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Assessment of fatty acids in cardiac tissue as 9-anthryldiazomethane esters by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic technique for the rapid assessment of fatty acids in cardiac tissue is described. A level of 50.4 ± 14.9 nmol fatty acids per g wet weight of rat myocardial tissue could be monitored. The content of the individual fatty acids $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{20:4}$ amounted to 1.9, 13.5, 0.6, 14.4, 6.1, 6.5 and 7.2 nmol/g wet weight, respectively. A comparison of this method with a well established gas chromatographic technique yielded good agreement. In contrast with time-consuming gas chromatographic techniques, there is no need to isolate (unesterified) fatty acids from the other lipid classes with column chromatography or thin-layer chromatography, because the derivatizing reagent 9-anthryldiazomethane reacts highly specifically with fatty acids.

INTRODUCTION

During recent years various assay techniques have been used to monitor (unesterified) fatty acids in biological samples with the use of high-performance liquid chromatography (HPLC) [1–6]. Since fatty acids lack strongly UV-absorbing chromophores, derivatization of fatty acids with UV-absorbing or fluorescent compounds is required [7,8]. Fluorogenic reagents that have been used successfully are dansyl semipiperazide [1], 9-anthryldiazomethane (ADAM) [2–4], 9-(hydroxymethyl)anthracene [5] and 4-bromomethyl-7-methoxycoumarin [6]. All but ADAM require extensive sample preparation and purification, with a high risk of introducing errors. Since ADAM is highly reactive towards carboxylic acids, it can be used for fatty acid derivatization without preliminary sample purification, catalysts or heating [4,9,10].

Most reports on fatty acid determination with HPLC techniques pertain to the assessment of the lipid moieties in plasma or serum. Although from a (patho-)physiological point of view, information on tissue levels of fatty acids is of vital importance, reports on the assessment of the tissue content of these lipids with HPLC techniques are scanty [4,6]. Since the content of fatty acids in normal tissue

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is low in general and in cardiac tissue in particular [11,12], compared with the esterified lipid pool, special attention has to be paid to potentially disturbing factors in the assay technique. Previous gas chromatographic (GC) studies of fatty acids in cardiac tissue have shown that tissue levels can easily be overestimated owing to various technical imperfections in the assay procedures, such as high blanks and intra-preparative hydrolysis of esterified fatty acids [11,13].

The aim of the present study was to apply an HPLC assay to the determination of fatty acids in normal cardiac tissue. For this purpose, fatty acids were esterified with the fluorescent labelling agent ADAM. Evaluation of the HPLC technique was achieved by assessment of the selectivity towards (unesterified) fatty acids, the separation efficiency, the recovery of standards added to biological samples, the linearity of calibration curves, the inter- and intra-assay coefficients of variation, blanks and by comparison of the cardiac tissue levels obtained with those obtained using a well established GC assay technique [14,15].

EXPERIMENTAL

Reagents

Standard fatty acids were obtained from Sigma (St. Louis, MO, USA), ADAM was purchased from Serva (Heidelberg, Germany) and LiChrosolv methanol and acetonitrile were obtained from Merck (Darmstadt, Germany).

Apparatus

A Varian modular HPLC system equipped with a Varian flow-through double-beam spectrofluorometer with variable wavelength settings as a fluorescent detector (maximum excitation wavelength 365 nm; maximum emission wavelength 415 nm) was used. The detector was connected to a Varian DS-600 data system. The separation was achieved on a 4 μm RP-18 LiChrocart Supersphere column, 250 mm × 4 mm I.D. (Merck), equipped with a 4 μm LiChrocart RP-8 precolumn, 4 mm × 4 mm I.D. (Merck). The column temperature was held constant by using a water-jacket (Omnifit, Cambridge, UK) connected to a water-bath at 25°C. Acetonitrile–water (93:7, v/v) was used as the mobile phase at an initial flow-rate of 0.6 ml/min or 25 min, followed by a linear increase to 1 ml/min in 5 min. Thereafter the flow-rate was kept constant at 1 ml/min. The injection volume was 10 μl.

Preparation of ADAM reagent

Since ADAM is a very labile reagent [16], it was prepared just before use. ADAM was used at a concentration of 1 mg/ml in acetonitrile. ADAM was weighed in a test-tube just before use, and dissolved by adding three drops of acetone followed by the appropriate amount of acetonitrile.

Handling of cardiac tissue

Routinely, a piece of freshly frozen normoxic rat heart, 100–200 mg wet weight, was pulverized in an aluminium mortar with a stainless-steel pestle, precooled with liquid nitrogen as described by Van der Vusse *et al.* [17], and extracted according to Folch *et al.* [18] with chloroform and methanol. The crude lipid extracts were dried at 37°C under a stream of nitrogen. The extracts were without further purification subjected to derivatization with ADAM to obtain fluorescent labelled fatty acids (see below).

Derivatization

To the screw-cap test-tubes, 0.5 ml of ADAM reagent was added. The tubes were closed under a stream of nitrogen and incubated either in the dark at room temperature overnight or at 40°C in a heating block during 40 min. After the incubation, 10 μl of the clear supernatant were transferred onto the column by using a Valco valve equipped with a 10-μl loop.

GC analysis

Fatty acids, isolated and purified by column and thin-layer chromatography as described previously [17], were methylated with 7% boron trifluoride–methanol according to Morrison and Smith [19], and analysed with a Hewlett-Packard Model 5890 gas chromatograph equipped with a flame ionization detector and a digital integrator. A WCOT fused-silica capillary column coated with 0.2 μm CP-Sil 88 (50 m × 0.22 mm I.D., Chrompack, Middelburg, Netherlands) was used for the separation of fatty acids under the conditions described previously [15].

RESULTS AND DISCUSSION

Separation of fatty acid derivatives by HPLC

Fig. 1 shows the elution pattern of a standard mixture of ten fatty acids, commonly present in cardiac tissue, esterified with the fluorophore ADAM. Excess ADAM and decomposed reagent were eluted within 12 min after the start of the run. Their peaks did not interfere with the peaks of the fatty acyl-ADAM esters of interest.

In the present study the mobile phase was acetonitrile–water (93:7, v/v). It was our experience that relatively small changes in the proportions of acetonitrile and water exerted a substantial effect on the retention times of the individual fatty acid derivatives, preventing a good separation of the compounds analysed. When a flow-rate of 0.6 ml/min was applied throughout, the total run-time was in the order of 60 min. Higher flow-rates hampered good separation of the first set of fatty acid derivatives eluting from the column (up to $C_{18:2}$, see Fig. 1). The total run-time was successfully shortened to 45 min when the flow-rate was increased after 25 min. A disadvantage of flow programming is that higher flow-rates re-

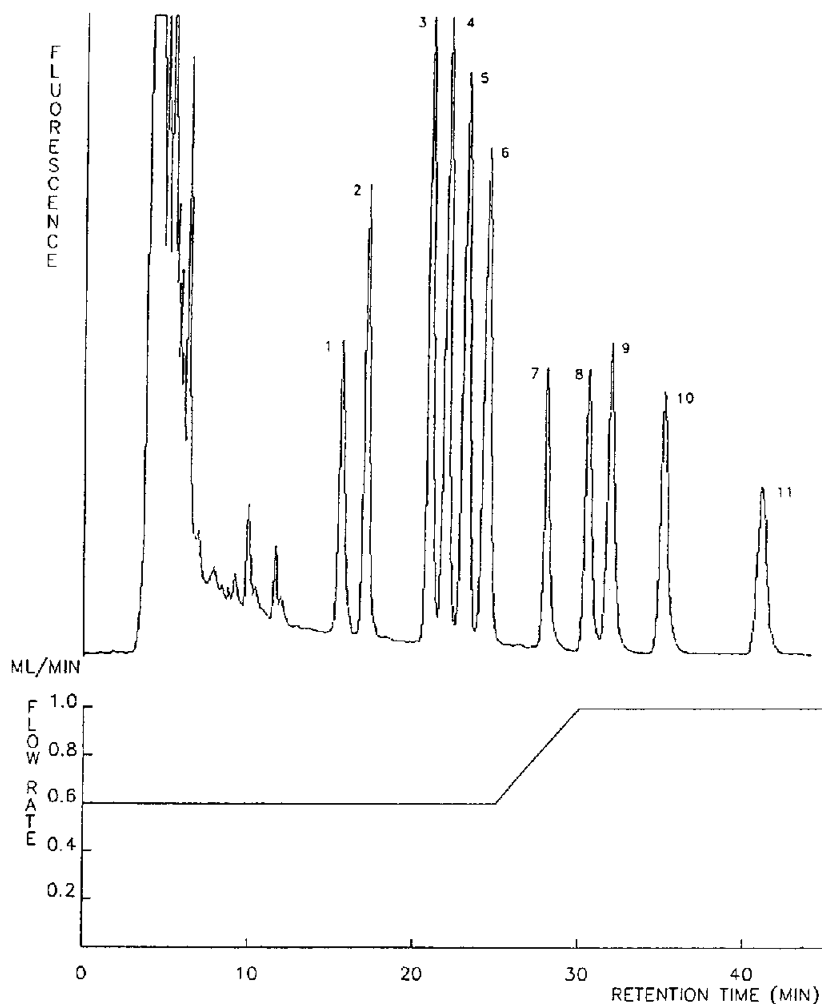

Fig. 1. Chromatogram of a mixture of ADAM derivatives of authentic fatty acids analysed by HPLC on an RP-18 column. Peaks: 1 = ADAM; 2 = $C_{22:6}$; 3 = $C_{20:4}$; 4 = $C_{14:0}$; 5 = $C_{16:1}$; 6 = $C_{18:2}$; 7 = $C_{22:4}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$; 10 = $_{17:0}$; 11 = $C_{18:0}$.

duce the response of the detector (lower peak-area over amount). This is because the detector used is a concentration detector, which renders the response flow-dependent.

Linearity of the calibration curve

For each fatty acid a calibration curve was plotted on the basis of the integrated area counts for nine different concentrations ranging from 4 to 400 pmol per injected sample (Fig. 2). Peak-area over amount was of the order of 54 000

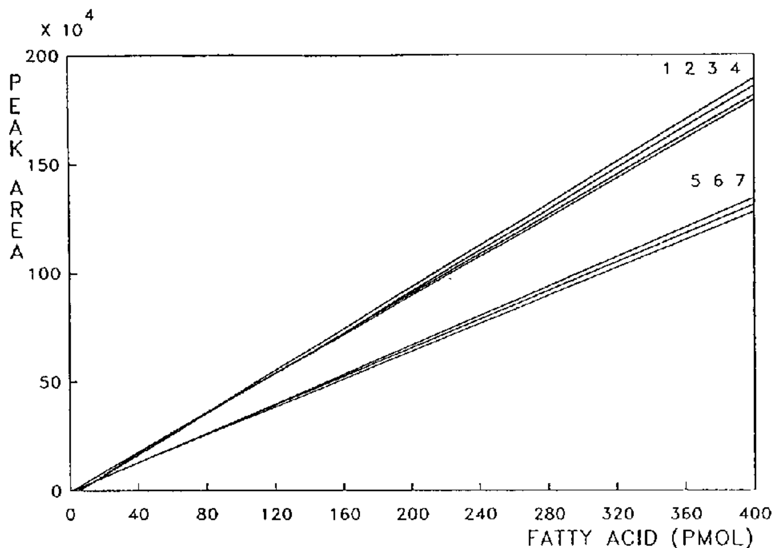

Fig. 2. Relationship between peak area and concentration in nmol/ml. Measurements were made as described in Experimental. The points represent means of duplicate estimations. The correlation coefficients (r^2) for the lines are 0.999 for $C_{18:2}$ and $C_{18:1}$ and 1.000 for $C_{20:4}$, $C_{14:0}$, $C_{16:1}$, $C_{16:0}$ and $C_{18:0}$. Lines (from top to bottom): 1 = $C_{18:2}$; 2 = $C_{20:4}$; 3 = $C_{16:1}$; 4 = $C_{14:0}$; 5 = $C_{18:1}$; 6 = $C_{18:0}$; 7 = $C_{16:0}$.

area counts per pmol for $C_{20:4}$ and 32 000 area counts per pmol for $C_{18:0}$. This difference was due to the increase in flow-rate, as described above. The correlation coefficients ranged from 0.999 to 1.000 for the fatty acid derivatives analysed.

Stability of fatty acyl-ADAM derivatives

To check the stability of the fatty acyl-ADAM derivatives, a standard mixture consisting of authentic fatty acids was derivatized with ADAM and stored at room temperature for 0, 24, 48 and 72 h. Analysis showed that no loss of fatty acyl-ADAM derivatives occurred during up to 72 h of storage (data not shown).

Blank value and detection limit

Since the level of fatty acids in rat heart tissue is very low [11], special attention should be given to the blank values. Throughout the whole procedure the blank values did not rise above 2.8 $\pm$ 1.2 nmol per assay.

The practical lower limit of detection is the amount of fatty acyl-ADAM derivative yielding a signal as high as four times the average detection noise. With an injection volume of 10 μl, a flow-cell volume of 15 μl and a peak volume of 1 ml, the detection limit of the HPLC system is of the order of 15 fmol, as calculated for stearic acid. For fatty acids with shorter retention times the detection limit is even lower. The practical lower limit of detection is *ca.* 50 times lower than that of the previously published GC analyses of fatty acids in biological samples [11,14].

TABLE I

PRECISION OF THE ADAM METHOD FOR ESTIMATION OF FATTY ACIDS

The data are arbitrary peak-area units expressed as mean and standard deviation of the mean and coefficient of variation.

Fatty acid	Intra-assay		Inter-assay	
	Mean ± S.D.	C.V. (%)	Mean ± S.D.	C.V. (%)
$C_{14:0}$	2 963 799 ± 71 865	2.4	735 333 ± 34 520	4.7
$C_{16:0}$	2 263 548 ± 23 455	1.0	586 133 ± 24 750	4.2
$C_{16:1}$	3 229 062 ± 34 334	1.1	764 467 ± 36 390	4.8
$C_{18:0}$	2 372 444 ± 52 986	2.2	658 300 ± 24 270	4.2
$C_{18:1}$	3 088 503 ± 49 318	1.6	692 917 ± 50 500	7.3
$C_{18:2}$	4 299 579 ± 24 309	0.6	972 783 ± 40 860	4.2
$C_{20:4}$	2 921 733 ± 77 755	2.7	594 467 ± 27 340	4.6

Recovery of standards and precision of the assay

Crude lipid extracts from four rat hearts were divided into two portions (A and B). To the first set a known amount of the standard mixture of fatty acids was added. A known amount of heptadecanoic acid was added to portions A and B to correct for losses during the assay. The recovery of added fatty acids was determined by substracting the content of fatty acids in portion B from A. The recovery of the added standard fatty acids varied from 92.8% for palmitoleic acid ($C_{16:1}$) to 104.3% for linoleic acid ($C_{18:2}$). The intra-assay precision was determined by analysing various fatty acyl-ADAM mixtures three times on the same day. The amount of each derivative injected into the column was of the order of 150 pmol. The coefficients of variation varied from 0.6% for linoleic acid ($C_{18:2}$) to 2.7% for arachidonic acid ($C_{20:4}$). The inter-assay precision was assessed by analysing the fatty acyl-ADAM mixture on three consecutive days. The coefficients of variation varied from 4.2% for linoleic acid ($C_{18:2}$) and stearic acid ($C_{18:0}$) to 7.3% for oleic acid ($C_{18:1}$) (Table I).

Determination of fatty acids in cardiac tissue

Fig. 3 shows a typical example of an ADAM-esterified extract of normal rat cardiac tissue. The peaks of the following fatty acyl moieties in the chromatogram were identified by comparing the retention times with those of the corresponding fatty acyl-ADAM derivatives in the standard mixture: myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), margaric acid ($C_{17:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$). Stearic acid ($C_{18:0}$) and palmitic acid ($C_{16:0}$) are the two most abundant fatty acids in the unesterified fatty acid pool in cardiac tissue (Table II). The average content of these fatty acids monitored in twelve normal rat hearts is also shown in

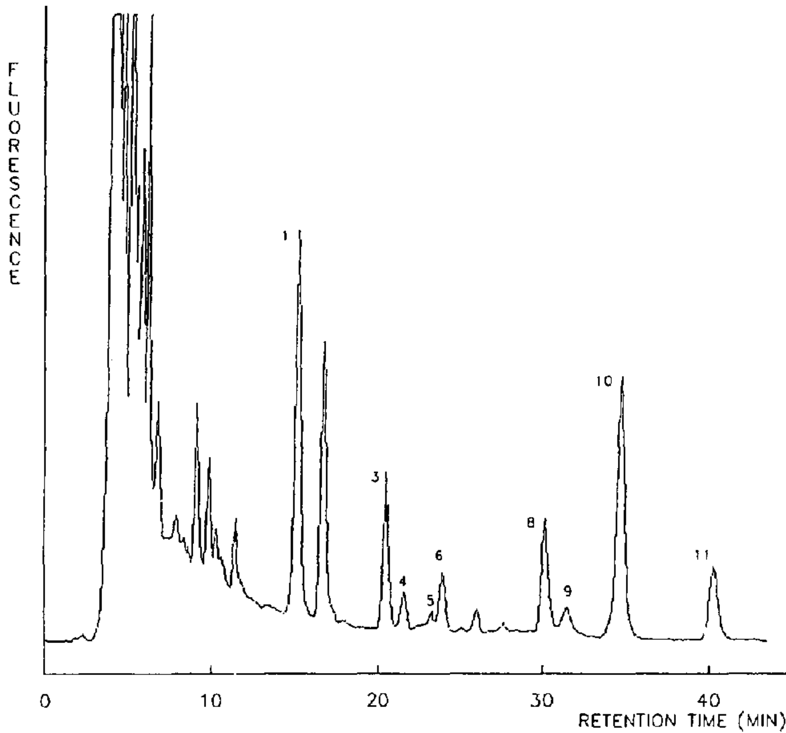

Fig. 3. Chromatogram obtained from fatty acids from a crude rat heart tissue extract. In this case, 10 μl of a 0.50-ml extract prepared from 150 mg wet weight of cardiac tissue was applied to the column. Peak identification as in Fig. 1.

TABLE II

COMPARISON OF FATTY ACIDS IN RAT HEART TISSUE ESTIMATED WITH HPLC AND GC

Values are expressed as nmol/g wet weight (n = 12, mean and standard deviation of the mean) and percentage of total.

Fatty acid	HPLC		GC	
	nmol/g	%	nmol/g	%
$C_{14:0}$	1.9 ± 1.4	3.9 ± 1.5	1.4 ± 1.0	2.0 ± 2.1
$C_{16:0}$	13.6 ± 7.1	25.3 ± 8.0	11.5 ± 3.7	25.0 ± 6.8
$C_{16:1}$	0.6 ± 0.6	1.1 ± 0.6	N.D.[a]	N.D.[a]
$C_{18:0}$	14.4 ± 6.0	28.8 ± 3.2	13.7 ± 3.8	31.9 ± 2.8
$C_{18:1}$	6.2 ± 4.0	11.7 ± 2.2	5.8 ± 3.2	13.1 ± 3.7
$C_{18:2}$	6.5 ± 3.5	11.8 ± 3.7	5.8 ± 2.6	12.8 ± 2.7
$C_{20:4}$	7.2 ± 3.3	14.3 ± 4.5	5.3 ± 2.2	12.4 ± 5.0
Total	50.4 ± 14.9		43.5 ± 12.8	

[a] N.D. = not detectable.

Table II. The sum of the fatty acids identified was of the order of 50 nmol per gram wet weight of tissue. This value is considerable lower than that reported by Miura *et al.* [4], as determined with a HPLC technique, but in the same range as GC values [17,20].

We compared this HPLC technique with a well established capillary GC technique [15]. An aliquot of the crude chloroform–methanol extract was separated on a silica gel column and by thin-layer chromatography to isolate the fatty acid fraction as described earlier [14,17]. Fatty acids were subsequently methylated [19] and quantitated by capillary GC (Table II) [15]. Statistical analysis failed to indicate a difference between the HPLC and GC values for individual fatty acids in normal rat heart tissue. A major advantage of using ADAM as a fluorescent label in the HPLC assay over the determination of fatty acyl methyl esters with capillary GC is the fact that time-consuming chromatographic separation of the various lipid classes is not required in the ADAM-based assay. ADAM reacts highly selectively with (unesterified) fatty acids in the crude chloroform–methanol extract of cardiac tissue. This notion was substantiated by incubating triacylglycerols and phospholipids isolated from *ca.* 250 mg of rat cardiac tissue with ADAM. No fatty acyl-ADAM derivatives could be detected in the triacylglycerol and phospholipid fractions.

CONCLUSION

Derivatization of fatty acids present in crude extracts of cardiac tissue with ADAM offers a reliable, sensitive and rapid technique to assess fatty acids by HPLC. Comparison between a capillary GC analytical technique and HPLC quantification of fatty acyl-ADAM esters showed good agreement. However, the HPLC technique is less time-consuming, because separation of the fatty acids from the esterified lipid classes, such as triacylglycerols and phospholipids, is not required owing to the selectivity of ADAM towards fatty acids.

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