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HIGHLY SENSITIVE DETERMINATION OF FREE FATTY ACIDS IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A highly sensitive and rapid high-performance liquid chromatographic method for the determination of free fatty acids in human serum is described The fatty acids are converted into the corresponding fluorescent derivatives by the reaction with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone in the presence of potassium carbonate and 18-crown-6 in acetonitrile The derivatives are separated simultaneously within 44 min on a reversed-phase column (YMC-Pack C_8) with a gradient elution of aqueous methanol and detected fluorimetrically The detection limits are 0 5—2 fmol in a 10-μl injection volume This sensitivity permits precise determination of free fatty acids including lauric, myristoleic and linolenic acids, which occur in serum at very low concentrations, in 5 μl of sera from healthy subjects and patients with diabetes

INTRODUCTION

Free fatty acids arise mainly from the hydrolysis of triacylglycerol in adipose tissues or from the action of lipoprotein lipase, and are released into the blood stream The amounts of the acids in human serum or plasma increase or

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decrease with the extent of diabetes [1], thyremphraxis and hepatic dysfunction [2]. Therefore, the determination of the acids is very useful for the diagnosis and treatment of these diseases

High-performance liquid chromatographic (HPLC) methods with UV detection [3—6] have been used for the simultaneous determination of free fatty acids in serum or plasma The methods have a limited sensitivity and thus require a large amount of serum or plasma (0.5—1 0 ml) Accordingly, fluorescence derivatization reagents for fatty acids, 9-anthryldiazomethane (ADAM) [7] and 9-aminophenanthrene (9-AP) [8] have been applied successfully to the determination of free fatty acids in serum or plasma. The fluorimetric HPLC methods are sensitive, and require 20—50 μl of serum or plasma. However, the methods do not permit the determination of lauric ($C_{12:0}$), myristoleic ($C_{14:1}$) and linolenic ($C_{18:3}$) acids, which occur in serum or plasma at very low concentrations Recently, a fluorimetric HPLC method using 4-bromomethyl-7-acetoxycoumarin (Br-MAC) has been developed for the determination of free fatty acids in plasma [9, 10] The method is based on the reaction of Br-MAC with free fatty acids to give esters, which are separated by HPLC The esters in the eluates are hydrolysed by mixing with an alkaline solution using a post-column system and the resulting fluorescence is detected The method is sensitive enough to determine free fatty acids including $C_{12:0}$, $C_{14:1}$ and $C_{18:3}$ acids in 10 μl of plasma However, the method requires both pre- and post-column techniques and thus is rather tedious

We have developed 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (Br-DMEQ) as a highly sensitive fluorescence derivatization reagent for carboxylic acids [11, 12] The purpose of the present research is to establish a sensitive, simple and rapid HPLC method utilizing Br-DMEQ for the microanalysis of free fatty acids in a minute amount of serum. Margaric ($C_{17:0}$) acid, which is not present in human physiological fluids, is used as an internal standard

EXPERIMENTAL

Reagents and materials

All chemicals and solvents were of analytical-reagent grade, unless otherwise noted. Deionized and distilled water was used Acetonitrile used for the derivatization reaction was purified as described previously [11] $C_{12:0}$, $C_{14:1}$, myristic ($C_{14:0}$), $C_{18:3}$, palmitoleic ($C_{16:1}$), arachidonic ($C_{20:4}$), linoleic ($C_{18:2}$), palmitic ($C_{16:0}$), oleic ($C_{18:1}$), $C_{17:0}$ and stearic ($C_{18:0}$) acids were purchased from Sigma (St Louis, MO, U S A) Br-DMEQ was prepared as described previously [11, 12] Br-DMEQ (1 3 m*M*), 18-crown-6 (5 7 m*M*) and $C_{17:0}$ acid (10.0 μ*M*, internal standard) solutions were prepared in acetonitrile The Br-DMEQ solution could be used for more than a week when stored in a refrigerator at 4°C

Serum specimens were obtained from fasting healthy volunteers in our laboratories and from patients with diabetes in hospital (Kyushu Cancer Centre Hospital, Fukuoka, Japan).

Apparatus and HPLC conditions

Infrared (IR) spectra were recorded with a Shimadzu 430 spectrophotometer

in potassium bromide pellets ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a JEOL FX-100 spectrometer at 99 5 and 25 1 MHz, respectively, using ca. 5% (w/v) solution in chloroform-d containing tetramethylsilane as an internal standard Splitting patterns were designated as follows s, singlet, d, doublet, t, triplet, q, quartet, m, multiplet. In ^{13}C NMR spectra, signals were assigned by both the complete decoupling and off-resonance decoupling techniques. Electron-impact mass spectra were taken with a JEOL DX-300 spectrometer.

A Hitachi 655A high-performance liquid chromatograph equipped with a high-pressure sample injector (20-μl loop) and a Hitachi F1000 fluorescence spectromonitor equipped with a 12-μl flow-cell operated at an excitation wavelength of 370 nm and an emission wavelength of 455 nm were used The column was a YMC-Pack C_8 (150 × 6 mm I D , particle size, 10 μm, Yamamura Chemical Labs , Kyoto, Japan) This column can be used for more than 1000 injections with only a small decrease in the theoretical plate number. The column temperature was ambient (20—27°C) For the separation of the DMEQ derivatives of the fatty acids on the column, a gradient elution with aqueous 80—100% (v/v) methanol (Fig 1) was carried out by using a Hitachi 833A solvent gradient device The flow-rate was 2.0 ml/min Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow-cell, the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

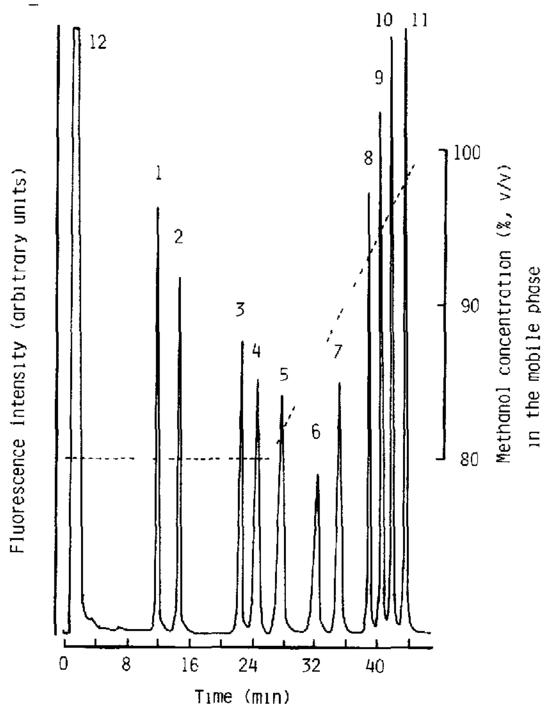

Fig 1 Chromatogram of the DMEQ derivatives of fatty acids A portion (5 μl) of a standard mixture of fatty acids (2 0 nmol each/ml) was treated according to the procedure Peaks 1 = $C_{12:0}$, 2 = $C_{14:1}$, 3 = $C_{14:0}$, 4 = $C_{18:3}$, 5 = $C_{16:1}$, 6 = $C_{20:4}$, 7 = $C_{18:2}$, 8 = $C_{16:0}$, 9 = $C_{18:1}$, 10 = $C_{17:0}$, 11 = $C_{18:0}$ acids, 12 = Br-DMEQ

Procedure

A 5-μl aliquot of serum was mixed with 200 μl of 0 5 M phosphate buffer (pH 6.5), 50 μl of the $C_{17\ 0}$ acid solution and 2.0 ml of a mixture of chloroform—n-heptane (1 1, v/v) The mixture was vortexed for ca 2 min and centrifuged at 1000 g for 5 min The organic layer was evaporated to dryness in vacuo and the residue was dissolved in 200 μl of acetonitrile A 100-μl portion of the resulting solution was placed in a screw-capped 1.5-ml vial, to which were added ca 20 mg of a mixture of finely powdered potassium carbonate and anhydrous sodium sulphate (1 1, w/w) and 50 μl each of the Br-DMEQ and 18-crown-6 solutions The vial was tightly closed and heated at 50°C for 20 min in the dark After cooling, 10 μl of the resulting mixture were injected into the chromatograph.

The calibration graph was prepared as in the procedure except that the $C_{17\ 0}$ acid solution was replaced with the solution containing 0.25 pmol to 5 0 nmol each of the fatty acids. The net peak-height ratios of the individual fatty acids and $C_{17\ 0}$ acid were plotted against the concentration of the fatty acids spiked

Preparation of the fluorescent compound from palmitic acid

Br-DMEQ (50 mg, 0 16 mmol), $C_{16\ 0}$ acid (40 mg, 0 16 mmol) and 18-crown-6 (21 mg, 0 08 mmol) were dissolved in 5 ml of acetonitrile To the solution placed in a screw-capped 10-ml test tube was added ca 500 mg of anhydrous potassium carbonate. The tube was tightly closed and heated at 80°C for ca 20 min and cooled. Potassium carbonate was removed by filtration and washed two to three times with small portions of acetone. The combined filtrates and washings were evaporated to dryness in vacuo. The residue dissolved in 5 ml of a mixture of n-hexane—ethyl acetate (3 2, v/v) was chromatographed on a silica gel 60 (ca. 75 g, 70—230 mesh, Japan Merck, Tokyo, Japan) column (25 × 2 cm I.D.) with the same solvent The main fraction was concentrated to dryness and the residue was recrystallized from n-hexane as pale yellow needles (m p. 84.5—86 5°C, 60 mg) Analytical data were as follows. IR ν_{max}^{KBr} (cm^{-1}) 1745 [O(C=O)], 1645 (C=O), 1635 (aromatic C=N); ^{1}H NMR ($CHCl_3$-d) δ 0.88—1 71 [29H, m, $(CH_2)_{13}CH_3$]; 2 48 [2H, t, $(C{=}O)CH_2$]; 3 71 (3H, s, N—CH_3), 3.95 and 4 02 (3H each, s each, O—CH_3), 7 28 and 6.69 (1H each, s each, aromatic H); ^{13}C NMR ($CHCl_3$-d) δ 14 3 (q), 22.8–32 0 (m), 34.4 (t), 56.4 (q), 56.5 (q), 63.1 (t), 95.8 (d), 111.3 (q), 127 7 (s), 128.6 (s), 146.4 (s), 150 6 (s), 152 1 (s), 178.5 (s), analysis calculated for $C_{28}H_{44}N_2O_5$ C, 68.82, H, 9.08; N, 5.73; found: C, 69.00; H, 9 25; N, 5 99. Mass spectra m/z 488 (M^+); 250 [M^+ − $CH_3(CH_2)_{14}CO$ + H, base peak].

RESULTS AND DISCUSSION

HPLC conditions

The simultaneous separation of the DMEQ derivatives of the eleven fatty acids was studied on reversed-phase columns, YMC-Pack C_8, LiChrosorb RP-8 and Radial-Pak cartridges C_{18}, C_8, phenyl and CN Isocratic elution was first examined using methanol, acetonitrile, water, 30 mM phosphate buffer (pH 4—8) and their mixtures as mobile phases The best separation of the deriva-

tives was achieved on YMC-Pack C_8 using aqueous 80% (v/v) methanol. However, the derivatives were eluted late (retention time for $C_{18\,0}$ acid was ca 72 min) with broadening of the peaks, especially those for $C_{16\,0}$, $C_{18\,1}$, $C_{17\,0}$ and $C_{18\,0}$ acids. Gradient elution with aqueous methanol served to minimize the elution times and also to sharpen the peaks Fig 1 shows a chromatogram obtained by a gradient elution with a methanol concentration between 80 and 100% (v/v) in the mobile phase The DMEQ derivatives of all the fatty acids tested were completely separated within 44 min The individual fatty acids gave single peaks in the chromatogram The change in methanol concentration actually had no effect on the fluorescence excitation (maximum, 370 nm) and emission (maximum, 455 nm) spectra and intensities of the DMEQ derivatives of all the fatty acids

Derivatization reaction conditions

The conditions for linear saturated fatty acids were described previously [11, 12] In this paper, the conditions for unsaturated fatty acids were examined using a mixture of $C_{14\,1}$, $C_{18\,3}$, $C_{16\,1}$, $C_{20\,4}$, $C_{18\,2}$ and $C_{18\,1}$ acids (20 nmol/ml each)

Br-DMEQ gave the most intense peaks at a concentration greater than ca. 0.9 m*M* in the solution for the fatty acids, 1 3 m*M* was used as a sufficient concentration Maximum and constant peak heights could be attained at 18-crown-6 concentrations in the solution in a range 3 0—12.0 m*M*; 5.7 m*M* was selected in the procedure. The peak heights reached maximum and constant values when the potassium carbonate content was higher than 2 mg, 10 mg was employed in the recommended procedure The peak heights of the DMEQ derivatives of the fatty acids were decreased slightly with increasing water concentration in the reaction mixture The addition of anhydrous sodium sulphate (50 mg) resulted in ca. 1 2 times the fluorescence intensity of that in its absence.

The derivatization reaction of $C_{18\,3}$ acid with Br-DMEQ apparently occurred

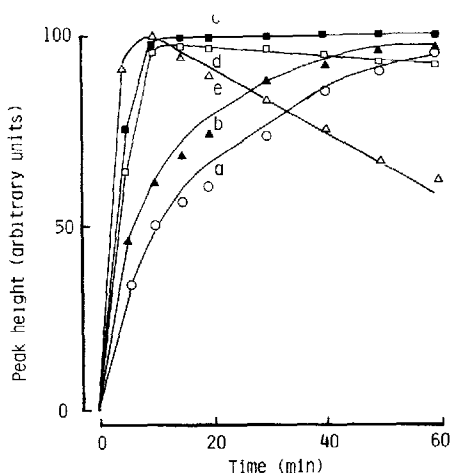

Fig 2 Effect of reaction time on the fluorescence development from $C_{18\,3}$ acid Portions (5 µl) of $C_{18\,3}$ acid (2 0 nmol/ml) were treated as in the procedure at various temperatures Temperatures a, 20°C, b, 37°C, c, 50°C, d, 80°C, e, 100°C

even at moderately low temperatures; higher temperature allowed the fluorescence to develop more rapidly (Fig 2). However, at 80—100°C, peak heights were decreased for a prolonged heating time (10—60 min) In addition, at these high temperatures, several unknown peaks, which may be due to decomposition products of the acids [1, 8], tended to appear At 50°C, the peak heights for all the fatty acids reached almost a maximum after heating for 15 min Thus, 20-min heating at 50°C was recommended in the procedure. The derivatization reaction proceeded in acetonitrile or acetone, acetonitrile was utilized tentatively because of easy purification. All the fatty acids gave similar peak heights. The DMEQ derivatives in the final mixture were stable for at least 72 h in daylight at room temperature

The detection limits were 0.5—2 fmol for the eleven fatty acids in 10 μl of injection volume at a signal-to-noise ratio of 2 The sensitivity is at least 100 times higher than that of the method with ADAM and 9-AP and ca 10 times higher than that with Br-MAC

Fluorescent products in the determination of fatty acids

In order to investigate the fluorescent product, $C_{16:0}$ acid was employed as a model compound The reaction product from the acid was confirmed as 3-hexadecanoyloxymethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone by the analytical data described under Experimental The fluorescence excitation (maximum, 370 nm) and emission (maximum, 455 nm) spectra of the product in aqueous 80—100% (v/v) methanol, which were independent of the concentration of water, were almost identical with those of the eluates for the other ten acids, respectively. These results indicate that the fluorescent products from the fatty acids should be the corresponding esters, 3-acyloyloxymethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinones

Determination of free fatty acids in serum

Free fatty acids in serum were extracted with a mixture of chloroform and *n*-heptane in the usual manner [10]. Typical chromatograms obtained with sera from a normal subject and from a patient with diabetes are shown in Figs 3 and 4, respectively. There were no interfering peaks in the chromatograms. All the peaks produced by the derivatization with Br-DMEQ were identified on the basis of their retention times and fluorescence excitation and emission spectra of the eluates in comparison with the standard compounds, and also by co-chromatography of the standards and the sera with aqueous 50—100% (v/v) methanol as the mobile phase

Free cholesterol and cephalin (as a representative phospholipid) give no fluorescent products under the derivatization conditions On the other hand, Br-DMEQ reacts with oxalic, malonic, succinic, adipic, lactic and malic acids, and acidic nucleosides to form the corresponding fluorescent derivatives [12]. However, these compounds did not interfere with both the detection and separation of the peak for all the fatty acids even when they were present at unusually high concentrations in serum (2 0 μmol/ml of serum), because DMEQ derivatives of the compounds were eluted at retention times between 1 and 8 min under the recommended HPLC conditions

Linear relationships were observed between the ratios of the peak heights of

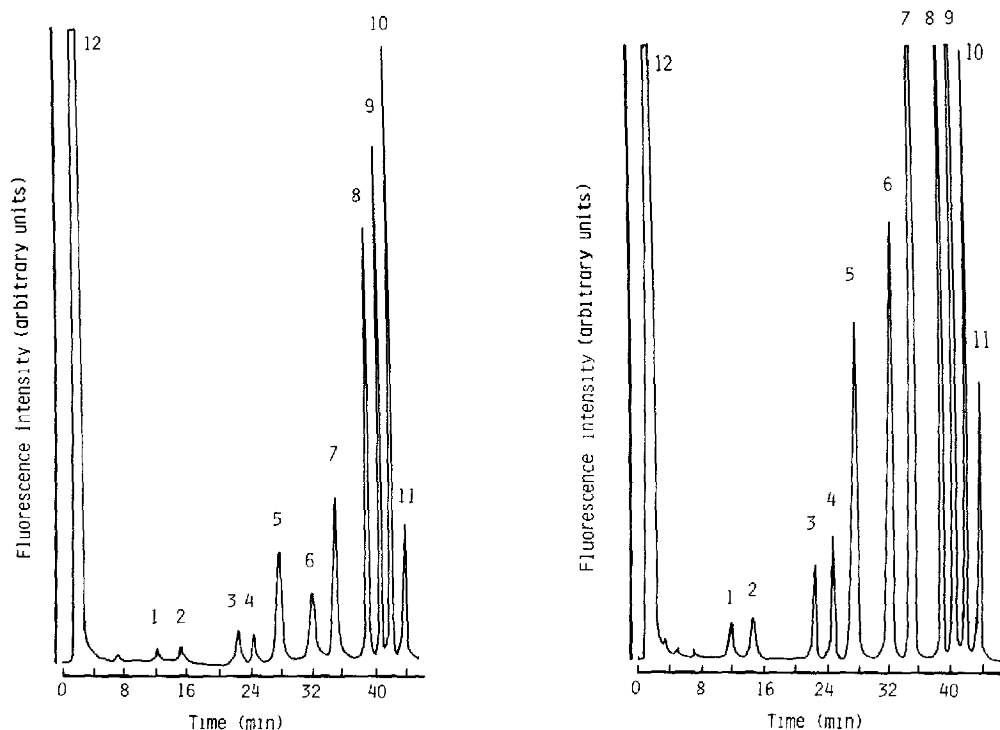

Fig 3 Chromatogram of the DMEQ derivatives of free fatty acids in serum from a normal subject A portion (5 μl) of normal serum was treated according to the procedure For peak identification, see Fig 1

Fig 4 Chromatogram of the DMEQ derivatives in serum from a patient with diabetes For peak identification, see Fig 1

the fatty acids to that of $C_{17.0}$ acid and the amounts of the fatty acids added in the range 0 25 pmol--5 0 nmol each to 5 μl of serum, no change of the slopes in the graphs of the relationships was observed, depending on the serum used. These facts indicate that the present internal standard method permits the determination of the fatty acids in serum over wide ranges of their concentrations

The recoveries (%, mean ± S.D., n = 8 each) of the fatty acids (0 2 nmol per 5 μl each, the compound in parentheses) added to a pooled normal serum were 98.3 ± 2.3 ($C_{12\ 0}$), 98.2 ± 2 8 ($C_{14\ 1}$), 100 0 ± 3.2 ($C_{14\ 0}$), 98.8 ± 1.8 ($C_{16\ 0}$), 96.2 ± 2 1 ($C_{16\ 1}$), 98.2 ± 2.8 ($C_{18\ 0}$), 103 1 ± 1 9 ($C_{18\ 1}$), 97 2 ± 2 4 ($C_{18\ 2}$), 95.4 ± 2 1 ($C_{18\ 3}$) and 98 4 ± 3.5 ($C_{20\ 4}$).

The precision was established by repeated determination (n = 20) using a normal serum The coefficients of variation were 4 5, 3.8 and 4 3% for $C_{12\ 0}$, $C_{14\ 1}$ and $C_{18\ 3}$ acids, respectively, and did not exceed 3% for the other fatty acids

The concentrations of free fatty acids in sera from healthy volunteers and from patients with diabetes were determined by this method (Tables I and II). The concentrations of all the free fatty acids were significantly increased in serum from patients with diabetes, as has also been demonstrated in another report [10] Comparison of the present method with the fluorimetric HPLC

TABLE I

CONCENTRATIONS OF FREE FATTY ACIDS IN SERA FROM HEALTHY VOLUNTEERS

Age	Sex	Concentration (nmol/ml)									
		$C_{12\ 0}$	$C_{14\ 1}$	$C_{18\ 3}$	$C_{14\ 0}$	$C_{16\ 1}$	$C_{20\ 4}$	$C_{18\ 2}$	$C_{16\ 0}$	$C_{18\ 1}$	$C_{18\ 0}$
21	M	0 3	0 8	1 2	3 0	2 6	3 6	19 8	25 1	33 1	6 2
22	M	0 3	0 8	3 9	11 2	3 7	5 8	61 4	90 7	78 9	17 9
22	M	0 1	0 4	4 1	3 5	6 6	3 2	29 3	60 0	57 5	15 1
23	M	0 9	1 7	4 3	6 3	3 2	3 0	67 1	78 1	131 1	22 8
27	M	0 7	1 9	8 5	11 5	3 6	2 3	98 3	125 8	165 8	45 9
35	M	0.7	0 3	2 6	3 7	5 6	4.5	45 1	73 2	82 5	18 6
37	M	0 7	1 0	2 5	3 3	7 8	3 6	30 7	62 1	40 1	13 8
21	F	4 4	3 7	4.3	13 4	5 5	4 0	75 0	125 1	150 8	21 8
22	F	2 1	1 3	6.4	5 1	4 7	5 8	70 4	115 6	115 7	22 6
22	F	0 7	1 0	5 0	4 9	5 8	4 4	58 1	62 8	74 7	19 1
24	F	1 2	0 8	1 0	9 1	3 1	4 1	92 1	131 4	108 1	27 0
25	F	6 4	3 7	8 5	19 4	4 4	7 8	65 5	152 6	45 6	22 8
Mean		1 5	1 5	4 4	7 9	4 7	4 3	59 4	91 9	90 3	21 2
S D		1 8	1 1	2 4	4 9	1 5	1 4	23 4	36 3	42 1	9 1

TABLE II

CONCENTRATIONS OF FREE FATTY ACIDS IN SERA FROM PATIENTS WITH DIABETES

Age	Sex	Concentration (nmol/ml)									
		$C_{12\ 0}$	$C_{14\ 1}$	$C_{18\ 3}$	$C_{14\ 0}$	$C_{16\ 1}$	$C_{20\ 4}$	$C_{18\ 2}$	$C_{16\ 0}$	$C_{18\ 1}$	$C_{18\ 0}$
32	M	7 5	4 8	25 1	36 1	30 0	87 4	216 1	387 1	876 0	85 8
40	M	5 4	5 9	36 5	25 8	25 5	90 5	386 0	487 5	502 9	65 0
45	M	9 1	4 9	17 6	22 7	16 4	64 7	225 6	298 5	886 6	63 1
55	M	4 8	11 6	33 7	42 9	66 2	44 3	350 1	505 1	420 6	75 9
57	M	8 3	7 8	27 1	21 8	17 8	53 7	355 5	255 8	388 9	90 4
43	F	4 7	5 7	32 8	35 4	76 1	119 8	401 8	365 9	554 8	52 8
47	F	5 9	8 6	25 8	37 5	17 8	154 8	286 4	387 2	563 9	68.0
55	F	6 6	3 9	30 4	37 1	56 4	42 6	300 5	234 8	336 3	45 9
Mean		6 5	6 7	28 6	32 4	38 3	82 2	315 3	365 2	566 3	68 9
S D		1 5	2 4	5 6	7 3	22 6	36 9	65 4	92 9	196 3	14 3

method using ADAM [7] was made for the normal and pathological sera The correlation coefficients were satisfactory (0 98—0 99, $n = 10$ in each case) for the fatty acids, except for $C_{12\ 0}$, $C_{14\ 1}$ and $C_{18\ 3}$ acids which could not be measured by the ADAM method. The mean values for the individual free fatty acids in normal serum (Table I) were in good agreement with the published data [10].

The present fluorimetric HPLC method using Br-DMEQ gave a satisfactory sensitivity in the quantitative analysis of free fatty acids including $C_{12\ 0}$, $C_{14\ 1}$

and $C_{18:3}$ acids, the sensitivity permits the use of only 5 μl of serum from a normal subject or a diabetic patient This method is also rapid and simple to perform and can therefore be applied for routine use

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