Identification of a diene conjugated component of human lipid as octadeca-9,11-dienoic acid

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Received 1 March 1984

The predominant diene conjugated acyl residue in triacylglycerols, cholesteryl esters and phospholipids in human serum was identified by high performance liquid chromatography and capillary gas chromatography-mass spectrometry. It is an octadeca-9,11-dienoic acid.

Diene conjugation *Serum* *Lipid peroxidation* *Free radical* *Octadeca-9,11-dienoic acid* *Duodenal juice*

1. INTRODUCTION

Diene conjugation (DC) has been used for many years as a non-specific index of free-radical-mediated lipid peroxidation in biological material [1–3]. More recently it has been shown that DC in human serum is associated with cholesteryl esters, triacylglycerols and phospholipids, and that on hydrolysis only one fatty acid with DC is detectable by high performance liquid chromatography (HPLC) [4]. The same fatty acid accounts for DC in bile and duodenal fluid. This study was undertaken to identify this fatty acid.

2. MATERIALS AND METHODS

Blood for serum was collected from 6 healthy volunteers aged 22–48 years. Two ml serum was hydrolysed by incubation for 15 min at 25°C with 2 ml Tris buffer, 0.1 mol/l (pH 7.7), containing phospholipase A_2 from *Naja naja* venom, 10000 units/l, cholesterol esterase, 10000 units/l, lipase, 20000 units/l and methanol, 1 mol/l (Sigma, Dorset). Eight ml of 0.5% acetic acid in methanol was added, the protein removed by centrifugation and the supernatant extracted using a disposable 'Bond Elut' octadecyl column no. 607203 (Jones Chromatography, Mid Glamorgan). The Bond Elut column was washed twice with 2.5 ml acetonitrile and twice with 2.5 ml of a wash solution comprising methanol/water/acetic acid (67:33:0.03, by vol.). The supernatant was added followed by 2.5 ml wash solution and the fatty acids were eluted with 1.1 ml acetonitrile and evaporated to dryness under nitrogen. The residue was redissolved in 20 μl acetonitrile and analysed by HPLC and subsequent gas chromatography-mass spectrometry (GC-MS). Duodenal fluid was collected from patients under investigation for pancreatic disease [5]. It was prepared for HPLC and subsequent GC-MS by chloroform–methanol extraction [4].

The synthetic isomer of 18:2 (9,11) was received as the methyl ester (Paint Research Association). One g was hydrolysed by boiling in 2 N KOH in methanol:water (90:10, v/v), acidified with concentrated HCl, extracted with chloroform and evaporated to dryness under nitrogen. The resulting fatty acid was isomerised with 2 mmol $NaNO_2$ followed by 50 μl concentrated HNO_3 under nitrogen [6]. The products were analysed by HPLC and subsequent GC-MS.

Linoleic acid (99% purity) and human fat-free albumin (Sigma, Dorset) were UV-irradiated for

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4 h in phosphate-buffered saline [4]. The protein was then precipitated with two volumes of 0.5% acetic acid in methanol and after centrifugation the supernatant was extracted with a Bond Elut column as described above.

HPLC was performed isocratically using apparatus which comprised a Constametric III pump, computing integrator 308, and a Constametric III variable wavelength UV detector set at 210 or 234 nm (Laboratory Data Control, Staffordshire), a Rheodyne injection valve (Corati, CA), with a 50 μl sample loop, and a Spherisorb ODS2 250 × 4 mm column containing 5 μm octadecyl-capped spherical particles (Hichrom, Reading, Berks). The mobile phase was acetonitrile/water/acetic acid (85:15:0.1, by vol.) (Rathburn Chemicals, Peebleshire) at a flow rate of 1.5 ml/min. Specimens to be analysed by gas chromatography (GC) were collected as peaks from the HPLC. Two collections were made. The first was selective for the peak of interest; the second was less selective and included most of the chromatogram. The mobile phase was extracted with chloroform to remove the water and evaporated to dryness under nitrogen. The residue was resuspended in 300 μl methanol.

Methyl esters of the fatty acids were prepared by treatment with diazomethane in ether (1.7 ml). The solution was dried under nitrogen and resuspended in 50 μl chloroform. The diazomethane reagent was prepared by adding a solution of 2 g KOH in 3.3 ml water to 7 g *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide dissolved in 43 ml ether and 12 ml diethyleneglycol monoethylether. The released diazomethane was condensed in 55 ml ether maintained at 0°C [7].

An attempt was made to localise the position of the double bonds in methyl 9,11-octadecadienoate by oxidation with osmium tetroxide followed by GC-MS of the trimethylsilylated products [8].

The pyrrolidides were synthesised from the methyl esters of the fatty acids by dissolving in 200 μl pyrrolidine (Aldrich, Gillingham) and adding 20 μl acetic acid. After heating at 100°C for 30 min the amide formed was extracted with methylene chloride, washed with 2 N HCl, evaporated under nitrogen and resuspended in 50 μl chloroform [9].

GC-MS was carried out on a Varian (Finnigan) MAT 112 instrument fitted with a Varian 1400 gas chromatograph housing a 20 m × 0.3 mm open tubular glass capillary column coated with Sil 88 (Chrompack, Middelburg, The Netherlands). Samples were introduced via an all-glass solid injection system [10]. The carrier gas was helium with an inlet pressure of 50 kPa which gave a flow rate through the column of 1 ml/min. Fatty acid methyl esters were separated isothermally at an oven temperature of 150°C and the pyrrolidide derivatives at 210°C. Methylene values were obtained by co-injection of a suitable hydrocarbon mixture with the samples. GC effluent was passed directly to the ion source via a heated line maintained at 250°C. The ion source temperature was 250°C, the electron energy was 70 eV, the ionisation current was 1.5 mA and the accelerating voltage 800 V. Repetitive magnetic scans (70 scans/min) over the mass range 50–500 amu were taken and the mass spectra acquired and processed by the Varian (Finnigan) MAT SS200 data system. Selective detection of single ions was also used to give greater sensitivity in some cases, e.g., monitoring mass/charge (m/z) 294, the molecular ion of the methyl esters of octadecadienoic acids (18:2).

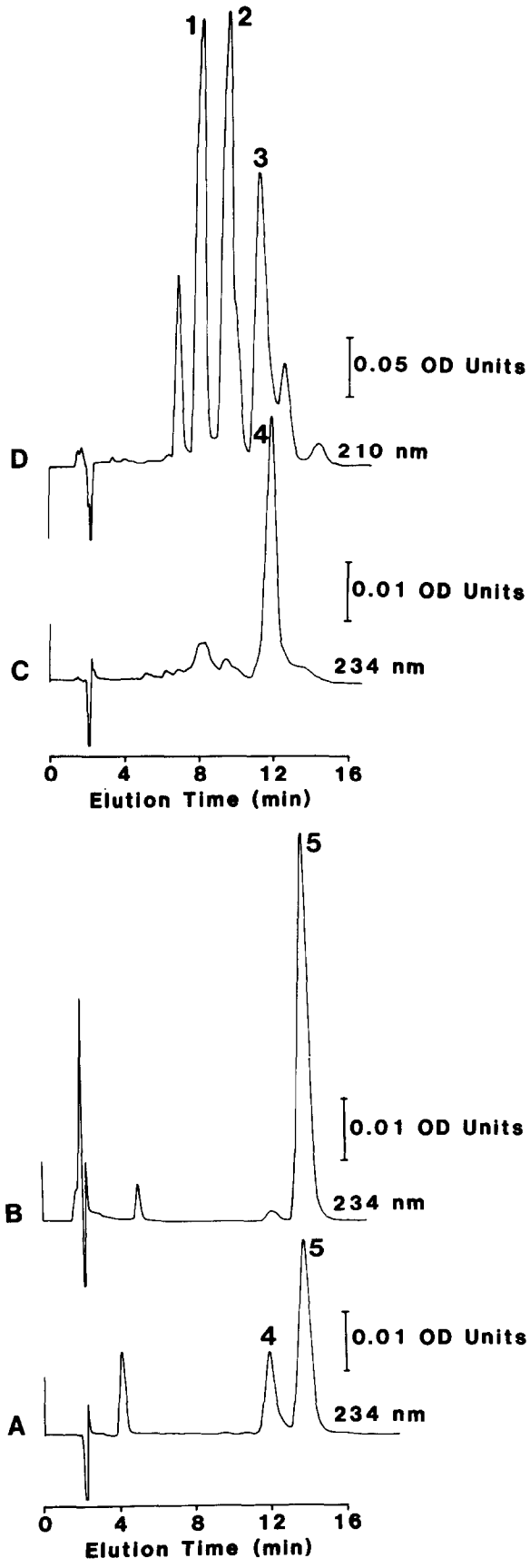
All identical chromatographic mobilities were demonstrated by coinjection. Matching of the GC and HPLC data was confirmed by the relative enrichment of GC peaks in the selective HPLC collections.

3. RESULTS

HPLC analysis of the UV irradiated linoleic acid and albumin preparation showed that two peaks with DC were produced during the irradiation. The second peak (peak 5, fig.1A) had the same HPLC mobility as the single peak produced by the synthetic 18:2 (9,11), but this peak was not present in the biological specimens (fig.1C). Peak 4 was the major peak in duodenal fluid as well as in hydrolysed serum.

HPLC analysis of the isomerised synthetic 18:2 (9,11) showed that one major peak with DC was produced by the isomerisation. This peak had the same HPLC mobility as peak 4 (fig.1).

GC-MS analysis of the methyl ester derivatives from the HPLC selective fractions of hydrolysed serum and duodenal fluid contained predominate-



ly one isomer of an 18:2. The relative amounts in individual samples as measured by HPLC and GC-MS agreed closely. The HPLC preparation of the isomerised synthetic 18:2 (9,11) contained two isomers of 18:2, the first of which had the same GC mobility as that from hydrolysed serum. The first HPLC peak from the UV irradiated linoleic acid and albumin preparation (peak 4, fig.1A) contained three isomers of 18:2, the first ofwhich had the same GC mobility as that from hydrolysed serum. The identity of the second HPLC peak from the UV irradiated linoleic acid and albumin preparation (peak 5, fig.1A) and the synthetic 18:2 (9,11) was confirmed.

The methyl ester of the biological conjugated diene eluted at 26.00 methylene units (MU) compared to methyl linoleate at 25.05 MU. The electron impact mass spectrum has a molecular ion at *m/z* 294 and fragmentation consistent with an 18:2 acid (fig.2A). The ions in the *m/z* series 81,95,109,123, etc., arise from the hydrocarbon end of the molecule and represent fragments containing 6,7,8,9, etc., carbon atoms, respectively. Differentiation of the many possible positional and configurational isomers directly from the spectrum is complicated by electron induced migration of the protons along the carbon chain.

Initial experiments with osmium tetroxide pretreatment of the fatty acids to localise the double bonds were successful with linoleic acid, but the oxidation reaction did not proceed with the conjugated 18:2 (9,11). However the successful hydroxylation of a conjugated double bond system with osmium tetroxide has been reported [11].

GC-MS analysis of the pyrrolidide of the conjugated 18:2 from serum permitted the position of the double bonds to be determined. This was due to the stabilisation of the electron impact fragments from the amide end of the molecule [12]. The mass spectrum of the pyrrolidide of the biological diene conjugated 18:2 (fig.2B) showed a regular series of ions representing stepwise

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Fig.1. HPLC chromatograms. (A) The diene conjugated products of the UV irradiation of linoleic acid and albumin. (B) The synthetic isomer of 18:2 (9,11). (C,D) Hydrolysed serum. 1, arachidonic acid; 2, linolenic acid; 3, linoleic acid; 4, biological isomer of 18:2 (9,11); 5, synthetic isomer of 18:2 (9,11).

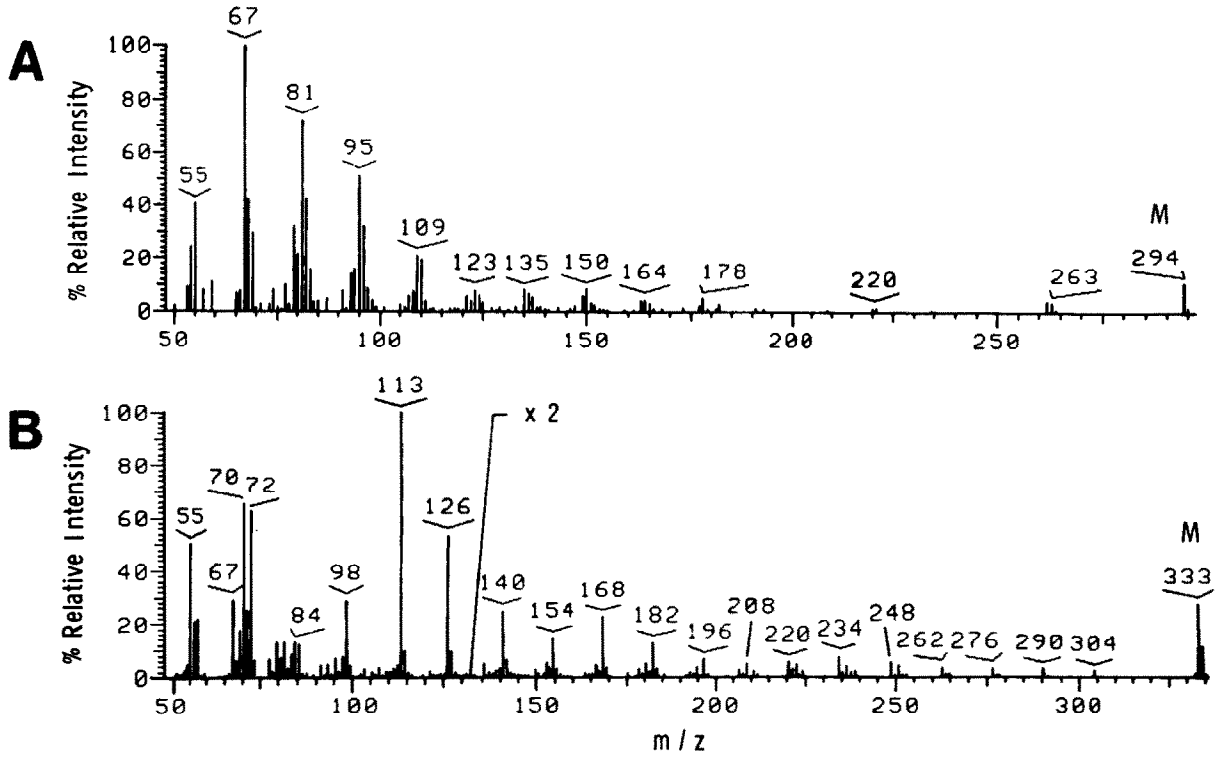

Fig.2. The mass spectra of the biological isomer of 18:2 (9,11), (A) as the methyl ester and (B) as the pyrrolidide.

fragmentation of the carbon chain. The position of the double bonds between carbon atoms 9 and 10 and carbon atoms 11 and 12 was indicated by the two consecutive 12 mass unit differences from *m/z* 196 to *m/z* 208 and from *m/z* 208 to *m/z* 220 within the remaining series of regular 14 mass unit differences [9]. The mass spectrum agreed closely with the published partial spectrum of the pyrrolidide of 9-*trans*,11-*trans*-octadecadienoic acid [12], although the configuration of the double bonds could not be confirmed from the spectrum.

Another two polyunsaturated fatty acids were observed in the HPLC fraction of serum. These were shown to be 20:3 and 22:5 acids. The mass spectra of the pyrrolidide derivatives indicated that they did not contain conjugated double bonds and hence did not contribute to the 234 nm response on HPLC. Their polarity would be expected to result in comparable HPLC mobility to 18:2 (9,11) and their concentration made it unlikely that they would contribute to the 210 nm response.

4. DISCUSSION

The results demonstrate that DC in our series of sera from healthy individuals represents a single isomer of 18:2 (9,11). They also confirm the prediction that this isomer does not contain oxygen other than in the carboxyl group [4]. In particular it is not a peroxide, epoxide, or hydroxyl as has been generally assumed on the basis of the peroxidation of pure polyunsaturated lipids [1–3]. The potential biological significance of the compound is 2-fold. On current evidence it is the most specific marker of free-radical-mediated lipid peroxidation measurable in clinical material [4,5,13]. Second, despite its similarity to linoleic acid the isomer has markedly different physical and perhaps biochemical properties [14,15]; and its presence even in low concentrations in cells could have significant functional consequences [16].

The stereospecificity of the biological isomer of 18:2 (9,11) might suggest an underlying enzymic

rather than a free-radical mechanism; but there is in fact no reason to assume that a free-radical reaction taking place in an organised biological structure and involving protein–lipid interaction should be any less specific.

A method for the measurement of serum esterified 18:2 (9,11) is currently being developed.

ACKNOWLEDGEMENT

The methyl ester of 18:2 (9,11) prepared from dehydrated castor oil was kindly donated by Mr A.F. Sherwood, Paint Research Association, Teddington, Middlesex, who regrets that further supplies are not available.

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