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SIMULTANEOUS SEPARATION AND SENSITIVE DETERMINATION OF FREE FATTY ACIDS IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The quantitative determination of saturated and unsaturated fatty acids (ranging from acetic acid to lignoceric acid) in biological samples is presented. The secondary amine group of 5-(dimethylamino)-1-naphthalenesulponyl-semipiperazide (dansyl-semipiperazide) reacts with the carboxyl group of the fatty acids to form an amide linkage in order to obtain fluorescent derivatives of the acids. The fluorescent derivatives are analysed by high-performance liquid chromatography (HPLC) using an internal standard.

INTRODUCTION

In spite of recent advances in gas chromatography, the quantitative determination of free low-molecular-weight fatty acids in biological samples is still difficult. The quantitative extraction of low-molecular-weight fatty acids from their aqueous solutions using hydrophobic organic solvents is difficult due to their low partition coefficients. Furthermore, condensation of the fatty acids by evaporation of the organic solvents cannot be used for quantitative determination because the fatty acids are highly volatile. In recent studies, derivatization with a suitable labelling reagent, i.e. 9,10-diaminophenanthrene

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[1], 9-anthryldiazomethane [2, 3] or 4-bromomethyl-7-methoxycoumarin [4—6], has been used for the sensitive determination of carboxylic acids. But in these studies, no consideration was given to the problems of low-molecular-weight fatty acids. In this work, therefore, these acids were not extracted using organic solvents, where extraction depends on their partition coefficients. A biological sample was mixed with a relatively large amount of acetonitrile, which is freely miscible with water, to obtain a homogeneous mixture. The protein contained in the sample was precipitated out of the mixture, leaving the filtrate from which the acids were extracted and deproteinized. The filtrate was dehydrated by adding anhydrous calcium chloride. The acids in the dried mixture were made to react with 5-(dimethylamino)-1-naphthalenesulphonyl semipiperazide (dansyl semipiperazide) by using dicyclohexylcarbodiimide (DCC) to produce non-volatile dansyl piperazides. These dansyl piperazides were analysed fluorometrically using high-performance liquid chromatography (HPLC).

EXPERIMENTAL AND RESULTS

Materials

Authentic fatty acids were purchased from Applied Science Laboratory (Tokyo, Japan). 5-(Dimethylamino)-1-naphthalenesulphonyl chloride (dansyl chloride) was recystallized several times, and anhydrous piperazine was recrystallized twice, from their respective acetone solutions. Dicyclohexylcarbodiimide, acetonitrile, chloroform (containing 0.5—0.9% ethanol as stabilizer) and methanol were purchased from Wako Pure Chemical Industries (Japan). All organic solvents were treated by descending chromatography through an alumina column (300 × 20 mm) and then distilled. The chloroform used in this experiment contained 1% ethanol. The ethanol was necessary for later Sep-Pak treatment.

Apparatus

For HPLC, a Nihon Seimitsu Kagaku Model 100 was used with a 250 × 4.6 mm reversed-phase column of Hitachi C_{18} 3056. For fluorescence detection, a Shimadzu RF 530 fluorescence spectromonitor was used (excitation wavelength 350 nm, emission wavelength 530 nm). The fluorescence intensity of HPLC eluate was measured using a Hitachi MPF-4 fluorescence spectrophotometer. The thin-layer chromatographic (TLC) plates (5 × 20 cm) were covered with Wako Gel B-10 of the silica gel (0.5 mm thick) and were activated for 24 h at 110°C. Toyo Roshi No. 5A filter paper was washed well with acetonitrile and stored in the solvent. The Sep-Pak silica cartridges used for the HPLC pretreatment were purchased from Waters Assoc. (Milford, MA, U.S.A.).

Preparation of dansyl semipiperazide

Piperazine (50 g) was dissolved in 500 ml of acetone and 8 g of dansyl chloride were added to the solution while stirring for 30 min at room temperature. The mixture was left overnight with continuous stirring. The mixture was then condensed to dryness by distillation of the solvent under reduced pressure. The residue was dissolved in 300 ml of chloroform, insoluble

matter was filtered off through filter paper No. 5A, and the filtrate was washed three times with a 5% solution of sodium bicarbonate and then with water. The washed chloroform solution was extracted three times with 100 ml of 1 *M* hydrochloric acid. The extracts were then combined and washed repeatedly with chloroform for depletion of any diazide present in the extract. The washed extract was then alkalinized by addition of a slight excess of powdered sodium carbonate, and the aggregate was extracted with benzene. The benzene extract was washed twice with a 5% solution of sodium bicarbonate and dried over anhydrous sodium sulphate. The solvent was distilled under reduced pressure to dryness, leaving 7 g of yellowish green crystals. The dansyl semipiperazide thus obtained was dissolved in a minimum amount of chloroform and applied to a column of Wako Gel C-200 (silica gel), 200 × 50 mm, prepared with chloroform and developed by the same solvent. When a first migrating yellow band reached the bottom of the column, the solvent was changed to methanol and the eluate corresponding to the yellow band was collected. The collected fraction was condensed by evaporation under reduced pressure. The condensed residue was dissolved in chloroform and stored in a dark place at ca. 5°C. The concentration of dansyl semipiperazide was determined spectrophotometrically by measuring the extinction at 340 nm. The molecular extinction coefficient in ethanol or methanol was 4300 M^{-1} cm^{-1}, according to Weber [7].

Preparation of the dansyl derivatives of the authentic fatty acids

The commercial form of authentic fatty acids contain other fatty acids as impurities, which could interfere with this experiment. To obtain pure dansyl derivatives of each fatty acid illustrated in Fig. 1, the following treatments were carried out. Several micromoles of each fatty acid shown in Table I were mixed with 22.5 μmol of dansyl semipiperazide in 5 ml of chloroform. To this mixture, ca. 150 mg of DCC were added and then it was allowed to stand for 30 min. The mixture was concentrated to ca. 0.1 ml in a rotary evaporator at a temperature of < 30°C. The concentrated matter was then spotted onto a TLC plate and developed into one dimension by a solvent of toluene—acetonitrile—chloroform—acetone (2:1:1:1). The main fluorescent spot on the plate was visualized under UV irradiation and then eluted with methanol and stored at 5°C.

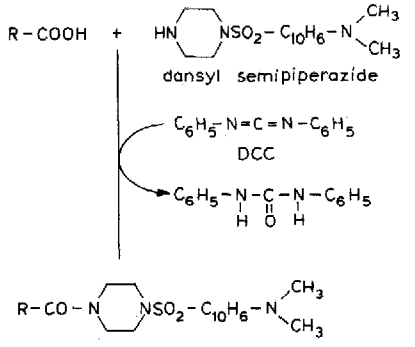

Fig. 1. Reaction scheme showing the derivatization of fatty acids to obtain dansyl derivatives.

TABLE I

THE AUTHENTIC FATTY ACIDS AND THEIR ABBREVIATIONS

Number of carbon atoms	Name of fatty acid	Abbreviation
2	Acetic acid	C_2
3	Propionic acid	C_3
3	Pyruvic acid	C_3-O
3	Lactic acid	C_3-OH
4	*n*-Butyric acid	C_4
4	Isobutyric acid	iso-C_4
4	β-Hydroxybutyric acid	C_4 β-OH
4	γ-Hydroxybutyric acid	C_4 γ-OH
5	*n*-Valeric acid	C_5
5	Isovaleric acid	iso-C_5
5	2-Pentenoic acid	$2C_{5:1}$
6	*n*-Caproic acid	C_6
6	Isocaproic acid	iso-C_6
6	2-Hexenoic acid	$2C_{6:1}$
7	Heptanoic acid	C_7
7	2-Heptenoic acid	$2C_{7:1}$
8	Caprylic acid	C_8
8	2-Octenoic acid	$2C_{8:1}$ (*trans*)
9	Nonanoic acid	C_9
10	Capric acid	C_{10}
11	Undecanoic acid	C_{11}
12	Lauric acid	C_{12}
13	Tridecanoic acid	C_{13}
14	Myristic acid	C_{14}
14	Myristoleic acid	$C_{14:1}$ (*cis*-9)
15	Pentadecanoic acid	C_{15}
16	Palmitic acid	C_{16}
16	Palmitoleic acid	$C_{16:1}$ (*cis*-9)
16	Palmitelaidic acid	$C_{16:1}$ (*trans*-9)
17	Heptadecanoic acid	C_{17}
18	Stearic acid	C_{18}
18	Petroselenic acid	$C_{18:1}$ (*cis*-5)
18	Oleic acid	$C_{18:1}$ (*cis*-9)
18	Elaidic acid	$C_{18:1}$ (*trans*-9)
18	Vaccenic acid	$C_{18:1}$ (*cis*-11)
18	Linoleic acid	$C_{18:2}$ (*cis*-9,12)
18	Linolelaidic acid	$C_{18:2}$ (*trans*-9,12)
18	γ-Linolenic acid	$C_{18:3}$ (*cis*-6,9,12)
18	Linolenic acid	$C_{18:3}$ (*cis*-9,12,15)
19	Nonadecanoic acid	C_{19}
20	Arachidic acid	C_{20}
20	*cis*-5-Eicosenoic acid	$C_{20:1}$ (*cis*-5)
20	*cis*-11-Eicosenoic acid	$C_{20:1}$ (*cis*-11)
20	11,14-Eicosadienoic acid	$C_{20:2}$ (*cis*-11,14)
20	Homo-γ-linolenic acid	$C_{20:3}$ (*cis*-8,11,14)
20	11,14,17-Eicosatrienoic acid	$C_{20:3}$ (*cis*-11,14,17)
20	Arachidonic acid	$C_{20:4}$ (*cis*-5,8,11,14)
20	Eicosapentaenoic acid	$C_{20:5}$ (*cis*-5,8,11,14,17)
21	Heneicosanoic acid	C_{21}
22	Behenic acid	C_{22}
22	Erucic acid	$C_{22:1}$ (*cis*-13)

TABLE I (continued)

Number of carbon atoms	Name of fatty acid	Abbreviation
22	Brassidic acid	$C_{22:1}$ (*trans*-13)
22	4,7,10,13,16,19-Decosahexaenoic acid	$C_{22:6}$ (*cis*-4,7,10,13,16,19)
23	Tricosanoic acid	C_{23}
24	Lignoceric acid	C_{24}
24	Nervonic acid	$C_{24:1}$ (*cis*-15)

Chromatography of the dansyl derivatives of the authentic fatty acids

Dansyl derivatives of the authentic fatty acids were applied to methanolic or acetonitrile HPLC systems and their retention times were measured. All authentic dansyl derivatives extracted from TLC plates were mixed and subjected to HPLC. The HPLC was developed stepwise, as shown in Fig. 2A and B, using the following solvents: methanolic system, methanol—water (65:35, 75:25, 85:15, 90:10, 95:5, 100:0) (flow-rate 0.8 ml/min, pressure from 90—150 kg/cm^2); acetonitrile system, acetonitrile—water (45:55, 60:40, 75:25, 85:15, 100:0) (flow-rate 0.8 ml/min, pressure from 40—105 kg/cm^2). The chromatograms of HPLC resulting from the mixture of the dansyl derivatives of the authentic fatty acids are shown in Fig. 2A and B. Fatty acids C_2—C_{24} (containing the unsaturated fatty acids) were able to be separated. The dansyl derivatives of the saturated fatty acids were eluted successively in the order of increasing carbon number of the fatty acid. With fatty acids having the same carbon number, but different chemical structures, the derivatives of the more-highly unsaturated acids were eluted faster than those of the lower unsaturated acids. Fatty acids of the same carbon number, which are unsaturated to the same degree but differ in the position of the double bond in the molecular structure, were also separated. In geometrical isomers, derivatives of the *cis* form were eluted faster than those of the *trans* form. In structural isomers, derivatives of the iso form were eluted faster than those of the normal form.

Excitation and emission spectra of the dansyl derivatives

Excitation and emission spectra of several authentic dansyl derivatives eluted from the HPLC column were examined using the fluorescence spectrophotometer. As shown in Fig. 3, there was virtually an overlap of the excitation and emission spectra of dansyl derivatives of the fatty acids.

Fluorescence intensities in solvents of different concentrations

Fatty acid C_{13} (obtained by the methanolic HPLC system) and fatty acid $2C_{7:1}$ (by the acetonitrile HPLC system) were used as the internal standards. As these HPLC were developed stepwise by solvents of different concentrations, the eluted dansyl derivatives were also dissolved in the corresponding eluents. The dansyl derivative of fatty acid C_{13} was eluted in the 90% methanol fraction by the methanolic HPLC system. The eluate corresponding to the peak was diluted 100 times with 65, 75, 85, 90, 95 and 100% methanol. Fluorescence intensities of each diluted solution were

TABLE II

VARIATION OF FLUORESCENCE INTENSITY WITH DIFFERENT CONCENTRATIONS OF METHANOL

Concentration of methanol (%)	Fluorescence intensity*	Ratio against the intensity in 90% methanol
65	29.9	1.85
75	40.1	1.38
85	50.0	1.10
90	55.4	1.00
95	60.6	0.914
100	65.9	0.840

*The eluate of the dansyl derivative of C_{19} on the HPLC system developed with 90% methanol solution was used as the test. Aliquots of the eluate were diluted 100 times with 65, 75, 85, 90, 95 and 100% methanol to prepare solutions of different methanol concentration. The fluorescence intensity of each diluted solution was measured at emission wavelength 530 nm and excitation wavelength 350 nm.

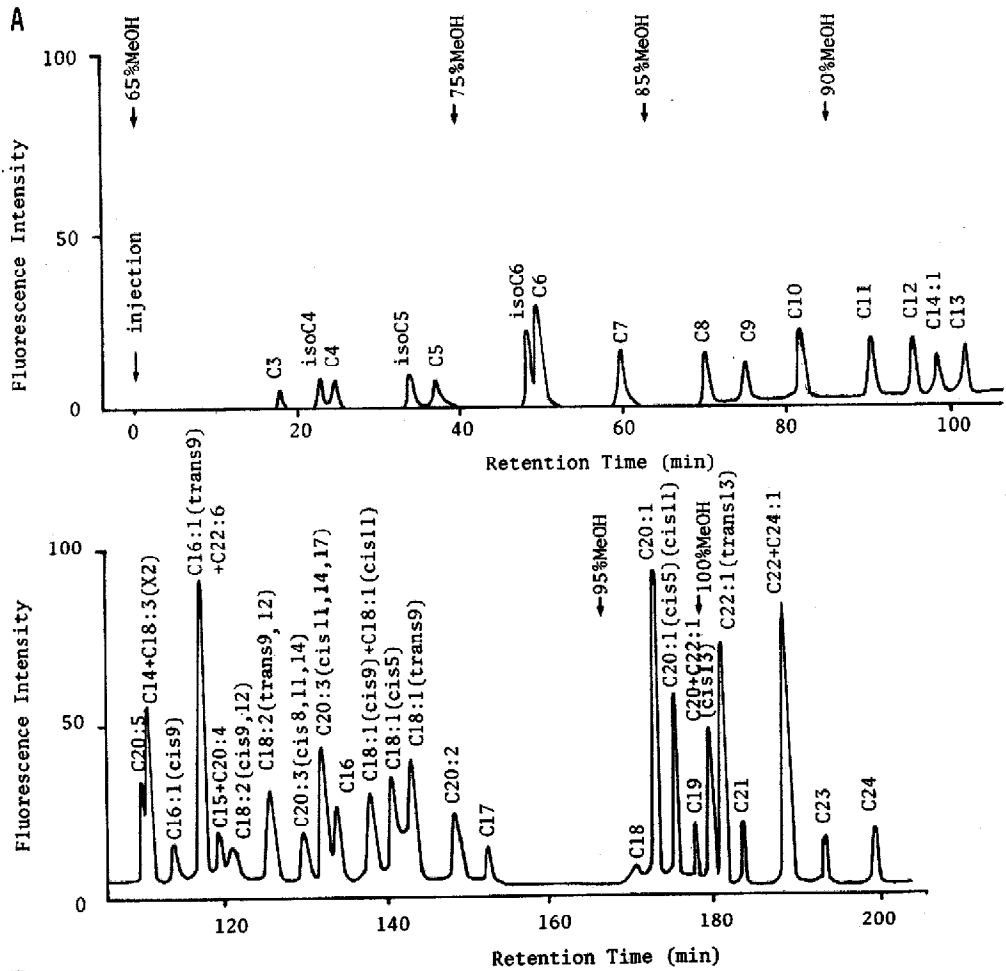

Fig. 2.

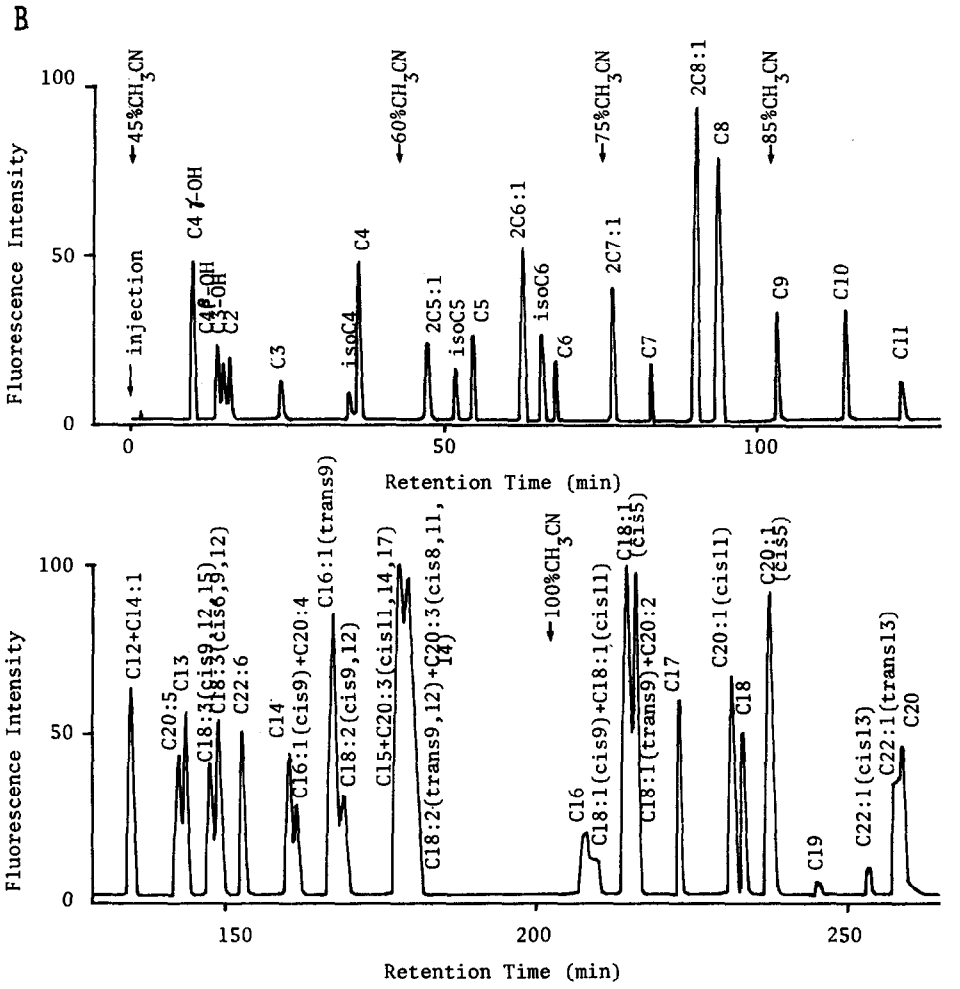

Fig. 2. Chromatograms of the dansyl derivatives of the authentic fatty acids. The HPLC conditions are described in the text. (A) Chromatogram developed by the methanolic solvent system; (B) chromatogram developed by the acetonitrile solvent system.

examined and the results are illustrated in Table II. The dansyl derivative of fatty acid $2C_{7:1}$ was eluted in the 60% acetonitrile fraction by the acetonitrile HPLC system. The eluate was diluted 100 times with 45, 60, 75, 85 and 100% acetonitrile. Each diluted solution was measured for fluorescence intensity as shown in Table III. Lower fluorescence intensities were observed at lower concentrations of solvent.

Molecular concentration and fluorescence intensity of dansyl derivatives of fatty acids

Fluorescence intensities differed in solvents of different concentrations. So, for the purpose of obtaining a constant fluorescence intensity, the eluates of each chromatographic peak were diluted 100 times with methanol or acetonitrile. These diluted solutions were observed for 40 fatty acids in methanol

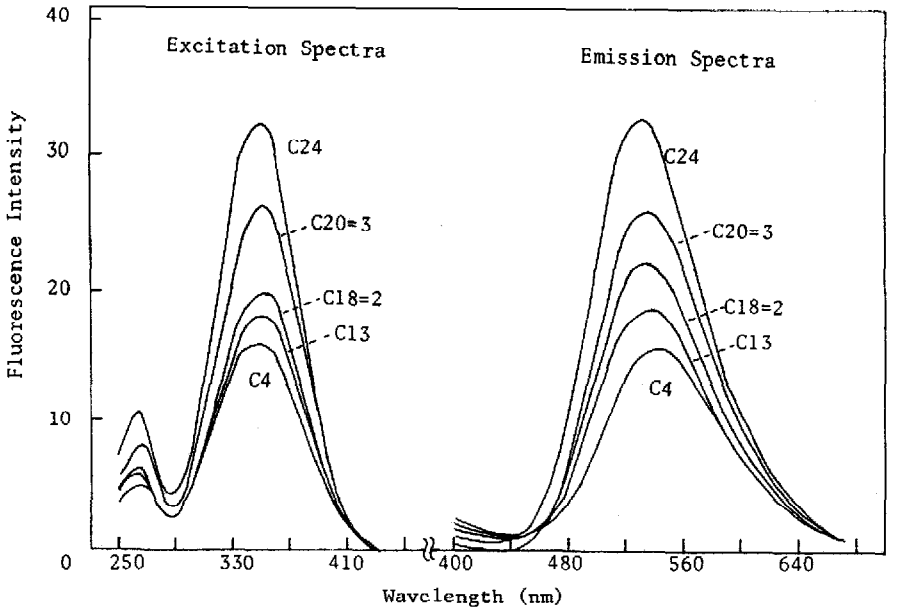

Fig. 3. Fluorescence spectra of the dansyl derivatives of the authentic fatty acids. The eluates of each dansyl derivative from the methanolic HPLC system were obtained and excitation spectra and emission spectra were measured. These derivatives were dissolved in different concentrations of the solvent corresponding to the chromatographic system.

TABLE III

VARIATION OF FLUORESCENCE INTENSITY WITH DIFFERENT CONCENTRATIONS OF ACETONITRILE

Concentration of acetonitrile (%)	Fluorescence intensity*	Ratio against the intensity in 60% acetonitrile
45	18.0	1.57
60	28.3	1.00
75	39.1	0.723
85	48.3	0.585
100	70.1	0.403

*The eluate of the dansyl derivative of $2C_{7:1}$ on the HPLC system developed with 60% acetonitrile solution was used as the test. Aliquots of the eluate were diluted 100 times with 45, 60, 75, 85 and 100% acetonitrile to prepare solutions of different acetonitrile concentration. The fluorescence intensity of each diluted solution was measured at emission wavelength 530 nm and excitation wavelength 350 nm.

and 41 fatty acids in acetonitrile. A proportional relationship was observed between molecular concentration and fluorescence intensity.

Calculation in practice. The fluorescence intensity values of the peaks eluted by 65, 75, 85, 90, 95 and 100% methanol were corrected by the ratios of 1.85, 1.38, 1.10, 1.00, 0.914 and 0.840, respectively, as illustrated in Table II. Similarly, in the acetonitrile HPLC system, these values were corrected by the ratios of 1.57, 1.00, 0.723, 0.585 and 0.403, respectively, as shown in Table III. Quantitative calculation of the fatty acids was estimated as follows.

The amount of each fatty acid in a sample was measured automatically by the Shimadzu Chromatopac C-R 1B integrator.

$$\text{Amount (nmol) of each fatty acid in serum} = \frac{\text{peak area of each fatty acid} \times \text{ratio as shown in Table II or III}}{\text{peak area of } C_{13} \text{ or } 2C_{7:1} \text{ as the internal standard}} \times \text{added amount (nmol) of } C_{13} \text{ or } 2C_{7:1}$$

In this way, the proportional relationship between molecular concentration and fluorescence intensity was observed from a few pmol/ml to 90 nmol/ml.

Analysis of human blood serum

To a glass-stoppered test-tube were pipetted 0.5 ml of the serum, 1.0 ml of chloroform solution containing 20.0 nmol of C_{13} and 9.53 nmol of $2C_{7:1}$ as internal standard, 0.1 ml of 0.5 *M* sulphuric acid, 5 ml of chloroform and 20 ml of acetonitrile. The mixture was shaken slowly and ca. 1.5 g of anhydrous calcium chloride were added. The tube was sealed with a glass stopper and

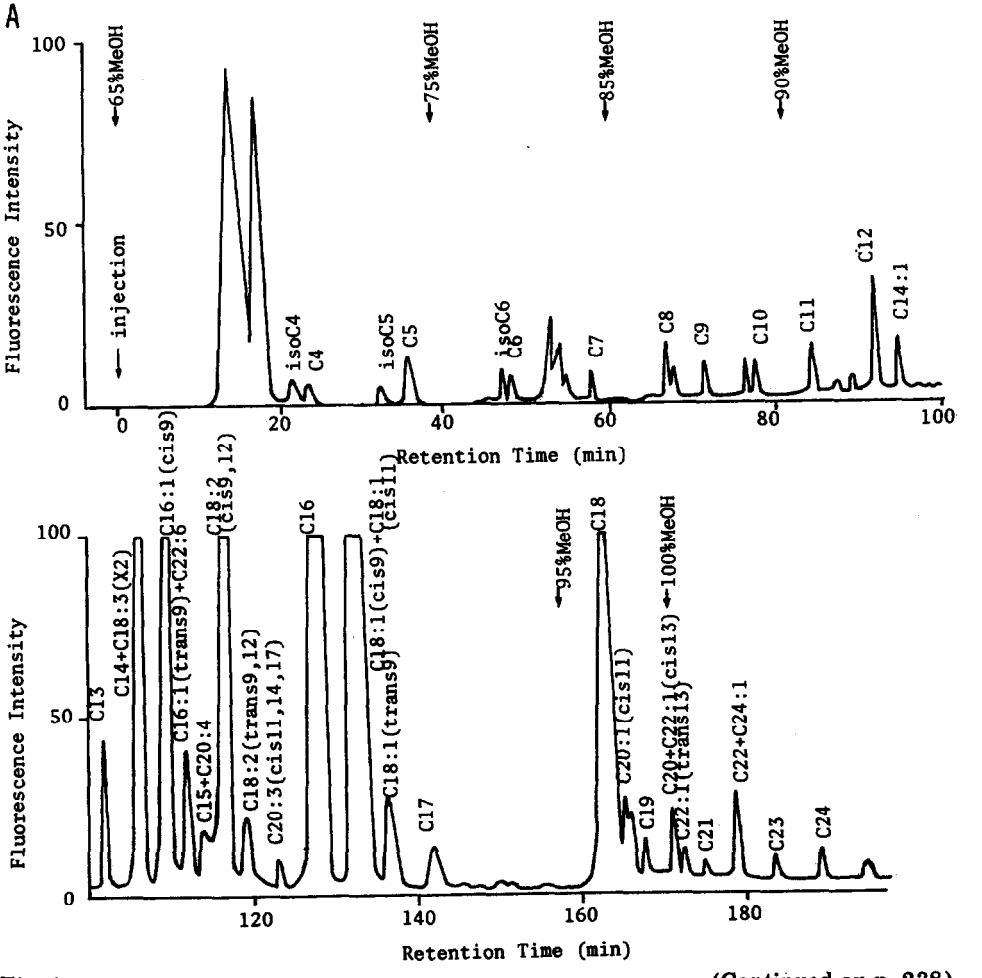

Fig. 4.

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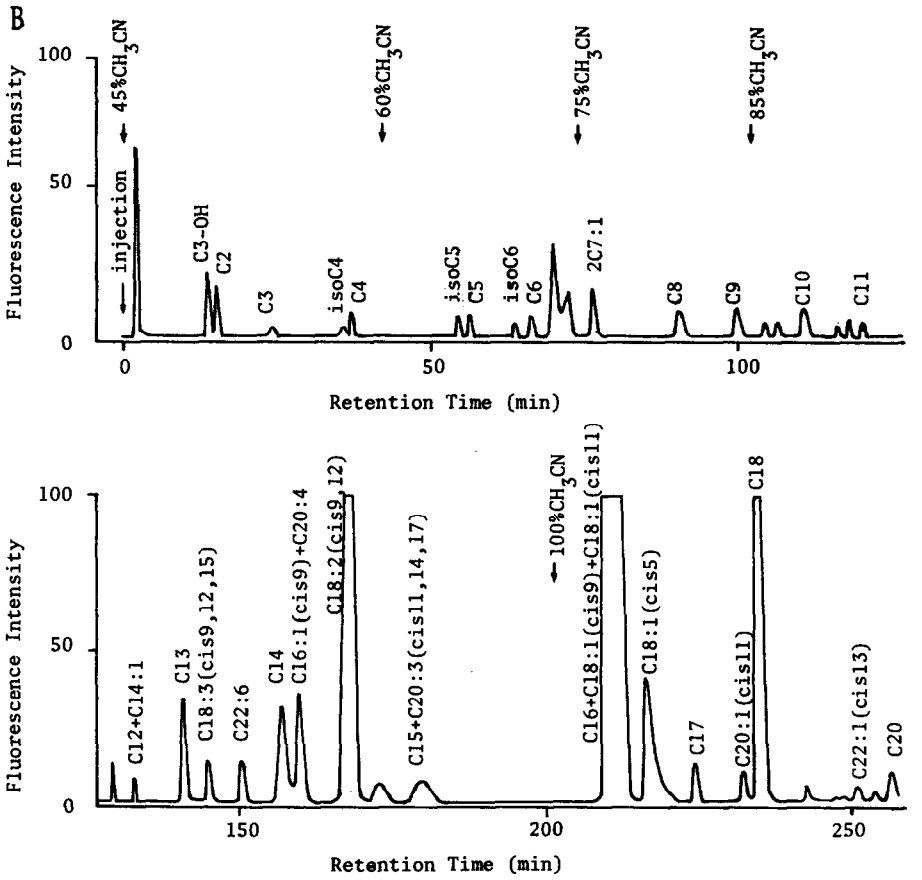

Fig. 4. Experiments applied to the serum free fatty acids. The HPLC conditions are described in the text. (A) Chromatogram developed by the methanolic solvent system; (B) chromatogram developed by the acetonitrile solvent system.

inverted slowly for mixing. After letting it stand for 30 min, the calcium chloride was precipitated out as moist, solid matter at the bottom. The upper layer was decanted for filtration through No. 5A filter paper. To the filtrate were added 22.5 μmol of dansyl semipiperazide in 0.5 ml of chloroform and ca. 150 mg of DCC. The mixture was allowed to stand for 30 min and the solvent was evaporated off at room temperature under reduced pressure. The residue was dissolved in a minimum amount of chloroform and applied to a Sep-Pak silica cartridge washed with chloroform. Then, the cartridge was developed with 1 ml of chloroform, three times. The eluate was collected and applied further to another Sep-Pak silica cartridge and developed twice with 1 ml of chloroform. The eluate was evaporated off under reduced pressure and the residue was dissolved in a minimum amount of methanol and applied to the two kinds of HPLC described above. The chromatograms are illustrated in Fig. 4A and B. The use of C_{13} or $2C_{7:1}$ as the internal standard was suitable because the fluorescent peak of the naturally contained substance did not occur near the fluorescent peak corresponding to C_{13} or $2C_{7:1}$ on the chromatogram.

TABLE IV

RECOVERY TEST

Fatty acid	Amount in 0.5 ml of serum (nmol)	Amount added to 0.5 ml of serum (nmol)	Recovery of amount added (%)
Acetonitrile HPLC system			
C_2	46.96	1.33	104.0
C_3	4.00	0.84	99.2
C_4	4.86	0.95	102.0
iso-C_5	2.37	2.88	101.0
C_5	7.02	2.54	100.3
iso-C_6	5.06	2.08	90.3
C_{12}	2.11	0.91	104.2
$C_{18:3}$ (*cis*-9,12,15)	13.75	7.02	97.5
$C_{22:6}$ (*cis*-4,7,10,13,16,19)	2.73	0.35	91.5
$C_{18:2}$ (*cis*-9,12)	27.02	12.17	101.0
$C_{20:3}$ (*cis*-11,14,17)	1.30	0.15	93.2
C_{16}	52.66	32.20	96.8
$C_{18:1}$ (*cis*-9)	42.10	4.26	94.2
C_{18}	60.01	37.20	101.8
$C_{22:1}$ (*cis*-13)	1.25	0.33	95.2
Methanolic HPLC system			
iso-C_5	5.04	3.64	92.8
iso-C_6	8.03	3.23	99.3
C_6	3.51	2.11	102.0
C_{12}	2.95	1.34	94.3
$C_{18:3}$ (*cis*-9,12,15)	18.40	3.28	91.7
$C_{18:2}$ (*cis*-9,12)	67.70	31.10	97.7
C_{16}	97.70	39.00	92.2
$C_{18:1}$ (*cis*-9)	107.60	17.82	96.4
C_{18}	24.00	7.16	101.4

Recovery test

As a control experiment, free fatty acids in 0.5 ml of the serum were analysed as described above. On the other hand, a methanol solution containing known amounts of 22 authentic fatty acids (not C_{16} or $C_{18:1}$; see Table IV) was added to 0.5 ml of the same serum and analysed for the recovery experiment. C_{16} and $C_{18:1}$ were examined separately by single additions to the serum. As shown in Table IV, the recoveries by the addition of each authentic fatty acid were over 90%. This relationship may be used for a quantitative estimation of the fatty acid in the serum.

DISCUSSION

In this experiment, gradient elution was not applied because quantitative calculation of fluorescence intensity was difficult. There is possible contamination of fatty acids in the commercial form of organic solvents and other materials, so they must be purified prior to the experiment as described in the text. The filter paper must also be washed by the solvent. In this experiment, a

good quantitative accuracy to low-molecular-weight fatty acid, which is difficult to detect by gas chromatographic techniques, was observed. In the first step of the experiment, the sample was acidified by the addition of 0.5 *M* sulphuric acid, and free fatty acids contained in the sample were converted into the non-ionic forms from the dissociated forms. The amount of 0.5 *M* sulphuric acid added to the sample must not exceed 0.3 ml in this system. An excess of the acid caused precipitation of dansyl piperazine and the effect of DCC was hindered. Formic acid and oxalic acid were not measured by this method. Both acids seemed not to have formed dansyl derivatives. Hydroxylated fatty acids (such as glycolic, lactic, β-hydroxybutyric and γ-hydroxybutyric acids) and keto acids (such as pyruvic and acetoacetic acids) were observed on the chromatogram, but these acids showed no quantitative value. DCC is known as a reagent used for the preparation of amides and esters. By DCC treatment, part of the hydroxyl group of these acids might be esterified with organic or inorganic acids in the sample. Thus, quantitative accuracy of these hydroxylated acids by the method would be hindered. When an isotopic dilution method is combined with it, quantitative estimation of these hydroxylated fatty acids is also possible. This method has an advantage in that synchronous analysis of fluorometric and radioisotopic measurement was possible in experiments on radioactive fatty acids. Furthermore, these dansyl derivatives could be analysed by TLC when developed by adequate solvents.

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