Journal of Chromatography, 341 (1985) 146—153
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2554

Note

Unusual fatty acids from amniotic fluid phospholipids

R.G. COOMBE*, JOHN H. VINE and HELEN YIP

Department of Pharmacy, University of Sydney, Sydney, N.S.W. 2006 (Australia)

(First received October 17th, 1984; revised manuscript received January 14th, 1985)

While looking at the possibility of using high-performance liquid chromatography (HPLC) to obtain accurate data on phospholipids contained in amniotic fluid, particularly phosphatidyl glycerol, the HPLC phospholipid fractions were collected, hydrolysed and their fatty acids were identified and quantified by gas—liquid chromatography (GLC) or gas chromatography—mass spectrometry (GC—MS) to see if there was any significant variation in fatty acid content with gestational age.

The expected $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ fatty acids were identified as their methyl esters as well as methyl oleate; however, in addition there appeared to be present two unsaturated fatty acids whose retention times suggested that their sites of unsaturation were not at the C-9—C-10 position usually reported for phospholipids.

EXPERIMENTAL

Materials and methods

Amniotic fluid was collected by trans abdominal amniocentesis as part of the routine management of complicated pregnancies in King George V Maternity Hospital and other New South Wales hospitals. Amniotic fluid (2 ml) was extracted following the method of Brown et al. [1]. About 0.5 ml of the phospholipid extract was used for thin-layer chromatographic (TLC) determination of lecithin/sphingomyelin (L/S) ratios and the remainder (about 1 ml) of the chloroform extract was evaporated to dryness at room temperature under a stream of nitrogen. The sides of the tube were washed with methanol and the washings also blown down to dryness with nitrogen, then 25 μl of methanol were added to the dried residue, vortex-mixed for 30 sec and 20 μl injected as single injection onto the HPLC column.

0378-4347/85/$03.30

Separation

The method used was based on that of Briand et al. [2] using a gradient system composed of acetonitrile and acetonitrile—water (80:20) set to deliver a water gradient from 2.4% to 15% at a flow-rate of 2 ml/min. After 9 min the elution was complete, the column was re-equilibrated and the next injection was made after 16 min. The UV detector was set at 206 nm. The purity of the separated phospholipids was checked by collecting the individual peaks and running them against the appropriate standards using two-dimensional silica gel TLC; solvent 1: chloroform—methanol—ammonium hydroxide—water (65:30:2.5:2); solvent 2: chloroform—methanol—glacial acetic acid—water (80:25:6:2); the spots were visualized by dipping the plates into 10% ethanolic sulphuric acid solution and heating at 150°C for 5—10 min. Phospholipids and other organic material charred dark brown.

Identification of phospholipid fatty acids

Separated peaks from the HPLC procedure were collected individually and evaporated to dryness in vacuo at room temperature. The residues were dissolved in 14% boron trifluoride—methanol complex and heated at 80°C for 20 min [3]. The resulting fatty acid methyl esters were extracted using a light petroleum (40—60°C)—water mixture (2:1). The organic layer was washed, dried, and evaporated to dryness under a stream of nitrogen at room temperature. The fatty acid methyl esters were dissolved in hexane (1—2 μl) and separated by GLC using 3% OV-101 on Gas-Chrom Q 120—140 mesh packed into a glass column (1.8 m x 6 mm I.D.).

A temperature programme of 160°C—230°C (6°C/min) was run and the column was held at 230°C until all the fatty acid esters were eluted.

Fatty acids were identified by comparison with retention times of reference standards obtained under the same conditions.

Gas chromatography—mass spectrometry

Analysis of fatty acid methyl ester mixtures was also carried out on a Finnigan 3200 quadrupole mass spectrometer fitted with a chemical ionisation (CI) source and interfaced to a Finnigan 9500 gas chromatograph. Data acquisition and processing were carried out on line using an Incos 2300 data system. The GLC column (2 m x 2 mm) used was packed with 3% OV-101 Gas-Chrom Q (120—140 mesh). Helium was used as the carrier gas at a flow-rate of 2.5 ml/min and held at 230°C until elution of peaks ceased. Injection port and interface temperatures were kept at 250°C. CI mass spectra were generated by adding reactant gas (methane) through a make-up T-piece at the end of the column. The reactant gas flow-rate was adjusted to give a source pressure of 67 Pa for helium and 133 Pa for methane.

Mass spectra were generated using an electron beam energy of 135 eV and source temperature was kept at 100°C.

2- and 3-dodecenoic acid

A mixture of 2- and 3-dodecenoic acids was produced via bromination of dodecanoic acid [4]. However, HPLC was found to be a more appropriate method to obtain small quantities of pure isomers than the fractional

distillation used in the reference [4]. Replicate injection of a mixture of isomers onto an ODS 10-μm HPLC column (250 × 4.5 mm), mobile phase acetonitrile—water (90:10), UV detector set at 206 nm, flow-rate 1.8 ml/min, gave a good separation of the components of the reaction mixture. Combining appropriate fractions gave sufficient material for GLC, GC—MS and UV spectral examination which were used to identify the 2- and 3-dodecenoic acids as their methyl esters.

2-Hexadecenoic acid

Tetradecanal (1.6 g) was refluxed with granulated zinc metal (0.5 g) and ethylbromoacetate (1.0 g) in benzene—diethyl ether (3:1) (15 ml) until the zinc had dissolved. The product, ethyl-3-hydroxyhexadecanoate, was obtained following the usual Reformatsky reaction product work up [5].

Ethyl-3-hydroxyhexadecanoate (1.0 g) was refluxed with 10% sulphuric acid—methanol (1:1) (40 ml) for 30 min. The reaction mixture was cooled, extracted with diethyl ether, the ether extract was then extracted with 10% sodium carbonate solution. The alkaline extract was acidified with hydrochloric acid and extracted with diethyl ether. The ether extract was washed with water, dried, and evaporated to dryness. The residue was refluxed with thionyl chloride (5 ml) for 60 min and the cooled mixture poured into ice water, then extracted with hexane. After removal of the solvent a portion of the reaction product (0.05 g) was reacted with boron trifluoride—methanol complex and separation of the esters by GLC and GC—MS showed the presence of 2- and 3-hexadecenoic acid methyl esters together with a small amount of starting material and an unidentified reaction product.

Purification of 2-hexadecenoic acid

The reaction mixture containing the hexadecenoic acid isomers was purified by HPLC following the procedure outlined above for dodecenoic acids. The mobile phase used was acetonitrile—water (95:5). The major HPLC peak was collected and methylated (boron trifluoride—methanol) and shown by the UV spectrum (λ_{max} 210 nm, log ϵ 3.9) and GC—MS to be methyl-2-hexadecenoate, molecular weight 268 a.m.u.

RESULTS AND DISCUSSION

Phospholipid fatty acids

The individual phospholipid classes extracted from amniotic fluid obtained from 36 patients were separated by HPLC, the collected fractions were hydrolysed, and the fatty acids chromatographed by GLC using the C_{12}—C_{22} even-numbered saturated fatty acids as reference standards, Fig. 1.

The expected tetradecanoic, hexadecanoic, octadecanoic and 9-octadecenoic acids were identified as their methyl esters. In some samples one or two unidentified fatty acids were noticed in addition to the normally occurring compounds.

Further investigation by GC—MS showed that the unknown compounds had molecular weights of 268 and 296 a.m.u., therefore they appeared to be hexadecenoic and octadecenoic acid methyl esters. However, their chromato-

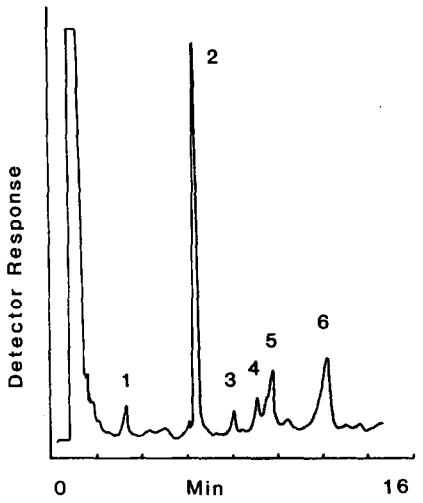

Fig. 1. Gas chromatogram of fatty acid methyl esters obtained from hydrolysis of phosphatidyl choline fraction. Column 3% OV-101 on Gas-Chrom Q (120—140 mesh), temperature programme 160°C—230°C at 6°C/min. Peaks: 1 = tetradecanoic acid methyl ester; 2 = hexadecanoic acid methyl ester; 3 = $C_{16:1}$ acid methyl ester; 4 = 9-octadecenoic acid methyl ester; 5 = octadecanoic acid methyl ester; 6 = $C_{18:1}$ acid methyl ester.

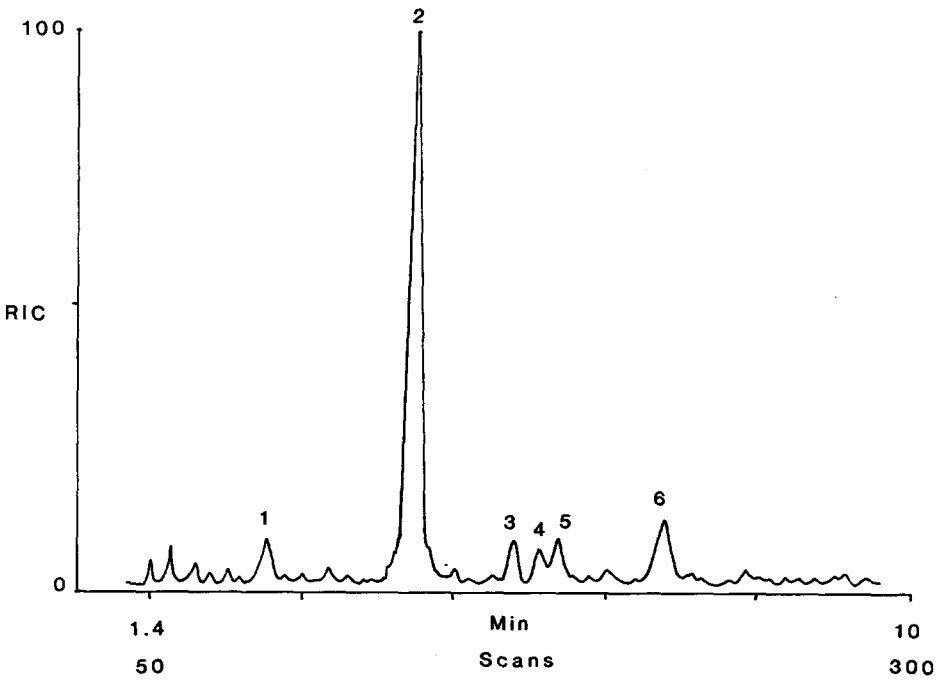

Fig. 2. GC—MS scan of fatty acid methyl esters obtained from hydrolysis of phosphatidyl choline fraction $(M + H)^+$. Peaks: 1 = *m/z* 243; 2 = *m/z* 271; 3 = *m/z* 269; 4 = *m/z* 297; 5 = *m/z* 299; 6 = *m/z* 297.

graphic characteristics did not point to their sites of unsaturation being at the usual C-9—C-10 position as in oleic acid (9-octadecenoic acid) since these isomers generally have shorter retention times than the saturated analogues (Fig. 2).

Comparison of the mass spectra by GC—MS of the two unknown compounds and their corresponding saturated analogues shows that the prominent peak *m/z* 74 ascribed to a McLafferty rearrangement in saturated fatty acid methyl esters is absent in the mass spectra of the unknown compounds indicating that the double bond is adjacent to the ester carbonyl group.

The readily accessible 2- and 3-dodecenoic acids [4] were synthesised for use as model compounds and it was found that the conjugated methyl-2-dodecenoate had a longer retention time than either the 3-dodecenoic or dodecanoic acid methyl esters. Using the Reformatsky procedure [5], ethyl-3-hydroxyhexadecanoate was synthesised from tetradecanal, then dehydration and hydrolysis produced a mixture of 2- and 3- hexadecenoic acids.

Separation of the mixture was easily followed since the conjugated chromophore in 2-hexadecenoic acid (λ_{max} 206 nm, log ϵ 3.9) allows this isomer to be readily distinguished from the unconjugated 3-hexadecenoic acid. Fig. 3 shows gas chromatograms of a mixture of hexadecanoic and 2-hexadecenoic acid methyl esters and Fig. 4 shows a GC—MS trace of a mixture containing hexadecanoic, octadecanoic, 2- and 3-hexadecenoic acid methyl esters together with specific ion scans at *m/z* 269 and 271 which show that the conjugated 2-hexadecenoate is clearly separated from the other components. The retention times for the unknown and synthetic samples relative to hexadecanoic acid methyl ester were the same and the CI mass spectra are shown in Fig. 5.

The mass spectrum of the second unknown component showed the

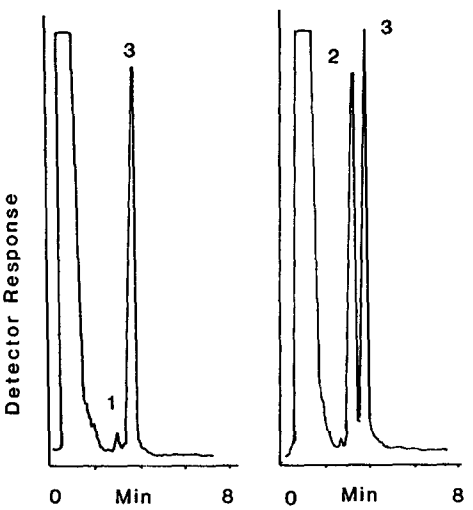

Fig. 3. Gas chromatograms of methyl esters of 2-hexadecenoic acid and hexadecanoic acid. Column 3% OV-101 on Gas-Chrom Q (120—140 mesh), 210°C. Peaks: 1 = 3-hexadecenoic acid methyl ester; 2 = hexadecanoic acid methyl ester; 3 = 2-hexadecenoic acid methyl ester.

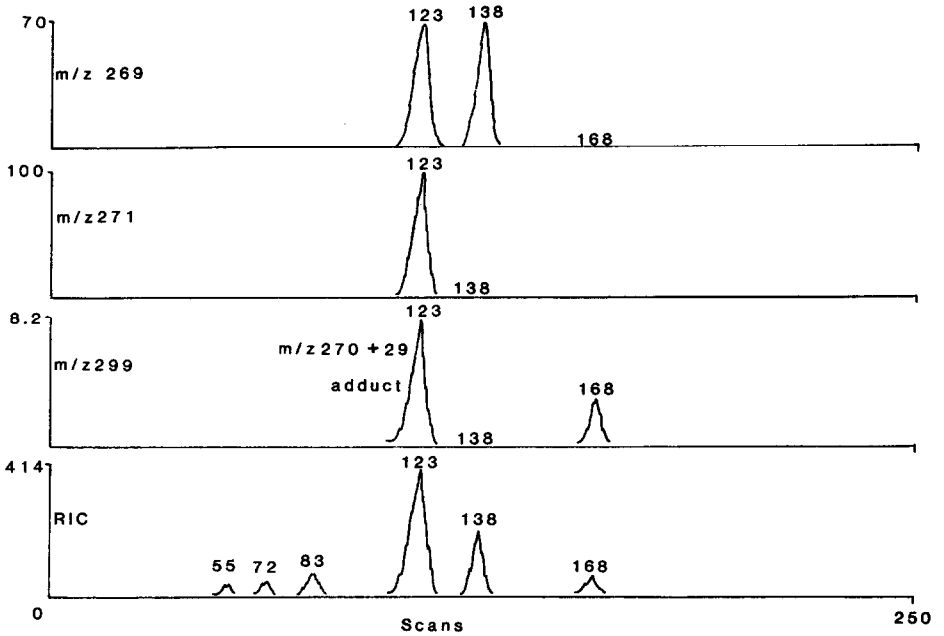

Fig. 4. Limited ion mass scan chromatogram of methyl esters of hexadecanoic acid, octadecanoic acid, 2- and 3-hexadecenoic acid.

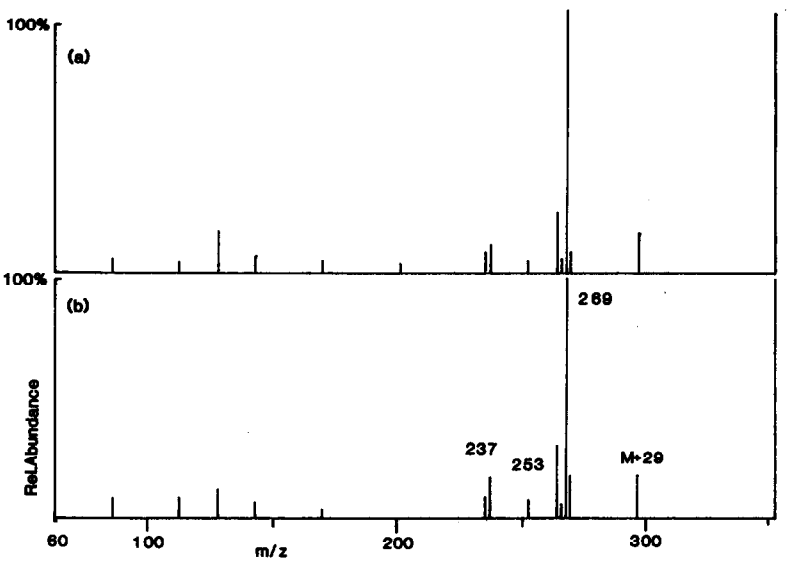

Fig. 5. Chemical ionisation mass spectra. (a) Unknown from amniotic fluid peak 3, Fig. 2; (b) synthetic 2-hexadecenoic acid methyl ester.

MH^+—CH_3OH peak characteristic of methyl esters but no *m/z* 74 peak, differed from the saturated C_{18} acid by 2 a.m.u., had a longer retention time analogous to 2-hexadecenoic acid methyl ester, and was separated from methyl-9-octadecenoate. Therefore it was concluded that the second unknown component was 2-octadecenoic acid.

TABLE I

PERCENTAGE COMPOSITION OF THE INDIVIDUAL PHOSPHOLIPID FATTY ACIDS COLLECTED FROM THE HPLC OF AMNIOTIC FLUIDS AT DIFFERENT STAGES OF GESTATION

Data are from 36 samples. Abbreviations: PG = phosphatidyl glycerol; PI = phosphatidyl inositol; PE = phosphatidyl ethanolamine; PC = phosphatidyl choline; SPH = sphingomyelin.

Fatty acid composition	< 35 Weeks gestation					35–37 Weeks gestation					> 37 Weeks gestation				
	PG	PI	PE	PC	SPH	PG	PI	PE	PC	SPH	PG	PI	PE	PC	SPH
$C_{14:0}$	3.5	8.5	20.0	2.5	4.5	11.0	12.1	13.6	6.0	9.2	6.0	11.6	9.4	6.9	11.4
$C_{16:0}$	45.7	43.7	40.0	72.6	64.0	43.4	41.1	41.1	74.8	64.0	47.0	37.1	44.1	74.6	74.1
$C_{16:1}$*				0.3		2.2	1.3	2.5	1.9	1.5	1.9	1.5	1.7	2.7	1.1
$C_{18:1}$	15.9	19.4	20.0	9.2	8.1	10.4	5.3	8.0	3.5	5.0	12.0	10.9	8.0	3.7	3.7
$C_{18:0}$	34.9	28.4	16.0	9.2	22.6	19.7	20.8	18.1	6.0	12.0	16.1	18.3	18.1	4.8	6.8
$C_{18:1}$*			4.0	6.2	0.8	13.3	19.4	16.7	7.8	8.3	17.0	20.6	18.7	7.3	2.9
Total saturated	80.6	80.6	76.0	84.3	91.1	74.1	74.0	72.8	86.8	85.2	69.1	67.0	71.6	86.3	92.3
Total unsaturated	19.4	19.4	24.0	15.7	8.9	25.9	26.0	27.2	13.2	14.8	30.9	33.0	28.4	13.7	7.7

*α–β Unsaturated fatty acids.

Table I shows the saturated and unsaturated acids identified in phospholipids from amniotic fluid. It is apparent that the conjugated unsaturated fatty acids are found in a range of phospholipids and in some phospholipids reach a significant proportion of the fatty acids present.

It is not clear how these fatty acids arise, however 2- and 3-hydroxy fatty acids are known to be intermediates in plant and animal metabolism of lipids and 3-hydroxy fatty acids are biosynthetic intermediates.

It is unlikely that the conjugated fatty acids identified arose by chemical degradation in the sample work-up since the synthetic work has shown that 3-hydroxy fatty acids are not as easily dehydrated as might be expected and when dehydration does occur chemically a mixture of mono unsaturated acids are formed. There was no sign of C_3—C_4 unsaturated isomers in the phospholipid extracts that would be expected to accompany the C_2—C_3 unsaturated acids if they were the result of chemical reactions.

It is not known what clinical or physiological significance, if any, can be attributed to these conjugated unsaturated fatty acids in amniotic fluid phospholipids; however, it is generally accepted that saturated phospholipids, especially dihexadecanoylphosphatidyl choline, are physiologically the most important phospholipids. This is reflected in the amounts of saturated phospholipids found in the samples analysed (Table I).

ACKNOWLEDGEMENTS

We would like to thank Dr. C.G. Duck-Chong for advice and assistance and Dr. Yvonne Holcombe and Louise Brown who very generously made available amniotic fluid samples used in this study.

REFERENCES

1 L.M. Brown, C.G. Duck-Chong and W.J. Hensley, Clin. Chem., 28 (1982) 344.
2 R.L. Briand, S. Harold and K.G. Blass, J. Chromatogr., 223 (1981) 277.
3 W.R. Morrison and L.M. Smith, J. Lipid Res., 5 (1964) 600.
4 N. Rabjohn (Editor), Organic Syntheses Collectivé, Vol. 4, Wiley, New York, 1963, p. 398.
5 A.I. Vogel, Practical Organic Chemistry, Longman, London, 3rd ed., 1956, p. 874.