A COMPARISON OF METHODS FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND CAPILLARY GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF FATTY ACID ESTERS

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SUMMARY

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the analysis of free fatty acids in plasma was compared with a method using capillary gas–liquid chromatography (GLC). The same extraction procedure was used for both assays. In the RP-HPLC method, the acids were separated as their anthrylmethyl esters on a C_{18} reversed-phase column, and detected by fluorescence. The coupling agent 2-bromo-1-methylpyridinium iodide was used with 9-(hydroxymethyl)anthracene. A mobile phase of acetonitrile–water (98:2) was used with flow programming. The derivatives of the C14:0, C16:1 and C18:2 acids could not be fully resolved. For capillary GLC, the acids were separated as their methyl esters following on-column injection into a 25-m OV-101 glass capillary column and detected using flame ionization detection. The esterifying agent used was diazomethane. The C18:2 and C18:3 esters were not fully resolved. The precision and sensitivity of both methods were similar.

In an application of the methods, the free fatty acid concentrations in the plasma of a group of diabetic patients and their age-matched controls were estimated. Fatty acid concentrations tended to be higher in the diabetic group but, in the small number of patients studied, wide inter-individual variations prevented a significant difference from being detected. Estimates of individual fatty acids were higher by the RP-HPLC method. The identity of the acids in the extract was confirmed by gas chromatography–mass spectrometry of their methyl esters.

INTRODUCTION

The analysis of fatty acids by gas chromatography is a long-established technique, greatly improved in recent years by the introduction of capillary columns with selective stationary phases. Analysis of plasma fatty acids by high-performance liquid chromatography (HPLC) is complicated by the lack of a strongly-UV-absorbing chromophore, and the majority of methods used for the analysis of plasma fatty

acids have relied on pre-column derivatization, combined in some cases with a post-column activation step, to produce a derivative with a suitable response. Many derivatives have been described in the recent literature and include UV-absorbing labels, such as phenacyl[1] and its p-bromo[2] or methoxy analogue[3], naphthacyl esters[4], p-methoxyacetanilides[5], 1-chloromethylisantin[6], naphthyldiazoalkanes[7] and naphthylamine[8]. Fluorescent labels have included 4-bromomethyl-7-methoxycoumarin[9,10], its 7-acetoxy analogue[11], 9-anthryldiazomethane[12-14], 9-chloromethylanthracene[15], 9-aminophenanthrene[16], 9,10-diaminophenanthrene[17], 2-nitrophenylhydrazides[18] and dansyl ethanolamine derivatives[19].

A second and perhaps more critical problem in the analysis is the actual HPLC separation of these derivatives, chiefly those of fatty acids with the same effective carbon number, e.g. palmitoleic (C16:1), linoleic (C18:2) and myristic (C14:0) acids.

We decided to prepare the anthrylmethyl ester derivatives of fatty acids, with a view to their detection by HPLC and liquid chromatography–mass spectrometry (LC–MS). In our initial work, we used the diazo derivative (ADAM), introduced by Barker et al.[13] and by Nimura and Konishita[12], but we prefer the derivatization method developed by Lingeman et al.[20] for a wide range of carboxylic acids and analogues. We have applied this method to the analysis of plasma fatty acids by HPLC. The method involves the coupling of the extracted acids with 9-(hydroxymethyl)anthracene (HMA, see Fig. 1) using the reagent 2-bromo-1-methylpyridinium iodide (BMP) prior to separation on a reversed-phase column, and analysis by fluorimetric detection. We have previously described the detection of these compounds by LC–MS[21]. To determine whether the HPLC method was adequate to deal with biological samples, we have compared it with a method based on the established procedure of capillary gas–liquid chromatography (GLC). The same sample extract was analysed by both methods.
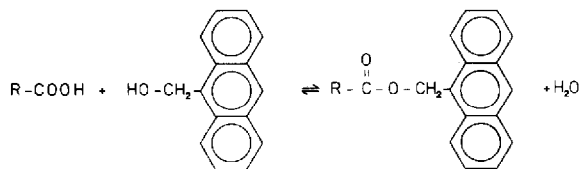

Fig. 1. Production of anthrylmethyl ester derivatives.

MATERIALS AND METHODS

The fatty acid standards (Table I) and reference samples of their methyl esters were obtained from Sigma, Poole, U.K.

9-(Hydroxymethyl) anthracene, methyl iodide, 2-bromopyridine and Diazald were obtained from Aldrich, Poole, Dorset, U.K. All solvents were of HPLC grade.

Gas–liquid chromatography

A Carlo-Erba 4160 instrument was used, with helium as the carrier gas. An on-column injection technique was used. The samples were injected at 110°C (i.e. 10°C above the solvent boiling point) using the secondary cooling on the injector,

TABLE I

CALIBRATION MIXTURE OF FATTY ACIDS

Acid	Concentration (μmol/l)
C14:0	10.9
C16:0	117.2
C16:1	21.4
C17:0 (internal standard)	215.6
C18:0	68.2
C18:1	185.7
C18:2	67.2
C18:3	8.8
C20:0	16.2
C20:4	79.2

which was switched on for at least 1 min prior to injection. Solutions were injected over 0.25 min. After completion of the injection, the syringe needle was held in the injector for 0.1 min, at which time the cooling air was switched off. An injection volume of 0.5–1.0 μl was used. Injections were made with a flexiquartz needle. Flame ionization detection (FID) was carried out at 280°C. After injection at 110°C, the column was held at this temperature for 1 min, then increased at 35°C/min to 150°C. It was then programmed at 5°C/min to 250°C and then held at 265°C for 45 min. This extended analysis time was found to be necessary when plasma extracts were analysed, in order to allow high-molecular-weight compounds to be eluted. If this was not done, the baseline in subsequent injections was not stable. The column (25 m × 0.35 mm I.D.) was a Jaeggi OV-101 column with a film thickness of 0.2 μm. A methyl silicone column, similar to OV-1 (CP-Sil 5, film thickness 14 μm, Chrompak U.K.), gave similar results.

High-performance liquid chromatography

An Altex 100A dual-piston pump was controlled by an Altex 420 programmer. A Rheodyne 7126 injection valve with a 20-μl loop, and a column (250 mm × 4.6 mm I.D.) packed with Zorbax ODS (5 μm particle size) were used. The effluent was led to a 20-μl flow-cell in a Perkin-Elmer LS-5 fluorimeter, set at an excitation wavelength of 365 nm and an emission wavelength of 412 nm. The output was analysed by a Hewlett-Packard 3388A integrator. The solvent was flow-programmed 1 min after injection, in a linear increase from 0.6 ml/min to 2.4 ml/min, and then held constant for 10 min.

Experimental techniques and methods

Blood samples were obtained from four male diabetics attending a monthly out-patient clinic and from four male age-matched controls. The plasma glucose values of the diabetic subjects ranged from 4.5 mmol/l (insulin-treated) to 7.2 mmol/l (maturity-onset diabetic). Two of these subjects were receiving oral hypoglycaemic agents. Plasma was extracted by the method of Dole[22]. To aliquots of plasma (500 μl) in a 15-ml round-bottomed tube, 25 μl of a C17 internal standard (9.93 mmol/l in methanol) were added. This gave an internal standard concentration of 472.66

μmol/l. Aliquots (200 μl) were extracted in duplicate with 5.0 ml of 2-propanol–n-heptane–1 M sulphuric acid (40:10:1) in 10-ml glass-stoppered centrifuge tubes. The mixture was shaken for 1 min and centrifuged for 5 min at 2000 g. The supernatant was poured into a second tube, and 1 M sulphuric acid (1 ml) and n-heptane (0.5 ml) were added. The tube was shaken and centrifuged as before. The upper heptane layer was transferred to a glass vial. This solution was then evaporated and analysed by the two methods.

Preparation of the methyl esters

The residue from the extraction was allowed to react with 1 ml of a 7.5 mmol/l solution of diazomethane in ether, prepared from N-methyl-N-nitroso-p-toluene sulphonamide (Diazald). After 30 min at room temperature, the ether was removed and the residue was taken up in 200 μl of heptane. Between 0.5 and 1 μl was injected into the gas chromatograph.

Preparation of the anthrylmethyl esters

BMP was prepared from 2-bromopyridine and methyl iodide following the method of Saigo et al.[23]. The pale yellow precipitate was washed with ether and the white crystals were used as a suspension in methylene dichloride at a concentration of 200 mg/ml. The dried residue from the extraction was heated with HMA in methylene dichloride (20 mg/ml), BMP in methylene dichloride (50 μl of the suspension) and triethylamine (10 μl), at 50°C for 30 min. The excess reagents were evaporated off under nitrogen at 50°C and the derivatized acids were taken up in 2 ml of acetonitrile prior to chromatography.

Calibration curves

A standard mixture of the acids shown in Table I was prepared in heptane. The concentrations of each acid were approximately twice as high as the normal plasma concentrations reported by Rogiers[24]. This solution was then diluted to produce five standard solutions each with a C17 acid concentration of 215.6 μmol/l. These solutions were used to calibrate both methods. Coefficients of variation (C.V.s) were calculated by the method of Barnett[25], the sum of the squares of the differences between duplicate observations being divided by the total number of observations. The square root of this number gives an estimate of the standard deviation from which the C.V. is obtained. Differences between means were evaluated using the Student t-test after prior use of the Fisher test. If the variance of the samples were significantly different, Cochrane's adaptation of the Behrens Fisher test, as described by Snedecor and Cochrane[26], was used.

RESULTS

The calibration lines for all the acids studied by the GC and HPLC methods were linear over the selected concentration range. For the GLC method, the worst correlation coefficient was produced with arachidonic acid (C28:4, $r = 0.9765$), while the remaining acids showed values in the range 0.9954–0.9984. The OV-101 phase was not able to separate the C18:2 and C18:3 acids, although the integrated signal did allow satisfactory calibration curves to be constructed and sample values to be
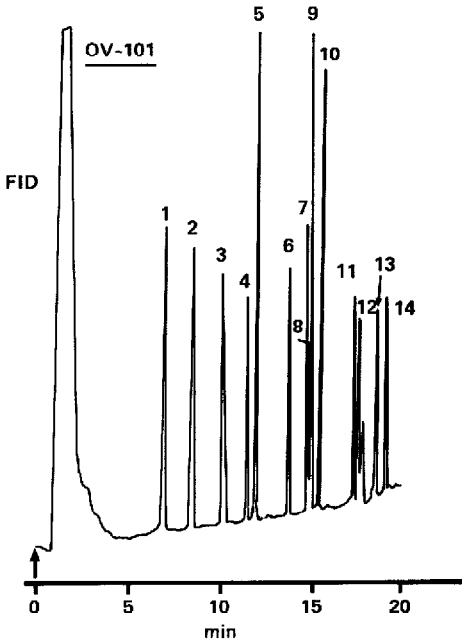
Fig. 2. Capillary GLC of standards of methyl esters of fatty acids. Peaks: 1 = C13:0; 2 = C14:0; 3 = C15:0; 4 = C16:1; 5 = C16:0; 6 = C17:0; 7 = C18:3; 8 = C18:2-; 9 = C18:1; 10 = C18:0; 11 = C19:0; 12 = C20:4; 13 = C20:1 and 14 = C20:0. For chromatographic conditions, see text.
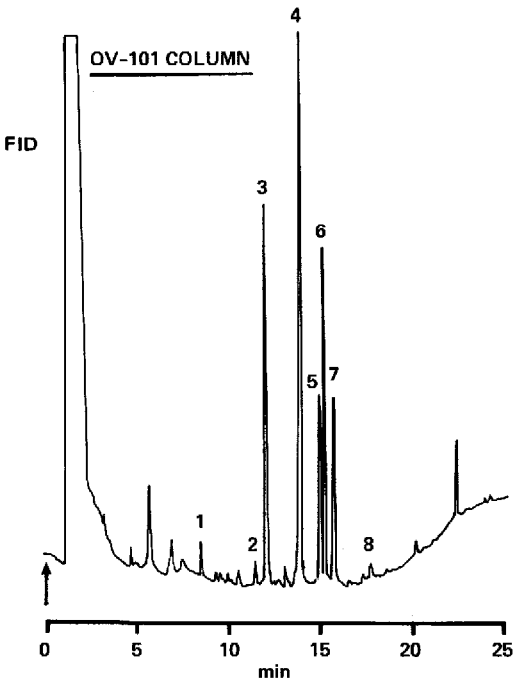

Fig. 3. GLC tracing of methyl esters in plasma from a diabetic subject. For conditions, see text. Peaks: 1 = C14:0; 2 = C16:1:3 = C16:0; 4 = C17:0 (internal standard); 5 = C18:2 + C18:3; 6 = C18:1:7 = C18:0; 8 = C20:4.

obtained for both acids. More selective phases, such as the cyanopropyl silicones, are available for this particular separation[27]. However, this was not felt to be a major problem in comparing the two techniques, since the HPLC method did not allow an accurate estimation of the 18:3 ester, and the 18:3 value recorded by the GLC method was not used in the comparison. The constitution of the peaks in the GLC analyses was checked by gas chromatography–mass spectrometry (GC–MS). Fig. 2 shows a typical chromatogram from a standard mixture, and Fig. 3 shows a GLC tracing from a diabetic subject. It was found that the anthrylmethyl esters of the fatty acids, known to be present in man, could not be fully resolved by isocratic separation, the optimum separation being achieved by flow programming, which allowed the best possible separation of the critical pair derivatives followed by rapid elution of the higher-carbon-number saturated acids. A typical chromatogram of a reference mixture of acids is shown in Fig. 4 and a chromatogram from a diabetic subject is shown in Fig. 5. The calibration data for the HPLC method also showed high correlation coefficients (r = 0.9986–0.9992). Because the C14:0 and C18:2 anthrylmethyl esters could not be resolved, they were measured as a mixed peak. The C16:1 acid, which occurs as a shoulder on the same peak, was measured via the integrated data signal.
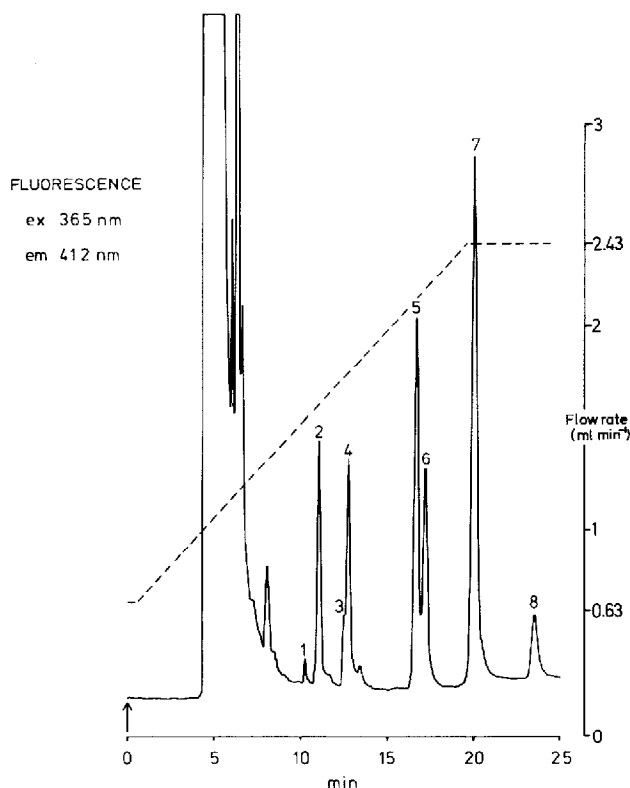

Fig. 4. HPLC tracing of anthrylmethylesters standards. Peaks: 1 = C18:3; 2 = C20:4; 3 = C16:1; 4 = C18:2 + C14:0; 5 = C18:1; 6 = C16:0; 7 = C17:0 (internal standard) + C18:0. For HPLC conditions, see text.
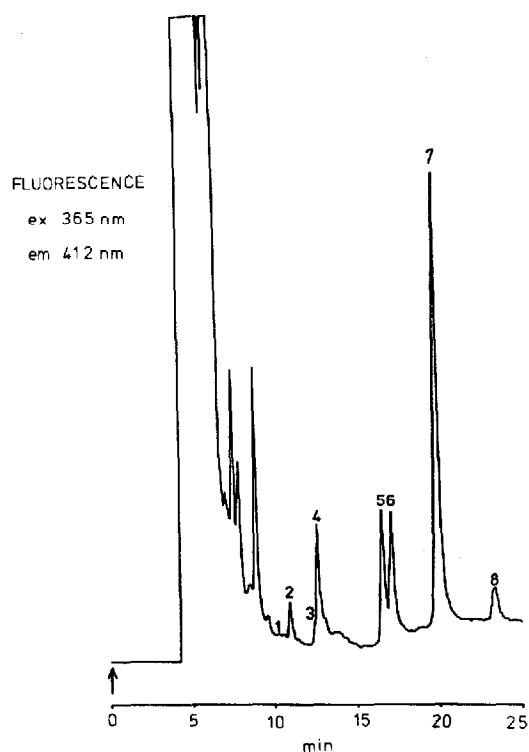
Fig. 5. HPLC trace of anthrylmethyl esters in plasma from a diabetic subject. Peak identification as in Fig. 4.

TABLE II

COMPARISON OF GLC AND HPLC IN THE ESTIMATION OF INDIVIDUAL FATTY ACID CONCENTRATIONS IN THE PLASMA OF EIGHT SUBJECTS (FOUR DIABETICS AND FOUR CONTROLS)

The diabetic and control values for each method are pooled.

Acid	Concentration ($\mu mol/l$) (mean ± S.D.)		
	GLC	HPLC	P*
C16:8	107.8 ± 90.0	127.8 ± 93.9	<0.001
C18:0	31.9 ± 17.5	44.7 ± 18.1	<0.001
C18:1	133.4 ± 133.8	129.9 ± 125.5	N.S.
C20:4	43.8 ± 2.4	15.8 ± 7.0	<0.0001
C14:0	10.6 ± 5.75	**	
C16:1	18.9 ± 16.4	**	

 * As estimated by the paired *t*-test.
 ** Values not presented, since C14:0, C16:1 and C18:2 were unresolved in HPLC and 18:2 and C18:3 were not fully resolved in GLC.

Although we were able to obtain values for these compounds, which compared well with the sum of the C16:1, C14:0 and C18:2 acid data obtained from our GLC data, we feel that despite the linear calibration graphs the sample values recorded for such compounds must be open to question, since, due to the lack of chromatographic resolution, they may well depend on the relative amounts of the other peak components. The levels of plasma fatty acids that we were able to measure in a comparative study of four control and four diabetic patients were not significantly different (due to large inter-individual variation). To compare the fatty acid values obtained by the HPLC and GLC methods, the control and diabetic data obtained by each method were thus used as a single group. They are presented in Table II. An estimate of the precision of each method was obtained from the duplicate values of total fatty acid obtained for each subject. The GLC method gave a value of 4.4% and the HPLC method a value of 4.9%.

DISCUSSION

While the extraction efficiency for the fatty acids measured was not studied in this work, there is evidence that the use of a calibration technique based on pure standards in heptane appears to be satisfactory. Using Dole's solvent mixture in a radiochemical method, Turnell et al.[28] reported a 100% recovery of palmitic acid added to plasma. Similar values have been reported for the majority of fatty acids[11]. The comparison of the two methods does not depend on the extraction parameter, since the same extract was analysed by each technique. The results obtained by the GLC method are in agreement with literature values[24], while the HPLC results are higher than those quoted by Ikeda et al., who used the naphthylamide derivative[8]. Because of the need to remove high-molecular-weight material from the column, the total GLC analysis time for plasma samples can be up to 1 h. However, the ability of capillary GLC to measure all the fatty acids of interest suggests that it is the superior technique. The HPLC method, while it is sensitive and specific, fails to separate certain critical pairs of acids, e.g. C14, C18:2 and C16:1. An attempt was made to improve the resolution of the derivatives by altering the composition of the mobile phase while maintaining a constant mobile-phase strength. Such an approach has been reported to be successful in the separation of the p-bromophenacyl esters[2]. The data used in the calculation of the mobile-phase strengths were those of Snyder and Kirkland[29]. As Table III indicates, none of the solvent systems was able to resolve the C16:0 and C18:1 derivatives fully. A major parameter in such separations appears to be the quality of the column itself. Halgunset et al.[2] reported an excellent separation of the p-bromophenacyl esters in isocratic elution from a Supelcosil 5-μm column. They state that two other columns were unable to perform the separation with the same mobile phase. Different elution orders have been reported for the anthrylmethyl esters. Barker et al.[13] found that on an MCH-10 C_{18} column, the C18:1 acid derivative was eluted before the C16:0. Nimura and Konishita[12] found the reverse elution pattern when using a LiChrosorb RP-8 5-μm column, but the same solvent combination. In our system, the C16:0 is eluted after the C18:1. To our knowledge, no separation of the anthrylmethyl esters of plasma fatty acids has been produced that gives baseline separation. The paper by Nimura and Konishita[12], although showing excellent resolution of most of the acids apart from C18:1 and

TABLE III

THE EFFECT OF COMPOSITION OF THE MOBILE PHASE ON THE RESOLUTION OF ANTHRYLMETHYL ESTERS OF C16:0 AND C18:1 ACIDS

Mobile phase composition	Solvent strength	Selectivity*
Acetonitrile–acetone (90:10)	3.13	1.05
Acetonitrile–2-propanol (91.9:8.1)	3.13	1.07
Acetonitrile–tetrahydrofuran (92.3:7.7)	3.2	1.06
Acetonitrile–methanol (88.7:11.3)	3.09	1.06
Methanol	3.0	1.0

* Selectivity $= k'$ C16:8/k' C18:1.

C16:0, does not include data on palmitoleic acid (C16:1) and arachidonic acid (C20:4), both significant acids in plasma. Although our HPLC estimate of the total amounts of each fatty acid recorded (including the acids measured as unresolved peaks) showed a remarkably good correlation with the corresponding value from the GLC method ($r > 0.99$, $n = 8$), the HPLC method is not optimized. A plasma sample with a particularly high or low acid value relative to an acid from which it is not well separated may produce erroneous data. We are currently attempting to improve the separation by using a combination of increased column efficiency (3 μm particle size), combined flow and gradient programming, a smaller detector flow-cell and the use of silver to interact with the unsaturated acid derivatives, since a system of acetonitrile–methanol–20 mM aqueous silver nitrate is reported to give excellent separation of the acyl dansyl ethanolamine derivatives of the saturated and unsaturated acids[19].

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