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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

Fatty acids are separated by reversed-phase high-performance liquid chromatography after derivatization with a fluorescence reagent, 4-bromomethyl-7-acetoxycoumarin. Each derivative eluted from a column is successively hydrolysed by mixing it with an alkaline solution, and the produced fluorescence is detected. The derivatives of series of both saturated and unsaturated fatty acids ($C_{6:0}$—$C_{20:4}$) are simultaneously separated by a continuous gradient elution method using a methanol-based solvent containing acetonitrile.

The quantitative detection of fatty acids is over a range of 5—1000 pmol per derivatization mixture. This method is applicable to the quantitative analysis of free fatty acids in normal human blood samples and blood samples from diabetic patients. Ten microlitres of

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blood plasma are sufficient to carry out the determination. The analytical results show good recovery and good reproducibility. This sensitive method is very useful for the analysis of fatty acids in very low concentrations.

INTRODUCTION

Free fatty acids in blood are derived mainly from the decomposition of triglycerides in adipose tissues or from the action of lipoprotein lipase. In spite of their concentration being lower than those of other lipids, free fatty acids are the most actively metabolized of the lipids. Thus, their analysis in biological samples is clinically of much importance. Various methods using high-performance liquid chromatography (HPLC) have been reported for this purpose [1, 2]. Although HPLC offers high resolution for the separation of a complicated mixture of fatty acids, their detection is not so easy because they generally show neither strong absorption nor fluorescence in the ultraviolet or visible region, which is widely used as the sensitive detection system for HPLC. The absorption near 200 nm has been adopted for the detection of non-derivatized fatty acids [2, 3]. However, such detection is neither sensitive nor selective. Furthermore, this method is markedly influenced by the properties of the mobile phase or impurities in it [3]. This is a disadvantage for effective application of a gradient elution technique to HPLC separation.

In order to increase sensitivity and selectivity, a number of derivatization methods for fatty acids prior to HPLC separation were developed [4—9]. In pre-column derivatizations, the use of fluorescence reagents can generally be expected to provide higher sensitivity. In this respect, 4-bromomethyl-7-methoxycoumarin [10—13], 9,10-diaminophenanthrene [14], and 9-anthryldiazomethane [15, 16], for example, were reported for the fluorometric detection of fatty acids. However, the methods using them were not very successful in the simultaneous separation of series of fatty acid derivatives. In addition, one characteristic of fluorometric detection, i.e. sensitive detection, was not sufficiently utilized for quantitative analyses such as the determination of free fatty acids in small amounts of blood samples.

A sensitive detection system for HPLC of carboxylic acids was recently developed using the fluorescence reagent 4-bromomethyl-7-acetoxycoumarin (Br-Mac) [17]. Thus, the simultaneous separation of fatty acid derivatives by this system and its application to the determination of free fatty acids in blood plasma were investigated in the present study.

EXPERIMENTAL

Reagents and apparatus

Standard fatty acids of even carbon numbers were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.) and Wako Pure Chemicals (Osaka, Japan), and those of odd carbon numbers from Nakarai Chemicals (Kyoto, Japan). Dibenzo-18-*crown*-6 was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. Br-Mac was prepared according to the method reported previously [17]. Methanol, acetonitrile, acetone, and water of

liquid chromatographic quality were used. All other reagents and solvents were of analytical grade.

The HPLC system was constructed in a manner basically similar to the one used in the previous work [17], except that fatty acids labelled with Br-Mac (FA-Mac) were successively mixed with 0.2 *M* sodium hydroxide in 80% methanol after they were eluted from the column, and the hydrolysis of FA-Mac was performed in a mixing coil (stainless-steel tube 3.5 m × 0.5 mm I.D.). Detailed chromatographic conditions are shown in Fig. 3.

Determination of free fatty acids in human blood plasma

Ten microlitres of plasma were placed in a stoppered glass vessel of 10 ml volume, and 200 μl of 0.5 *M* phosphate buffer (pH 6.5), 50 μl of a methanol solution of $C_{17:0}$ (1 nmol per 50 μl) as an internal standard, and 2.0 ml of an extraction solution (chloroform—*n*-heptane, 1:1, v/v) were added to the vessel. The mixture was vortexed for 2 min and centrifuged (1000 *g*, 10 min). The lower organic phase was evaporated to dryness. The residue was dissolved with 200 μl of acetone (100 μl × 2), and the acetone solution was transferred into a glass ampoule. After evaporating the solvent, 2—3 mg of a finely powdered mixture of potassium bicarbonate and sodium sulphate (1:1, w/w), 50 μl of dibenzo-18-*crown*-6 acetone solution (40 nmol per 50 μl), and 50 μl of Br-Mac acetone solution (100 nmol per 50 μl) were added sequentially to the ampoule. The stock solution of Br-Mac was protected from light, stored at 4°C, and renewed every three days. The ampoule was sealed and covered with aluminium foil. The reaction mixture was warmed for 30 min at 50°C in the dark. About 50 μl of the resulting solution were injected onto the column.

Determinations were carried out based on the calibration graph obtained from a standard mixture of fatty acids treated in the same manner as plasma samples.

When the concentration of free fatty acids in plasma was higher than the quantitative range, the plasma was appropriately diluted with water and then treated. On the other hand, more than 10 μl of plasma were used in the case of very low concentrations of free fatty acids.

RESULTS AND DISCUSSION

The simultaneous separation of FA-Mac was tried using continuous gradient elution according to the elution methods used by other workers [12, 13, 17]. When using a mobile phase composed of acetonitrile and water, the derivatives of fatty acids not only of the same chain length and different degrees of unsaturation but also of different chain lengths and the same degree of unsaturation could be readily separated. However, it was not easy to separate the derivatives of fatty acids having different chain lengths and different degrees of unsaturation, such as the separation between $C_{18:2}$ and $C_{14:0}$, and between $C_{20:4}$ and $C_{16:1}$.

It was reported that the concentration ratios of methanol and acetonitrile in the mobile phase significantly influenced the resolution in the isocratic separation of fatty acid phenacyl esters [18, 19]. So, the conditions to separate series of FA-Mac simultaneously were investigated by the gradient elution

method using a mobile phase of various compositions of methanol and acetonitrile. The second solvent of 90% aqueous methanol solution was continuously added to the first solvent containing different concentrations of methanol and acetonitrile, in which the water content was fixed at 30%. Fig. 1 shows the variation in retention times (t_R) of FA-Mac. Although t_R decreased with increasing acetonitrile concentration, the slopes of the curves differed among the fatty acids, as is apparent for between $C_{18:3}$ and $C_{14:0}$, and between $C_{20:4}$ and $C_{16:1}$. The separation of $C_{18:3}$ and $C_{14:0}$ was better with decreasing methanol concentration, whereas the separation of $C_{20:4}$ and $C_{16:1}$ was better with increasing methanol concentration. From these results, the first solvent of 35% methanol and 35% acetonitrile was adopted.

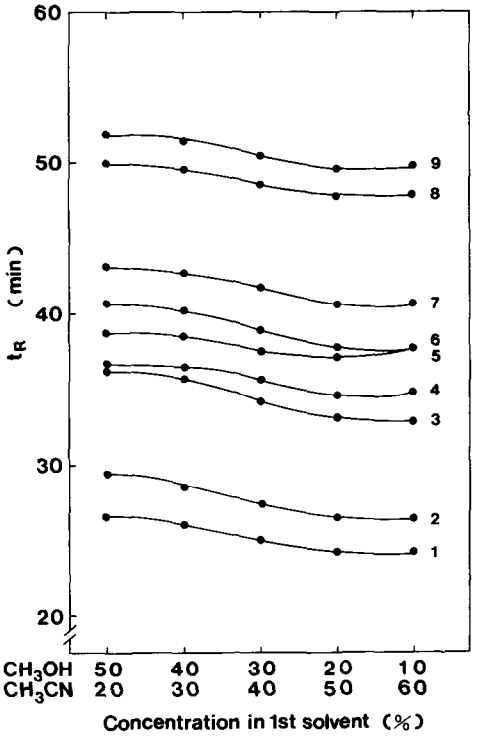

Fig. 1. Effect of methanol and acetonitrile concentrations in the first solvent on retention times (t_R) of FA-Mac. A standard mixture of FA-Mac was separated by the gradient elution method using a gradient prepared by adding the second solvent (90% aqueous methanol solution) to the first solvent (water content was fixed at 30%). The concentrations of both organic solvents are indicated. 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{20:4}$; 6 = $C_{16:1}$; 7 = $C_{18:2}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$.

As shown in Fig. 2A, t_R decreased linearly with increasing column temperature. By increasing the temperature, t_R decreased more for $C_{16:1}$ than it did for $C_{20:4}$. A similar phenomenon tended to be observed between $C_{18:3}$ and $C_{14:0}$. Fig. 2B shows typical resolution factors between adjacent peaks. A decrease of the column temperature was important for the separation of FA-Mac, especially between $C_{18:3}$ and $C_{14:0}$, and between $C_{20:4}$ and $C_{16:1}$. A column temperature of 40°C was used to shorten the analysis time and to keep the column pressure as low as possible.

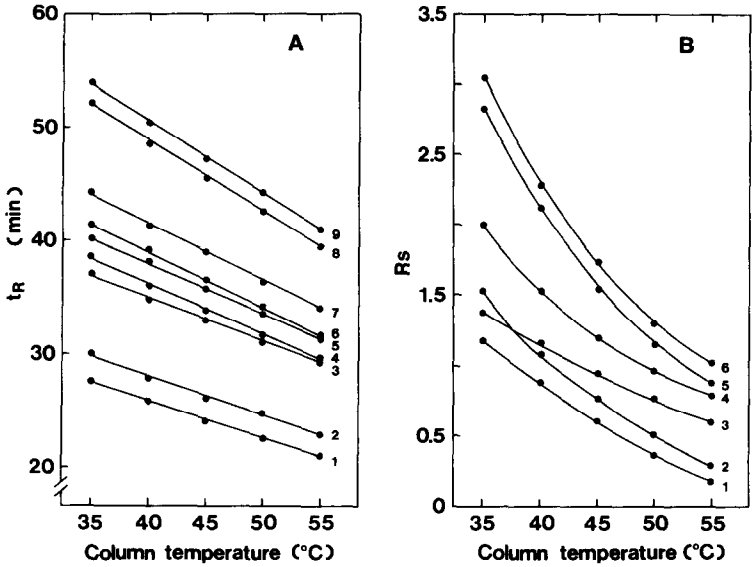

Fig. 2. Effect of column temperature on retention times (t_R) of FA-Mac (A) and on resolution factors (Rs) between adjacent peaks (B). Chromatographic conditions were the same as in Fig. 3, except for the column temperature. (A) 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{20:4}$; 6 = $C_{16:1}$; 7 = $C_{18:2}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$. (B) 1 = $C_{20:4}/C_{16:1}$; 2 = $C_{18:3}/C_{14:0}$; 3 = $C_{16:0}/C_{18:1}$; 4 = $C_{14:0}/C_{20:4}$; 5 = $C_{16:1}/C_{18:2}$; 6 = $C_{12:0}/C_{14:1}$.

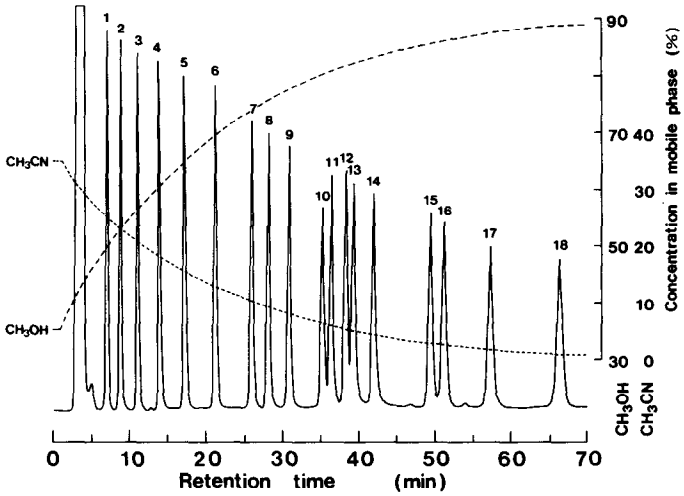

Fig. 3. High-performance liquid chromatogram obtained from a standard mixture of FA-Mac. Peaks: 1 = $C_{6:0}$; 2 = $C_{7:0}$; 3 = $C_{8:0}$; 4 = $C_{9:0}$; 5 = $C_{10:0}$; 6 = $C_{11:0}$; 7 = $C_{12:0}$; 8 = $C_{14:1}$; 9 = $C_{13:0}$; 10 = $C_{18:3}$; 11 = $C_{14:0}$; 12 = $C_{20:4}$; 13 = $C_{16:1}$; 14 = $C_{18:2}$; 15 = $C_{16:0}$; 16 = $C_{18:1}$; 17 = $C_{17:0}$; 18 = $C_{18:0}$. Chromatographic conditions: column, 250 × 4.0 mm I.D., LiChrosorb RP-18 (5 μm); column temperature, 40°C; mixing coil temperature, 50°C; mobile phase first solvent, methanol—acetonitrile—water, (35:35:30), second solvent 90% aqueous methanol solution (the gradient was prepared by adding the second solvent to the first solvent; the dotted lines show the concentrations of organic solvents in the mobile phase); mobile phase flow-rate, 1.2 ml/min; flow-rate of alkaline solution for hydrolysis, 0.4 ml/min; detector, spectrofluorometer (excitation 365 nm, emission 460 nm).

The chromatogram shown in Fig. 3 was obtained from a standard mixture of fatty acids. A methanol solution of standard fatty acids (1 nmol of each) was placed in a glass ampoule and subjected to derivatization after evaporation of the solvent. There were few significant peaks on the chromatogram other than those of fatty acids, except for the presence of the front peaks derived from an excess of Br-Mac and its decomposition products.

Fig. 4 shows the amount of FA-Mac formed (relative units) versus the reaction time at different reaction temperatures. The amount of derivative formed is proportional to the peak height ratio, i.e. the height of the FA-Mac peak relative to that of internal standard (anthracene). Even at 30°C, the reaction was completed within 40 min. The reaction was completed within 10 min at 70°C, but several unknown peaks tended to appear. In order to shorten the reaction time and minimize the production of unknown peaks, a reaction temperature of 50°C and a reaction time of 30 min were chosen in this study.

The effect of a catalyst on the reaction yield of FA-Mac is shown in Fig. 5. Although dibenzo-18-*crown*-6 is widely used as a catalyst for the derivatization of fatty acids, the effect of this reagent was not so significant in comparison with other derivatizing reagents [11, 20]. Even in the absence of the catalyst, the reaction proceeded smoothly.

A typical result of the quantitative investigation is shown in Fig. 6, which is

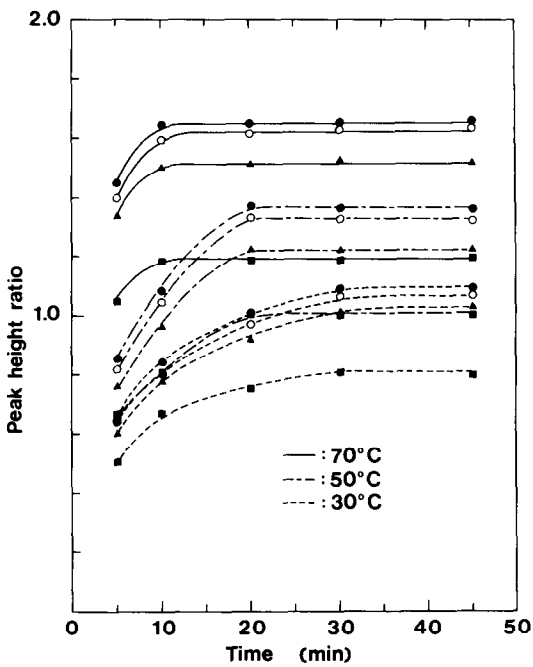

Fig. 4. Effects of reaction time and temperature on fluorescence intensity. A mixture of fatty acids (1 nmol of each) was subjected to derivatization. Chromatographic conditions were the same as in Fig. 3, except that the gradient was prepared by adding 80% aqueous acetonitrile solution to 65% aqueous acetonitrile solution. Peak height ratios were determined by dividing the peak heights of FA-Mac by the peak height of anthracene added in the reaction mixture. (●), $C_{14:0}$; (○), $C_{16:1}$; (▲), $C_{18:2}$; (■), $C_{18:3}$.

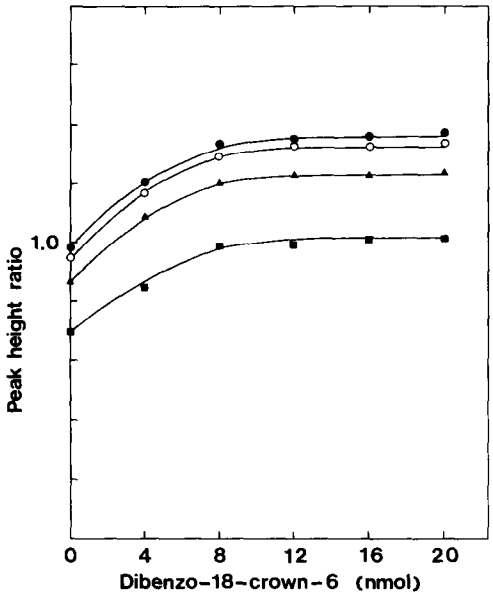

Fig. 5. Effect of dibenzo-18-*crown*-6 concentration in a reaction mixture on fluorescence intensity. A mixture of fatty acids (1 nmol of each) was subjected to derivatization for 30 min at 50°C. Chromatographic conditions as in Fig. 4. Peak height ratios were determined in the same manner as in Fig. 4. (●), $C_{14:0}$; (○), $C_{16:1}$; (▲), $C_{18:2}$; (■), $C_{18:3}$.

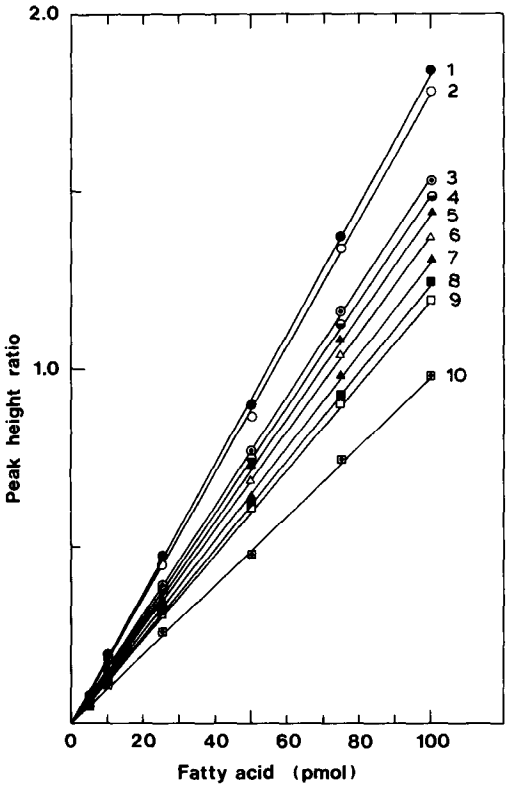

Fig. 6. Calibration graph of fatty acids. The separation was performed under chromatographic conditions shown in Fig. 3. Graphs: 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{20:4}$; 4 = $C_{14:0}$; 5 = $C_{16:1}$; 6 = $C_{18:2}$; 7 = $C_{18:3}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$; 10 = $C_{18:0}$.

a plot of the ratio of the peak height of each FA-Mac to that of internal standard ($C_{17:0}$). The plot gave linearity in a range from at least 1000 to 5 pmol (only less than 100 pmol are shown). This result indicates that the derivatization method described here can be used to quantitate fatty acids.

The method was applied to the determination of free fatty acids in human blood plasma samples. Solvent extraction was chosen for pre-purification of fatty acids in the samples because of its simplicity. The chromatograms obtained from normal human plasma and from plasma of a diabetic patient are shown in Figs. 7 and 8, respectively. There were few interfering peaks other

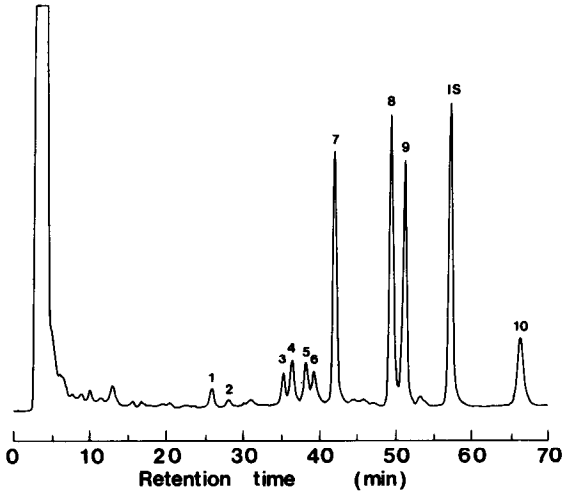

Fig. 7. High-performance liquid chromatogram obtained from a normal human plasma sample. Peaks: 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{20:4}$; 6 = $C_{16:1}$; 7 = $C_{18;2}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$; 10 = $C_{18:0}$; IS (internal standard) = $C_{17:0}$. Chromatographic conditions as in Fig. 3.

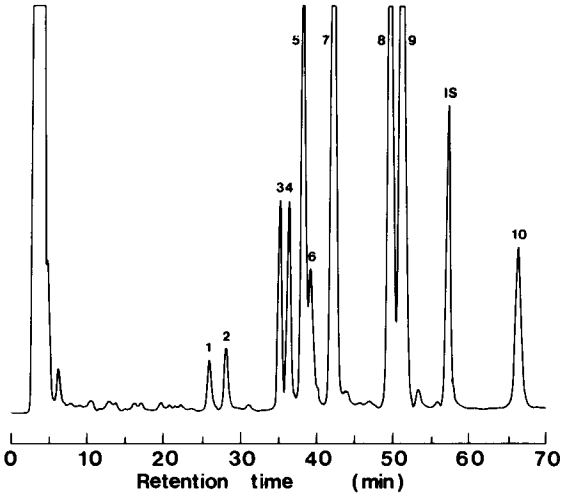

Fig. 8. High-performance liquid chromatogram obtained from the plasma sample of a diabetic patient. Peak numbers as in Fig. 7. Chromatographic conditions as in Fig. 3.

than free fatty acid derivatives, which were identified by comparing their retention times with those of standard derivatives.

Analytical recovery and reproducibility were estimated by adding a standard mixture of fatty acids ($C_{18:2}$, $C_{16:0}$, and $C_{18:1}$ = 1.00 nmol; others = 0.30 nmol) to 10 μl of plasma (n = 7). The percentage recoveries and coefficients of variation (C.V., %) were as follows: $C_{12:0}$ = 100 (2.7); $C_{14:1}$ = 97 (3.7); $C_{18:3}$ = 97 (3.7); $C_{14:0}$ = 93 (4.3); $C_{20:4}$ = 93 (3.0); $C_{16:1}$ = 97 (3.4); $C_{18:2}$ = 99 (2.0); $C_{16:0}$ = 96 (1.8); $C_{18:1}$ = 97 (2.4); $C_{18:0}$ = 90 (4.5).

The results of determinations of free fatty acids in plasma samples are summarized in Table I. They agreed with the report of other workers [9]. The concentrations of certain kinds of free fatty acids such as $C_{20:4}$, $C_{18:2}$, $C_{16:0}$, and $C_{18:1}$ significantly increased in the samples of diabetic patients. Their composition ratios in free fatty acids also differed from those of normal samples.

TABLE I

FREE FATTY ACIDS IN HUMAN PLASMA

Results are expressed as nmol/ml; — indicates that the peak was detectable but the determination was impossible.

Sample	$C_{12:0}$	$C_{14:1}$	$C_{18:3}$	$C_{14:0}$	$C_{20:4}$	$C_{16:1}$	$C_{18:2}$	$C_{16:0}$	$C_{18:1}$	$C_{18:0}$
Normal										
1	5.6	2.7	6.4	14.9	15.6	6.3	57.5	118	145	38.4
2	1.6	—	2.5	5.3	4.6	4.2	18.7	46.6	48.5	15.3
3	2.2	—	2.3	7.0	3.2	8.7	30.5	49.2	45.7	15.2
4	3.7	—	5.1	8.2	12.0	8.8	73.3	68.3	80.3	26.7
5	1.9	—	6.4	5.7	4.2	5.9	42.7	46.7	44.3	18.1
6	2.2	—	5.5	7.0	5.2	4.5	39.0	61.4	54.6	21.9
7	1.5	—	12.8	5.7	4.8	5.6	141	61.2	221	27.7
8	3.0	—	2.2	9.4	3.3	5.2	16.6	54.8	46.8	17.6
9	2.3	—	5.1	14.5	5.4	6.5	32.7	61.7	69.3	27.8
10	2.2	—	3.9	7.0	7.0	5.2	27.5	56.2	55.4	20.4
11	3.0	1.7	5.6	10.8	4.0	13.1	56.2	95.9	135	32.4
12	2.6	1.8	7.3	8.4	13.2	7.0	60.3	74.0	129	22.3
13	2.7	2.5	6.2	12.6	7.4	21.8	36.7	71.3	91.8	20.4
14	1.8	—	2.8	6.1	2.5	7.1	24.1	41.8	46.8	13.9
15	1.6	—	3.4	7.9	5.0	8.7	25.3	70.6	59.8	22.3
Diabetic patient										
1	6.3	4.9	22.6	22.2	75.5	12.6	218	258	750<	71.8
2	4.6	4.6	23.1	23.8	74.5	20.9	300	289	750<	66.7
3	5.2	4.1	38.2	38.8	60.0	15.3	240	396	506	61.0
4	9.3	13.2	26.4	42.4	172	21.9	239	454	750<	83.8
5	5.9	6.9	36.0	29.4	102	16.0	380	321	527	54.3
6	8.1	8.4	10.4	23.2	32.6	70.9	307	426	437	114

Although HPLC separation of fatty acids has been widely attempted using ultraviolet-absorbing reagents [4—10, 19], the sensitivity was not sufficient for treating very small amounts of blood samples. With such methods, at least 0.5—1.0 ml of plasma or serum was necessary for the determination [9, 19]. In this study, a plasma sample volume of 10 μl was used for precision of sampling. If precise quantitation is not required, the determination can be performed using plasma sample volumes of less than 10 μl.

CONCLUSIONS

A sensitive determination method for fatty acids was developed by employing fluorometric detection. It is considered that the high reactivity of Br-Mac and the high sensitivity are useful for treating samples containing very small amounts of fatty acids.

The simultaneous separation of series of fatty acids was achieved, which was difficult for HPLC methods using other fluorescence reagents. The analysis time per sample, however, became relatively long to obtain better resolution. It might be reduced by using another gradient elution mode or a different reversed-phase column.

REFERENCES

1 M.J. Cooper and M.W. Anders, J. Chromatogr. Sci., 13 (1975) 407.
2 M. VanRollins, M.I. Aveldano, H.W. Sprecher and L.A. Horrocks, Methods Enzymol., 89 (1982) 618.
3 M.I. Aveldano, M. VanRollins and L.A. Horrocks, J. Lipid Res., 24 (1983) 83.
4 R.F. Borch, Anal. Chem., 47 (1975) 2437.
5 P.-T.S. Pei, W.C. Kossa, S. Ramachandran and R.H. Henly, Lipids, 11 (1976) 814.
6 H.C. Jordi, J. Liquid Chromatogr., 1 (1978) 215.
7 D.P. Matthees and M.C. Purdy, Anal. Chim. Acta, 109 (1979) 61.
8 J.F. Lawrence, in R.W. Frei and J.F. Lawrence (Editors), Chemical Derivatization in Analytical Chemistry, Vol. 2, Plenum, New York, 1982, p. 191.
9 M. Ikeda, K. Shimada and T. Sakaguchi, J. Chromatogr., 272 (1983) 251.
10 W. Dünges and N. Seiler, J. Chromatogr., 145 (1978) 483.
11 S. Lam and E. Grushka, J. Chromatogr., 158 (1978) 207.
12 S.G. Zelenski and J.W. Huber, III, Chromatographia, 11 (1978) 645.
13 W. Voelter, R. Huber and K. Zech, J. Chromatogr., 217 (1981) 491.
14 J.B.F. Lloyd, J. Chromatogr., 189 (1980) 359.
15 N. Nimura and T. Kinoshita, Anal. Lett., 13 (1980) 191.
16 S.A. Barker, J.A. Monti, S.T. Christian, F. Benington and R.D. Morin, Anal. Biochem., 107 (1980) 116.
17 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, J. Chromatogr., 234 (1982) 121.
18 J. Halgunset, E.W. Lund and A. Sunde, J. Chromatogr., 237 (1982) 496.
19 M. D'Amboise and M. Gendreau, Anal. Lett., 12 (1979) 381.
20 G. Gübitz, J. Chromatogr., 187 (1980) 208.