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Note

Silicic acid column chromatography of phosphonolipids

I. Separation of phosphonosphingomyelin from cardiolipin and other phospholipids

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Silicic acid column chromatography has been widely employed for the chromatographic fractionation of simple and complex lipids and particularly phospholipids[1-7]. Elution of the columns with diethyl ether–light petroleum, chloroform–methanol and benzene–diethyl ether mixtures has been used for separations of neutral lipids, phospholipids, glycolipids etc.

This paper describes the use of silicic acid column chromatography for the efficient separation of phosphonosphingomyelin from cardiolipin, α-lecithin, lysolecithin and sphingomyelin. The collected fractions were analysed by thin-layer chromatography (TLC) and IR spectroscopy to confirm species identification. This method provides a means for separating, isolating and identifying phosphonosphingomyelin from a naturally occurring mixture of related phospholipids.

EXPERIMENTAL

Instrumentation

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

Reagents

Solvents for column chromatography and TLC were of analytical-reagent grade (Merck, Darmstadt, G.F.R.) and were distilled before use. TLC was conducted on 20 × 20 cm plates with a 0.25 mm thick layer of silica gel G or 60 F_{254} (Merck).

Standards

Phosphonosphingomyelin was a synthetic product[8,9] and cardiolipin was purchased from Koch-Light (Colnbrook, Great Britain). Sphingomyelin was isolated from ox brain chromatographically pure[8,9] and α-lecithin and lyso-lecithin were purchased from Aldrich (Milwaukee, WI, U.S.A.). Silicic acid for column chromatography was purchased from Sigma (St. Louis, MO, U.S.A.).

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Procedure

The chromatographic column, with a glass-wool plug at the bottom, was loaded with a slurry of 10 g of regenerated silicic acid in 50 ml of chloroform to a height of 10.5 cm and a total column volume of 25 ml. The column was washed with two column volumes of chloroform and the flow-rate during elution was 1.0–1.8 ml/min. The volume of the eluate collected by the fraction collector was about 5.0 ml. When the total weight was desired, a total of 20–30 ml of the eluates was obtained by pooling of fractions. Evaporation of the solvents was accomplished under vacuum and at a bath temperature of 35°C or under nitrogen (Table I).

TABLE I

ELUTION OF THE CHROMATOGRAPHIC COLUMN

The column (35 cm × 1.6 cm I.D.) was packed with 10 g of regenerated silicic acid (Sigma) to a height of 10.5 cm. Column volume, 25 ml; flow-rate, 1.0–1.8 ml/min. Fractions of about 5.0 ml each collected.

Concentration of methanol in chloroform (%)	*Column volumes*	*Total volume of solvent (ml)*	*Fractions collected*
5	3	80	1–18
20	5	130	19–44
40	9	180	45–75
80	5	110	76–99

IR spectra of the various fractions were run as chloroform solutions or potassium bromide discs. TLC was run on silica gel G F_{254} plates (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 about 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) (system B). Detection was effected with molybdenum blue, iodine vapour and UV irradiation. Standards were also spotted on the plates to assist in the identification of the developed spots.

RESULTS

The chromatographic column was loaded with phospholipids as shown in Fig. 1. Elution was effected with combinations of methanol in chloroform mixtures as indicated 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 depicted in Fig. 1. With the solvents used, 99.33% of the lipids applied to the column could be recovered.

DISCUSSION

This work was initially prompted by the observation that phosphonosphingomyelin migrated to high R_F values in TLC solvent system A and had an R_F value

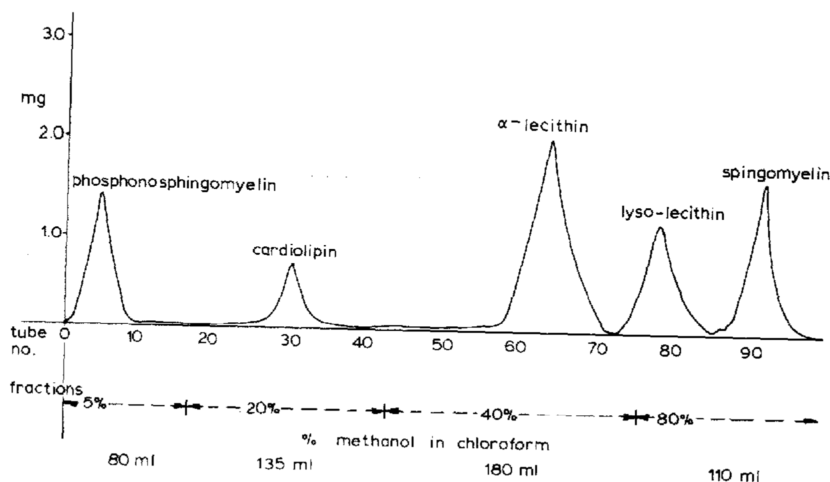

Fig. 1. Chromatography of phospholipids on silicic acid. Solvents: (a) 5%, (b) 20%, (c) 40% and (d) 80% methanol in chloroform. The column was loaded with a total of 23.90 mg of phospholipids in 5.0 ml of chloroform–methanol (1:1), plus 4.80 mg of sphingomyelin, 8.60 mg of α-lecithin, 2.10 mg of cardiolipin, 3.20 mg of phosphonosphingomyelin and 5.20 mg of lyso-lecithin.

of 0.96. A similar value was obtained in a corresponding system[10] containing glacial acetic acid.

Preliminary DEAE-cellulose column chromatography of the same class of phospholipids indicated no separation of phosphonosphingomyelin from α-lecithin, lyso-lecithin and sphingomyelin (which were eluted with chloroform in fractions 1–17; volume of the fractions collected = 6.0 ml), but an effective separation from cardiolipin [which was eluted with chloroform–methanol (4:1)–ammonia salt in fractions 82–92].

It was therefore decided to use silicic acid to carry out this separation, as pilot experiments using a silicic acid column with plain phosphonosphingomyelin indicated that the latter was eluted early in the process and collected in the cardiolipin fraction. Thus phosphonosphingomyelin was separated effectively from cardiolipin, being elut-

TABLE II

COMPOSITION OF FRACTIONS COLLECTED FROM CHROMATOGRAPHY OF PHOSPHOLIPIDS ON SILICIC ACID

23.90 mg of lipids were applied to 10 g of silicic acid and the total recovery was 23.74 mg (99.33%).

Concentration of methanol in chloroform (%)	*Fractions collected*	*Component identified from IR spectrum*	*TLC R_F value*	
			System A	*System B*
5	1– 9	Phosphonosphingomyelin	0.96	
20	24–37	Cardiolipin	0.68	
40	59–69	α-Lecithin	0.29	
80	74–84	Lyso-lecithin/		0.10
	85,86	sphingomyelin	0.15	0.10
	89–98	Sphingomyelin	0.16	

ed early in the process with only 5% methanol in chloroform and from the other phospholipids, which follow much later in this elution.

This behaviour of phosphonosphingomyelin was unusual, as also was the fact that its R_F value in system A was 0.96[8]. This is in contrast to other observations[11–14] that under normal circumstances phospholipids and phosphonolipids possess similar R_F values in chloroform–methanol–water (65:25:4). An effective separation was accomplished later[10], but the column chromatographic separation of structurally similar individual phospholipids and phosphonolipids has not previously been attempted.

Sphingomyelin and phosphonosphingomyelin were separated effectively with system A and also by silicic acid column chromatography. It can therefore be postulated that phosphonosphingomyelin exists as an ion carrying a net positive charge, which would then explain its unusual chromatographic behaviour.

The possibility also of the existence of intramolecular bonding is currently being investigated. Another interesting observation is the effective separation of lyso-lecithin from sphingomyelin, which is of practical interest and was not originally an objective of this work.

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