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ISOLATION OF GOAT SPLEEN PHOSPHONOLIPIDS BY THIN-LAYER CHROMATOGRAPHY: THEIR IDENTIFICATION AND SILICIC ACID COLUMN CHROMATOGRAPHIC SEPARATION

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SUMMARY

Goat spleen phosphonolipids were isolated by preparative thin-layer chromatography (TLC) using the solvent system methanol–water (2:1) and were identified by TLC, nitrogen–phosphorus determinations and IR spectroscopy. Silicic acid column chromatography of the isolated phosphonolipids confirmed the initial assignments.

INTRODUCTION

The presence of phosphonolipids in nature is well established[1–17]. Most of this work has involved *Tetrahymena pyriformis*, snails and various other marine species. In this paper is reported the isolation of phosphonolipids from goat spleen by preparative thin-layer chromatography (TLC) in methanol–water (2:1)[18]. By these means the phosphonolipids are obtained quantitatively and chromatographically pure from the original phospholipid fraction.

The isolated phosphonolipids were identified by TLC and IR spectroscopy; they were then subsequently subjected to silicic acid column chromatography, and were fully fractionated and identified.

EXPERIMENTAL

Materials

The solvents used were of pro analysi or analytical-reagent grade and were distilled before use. Silica gel G was purchased from Merck (Darmstadt, F.R.G.) and goat spleens were purchased from Schimatari (Viotias, Greece). Silicic acid for column chromatography was purchased from Sigma (St. Louis, MO, U.S.A.).

Methods

The goat spleen sample was obtained from young animals just after slaughter and weighed 280 g as fresh spleen. It was dried in a vacuum oven at 45°C to constant weight and was subsequently homogenised in chloroform–methanol (2:1) with a Sorvall homogeniser.

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Preparative TLC was performed on glass plates coated with silica gel G to a thickness of 0.75 mm. The chromatograms were developed in methanol–water (2:1) (system A) and the run normally took *ca.* 80 min for full development. The solvent system, chloroform–methanol–water (65:25:4) (system B) was also used for identification purposes and also for quantitative isolation of the phosphonolipid classes.

The spots and bands were made visible with iodine, ammonium molybdate and ninhydrin spray reagents and the Stillway–Harmon procedure[20].

IR spectra were recorded on a double-beam Perkin-Elmer 197 grating IR spectrophotometer as thin films from dry chloroform.

Total phosphorous and phosphono-phosphorus were determined by the procedure of Kapoulas[21] and total nitrogen and lipid nitrogen by the procedures of Kjeldahl and Lea *et al.*[22].

A glass column of length 40 cm and I.D. 2.4 cm was used for the chromatographic separation of the isolated total phosphonolipids.

Procedure

The lipids from the homogenised spleen sample were extracted according to the method of Folch and Lees[23,24] and the solvents were evaporated under vacuum using a bath at 35°C. The total lipids were then repeatedly extracted with acetone to constant phospholipid weight and the total phospholipids were dried in a vacuum desiccator over phosphorus pentoxide for 24 h.

The total phospholipids were dissolved in 34 ml of chloroform–methanol (2:1) and subjected to preparative TLC with solvent system A. The band of R_F 0.75–0.98 was scraped off and the phosphonolipids were obtained from the silica gel with chloroform–methanol (2:1).

The phospholipids were similarly obtained from the silica gel with the same solvents.

The phosphonolipids were checked for purity by re-chromatographing a small sample in solvent system A; no phosphorus or other lipid could be detected at the origin. Following this, the phosphonolipids were subjected to TLC analysis to identify the lipids present. They were chromatographed on glass plates coated with silica

TABLE I

AMOUNTS AND PERCENTAGES OF PHOSPHOLIPIDS AND PHOSPHONOLIPIDS OBTAINED FROM GOAT SPLEEN

Parameter	*Value*
Weight of goat spleen	280 g
Weight of total phospholipids isolated	0.816 g
Phospholipids in initial spleen sample	0.291%
Weight of phosphonolipids obtained after preparative TLC	0.028 g
Phosphonolipids in total phospholipids	3.43%
Phosphonolipids in initial goat spleen sample	0.010%

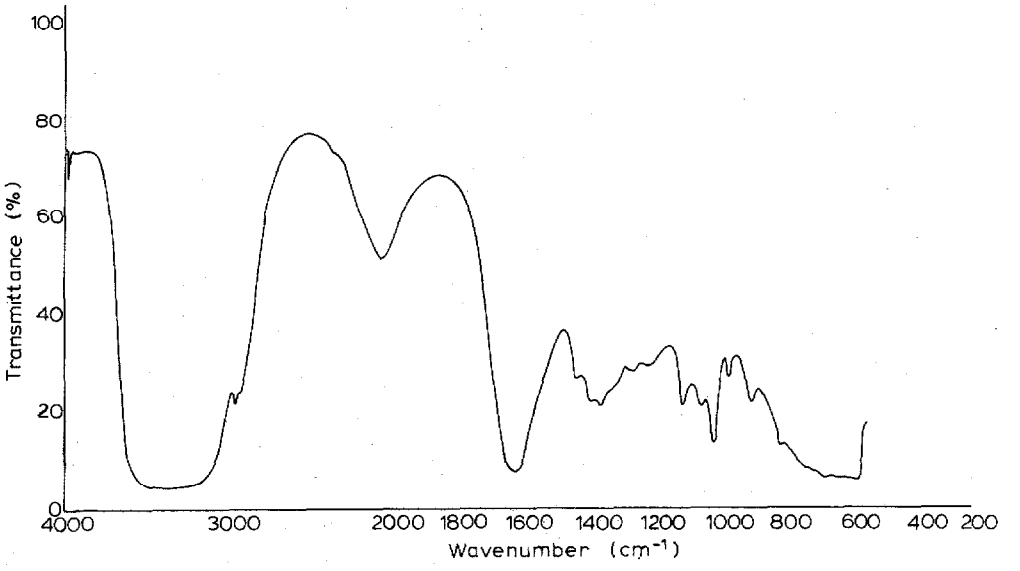

Fig. 1. IR spectrum of the total phosphonolipids isolated from goat spleen by preparative TLC.

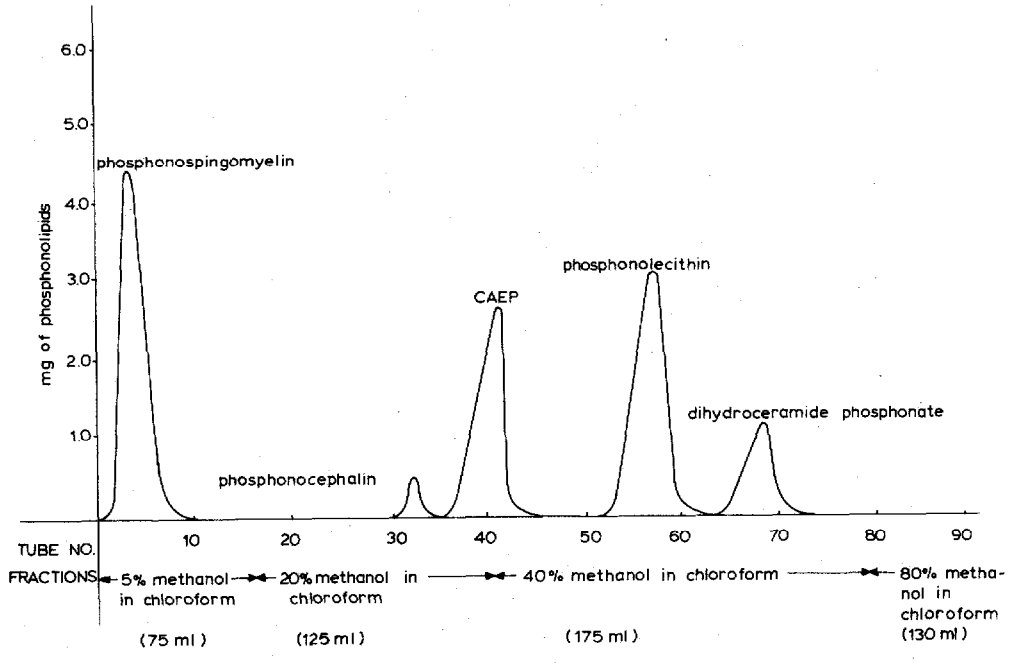

Fig. 2. Chromatographic elution pattern of the natural phosphonolipids isolated from goat spleen. Solvent: methanol–chloroform. The composition of the eluted fractions was phosphonosphingomyelin 11.96 mg, phosphonocephalin 0.58 mg, CAEP 6.36 mg, phosphonolecithin 7.88 mg and dihydroceramide phosphonate 1.16 mg. The phosphonolipids were applied to the column in 3.0 ml of chloroform.

TABLE II

ANALYTICAL AND OTHER DATA RELATING TO THE ISOLATED PHOSPHONOLIPIDS AFTER COLUMN CHROMATOGRAPHIC FRACTIONATION

Phosphonolipid	*R_F in system B*	*Nitrogen found (%)*	*Phosphorus found (%)*	*Total abundance in total phosphonolipids (%)*
Phosphonolecithin	0.23	2.08	4.30	28.00
Phosphonocephalin	0.69	2.51	4.61	1.99
CAEP	0.76	4.14	4.84	22.50
Phosphonosphingomyelin	0.88	3.97	4.14	42.50
Dihydroceramide phosphonate	0.17	4.05	4.81	5.05

gel G to a thickness of 0.25 mm in solvent system B. After initial identification the phosphonolipids were fractionated on a silicic acid column as described previously[25–29]; 10 g of silicic acid were used and the column was loaded to a height of 5.5 cm and a total volume of 25 ml. The flow-rate maintained in the elution was 1.8–2.0 ml/min.

RESULTS

Table I gives the amounts of phosphono- and phospholipids obtained from the goat spleen sample.

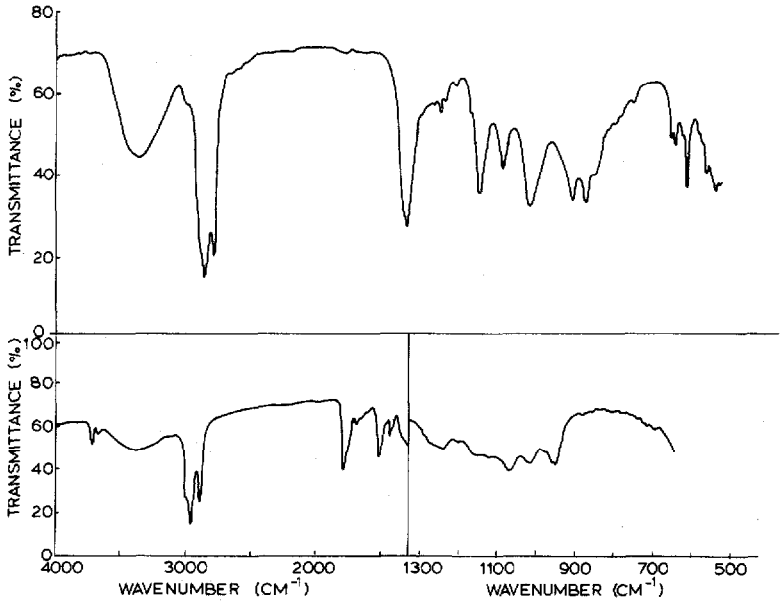

Fig. 3. IR spectrum of phosphonosphingomyelin isolated from goat spleen and fractionated on a silicic acid column (upper spectrum), compared with synthetic phosphonosphingomyelin (lower spectrum). The spectra are identical.

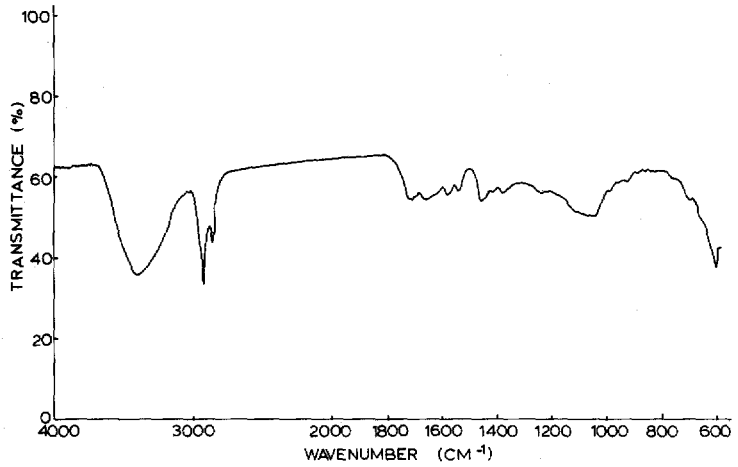

Fig. 4. IR spectrum of CAEP isolated from goat spleen and fractionated on a silicic acid column.

Chromatography of the phosphonolipid-free spleen phospholipids (TLC in solvent system B) provided evidence for the presence of the following phospholipids: lysolecithin (R_F = 0.13), sphingomyelin (R_F = 0.16), phosphatidylcholine (R_F = 0.34), phosphatidylethanolamine (R_F = 0.66) and cardiolipin (R_F = 0.86). In addition to the above, TLC of the total lipids in solvent system B furnished evidence for the presence of cerebrosides (R_F = 0.72), which were not isolated from the total lipids.

The IR spectrum of total goat spleen phosphonolipids is shown in Fig. 1.

TLC of the isolated phosphonolipids in solvent system B provided evidence for the presence of the following phosphonolipids in goat spleen: dihydroceramide phosphonate (R_F = 0.17), phosphonolecithin (R_F = 0.23), phosphonocephalin (R_F = 0.69), CAEP (R_F = 0.76) and phosphonosphingomyelin (R_F = 0.88).

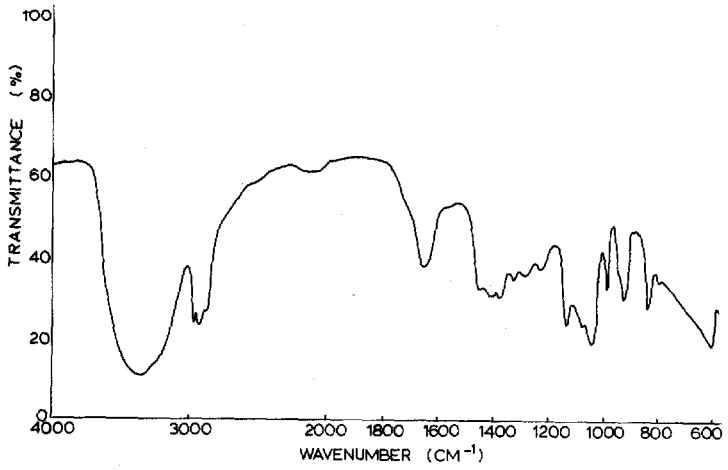

Fig. 5. IR spectrum of phosphonolecithin isolated from goat spleen and fractionated on a silicic acid column. The possible presence of glycerophosphonic acid is also postulated.

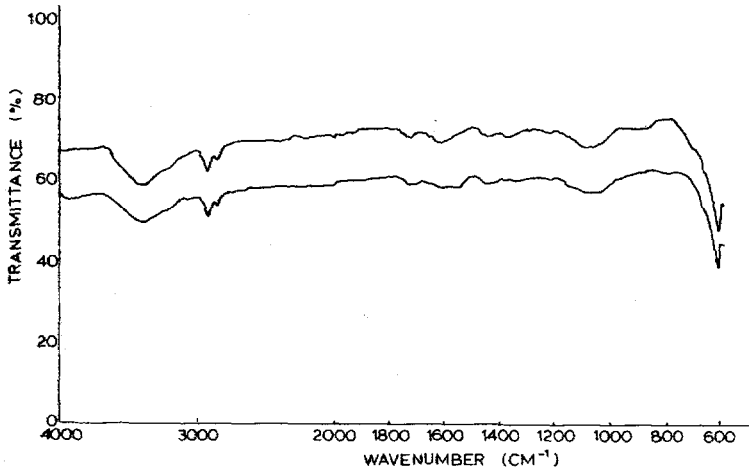

Fig. 6. IR spectrum of N-acyldihydroceramide phosphonate isolated from goat spleen and fractionated on a silicic acid column.

TABLE III

ELUTION OF THE CHROMATOGRAPHIC COLUMN WITH DIMENSIONS OF LENGTH 40 cm AND I.D. 2.4 cm, LOADED WITH 10.0 g OF SILICIC ACID TO A HEIGHT OF 5.5 cm AND A TOTAL COLUMN VOLUME OF 25 ml

Flow-rate: 1.8–2.0 ml/min. Fractions of approximately 5.0 ml were collected.

Methanol in chloroform (%)	*Column volumes*	*Total volume of solvent (ml)*	*Fractions collected*
5	3	75	1– 15
20	5	125	16– 42
40	7	175	43– 80
80	5	130	81–107

TABLE IV

COMPOSITION OF FRACTIONS OBTAINED FROM CHROMATOGRAPHY OF NATURAL PHOSPHONOLIPIDS ISOLATED FROM GOAT SPLEEN ON SILICIC ACID

28.00 mg of phosphonolipids were applied to the column. The total recovery was 27.94 mg (99.80%).

Solvent: methanol in chloroform (%)	*Fractions collected*	R_F		*IR spectral data: component identified*
		System A	*System B*	
5	2– 9	0.95	0.97	Phosphonosphingomyelin
20	30– 34	0.86	0.71	Phosphonocephalin
	34– 44	0.78	0.65	CAEP
40	52– 64	0.80	0.33	Phosphonolecithin
	65– 75	0.75	0.17	Dihydroceramide phosphonate
80	77–107	–	–	–

The total phosphonolipids were then chromatographed on a silicic acid column and thus separated and fractionated. The phosphonolipids appeared as shown in Fig. 2; their relative abundance in the total phosphonolipid fraction is indicated in Table II.

The phosphonolipids thus fractionated were analysed for total nitrogen, total phosphorus, phosphono-phosphorus and lipid nitrogen for the ethanolamine phosphonolipids. The IR spectra of the separated phosphonolipids compared well with those reported in the literature[14,30–34] (Figs. 3–6).

Further to the evidence provided earlier[18], TLC of various other standards in solvent system A indicated that cholesterol, oestrogens, glycerides, diglycerides and monoglycerides have $R_F = 0.00$.

DISCUSSION

The TLC method employed provides quantitative isolation and efficient separation of phosphonolipids from the total phospholipid fraction. There were no phospholipids in the phosphonolipid fraction as no phospholipid phosphorus could be detected[21]. Also, no phosphonolipid phosphorus was detected in the phospholipid fraction[21]. From data already published[18] and the present results it can be deduced that phosphonolipids can be completely separated from phospholipids using methanol–water (2:1) (system A). TLC of the total phosphonolipids provided evidence for the presence of phosphonocephalin, phosphonolecithin, phosphonosphingomyelin, CAEP and N-acyldihydroceramide phosphonate.

The fractionation pattern of the aforementioned natural phosphonolipids resembles that of the synthetic phosphonolipids[25–29]. The IR spectra of the respective fractions confirmed the initial assignments. With regard to Fig. 4, it can be postulated that an unidentified glycerophosphonic acid salt has also been isolated. With regard to phosphonosphingomyelin, this is the second instance that this phosphonolipid has been reported to have been isolated from natural sources[15].

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