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Note

Separation of the 9-anthryldiazomethane derivates of fatty acids by high-performance liquid chromatography on a Fatty Acid Analysis Column®

Application to albumin-bound fatty acid analysis

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Methods for the separation of fatty acids (FAs) by high-performance liquid chromatography (HPLC) after chemical derivatization in fluorescent compounds have been developed successfully in the last few years. The principal derivatizing reagents reported so far are phenacyl bromide [1], 2-naphthacyl bromide [2], *p*-bromophenacyl bromide [3], 1-benzyl-2-talyl-triazene [4], O-*p*-nitrobenzyl-N,N′-diisopropylisourea [5], 4-bromomethyl-7-methoxycoumarin [6], pentafluorobenzyl bromide [7], 1-naphthylamine [8], Dns-ethanolamine [9], panacyl bromide [10] and 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone [11]. While these methods give the main advantage of a very low limit of detection in the picogram range, they allow nevertheless the quantitation of only the major classes of FAs, generally from C_{14} to C_{20}. In 1966, Nakaya et al. [12] developed a very sensitive

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fluorescent technique for labelling FAs, which has been applied subsequently to a traditional reversed-phase HPLC system (μBondapak C_{18}) by Barker et al. [13] and by Nimura and Kinoshita [14] to separate about ten different kinds of FAs. However, this method still presents incomplete separation of some important critical pairs, such as palmitic acid and oleic acid. Furthermore, there is a considerable limitation to the application of this system for studying FAs bound to serum albumin since this protein contains, in addition to the main classes of FAs from C_{16} to C_{18}, discrete amounts of medium- and short-chain FAs from C_6 to C_{14}. This has led us to develop a new HPLC system for the separation of a wide range (from C_6 to C_{20}) of fluorescent FAs, derivatized by 9-anthryldiazomethane (ADAM) [12]. The new technique was successfully applied to the study of albumin-bound FAs in normal children.

EXPERIMENTAL

Reagents

9-Anthraldehyde, hydrazine hydrate and manganese dioxide were obtained from Aldrich (Steinheim, F.R.G.); high-purity FA standards were from Serva (Heidelberg, F.R.G.); spectrograde acetonitrile, methanol, 2-propanol and water were from Merck (Darmstadt, F.R.G.); Affi-Gel-Blue F_3-GA was from Bio-Rad (Richmond, CA, U.S.A.).

Purification of human serum albumin (HSA)

HSA was purified from sera of six normal, fasting children (mean age 5.5 ± 2.1 years) by pseudo-ligand chromatography on Affi-Gel-Blue F_3-GA (4.5 μg/ml), packed in a 7 × 1.5 cm I.D. column according to a modification [15] of the original method of Travis et al. [16]. A 1-ml aliquot of serum was applied to the column and processed further.

Apparatus

For HPLC separation of FAs, an isocratic Waters (Milford, MA, U.S.A.) system was used, equipped with a Model 45 pump, a Rheodyne injector with a 20-μl loop, a Model 420 fluorescence detector with an excitation filter at 365 nm and an emission filter at 415 nm, and a Model 740 data module.

HPLC conditions

Separation of FAs was carried out on a Fatty Acid Analysis Column® (300 × 3.3 mm) (Waters) at room temperature. Final conditions for the mobile phase were: (i) acetonitrile—methanol—2-propanol—water (100:5:5:60) for 150 min and (ii) acetonitrile—water (100:40) for 100 min at a constant flow-rate of 0.6 ml/min. For comparison, a μBondapak C_{18} (Waters) 10-μm column (300 × 3.9 mm) was used, with the conditions originally developed by Barker et al. [13]. Identification of peak components was performed by comparison with the retention times of known individual standards.

Preparation of 9-anthryldiazomethane

9-Anthraldehyde hydrazone was prepared by the reaction of 9-an-

thraldehyde with hydrazine hydrate according to the method of Nakaya et al. [12]. The hydrazone (0.22 g; 0.001 mol) was dissolved in 100 ml of anhydrous diethyl ether; 0.8 g of activated manganese dioxide and 0.6 ml of a saturated ethanol solution of potassium hydroxide were then added. The reaction was stirred and stopped by filtering the manganese dioxide off after 15 min. The evaporated solid crystals were kept at −20°C.

FAs were derivatized by incubating 0.25 ml of the mixture with 0.25 ml of ADAM solution (1 mg/ml) for 3 h in the dark.

Extraction of FAs

For the extraction of free FAs from serum we adopted the method of Bligh and Dyer [17], according to which 1 ml of serum was mixed with 10 ml of methanol—chloroform (2:1) and the extract after centrifugation was diluted with chloroform—water (2:1) to form a biphasic system.

To extract FAs hydrophobically bound to albumin, 4 ml of albumin solution (1 mg/ml) were incubated with 10 ml of chloroform—methanol (2:1); insoluble protein was separated by centrifugation at 15 000 *g* for 15 min and the precipitate was washed four times with the same solution [18]. Excess solvent was removed with a stream of nitrogen at room temperature.

RESULTS AND DISCUSSION

Effects of mobile phase composition

Preliminary studies demonstrated that FAs can be separated isocratically on a Fatty Acid Analysis Column (Waters) with a mobile phase of acetonitrile—water—tetrahydrofuran (THF). We tried to separate, on the same column, the ADAM derivates of FAs in order to take advantage of the increased sensitivity given by the fluorescence technique and to develop a method for analysing FAs in biological fluids. This aim was achieved by using two mobile phase systems: (i) acetonitrile—methanol—2-propanol—water (100:5:5:60) for 150 min and (ii) acetonitrile—water (100:40) for 100 min. Even small changes from this composition were found to produce a marked variation of FA retention times. For example, increasing acetonitrile, methanol or propanol resulted in an unsatisfactory separation of palmitic acid with oleic acid and of myristoleic acid with palmitoleic acid. In general, the retention times of all single ADAM derivatives of the FAs increased directly as a function of the water content of the mixture and decreased when the percentage of acetonitrile was increased.

Separation of a mixture of ADAM—FA

Fig. 1 shows that a mixture of the ADAM derivatives of major classes of human FAs can be separated, using the conditions described here, in addition to a lot of non-human FAs. For human FAs, all the major serum constituents such as myristic, linoleic, palmitic, linolenic, oleic and stearic acids are readily separated together with shorter FAs such as caproic, caprylic, capric and lauric acids. For non-human FAs, the major species separated are pelargonic acid, erucic and nervonic acids. For their positions on the chromatogram, each of these plant FAs can be used as an internal standard in biological assays; we

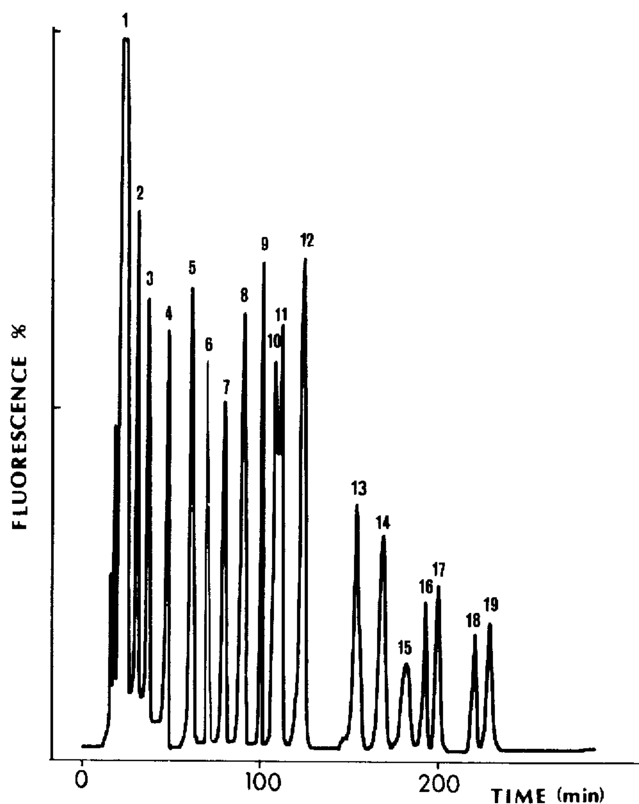

Fig. 1. Chromatogram of a mixture of ADAM derivates of FAs. Authentic FAs were derivatized with ADAM and analysed by HPLC on a Fatty Acid Analysis Column (Waters). Mobile phases were: (i) acetonitrile—methanol—2-propanol—water (100:5:5:60) for 150 min; (ii) acetonitrile—water (100:60) for 100 min. Peaks: 1 = ADAM; 2 = caproic acid; 3 = caprylic acid; 4 = capric acid; 5 = lauric acid; 6 = myristolic acid; 7 = myristic acid; 8 = linolenic acid; 9 = linoleic acid; 10 = palmitic acid; 11 = arachidonic acid; 12 = oleic acid; 13 = stearic acid; 14 = *cis*-11-eicosanoic acid; 15 = unknown; 16 = arachidic acid; 17 = erucic acid; 18 = behenic acid; 19 = neuronic acid. The identification of each peak component was performed by comparison with retention times of known individual standards.

chose erucic acid, eluting after stearic acid. Finally, *cis*-2-endocanoic acid ($C_{20:1}$)*, an FA of unclear occurrence in human serum, may also be determined by our method. Recovery for each individual FA added separately to serum was nearly 100%. Retention times for each individual FA are reported in Table I. The lower limit of detection, based on a peak-height versus baseline-noise ratio of 3:1, was 5 pmol/μl for FAs from C_6 to C_{14} and 7 pmol/μl for longer molecules owing to peak broadening. The highest limit of detection experimentally determined was 120 pmol/μl. A good linearity between the two limits was found by analysing four intermediate concentrations for each individual FA ($r > 0.9$ on 40 determinations). The intra-assay coefficient of variation evaluated by ten successive analyses of the same mixture was < 2.5%.

*Fatty acids are denoted by the numbers of carbon atoms and the number of double bonds; thus, 20:1 represents 20 carbon atoms and 1 double bond.

TABLE I

RETENTION TIMES OF 9-ANTHRYLDIAZOMETHANE DERIVATES OF FAs

Stationary phase: Fatty Acid Analysis Column (Waters). Mobile phase: (i) acetonitrile—methanol—2-propanol—water (100:5:5:60) for 150 min. (ii) acetonitrile—water (100:60) for 100 min.

Fatty acid	Retention time (min)	Fatty acid	Retention time (min)
C_6	18.7	C_{16}	101.8
C_8	25.3	$C_{18:1}$	114.1
C_{10}	34.8	C_{18}	148.1
C_{12}	49.1	$C_{20:1}$	163.9
$C_{14:1}$	54.7	C_{20}	191.3
C_{14}	70.0	$C_{22:1}$	197.4
$C_{18:3}$	78.1	C_{22}	219.8
$C_{18:2}$	91.8	$C_{24:1}$	227.5
$C_{20.4}$	95.6		

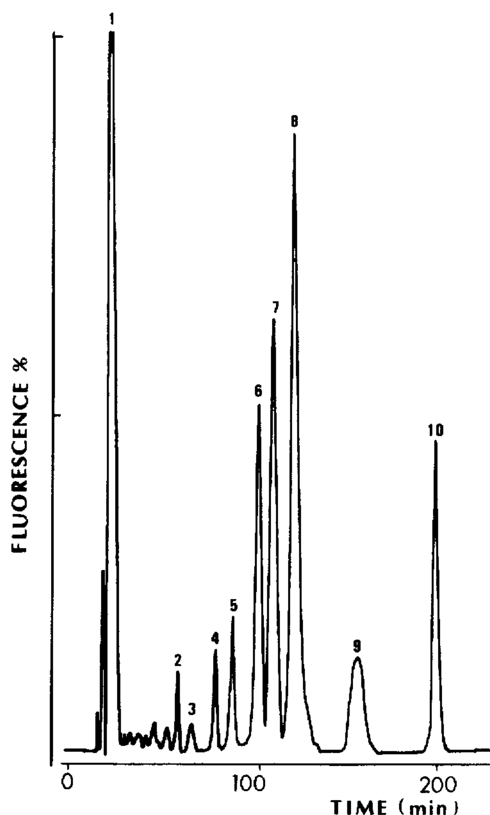

Fig. 2. Chromatogram of ADAM derivates of serum FAs extracted by the procedure of Bligh and Dyer [17] from a normal child (aged 5 years). Peaks: 1 = ADAM; 2 = lauric acid; 3 = myristoleic acid; 4 = myristic acid; 5 = linolenic acid; 6 = linoleic acid; 7 = palmitic acid; 8 = oleic acid; 9 = stearic acid; 10 = erucic acid.

Comparison of Fatty Acid Analysis Column with traditional reversed-phase systems for the analysis of ADAM derivates of FAs

The technique for labelling FAs with ADAM was first applied to a traditional

TABLE II

FREE FATTY ACID COMPOSITION OF SERUM FROM SIX NORMAL CHILDREN

Results are given as means ± standard error of the mean.

Fatty acid	Composition (nmol/ml)
C_{12}	29.8 ± 7.8
$C_{14:1}$	15.9 ± 7.3
C_{14}	38 ± 22.1
C_{16}	360.3 ± 80.3
$C_{18:3}$	96.2 ± 45.1
$C_{18:2}$	200.3 ± 35.5
$C_{18:1}$	666.7 ± 187.1
C_{18}	126.5 ± 22.6
$C_{20:4}$	16 2 ± 2.1

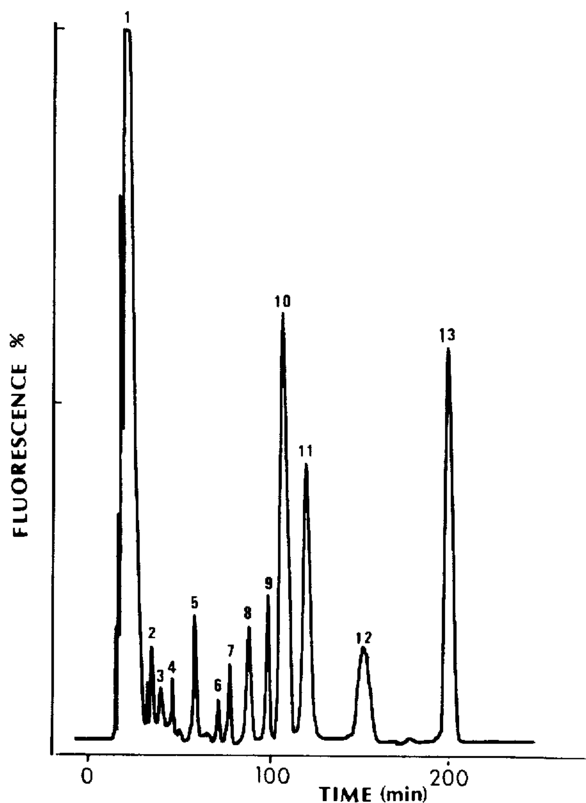

Fig. 3. Chromatogram of ADAM derivates of FAs extracted from serum albumin after purification of the protein from the same child of Fig. 1. Peaks: 1 = ADAM; 2 = caproic acid; 3 = caprylic acid; 4 = capric acid; 5 = lauric acid; 6 = myristoleic acid; 7 = myristic acid; 8 = linolenic acid; 9 = linoleic acid; 10 = palmitic acid; 11 = oleic acid; 12 = stearic acid; 13 = erucic acid.

reversed-phase HPLC system (C_{18}) by Barker et al. [13] and Nimura and Kinoshita [14], who obtained a good separation of most of the serum FAs but an incomplete resolution of palmitic acid and oleic acid, the most abundant of human serum FAs. Using a μBondapak C_{18} (Waters) 10-μm column, we have found the same disadvantages as those authors [13, 14] and tried therefore to develop better conditions of separation. The method reported here offers considerable advantages on the traditional reversed-phase system, which may be summarized as follows: (i) good separation of the most abundant pair of human serum FAs (i.e. palmitic acid and oleic acid); (ii) good separation of medium-length FAs such as lauric acid, myristic acid and myristoleic acid, which are not readily resolved by the reversed-phase system; (iii) satisfactory separation of shorter FAs such as caproic acid, caprylic acid and capric acid.

Free and albumin-bound FAs in normal serum

Fig. 2 reports an analysis of free FAs of normal serum. Results obtained from six normal children (Table II) are comparable with literature data on age-matched subjects [19].

The pattern of FAs bound to serum albumin is more complex, since under normal conditions serum albumin is the major carrier. As shown in Fig. 3, FAs from C_{16} to C_{18}, both saturated and unsaturated, are the main FAs transported by the protein; however, shorter FAs, such as C_6, C_8 and C_{12}, are easily detected and account for a discrete amount of total transported FAs.

Finally, lauric acid, which is only detectable in trace amounts in its free form in serum, is one of the principal FAs transported.

By current condition of chromatography, it is possible to analyse the amount of FAs bound to the albumin content of about 0.2 ml of serum.

CONCLUSION

In this report we present results showing that the Fatty Acid Analysis Column (Waters) provides a very good stationary phase for the separation of ADAM derivates of FAs. The combination of high sensitivity owing to the fluorescent label together with the highly selective separation power of the column allows the determination of all major classes of human FAs and a lot of plant FAs.

The main application of the new technique was the determination of FAs bound to serum albumin in children, which is a particular case of analysis since all FAs from C_6 to C_{18} are bound to this protein. In this case, our technique offers remarkable advantages on the traditional reversed-phase systems described by other authors [13, 14].

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