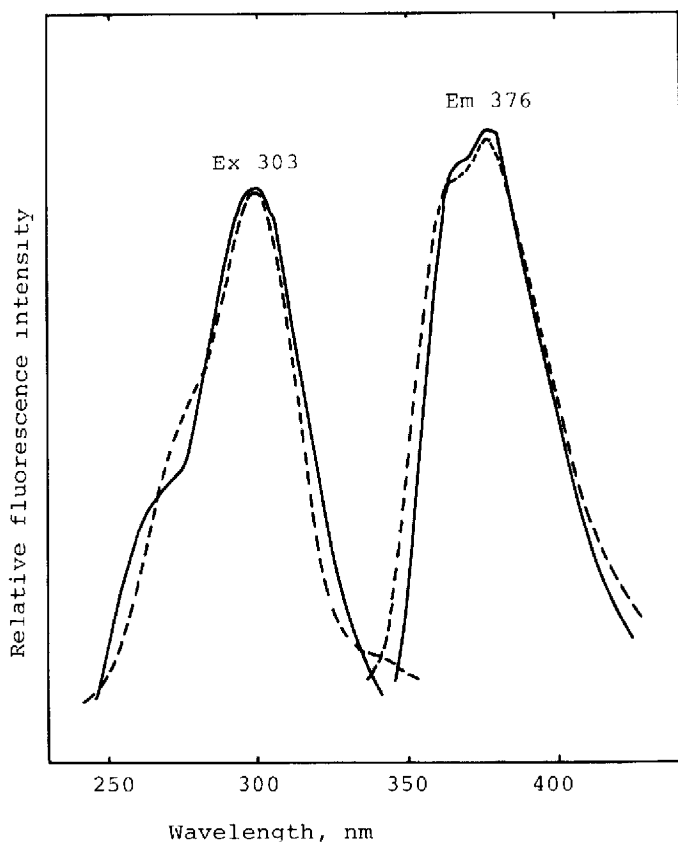

Fig. 1. Fluorescence spectra of N-palmitoyl-9-aminophenanthrene (——) and N-linoleoyl-9-aminophenanthrene (- - -) in methanol—water (81:19).

Ll · AP have a strong fluorescence around excitation wavelength of 303 nm and emission wavelength of 376 nm (Fig. 1). These derivatives also had strong absorption in the UV region, and maximal absorption at around 254 nm.

These results showed that FFA can be converted to the fluorescent FFA derivative with 9-AP. Secondly, reaction conditions necessary for the introduction of 9-AP into FFA were studied. The optimum conditions necessary for the introduction of 9-AP into an acid chloride of FFA were investigated in this paper, since the reaction conditions for the derivatization of FFA to an acid chloride form had already been evaluated [13]. In the reaction of PT · Cl and Ll · Cl with 9-AP in benzene, increase of reaction time from 15 to 30 min and to 45 min increased the production of PT · AP and Ll · AP; and increase of reaction temperature from 30°C to 50°C and 70°C also increased the production of these derivatives. The reaction for 45 min at 70°C produced a maximum amount of PT · AP and Ll · AP. The recovery rates of their derivatives were 97% in PT · AP, and approximately 100% in Ll · AP. However, it is supposed that a further increase of reaction temperature does not facilitate production, but breaks FFA down by the heat [18]. Therefore, the reaction conditions for FFA acid chloride with 9-AP were decided to be 45 min at 70°C. Under these conditions, derivatization of seven FFA (C_{14}—C_{20}) with

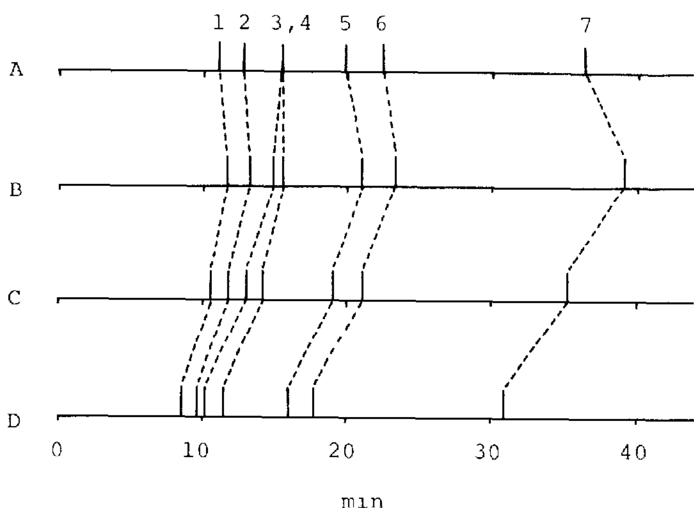

Fig. 2. Separation of a mixture of 9-AP-derivatized FFA. Column: μBondapak C_{18}. Flow-rate: 2.0 ml/min. Fluorescence excitation at 303 nm, fluorescence emission at 376 nm. Temperature: 40°C. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{20:4}$, 4 = $C_{18:2}$, 5 = $C_{16:0}$, 6 = $C_{18:1}$, 7 = $C_{18:0}$ Mobile phase composition (methanol—acetonitrile—water): (A) 80:0:20, (B) 55:25:20, (C) 53.27:20, (D) 50:30:20.

9-AP was carried out. Subsequently, HPLC separation conditions for these derivatized FFA were discussed. It was decided to use a μBondapak C_{18} column at 40°C, and a flow-rate of 2.0 ml/min. Under these conditions, resolution of each derivatized FFA was studied using different eluates (Fig. 2). With methanol—water (80:20), AR and Ll remained in a same location unseparated. With an eluate of decreased methanol and acetonitrile instead of the decrease in methanol, AR and Ll were separated from each other. It was confirmed that FFA of PL, AR, and Ll were most favourably separated with methanol—acetonitrile—water (53:27:20) (Fig. 3). D'Amboise and Gendeau [19] separated phenacyl derivatives of FFA by HPLC with the solvent system methanol—acetonitrile—water on LiChrosorb RP-8 (10 μm).

For quantitative determination of FFA, calibration curves of seven kinds of saturated and unsaturated FFA (C_{14}—C_{20}) were drawn. From chromatograms obtained, the concentration of FFA was calculated from the ratio of the peak height of samples to that of the internal standard ($C_{17:0}$). All seven FFA showed straight lines in the range 10—100 ng. The detection limit of FFA, assuming a signal-to-noise ratio of 3, was 10 pmol for ML, PT, PL, OL and Ll, and 15 pmol for ST and AR. This result showed almost the same sensitivity as the other fluorescent-labeling reagents, 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [20] or 9-anthryldiazomethane (ADAM) [6, 7], and showed ten times more sensitivity than that of the UV-labeling reagent, *p*-bromophenacyl bromide (PBPB) which was developed by Durst et al. [21].

Before applying the determination method of FFA to biological samples, seven kinds of FFA were added to human serum and recovered from it. The same amount as an average FFA level in 0.5 ml of normal serum was added. Each FFA was recovered 96—107%, and the coefficient of variation was 1.4—5.0% (n = 4), showing a high sensitivity (Table I).

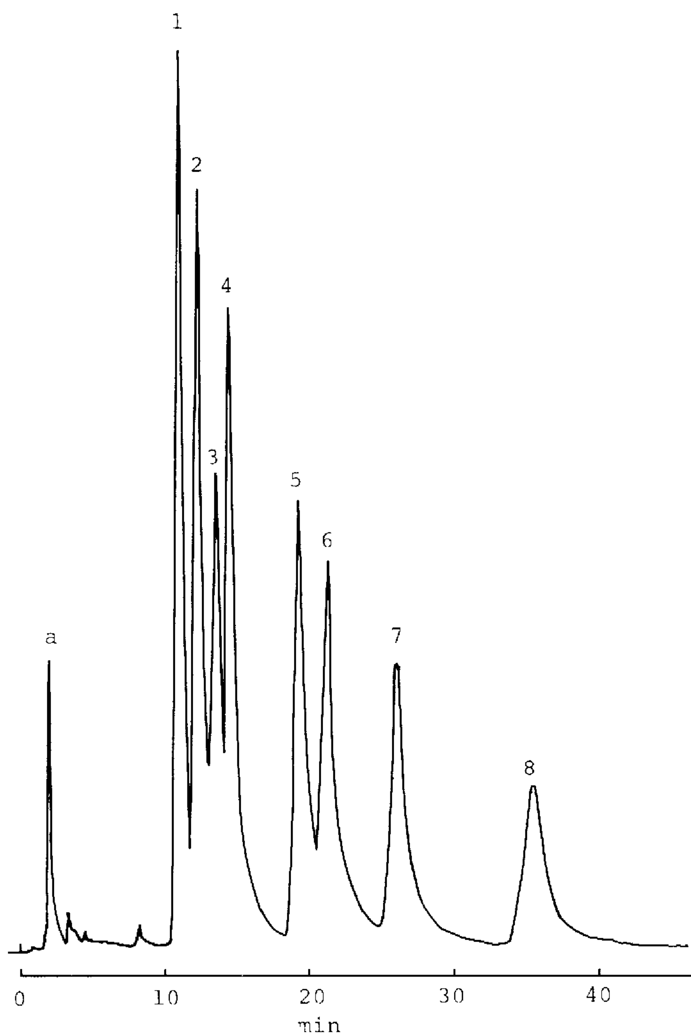

Fig. 3. Separation of the 9-AP derivatives of an FFA mixture. Mobile phase: methanol—acetonitrile—water (53:27:20). Flow-rate: 2.0 ml/min. a = 9-AP, 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{20:4}$, 4 = $C_{18:2}$, 5 = $C_{16:0}$, 6 = $C_{18:1}$, 7 = $C_{17:0}$ (I S.), 8 = $C_{18:0}$.

With 0.5 ml of serum separated immediately from the blood collected from six volunteers (adult, both sexes), FFA was quantitatively analysed by the present method (Table II). These results were almost the same as the average normal FFA level reported by Rogiers [22]. In order to determine the FFA level in serum with UV-labeling reagents, especially to detect PL and AR which are scarcely liberated in the blood, the extract from at least 50 μl of serum has to be injected into the HPLC column. However, the amount of extract injected into the column can be reduced to approximately one-tenth by the use of the present method. This is also good for maintaining higher column efficiency. In order to compare 9-AP with ADAM, a derivatization experiment for three kinds of FFA with the two reagents was performed. The derivatives obtained were applied to a μBondapak C_{18} column, and the recovery was measured using HPLC under optimum conditions. For comparison with the GC method, the same amounts of FFA in a specimen were measured, after

TABLE I

ANALYTICAL RECOVERY OF FFA ADDED TO HEALTHY HUMAN SERUM

The mixture of FFA was added to 0.5 ml of serum.

Fatty acid	Added (μg)	Found* (μg)	Recovery* (%)	C.V. (%)
$C_{14:0}$	2.0	1.9 ± 0.1	96.3 ± 4.8	5.0
$C_{16:0}$	20.0	21.5 ± 0.3	107.3 ± 1.5	1.4
$C_{16:1}$	4.0	4.0 ± 0.1	100.0 ± 2.0	2.0
$C_{18:0}$	4.0	3.9 ± 0.1	97.5 ± 3.5	3.6
$C_{18:1}$	40.0	38.9 ± 0.2	97.3 ± 0.5	0.6
$C_{18:2}$	10.0	9.7 ± 0.2	96.8 ± 1.7	1.8
$C_{20:4}$	4.0	4.0 ± 0.1	101.0 ± 1.5	1.5

*Mean ± S.D., $n = 4$.

TABLE II

FFA CONCENTRATION IN HEALTHY HUMAN SERUM DETERMINED BY HPLC

Volunteer	FFA (μM)						
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
M.I.	13	97	5	16	109	41	8
N.E.	9	40	3	10	48	36	3
A.S.	9	77	1	13	73	58	5
Y.A.	8	31	2	10	31	24	N.D *
J.I.	16	68	6	26	72	36	5
K T.	9	65	4	6	58	26	5
Ref. 22	5 ± 4	62 ± 23	11 ± 7	26 ± 11	88 ± 42	42 ± 22	—

*N.D. = not detectable.

methylation, using GC with a 15% DEGS column. An average value of quadruplicate measurements suggested that in the GC method FFA was recovered almost 90—100%. However, the coefficient of varation was as large as 2.1—7.6%, and this suggested low reliability of measurement. On the other hand, two methods of fluorescence HPLC showed a small distribution in the results of quadruplicate measurements compared with that by the method of GC. Especially the 9-AP derivatization method showed a coefficient of variation of 1.4—3.4%, with high reliability (Table III).

These results showed that FFA detection at the picomole level has become possible, and that FFA in serum can be precisely assayed. With the establishment of this method, the usefulness of the primary amine has been established as a labeling preparation, and it may be used for the development of a new FFA derivatization reagent. For most FFA derivatization reagents which have been developed up to now, polar and aprotic solvents were used for reaction with FFA [21], but crown ethers were used for the reaction of higher FFA with low solubility [23—25]. Although the present derivatization method has the disadvantage that the process is slightly complicated, acid chlorides of FFA

TABLE III

COMPARISON OF 9-AP DERIVATIZATION METHOD WITH THE OTHER METHODS FOR THE DETERMINATION OF FFA

Fatty acid	Added (ng)	Proposed method (9-AP)			Method A**			Method B***		
		Found* (ng)	Recovery* (%)	C.V. (%)	Found* (ng)	Recovery* (%)	C.V. (%)	Found* (ng)	Recovery* (%)	C.V. (%)
$C_{14:0}$	30	29.6 ± 0.6	98.6 ± 2.0	2.1	29.0 ± 1.1	96.8 ± 3.7	3.8	29.3 ± 1.3	97.5 ± 4.3	4.4
	50	46.4 ± 1.2	92.7 ± 2.4	2.6	44.8 ± 2.0	89.6 ± 4.0	4.5	48.7 ± 3.5	97.3 ± 7.0	7.2
	70	65.6 ± 1.6	93.7 ± 2.2	2.4	65.6 ± 2.0	93.7 ± 2.8	4.5	62.6 ± 3.3	89.4 ± 4.6	5.2
	90	90.3 ± 1.5	100 3 ± 1.7	1.7	88.3 ± 2.5	98.1 ± 2.8	2.8	86.6 ± 4.2	96.3 ± 4.5	4.6
$C_{18:1}$	30	31.7 ± 0.8	105.8 ± 2.7	2.6	30.9 ± 1.1	103.1 ± 3.7	3.6	29.1 ± 0.6	97.0 ± 2.0	2.1
	50	46.4 ± 0.8	92.8 ± 1.5	1.6	51.8 ± 2.1	103.5 ± 4.2	4 0	50.5 ± 2.8	101.0 ± 5.9	5.8
	70	63.5 ± 1.8	90.6 ± 2.5	2.8	66.3 ± 1.1	94.8 ± 1.5	1.6	70.4 ± 4.3	100.5 ± 6.1	6.1
	90	90.0 ± 3.1	100.0 ± 3.4	3.4	86.2 ± 1.6	95.8 ± 1.8	1.9	89.1 ± 5.1	98.9 ± 5.7	5.8
$C_{18:2}$	30	30.6 ± 0 8	102.1 ± 2.7	2.6	25.9 ± 0.6	86.4 ± 1.9	2.2	30.7 ± 0.7	102.2 ± 2.3	2.3
	50	46.8 ± 0.7	93.6 ± 1.4	1.5	46.1 ± 2.3	92.2 ± 4.6	5.0	48.3 ± 1.8	96.5 ± 3.5	3.7
	70	65.1 ± 1.1	92 9 ± 1 6	1.7	67 9 ± 3.3	97.0 ± 4.7	4.9	61.6 ± 2.7	88 1 ± 3.9	4.4
	90	92.9 ± 1.3	103.2 ± 1.4	1.4	82.8 ± 2.1	92.0 ± 2.3	2.5	85 6 ± 6 5	95.1 ± 7.2	7.6

*Mean ± S.D., $n = 4$.

**Method A: The FFA were dissolved in methanol. To the solutions 0.05% methanolic ADAM solution was added and the mixture was allowed to stand for 2 h at room temperature. The reaction mixture was injected directly into the column. The HPLC conditions are given in the experimental section.

***Method B: The FFA were methylated with the ethereal diazomethane solution. The methyl ester solution obtained was injected into the column The GC conditions are given in the experimental section.

and primary amine react easily to produce 9-AP derivatives of FFA in high yield. As the reaction of the present method is conducted with non-polar solvents, it is convenient for the higher FFA with high solubility for such solvents. The 9-AP solution is very stable when protected from light, and can be used for more than one month.

By using the preseparation technique, it is not necessary to remove excess reagents or degradation products which may interfere with the determination.

It has been reported recently that the intake of unsaturated higher FFA which are said to be precursors of prostaglandin or thromboxane in platelets [26, 27] or vascular smooth muscle [28], has something to do with myocardial infarction or thrombosis. To clarify the mechanisms, the application of this method for FFA determination with such high sensitivty is expected to determine FFA at the cellular level and to provide much information.

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