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ANALYSIS OF FATTY ACIDS AS THEIR ANTHRYLMETHYL ESTERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

J. D. BATY* and SIMA PAZOUKI
Department of Biochemical Medicine, Ninewells Hospital, Dundee (U.K.)
and
J. DOLPHIN
Philips Analytical, Cambridge (U.K.)

SUMMARY

A reversed-phase high-performance liquid chromatographic method for the analysis of free fatty acids in plasma has been developed which allows the resolution of the major plasma fatty acids in man. A mixture of thirteen fatty acid anthrylmethyl esters is resolved on a Spherisorb 3-μm C_8 column at a flow-rate of 1 ml/min by gradient elution. The solvent system consists of acetonitrile–water (93:7) for 12 min, followed by 5 min at acetonitrile–water (86:14) and 23 min at 100% acetonitrile. The eluent is monitored with a fluorescence detector (excitation 360 nm, emission 420 nm). Three different C_{18} columns were evaluated, and none were able to resolve all the esters. To optimize the separation on the C_8 columns it was necessary to construct plots of log capacity factor *vs.* percent water in the mobile phase. The slope of the line for arachidonic acid ($C_{20:4}$) was markedly different from that for the saturated acid derivatives.

The method was applied to the analysis of plasma free fatty acids in normal and diabetic subjects. As expected, elevated levels of free fatty acids were found in diabetic subjects. The results were shown to correlate well with an established gas chromatographic assay.

INTRODUCTION

The analysis of plasma fatty acids by high-performance liquid chromatography (HPLC) is complicated by the lack of a strongly absorbing UV chromophore. The majority of HPLC methods used for the analysis of plasma fatty acids have relied on some form of derivatization to produce a compound with a suitable response. Many derivatives have been described in the recent literature and include UV-absorbing adducts, such as phenacyl[1] and its *p*-bromo[2] and methoxy analogue[3], naphthacyl esters[4], *p*-methoxyacetanilides[5], 1-chloromethylisatin derivatives[6], naphthyl esters[7] and naphthylamides[8]. Fluorescent reagents include 4-bromomethyl-7-methoxycoumarin[9,10], its 7-acetoxy analogue[11], 9-anthryldiazomethane[12–14], 9-chloro-

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methylanthracene[15], 9-aminophenanthrene[16], 9,10-diaminophenanthrene[17], 2-nitrophenylhydrazine[18], dansyl semipiperazine[19] and dansyl ethanolamine[20]. Few of these methods produce a separation of all the major fatty acids known to be present in man, because of the difficulty in resolving the derivatives of fatty acids with the same effective carbon number, *e.g.*, palmitoleic ($C_{16:1}$), linoleic ($C_{18:2}$) and myristic ($C_{14:0}$).

We have previously reported the preparation of the anthrylmethyl ester derivatives of several fatty acids with a view to their analysis by HPLC and liquid chromatography–mass spectrometry (LC–MS)[22]. We have used the derivatization method developed by Lingeman *et al.*[21]. The reaction is shown in Fig. 1. We previously reported the LC–MS analysis of these compounds[22] and an initial attempt to resolve the derivatives by gradient elution on a Zorbax 5-μm C_{18} column[23]. This method did not allow the resolution of the $C_{16:1}$, $C_{18:2}$ and $C_{14:0}$ esters, although HPLC data obtained for the other acids correlated well with that obtained by capillary gas–liquid chromatography (GLC).

The analytical problem revolves around the separation of six compounds, namely the derivatives of the $C_{12:0}$, $C_{18:3}$, $C_{20:4}$, $C_{14:0}$, $C_{16:1}$ and $C_{18:2}$ acids. In an attempt to solve this problem, we have evaluated different C_{18} and C_8 columns. The variation in capacity factors with the proportion of water in the mobile phase was studied for all six acid derivatives. The method eventually developed resolved all the major fatty acids in man and was used to analyse plasma free fatty acids from normal and diabetic subjects.

EXPERIMENTAL

The following fatty acid standards were obtained from Sigma (Poole, U.K.): $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$ (used as internal standard), $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:0}$, $C_{20:4}$, $C_{22:0}$ and $C_{24:0}$. 9-Hydroxymethylanthracene, methyl iodide and 2-bromopyridine were obtained from Aldrich (Poole, U.K.). All solvents were of HPLC grade.

Instrumentation

A PU4003 solvent delivery system comprising a dual piston pump and controller (Philips Analytical, Cambridge, U.K.) was used. This system allows gradient programming to be performed. A 20-μl loop injector (Rheodyne, Cotati, CA, U.S.A.)

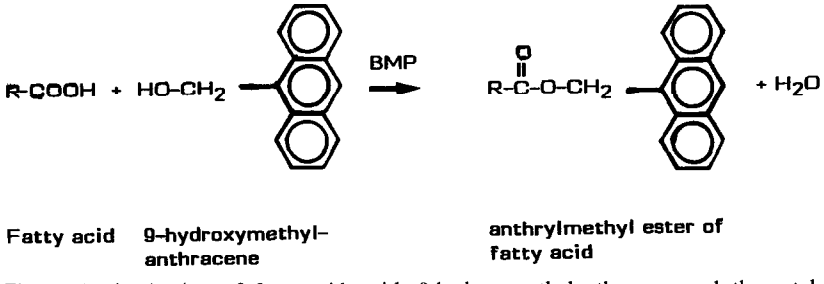

Fig. 1. Derivatization of fatty acids with 9-hydroxymethylanthracene and the catalyst 2-bromo-1-methylpyridinium iodide (BMP).

was used and the detector was a Philips Analytical PU 4024 fluorescence detector, equipped with a 360-nm excitation filter and a 420-nm emission filter. The total cell volume is nominally 25 μl. Columns were obtained from the manufacturers listed in Table I.

Subjects

Blood samples were obtained from ten diabetic subjects attending a monthly out-patient clinic and from four control subjects. The plasma glucose values of the diabetic subjects ranged from 5.8 mmol/l to 16.0 mmol/l. The diabetic subjects were being treated either by diet or drug therapy.

Fatty acid analysis

Plasma was extracted by the method of Sampson and Henley[24]. To aliquots of plasma (200 μl) in a 10-ml tube was added 3 ml of chloroform–*n*-heptane–methanol (28:21:1, v/v/v) containing 3.9 μg/ml (14.4 μmol/l) of a $C_{17:0}$ internal standard. This gave an internal standard concentration of 58.6 μg/ml (215.6 μmol/l). The mixture was vortex-mixed for 2 min and centrifuged for 20 min at 2000 *g*. The supernatant was removed, and 2 ml of the organic layer transferred to a glass vial. This solution was then evaporated under nitrogen at room temperature.

Preparation of the anthrylmethyl esters

2-Bromo-1-methylpyridinium iodide (BMP) was prepared as follows. 2-Bromopyridine (5 ml) was dissolved in 20 ml of dried diethyl ether. Methyl iodide (7 ml) was added, and the solution was gently refluxed on a water bath for 1 h. The pale yellow precipitate which formed was washed with ether, and the white crystals were used as a suspension in dichloromethane at a concentration of 20 mg/ml. The dried residue from the extraction was mixed with 50 μl of a solution of 9-hydroxymethylanthracene in dichloromethane (2 mg/ml), BMP in dichloromethane (50 μl of the suspension) and triethylamine (10 μl). After mixing (0.25 min sonication, followed by 0.25 min vortex mixing), the mixture was heated at 50°C for 30 min. The excess reagents were evaporated under nitrogen at 50°C, and the derivatised acids were taken up in 1 ml of mobile phase prior to chromatography.

Calibration curves

A standard mixture of the acids listed in the materials and methods section was prepared in heptane. The concentrations of each acid were approximately twice the normal plasma concentrations reported by Rogiers[25]. This solution was then diluted to produce five standard solutions.

RESULTS

We were unable to resolve derivatives of the following six acids: $C_{12:0}$, $C_{18:3}$, $C_{20:4}$, $C_{14:0}$, $C_{16:1}$ and $C_{18:2}$ on three different C_{18} 5-μm columns, despite using a wide range of solvent compositions. Table I shows capacity factor values for three critical acid derivatives on a number of columns. When a C_8 5-μm column was used with an isocratic solvent system [acetonitrile–water (92:8, v/v)] we observed a marked improvement in the resolution of the six derivatives (Fig. 2). However, it was not

TABLE I

CAPACITY FACTORS FOR THE $C_{12:0}$, $C_{18:3}$ AND $C_{20:4}$ DERIVATIVES ON COLUMNS WITH DIFFERENT % CARBON LOAD

Spherisorb columns from Phase Separations (Queensferry, U.K.); LiChrosorb columns from Merck (Darmstadt, F.R.G.); Nucleosil columns from Macherey-Nagel (Duren, F.R.G.); APEX-MF columns from Jones Chromatography (Llanbradach, U.K.).

Mobile phase	*Column*	*Load (%)*	*Surface area (m^2/g)*	*k'*		
				$C_{12:0}$	$C_{18:3}$	$C_{20:4}$
Acetonitrile–water (85:15)	*Spherisorb* C_8	6	220	6.77	8.66	10.08
	Apex-MF_8	7–8	170	6.61	9.33	11.48
	LiChrosorb C_8	12	250	4.41	5.32	5.97
Acetonitrile–water (92:8)	Spherisorb ODS	8	220	6.01	6.65	7.23
	Nucleosil ODS	16	300	18.12	22.71	26.80
	LiChrosorb ODS	22	150	11.51	13.65	15.84

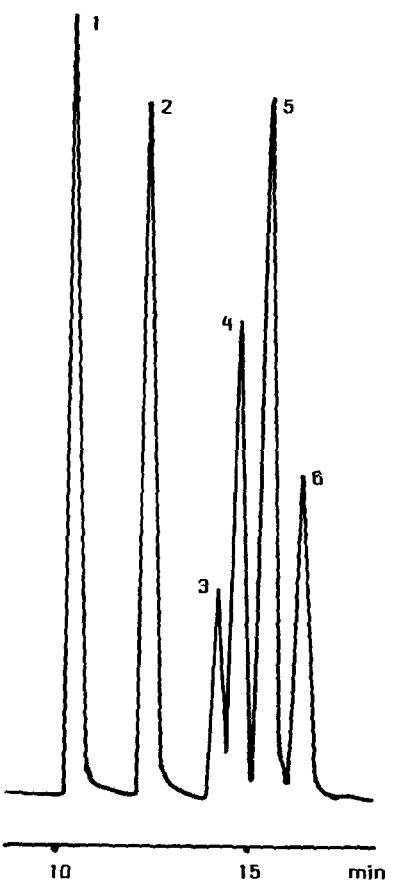

Fig. 2. Separation of the "critical" set of acid derivatives. Column, Apex MF_8; 250 mm × 4.6 mm I.D.; particle size, 5 μm; flow-rate, 1 ml/min; mobile phase, acetonitrile–water (92:8, v/v); detection, fluorescence (excitation, 360 nm, emission 420 nm). Peaks: 1 = $C_{12:0}$, 2 = $C_{18:3}$, 3 = $C_{20:4}$, 4 = $C_{14:0}$, 5 = $C_{16:1}$, 6 = $C_{18:2}$.

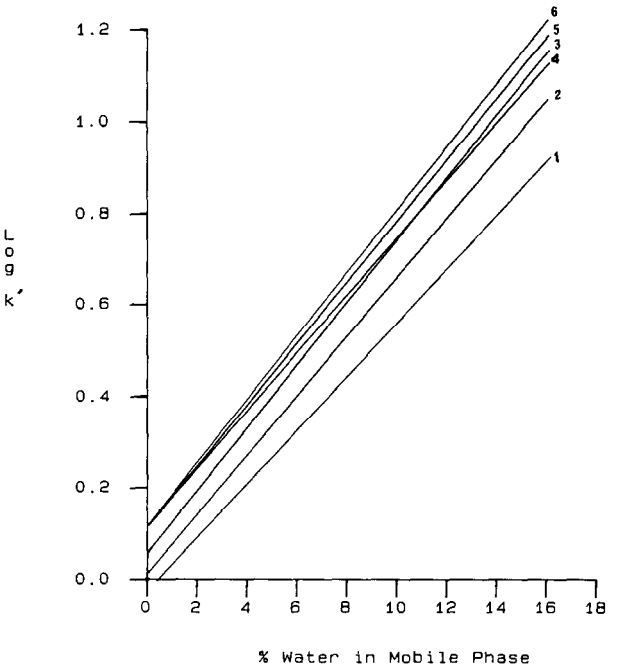

Fig. 3. Plot of log k' *vs.* percentage water in the mobile phase for the critical six acid derivatives. Column, Apex MF_8; 250 mm × 4.6 mm I.D.; particle size, 5 μm; flow-rate, 1 ml/min; mobile phase, acetonitrile–water; detection, fluorescence (excitation. 360 nm, emission. 420 nm). Lines: 1 = $C_{12:0}$, 2 = $C_{18\,3}$, 3 = $C_{20:4}$, 4 = $C_{14:0}$, 5 = $C_{16\,1}$, 6 = $C_{18\,2}$.

possible to achieve complete resolution of the six compounds under isocratic conditions, despite numerous experiments with a range of organic solvent mixtures consisting of acetonitrile, methanol, tetrahydrofuran and water. Modification of the isocratic solvent composition shown in Fig. 2 by increasing the percentage of water did not improve matters, since this caused the $C_{20:4}$ acid derivative to be eluted with the $C_{14:0}$ derivative. This led us to investigate the variation in capacity factors of all six derivatives with different acetonitrile–water mixtures. Fig. 3 shows the effect of percent water on log k' for the six acid derivatives. Fig. 4 shows the separation finally achieved.

Fatty acid levels for normal and diabetic plasma samples are shown in Table II.

To determine the precision of the method, five aliquots of a plasma sample taken from a healthy male volunteer were analysed consecutively. Values for the coefficient of variation (C.V.) for the assay, performed with fresh BMP, ranged from 8.8% for stearic acid to 10.9% for arachidic acid. These values increased to 18–26% with increasing age of the catalyst.

DISCUSSION

Much of the recent work in the field of fatty acid analysis by HPLC shows that the resolution of many critical pairs is incomplete. Some workers have not in-

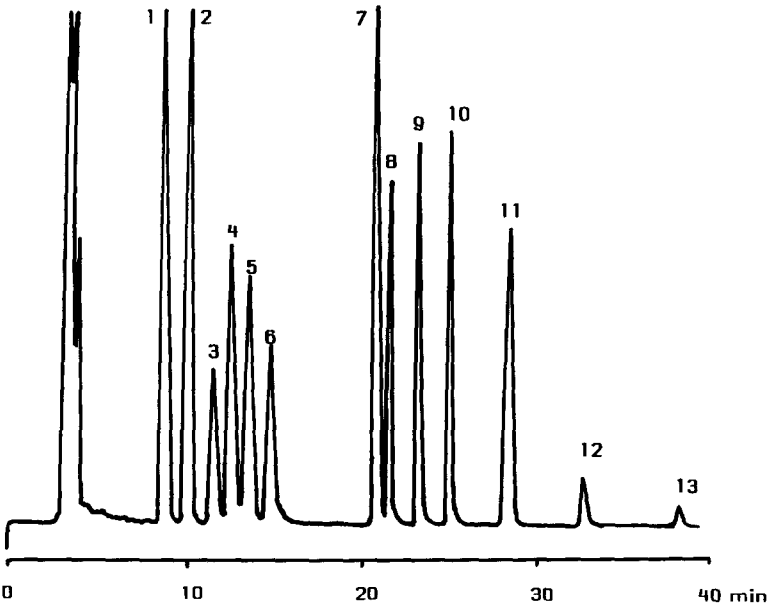

Fig. 4. Separation of a standard mixture of saturated and unsaturated anthrylmethyl derivatives. Column, Spherisorb C_8, 250 mm × 4.5 mm I.D.; particle size, 3 μm; flow-rate, 1 ml/min; gradient elution, mobile phase: acetonitrile–water, 93:7 (v/v) for 12 min, 86:14 (v/v) for 5 min, 100:0 (v/v) for 23 min; detection, fluorescence (excitation 360 nm, emission 420 nm). Peaks: 1 = $C_{12:0}$, 2 = $C_{18:3}$, 3 = $C_{20:4}$, 4 = $C_{14:0}$, 5 = $C_{16:1}$, 6 = $C_{18:2}$, 7 = $C_{16:0}$, 8 = $C_{18:1}$, 9 = $C_{17:0}$, 10 = $C_{18:0}$, 11 = $C_{20:0}$, 12 = $C_{22:0}$, 13 = $C_{24:0}$.

cluded certain physiologically important acids, such as arachidonic ($C_{20:4}$) and palmitoleic ($C_{16:1}$) acids, in the mixtures they have analysed. Where apparently good resolution of all the acids has been reported, the method has not been applied to physiological matrices[20]. The results of the present study show a considerable improvement over the existing analyses. Using a Spherisorb 3-μm C_8 column and gra-

TABLE II

FREE FATTY ACID LEVELS IN FOUR NORMAL AND TEN DIABETIC SUBJECTS

Results are given in μmole/l as mean ± 95% confidence limits. Each sample was analysed in duplicate.

Fatty acid	*Normal*	*Diabetic*
$C_{12:0}$	1.9 ± 0.8	4.1 ± 1.7
$C_{14:0}$	4.7 ± 2.2	10.8 ± 3.1
$C_{16:0}$	50.7 ± 3.8	137.6 ± 31.9
$C_{16:1}$	11.5 ± 1.9	21.1 ± 7.2
$C_{18:0}$	25.2 ± 6.9	42.0 ± 8.7
$C_{18:1}$	73.4 ± 17.3	176.4 ± 30.3
$C_{18:2}$	20.6 ± 7.1	57.9 ± 12.0
$C_{18:3}$	2.4 ± 1.3	7.8 ± 3.0
$C_{20:4}$	10.9 ± 2.0	27.9 ± 4.9

dient elution with acetonitrile–water solvents, a complete separation of all the acids of physiological importance has been achieved.

Fig. 3 shows that the logarithm of the capacity factor for the six "critical" derivatives shows a linear dependance on the percent water composition of the solvent. The slopes of the lines relating to saturated and unsaturated acid derivatives are however different, the most pronounced difference being in the slope of the $C_{20:4}$ acid derivative. At water compositions of 10–14% the arachidonic acid derivative and the $C_{14:0}$ compounds were unresolved, while at percentage water compositions below 3% the $C_{14:0}$ was eluted together with the $C_{16:1}$ and $C_{18:2}$ derivatives. It was found necessary to employ a gradient which started with 7% water and then to introduce more water for a short time (using a concave elution program) to allow adequate separation of the six derivatives. Following the separation of the six "critical" compounds, the elution of the remaining derivatives was straightforward and was accomplished by using 100% acetonitrile (Fig. 4).

Of the C_{18} columns used, none were successful at separating all the derivatives, and of the C_8 columns used, a low percent carbon load appeared to be the optimum

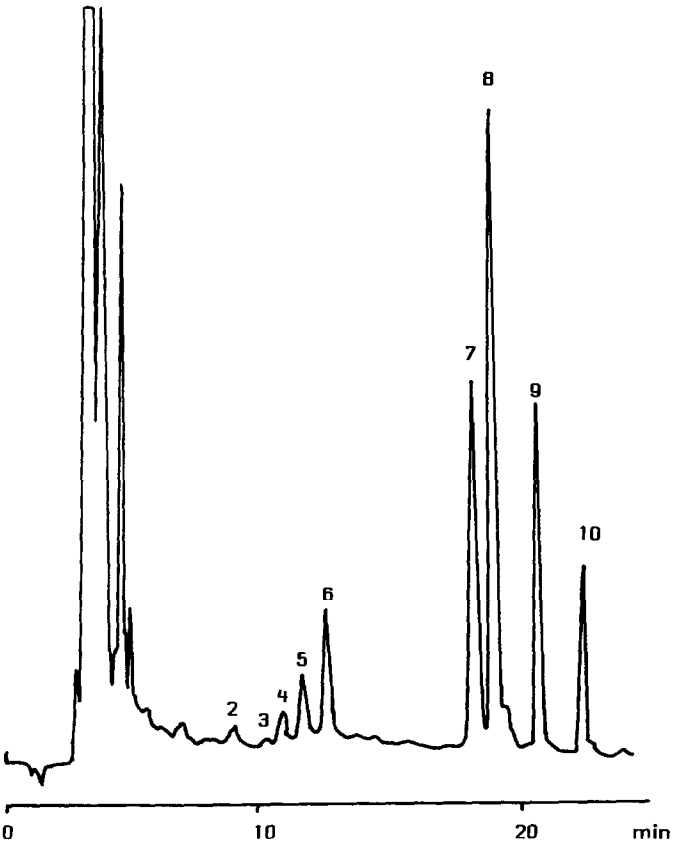

Fig. 5. Separation of the plasma free fatty acids from a healthy male volunteer as their anthrylmethyl esters. Conditions as in Fig. 4.

choice. The importance of the amount of carbon load on the column, which varies widely between columns from different manufacturers, has been discussed for the reversed-phase (RP) HPLC separation of polycyclic aromatic hydrocarbons on ODS columns by Wise and May[26]. They suggest that the differences in chromatographic retention and selectivity are a result of the utilization of different silica materials as supports and a variety of reagents and procedures to produce the bonded phases. Several studies have shown that the capacity factor, k', generally increases with increasing carbon content[27,28]. However, our results show that k' values are not always correlated with the differences in carbon content. This may be explained, as Unger *et al.*[29], and Engelhardt and Ahr[27] illustrate, by the fact that the carbon content alone is often misleading in the comparison of columns because of differences in the surface area of the original silica, which results in different surface coverage of the bonded alkyl groups, and also differences in the packing density of the stationary phase.

The precision of the assay was found to be acceptable when the catalyst, BMP, was fresh (C.V. values 8.8%–10.9%). However, with increasing age of the catalyst, the values for precision decreased (C.V. values 18–26%). This suggests that BMP is unstable and requires rigorous storage conditions. The derivatization limit of 50 ng is approximately ten times that found for the lowest concentration of fatty acid in the 200-μl sample of plasma. Results from normal and diabetic plasma samples were similar to those of other researchers[11,23,30]. A typical chromatogram from a normal subject is shown in Fig. 5. Overall, the levels of the acids were found to be higher in diabetic than in normal subjects.

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