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Note

Silicic acid column chromatography of phosphonolipids

II. Separation of 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphono choline from 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosporyl choline, cardiolipin and other related phospholipids

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Ether phospholipids are among the most potent biologically active phospholipid derivatives[1,2]. Procedures for the preparation of individual alkyl-acetyl-glycerophospho cholines[3] and mixtures of these compounds[1] have been described. The synthesis of alkyl-acetyl-glycero-phosphocholines has recently been described[4]. In this paper use is made of silicic acid column chromatography for the efficient separation of 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphono choline (phosphono-AGEPC) from its analogue 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphoryl choline (phosphoryl-AGEPC) and also from cardiolipin, lyso-lecithin, sphingomyelin and partially from phosphono-sphingomyelin. Collected fractions were analysed by thin-layer chromatography (TLC) and infrared (IR) spectroscopy to confirm species identification. This method then provides a means for the separation, isolation and identification of 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphono choline from its phosphoryl analogue and from other related phospholipids.

EXPERIMENTAL

Instrumentation

IR spectra were recorded on a double-beam Perkin-Elmer spectrometer. A glass column (35 × 1.6 cm I.D.) was employed for the separation.

Reagents

Solvents for column chromatography and TLC were analytical-reagent grade (E. Merck, Darmstadt, G.F.R.) and were distilled before use. TLC was conducted on 20 × 20 cm chromatoplates of 0.25 mm thickness silica gel G or 60 F_{254} (E. Merck) and visualisation was effected with molybdenum blue, iodine vapour, UV irradiation and ninhydrin spray.

Standards

Phosphono-AGEPC was a synthetic product[4], phosphoryl-AGEPC was synthesized in this laboratory and phosphono-sphingomyelin was also a synthetic prod-

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uct[5,6]. Cardiolipin was purchased from Koch-Light, Colnbrook, Great Britain. Sphingomyelin was isolated from ox brain and was chromatographically pure[5,6] and lyso-lecithin was purchased from Aldrich, Milwaukee, WI, U.S.A. Silicic acid for column chromatography was purchased from Sigma, St. Louis, MO, U.S.A.

Procedure

The chromatographic column, which was fitted at the bottom with a glass-wool plug, was loaded with a slurry of 11.0 g of regenerated silicic acid in 60 ml of chloroform, to a column height of 11.0 cm and a total column volume of 27.5 ml. The column was washed with two column volumes of chloroform and the flow-rate maintained in the elution was 1.5–1.9 ml/min. The volume of the eluate collected by the fraction collector was *ca.* 5.0 ml. When the total weight was desired, a total of 10–25 ml of the eluates was obtained by pooling of fractions. Evaporation of the solutions was accomplished either under vacuum with a bath temperature of 35°C or under nitrogen.

Column elution was effected with combinations of methanol–chloroform mixtures as shown in Table I.

TABLE I

ELUTION OF THE CHROMATOGRAPHIC COLUMN

Column (35 × 1.6 cm I.D.) packed with 11.0 g of regenerated silicic acid (Sigma), to a height of 11.0 cm. Column volume, 27.5 ml; flow-rate, 1.5–1.9 ml/min. Fractions of *ca.* 4.0 ml were collected.

Methanol–chloroform (%)	*No. of column volumes*	*Total volume of solvent (ml)*	*No. of fractions collected*
5	3	75	1–19
20	5	130	20–44
40	7	180	45–80
80	5	110	81–105

IR spectra of the various pilot fractions were run as solutions in chloroform or as potassium bromide discs. TLC chromatograms were run on silica gel G F_{254} plates (E. Merck) and also on glass plates coated in this laboratory to a thickness of 0.30 mm. Development of the chromatograms was effected in two chambers of dimensions 20.5 × 8 cm (Desaga) and the run normally took *ca.* 45 min. The plates were developed in chloroform–methanol–water (65:25:4) (system A) and chloroform–methanol–acetic acid–water (25:15:4:2) (sytem B). Visualisation was effected with molybdenum blue, iodine vapour, and UV irradiation. Standards were also spotted onto the plates to ease in the detection of the developed spots.

RESULTS

Column elution was effected with combinations of methanol–chloroform mixtures as shown in Fig. 1. Fractions were identified by TLC and IR spectroscopy (Table II) and the nature of the fractionation pattern of the phospholipids under examination is graphically depicted in Fig. 1. With the solvents used 99.90% of the lipids applied could be recovered.

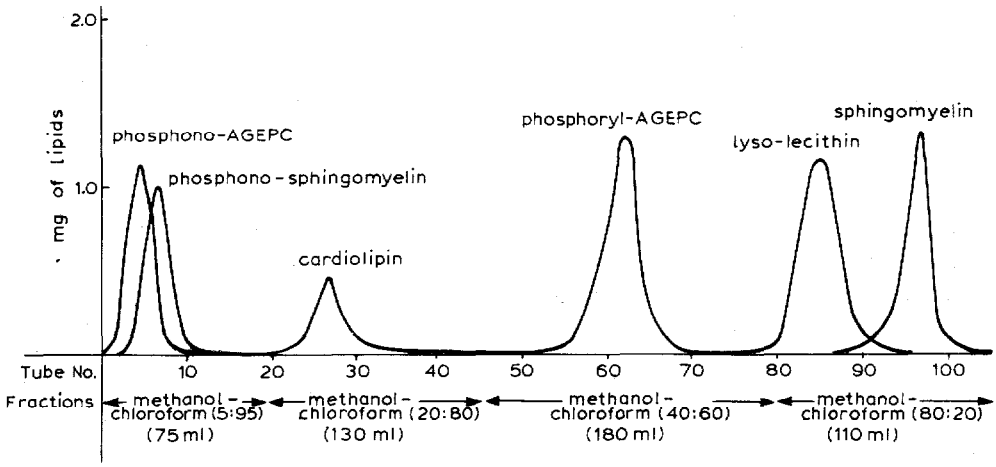

Fig. 1. Chromatography of various indicated phospholipids on silicic acid. Solvents were methanol–chloroform (a) (5:95), (b) (20:80), (c) (40:60) and (d) (80:20). Composition of the various fractions was: Phosphono-AGEPC, 4.5 mg; phosphoryl-AGEPC, 6.0 mg; cardiolipin, 3.0 mg; lyso-lecithin, 5.0 mg; sphingomyelin, 6.0 mg; phosphono-sphingomyelin, 3.0 mg. The lipids were loaded on to the column in 5 ml of chloroform–methanol (1:1).

TABLE II

COMPOSITION OF FRACTIONS OBTAINED FROM CHROMATOGRAPHY OF PHOSPHOLIPIDS ON SILICIC ACID

Weight of lipids applied to the column was 27.5 mg. Total recovery was 27.47 mg (99.89%). Components identified by IR spectral data.

Solvent	*No. of fractions collected*	*TLC R_F*		*Identified components*
		System A	*System B*	
Methanol–chloroform (5:95)	1– 9	0.96,0.78		Phosphono-sphingomyelin, phosphono-AGEPC
Methanol–chloroform (20:80)	20– 35	0.68		Cardiolipin
Methanol–chloroform (40:60)	55– 67	0.30		Phosphoryl-AGEPC
Methanol–chloroform (80:20)	82– 89		0.10	Lyso-lecithin
	90– 92	0.15	0.10	Lyso-lecithin–sphingomyelin
	93–105	0.15		Sphingomyelin

DISCUSSION

The unusual chromatographic behaviour of phosphono-sphingomyelin has been noted in a previous paper[7], where it was proposed that in fact this compound carries a net positive charge.

Phosphono-AGEPC apparently possesses chromatographic properties comparable to those of phosphono-sphingomyelin. In solvent system A it possesses an R_F value of 0.78 and in methanol–water (2:1) one of 0.64. In addition preliminary column chromatographic examination indicated that phosphono-AGEPC was also collected in the cardiolipin fraction (as was also the case with phosphono-sphingomyelin). Phosphoryl-AGEPC on the other hand exhibited the usual chromatographic behaviour for this class of phospholipids. Thus in TLC with a solvent system of methanol–water (2:1) it had an R_F value of 0.54 while using solvent system A it had an R_F value of 0.30. As is also seen from the column chromatographic results, it is eluted in the lecithin fraction.

Phosphono-AGEPC therefore separates effectively from phosphoryl-AGEPC, cardiolipin, lyso-lecithin and sphingomyelin and partially from phosphono-sphingomyelin, being eluted early in the process with only 5% methanol–chloroform. The effective column chromatographic separation of phosphono-AGEPC and phosphonosphingomyelin is currently under investigation and the results will be published in a future paper.

Phosphono-AGEPC and phosphoryl-AGEPC can thus be effectively separated by TLC and silicic acid column chromatography. This is due to an unusual property of phosphono-AGEPC, namely that this compound also carries a net positive charge (*cf.* phosphono-sphingomyelin). It is therefore evident that phosphono-sphingomyelin is not the only species possessing a net positive charge and that this property is shared by other naturally occurring substances, in this case phosphono-AGEPC.

In addition, we have been successful in separating phosphoryl-AGEPC from sphingomyelin, lyso-lecithin and phosphatidyl choline, which in a previous work[9] proved to be the major contaminants.

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