Journal of Chromatography, 80 (1973) 233–240

CHROM. 6678

CHROMATOGRAPHIC RESOLUTION OF MOLECULAR SPECIES OF PHOSPHATIDYLETHANOLAMINE AS N-ACYL-O-METHYL DERIVATIVES

R. SUNDLER and B. ÅKESSON

Department of Physiological Chemistry, Chemical Centre, University of Lund, Lund (Sweden)

(Received January 31st, 1973)

SUMMARY

Phosphatidylethanolamines were converted into the N-acetyl-O-methyl or N-benzoyl-O-methyl derivatives by treatment with acetic anhydride/benzoyl chloride and diazomethane. Methods for the separation of these derivatives by argentation thin-layer chromatography and reversed-phase partition thin-layer chromatography are described. The procedure is especially advantageous for the analysis of phosphatidylethanolamines that are radioactively labelled in the ethanolamine moiety.

INTRODUCTION

Several methods for the separation of molecular species of phosphatidylethanolamines are available, including argentation chromatography, reversed-phase partition chromatography and countercurrent distribution (for a recent review, see ref. 1). Argentation chromatography of intact phosphatidylethanolamines separates only major species[2–4], whereas more detailed resolution is obtained after conversion to diacylacetylglycerols[5,6], diacylglycerols[7], dimethyl phosphatidates[8] or N-dinitrophenyl-O-methyl derivatives[9]. Only the last derivative contains the ethanolamine part of the molecule, but because of colour quenching of the liquid scintillation process it is unsuitable for the analysis of phosphatidylethanolamines that are radioactively labelled in this part of the molecule. For this analysis we have developed a method for the separation of ethanolamine phosphoglycerides as N-acyl-O-methyl derivatives by adsorption chromatography, argentation chromatography and reversed-phase partition chromatography.

MATERIALS AND METHODS

Preparation of N-acetyl-O-methylphosphatidylethanolamines

Ethanolamine phosphoglycerides from different sources were extracted according to the method of Folch *et al.*[10] and were isolated by thin-layer chromatography (TLC) on Silica Gel H (Merck) plates (developing solvent: chloroform–methanol–water, 70:30:5). 2,6-Di-*tert.*-butyl-*p*-cresol (0.05%) was added to the lipid extracts and also to the lipid samples in other procedures. After elution from the silica gel and washing[2], the phospholipid (up to 10 μmoles) was dissolved in 2 ml of freshly distilled chloroform (free from alcohol). Triethylamine (0.1 ml) and 0.1 ml of acetic

anhydride were added and the mixture was allowed to stand for 2 h at room temperature. After two washes with 3 ml of methanol–0.1 *M* HCl (1:3) and two washes with 3 ml of methanol–water (1:1), the chloroform solution was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 5 ml of methanol–diethyl ether (5:95). A 1-ml volume of 0.2–0.3 *M* diazomethane, freshly prepared from N-methyl-N-nitrosotoluene-4-sulphonamide[11], was added. After 30 min at room temperature, the solvent was evaporated and the phospholipid was dissolved in chloroform. N-Acetyl-O-methylphosphatidylethanolamines were isolated by TLC on Silica Gel H with chloroform–methanol–acetic acid (96:4:0.5) as developing solvent (R_F approximately 0.4).

Preparation of N-benzoyl-O-methylphosphatidylethanolamines

Phosphatidylethanolamines (up to 10 μmoles) were dissolved in 2 ml of freshly distilled chloroform. Triethylamine (0.1 ml) and benzoyl chloride (0.01 ml) were added and the mixture was allowed to stand at room temperature for 2 h. Thereafter, the procedure was carried out as for the preparation of the N-acetyl-O-methyl derivatives, except that the developing solvent for TLC was chloroform–methanol (98:2). We also tried to separate the diacyl derivative from the 1-alkyl-2-acyl and the 1-alk-1-enyl-2-acyl derivatives. Ethanolamine phosphoglycerides from bovine brain were treated in the same manner with benzoyl chloride and diazomethane. The lipids were applied to a silica gel plate, which was developed approximately ten times in benzene–chloroform–methanol (70:30:2). This procedure gave a clear separation between the diacyl derivative and the 1-alk-1-enyl-2-acyl derivative, which was identified by its lability to acid[12]. When the N-benzoyl-O-methyl derivatives of brain ethanolamine phosphoglycerides were hydrogenated over PtO_2 (ref. 13) and chromatographed as above, a spot with an R_F value between those of the diacyl and the 1-alk-1-enyl-2-acyl derivatives was visible, probably representing the 1-alkyl-2-acyl derivative. This separation is very similar to that obtained with N-dinitrophenyl-O-methylethanolamine phosphoglycerides[14].

Argentation chromatography

Thin-layer plates (20 × 20 cm and 0.5 mm thick) containing 17.5 g of $AgNO_3$ per 100 g of Silica Gel H were activated at 170° for 2 h and stored over P_2O_5. N-Acetyl-O-methylphosphatidylethanolamines or N-benzoyl-O-methylphosphatidylethanolamines in chloroform solution were applied to the plate (approximately 0.5 μmole/cm). The developing solvents are given later in Figs. 1 and 2. After chromatography, the phospholipid fractions were eluted, washed with 0.5% NaCl in 1:1 methanol–water[2] and analyzed for fatty acid composition. They were quantitated by gas–liquid chromatography after the addition of pentadecanoic acid as internal standard[15]. The identity of the major fatty acids (>3% in total rat liver phosphatidylethanolamines) was checked by comparison with authentic standards obtained from the Hormel Institute. Minor components were identified by comparison with published values of relative retention times[16].

Reversed-phase partition chromatography

Kieselguhr G plates (20 × 20 cm and 0.5 mm thick) were activated at 120° for 1 h and stored over P_2O_5. Immediately before use, they were impregnated with

undecane by developing in 7.5% undecane in light petroleum[17]. The N-acetyl-O-methylphosphatidylethanolamines isolated by argentation chromatography were dissolved in chloroform and were applied to the plate as a band (15–25 μg/cm). The developing solvent was methanol–water (85:15), 70% of which was saturated with undecane. After development at 20–25°, the grey bands visible against a white background[18] were eluted with 12 ml of chloroform–methanol (2:1). A volume of 4 ml of 1% NaCl was added and the chloroform phase was washed with methanol–water (1:1).

Other methods

Liquid scintillation counting was performed as described elsewhere[19]. Phosphorus was determined according to the method of Chen *et al.*[20].

RESULTS AND DISCUSSION

The efficiency of the conversion into N-acyl-O-methyl derivatives was checked with liver phosphatidylethanolamines isolated from rats injected with [^{14}C]ethanolamine[19]. After treatment with acetic anhydride and diazomethane, 92–104% of the original phosphatidylethanolamine radioactivity was recovered from the thin-layer plate, of which 97–98% was located in the only visible spot. The corresponding figures after treatment with benzoyl chloride and diazomethane were 95–102 and 96–97%, respectively. We also investigated whether diacyl-lipids that could possibly contaminate phosphatidylethanolamines after chromatographic isolation in different systems gave rise to derivatives migrating as N-acetyl-O-methylphosphatidylethanolamines. Less than 2% of the original phosphorus in phosphatidic acid or phosphatidylserine migrated as this derivative when treated with acetic anhydride and diazomethane. Dimethylphosphatidates can be formed when different phospholipids are treated with diazomethane[8,21]. These compounds, prepared from phosphatidic acids[17], had a higher R_F value than those of the N-acetyl-O-methyl- and N-benzoyl-O-methylphosphatidylethanolamines. Insignificant amounts of radioactivity were recovered as dimethyl phosphatidates when phosphatidylethanolamines labelled with [^{3}H]-

TABLE I

FATTY ACID COMPOSITION IN DIFFERENT DERIVATIVES OF RAT LIVER PHOSPHATIDYLETHANOLAMINES

A sample of rat liver phosphatidylethanolamines was divided into three portions. Two of them were converted into N-acetyl-O-methyl or N-benzoyl-O-methyl derivatives, and purified by TLC as described in the text.

Derivative	*Fatty acid composition (mole-%)*								
	16:0	*18:0*	*18:1*	*18:2*	*20:3*	*20:4*	*20:5*	*22:5*	*22:6*
Original phosphatidylethanolamine	19.8	23.2	4.9	9.3	1.0	25.5	0.7	2.3	13.2
N-acetyl-O-methyl	20.2	23.1	4.7	9.0	1.1	25.9	0.6	2.3	13.1
N-benzoyl-O-methyl	20.1	23.2	5.8	9.3	1.1	24.9	0.8	2.1	12.8

TABLE II

FATTY ACID COMPOSITION OF RAT LIVER PHOSPHATIDYLETHANOLAMINES SEPARATED AS N-ACETYL-O-METHYL DERIVATIVES ACCORDING TO DEGREE OF UNSATURATION

Phosphatidylethanolamines were isolated from a male fed Sprague–Dawley rat, weighing 200 g, and converted into the N-acetyl-O-methyl derivatives as described in the text. The separation was performed in the system shown in Fig. 1b and six fractions were analyzed.

Phosphatidylethanolamine fraction	*Proportion of total phosphatidylethanolamines (%)*	*Fatty acid composition (mole-%)*									
		16:0	*16:1*	*18:0*	*18:1*	*18:2*	*20:3*	*20:4*	*20:5*	*22:5*	*22:6*
Saturated monoenoic	2.0	36.2	3.5	18.6	41.7						
Saturated dienoic	12.8	22.0		21.2	7.4	49.4					
Trienoic	2.1	17.4	1.7	40.3	6.1	12.8	21.7				
Saturated tetraenoic	46.6	12.2		32.9	3.6		0.7	50.6			
Pentaenoic	6.0	20.2		20.4	8.0	1.3		27.1	4.2	18.8	
Saturated hexaenoic	30.5	30.2		16.8	2.7			2.1		3.3	44.9
Total, calculated*		20.0	0.1	25.6	4.9	6.7	0.8	25.8	0.3	2.1	13.7
Total, found		19.5		26.5	4.6	6.3	0.5	25.9	1.1	2.2	13.3

* Calculated from the relative amounts and the fatty acid composition of the different phosphatidylethanolamine fractions.

glycerol were treated with benzoyl chloride and diazomethane. The fatty acid composition of the phosphatidylethanolamine derivatives was the same as that in the original phospholipid (Table I), indicating that no selective loss of fatty acids during derivatization took place.

The separation on $AgNO_3$-containing plates is shown in Figs. 1 and 2 and a quantitative analysis of rat-liver phosphatidylethanolamines is given in Table II.

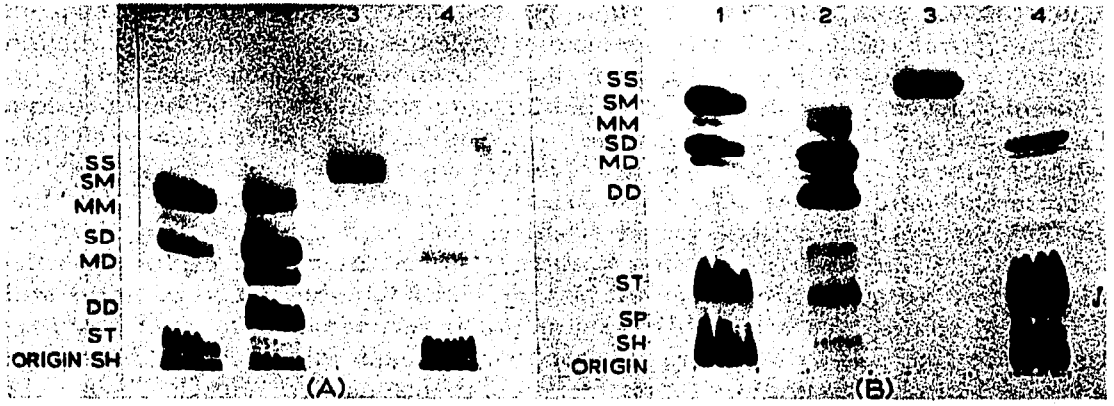

Fig. 1. Separation of phosphatidylethanolamines as N-acetyl-O-methyl derivatives by argentation chromatography. Phosphatidylethanolamines from the following sources were separated: 1 = egg yolk; 2 = commercial soyabean lecithin; 3 = hydrogenated egg yolk phosphatidylethanolamines; 4 = rat liver. Developing solvents: A, chloroform–methanol (95:5); B, the plate was first developed for 10 cm in chloroform–methanol–water (80:15:2) and then for 17 cm in chloroform–methanol (97:3). The spots were rendered visible by spraying with 20% NH_4HSO_4 solution[5] and heating at 120°. The fatty acid content in each band is indicated by the abbreviations at the left-hand side. Abbreviations: S = saturated; M = monoenoic; D = dienoic; T = tetraenoic; P = pentaenoic; H = hexaenoic. The two bands below the dienoic–dienoic fraction in the soyabean sample contained dienoic and trienoic fatty acids.

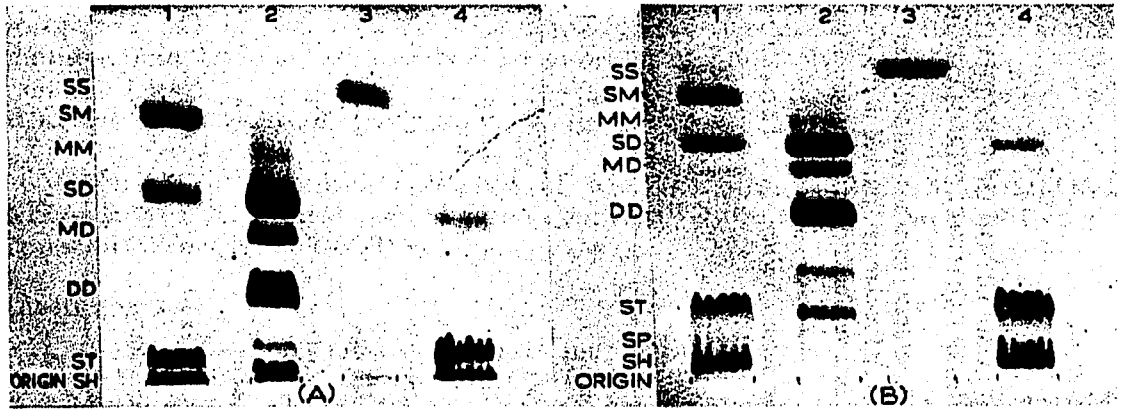

Fig. 2. Separation of phosphatidylethanolamines as N-benzoyl-O-methyl derivatives by argentation chromatography. The sources of phosphatidylethanolamines were as in Fig. 1. Developing solvents: A, chloroform–methanol (96:4); B, the plate was first developed for 10 cm in chloroform–methanol–water (90:10:1) and then for 17 cm in chloroform–methanol (98:2). Abbreviations as in Fig. 1.

TABLE III

FATTY ACID COMPOSITION OF PHOSPHATIDYLETHANOLAMINES SEPARATED AS N-ACETYL-O-METHYL DERIVATIVES BY REVERSED-PHASE PARTITION CHROMATOGRAPHY

Saturated dienoic, saturated tetraenoic and saturated hexaenoic N-acetyl-O-methyl phosphatidylethanolamines were isolated by argentation chromatography (Fig. 1b) and re-chromatographed in the same system. They were then separated by reversed-phase partition chromatography as described in the text and analyzed for fatty acid composition (sample A), or further purified by re-chromatography in the same system (sample B).

Phosphatidylethanolamine fraction	*Fatty acid composition (mole-%)*											
	Sample A						*Sample B*					
	16:0	*18:0*	*18:1*	*18:2*	*20:4*	*22:6*	*16:0*	*18:0*	*18:1*	*18:2*	*20:4*	*22:6*
Saturated dienoic	33.2	16.1	0.6	50.1								
Palmitoyllinoleoyl	50.4	1.9	1.8	45.9			49.3	0.8	1.6	48.3		
Stearoyllinoleoyl	9.2	44.1	3.0	43.7			2.6	47.7		49.7		
Saturated tetraenoic	15.8	32.6	0.7	1.1	49.9							
Palmitoylarachidonoyl	43.1	6.3	1.8		48.8		39.8	2.9	7.0		50.2	
Stearoylarachidonoyl	1.4	47.6			51.0		1.0	47.6	1.5		49.9	
Saturated hexaenoic	31.6	15.0	2.9			50.5						
Palmitoyldocosahexaenoyl	47.9	2.2	2.7			47.2	48.1	1.6	2.5			47.8
Stearoyldocosahexaenoyl	7.6	45.3	0.6			46.5	3.1	48.3				48.6

Development of the plate in one step yielded separations very similar to those reported for diacylacetylglycerols[5,6], diacylglycerols[7], dimethyl phosphatidates[8] and N-dinitrophenyl-O-methylphosphatidylethanolamines[9]. Two-step development (Figs. 1b and 2b) also resolved the fractions with 4–6 double bonds and was therefore used in metabolic studies of radioactively labelled rat liver phosphatidylethanolamines[19]. The composition of phosphatidylethanolamines in this organ (Table II) was similar to that found by other workers[2,22–24]. The calculated fatty acid composition of total phosphatidylethanolamines, obtained by summing the fatty acid contents in the individual fractions, was in excellent agreement with that of the starting material.

The three major fractions of rat liver phosphatidylethanolamines contained approximately 50% each of one unsaturated fatty acid and palmitic acid plus stearic acid. They were further resolved into two fractions by reversed-phase partition TLC (Table III). The isolated fractions contained almost exclusively one saturated fatty acid and one unsaturated fatty acid. Therefore they are practically unimolecular, as the positional distribution of fatty acids is highly specific with unsaturated fatty acids at position 2 and saturated fatty acids at position 1 (refs. 3 and 24). This procedure seems to be the only published method for such a resolution by reversed-phase partition TLC of phosphatidylethanolamines with the ethanolamine part of the molecule retained. Similar separations of derivatives devoid of this part of the molecule, such as dimethyl phosphatidates[17,25] and diacylacetylglycerols[17], have been reported. The separation of phosphatidylethanolamines with a retained ethanolamine moiety according to the chain-length of the saturated fatty acids can also be accomplished by countercurrent distribution of the N-dinitrophenyl-O-methyl derivatives[26,27].

The procedures described in this paper are complementary to other methods for phosphatidylethanolamine analysis. We have found them to be at least as rapid and simple as the methods that involve conversion to diacylacetylglycerols and dimethylphosphatidates. The main advantage of the method is the possibility of detailed resolution of phosphatidylethanolamine mixtures that are labelled in the ethanolamine moiety. If radioactively labelled acylating agents are utilized, the procedures can be used as a simple method for the quantitation of individual phosphatidylethanolamines, as described for phosphatidylinositols[28].

ACKNOWLEDGEMENTS

The skilful technical assistance of Miss Birgitta Mårtensson is gratefully acknowledged. This investigation was supported by the Swedish Medical Research Council (Project No. 03X-3958).

REFERENCES

1 A. Kuksis, *Progr. Chem. Fats Other Lipids*, 12 (1972) 1.
2 G. A. E. Arvidson, *Eur. J. Biochem.*, 4 (1968) 478.
3 S. M. Hopkins, G. Sheehan and R. L. Lyman, *Biochim. Biophys. Acta*, 164 (1968) 272.
4 C. V. Viswanathan, S. P. Hoevet and W. O. Lundberg, *J. Chromatogr.*, 35 (1968) 113.
5 O. Renkonen, *Biochim. Biophys. Acta*, 125 (1966) 288.
6 B. J. Holub and A. Kuksis, *Lipids*, 4 (1969) 466.
7 L. M. G. Van Golde and L. L. M. Van Deenen, *Chem. Phys. Lipids*, 1 (1967) 157.
8 O. Renkonen, *Biochim. Biophys. Acta*, 152 (1968) 114.

9 O. Renkonen, *Acta Chem. Scand.*, 21 (1967) 1108.
10 J. Folch, M. Lees and G. H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
11 S. R. Lipski and R. A. Landowne, *Methods Enzymol.*, 6 (1963) 515.
12 R. E. Anderson, R. D. Garrett, M. L. Blank and F. Snyder, *Lipids*, 4 (1969) 327.
13 F. Snyder and M. L. Blank, *Arch. Biochem. Biophys.*, 130 (1969) 101.
14 O. Renkonen, *J. Lipid Res.*, 9 (1968) 34.
15 B. Åkesson, J. Elovson and G. Arvidson, *Biochim. Biophys. Acta*, 210 (1970) 15.
16 R. G. Ackman and R. D. Burgher, *J. Amer. Oil Chem. Soc.*, 42 (1965) 38.
17 B. Åkesson, *Biochim. Biophys. Acta*, 218 (1970) 57.
18 G. A. E. Arvidson, *J. Lipid. Res.*, 8 (1967) 155.
19 R. Sundler, *Biochim. Biophys. Acta*, 306 (1973) 218.
20 P. S. Chen, T. Y. Toribara and H. Warner, *Anal. Chem.*, 28 (1956) 1756.
21 H. D. Crone, *Biochim. Biophys. Acta*, 84 (1964) 665.
22 E. E. Hill, D. R. Husbands and W. E. M. Lands, *J. Biol. Chem.*, 243 (1968) 4440.
23 B. Åkesson, *Eur. J. Biochem.*, 9 (1969) 463.
24 B. J. Holub and A. Kuksis, *Can. J. Biochem.*, 49 (1971) 1347.
25 C. F. Wurster and J. H. Copenhaver, *Lipids*, 1 (1966) 422.
26 M. A. Trewhella and F. D. Collins, *Lipids*, 4 (1969) 305.
27 F. D. Collins and M. A. Trewhella, *Lipids*, 6 (1971) 355.
28 M. G. Luthra and A. Sheltawy, *Biochem. J.*, 126 (1972) 1231.