*Journal of Chromatography*, 298 (1984) 366–371
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,836

## Note

# Sheep and goat brain phosphonolipids: isolation by thin-layer chromatography, identification and column chromatography

MICHAEL C. MOSCHIDIS
*A. Dedoussi E.E. Pharmaceutical Company, Schimatari, Vietias (Greece)*
(Received April 19th, 1984)

This work constitutes part of a project concerned with the isolation and identification of phosphonolipids from animal tissues and plants. So far the isolation and identification of the phosphonolipids from goat spleen have been reported[1], using preparative thin-layer chromatography (TLC) with methanol–water (2:1) as the solvent[2]. In a subsequent paper evidence was provided for the presence of phosphonolipids in apricot kernel[3].

This paper reports the quantitative isolation of the chromatographically pure phosphonolipids from whole sheep and goat brains. The isolated phosphonolipids were identified by TLC and IR spectroscopy, then subjected to silicic acid column chromatography, fractionated and identified.

## EXPERIMENTAL

### *Materials*

The solvents 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 silicic acid for column chromatography from Sigma (St. Louis, MO, U.S.A.). Sheep and goat brains were purchased locally.

### *Methods*

Sheep brain was obtained from a 2-year old animal just after slaughter and weighed 179.9 g as fresh brain; goat brain was obtained from a 1-year old animal just after slaughtter and weighed 105 g as fresh brain. The brain samples were homogenized in water–methanol–chloroform, (0.3:2.0:1.0) using a suitable homogenizer.

Preparative TLC was performed on glass plates coated with silica gel G to a thickness of 0.75 mm. The chromatograms were developed using methanol–water (2:1) as the solvent (system A) and the run took approximately 85 min for full development. Chloroform–methanol–water (65:25:4) (system B) was also used for identification purposes and also for the quantitative isolation of the respective phosphonolipid classes.

The spots and bands were rendered visible with iodine, ammonium molybdate and ninhydrin spray reagents and by the Stillway and Harmon procedure[4].

0021-9673/84/$03.00 © 1984 Elsevier Science Publishers B.V.

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

Total phosphorus, phosphono-phosphorus and nitrogen were determined as described previously[1,2].

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 homogenized whole brain samples were subsequently extracted according to the procedure of Bligh and Dyer[5] and the solvents were evaporated under vacuum from a bath at a temperature of 35°C. The residue was dissolved in 150 ml of chloroform and rapidly extracted twice with saline water. The chloroform layer was filtered dry through anhydrous sodium sulphate and again evaporated to dryness. The total lipids were extracted with acetone to constant phospholipid weight and 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 using solvent system A. The band whose $R_F$ ranged from 0.80 to 0.98 was scraped off and the phosphonolipids were obtained from the silica gel with chloroform. The phospholipids were similarly obtained from the silica gel with chloroform–methanol (2:1). The phosphonolipids were checked for purity by rechromatographing a small sample in solvent system A; no phosphorus or other lipid could be detected at the origin.

The phosphonolipids were then subjected to TLC to identify the lipids present. They were chromatographed using system B on glass plates coated with silica gel G to a thickness of 0.25 mm. The chromatograms were additionally tested for carbohydrates using an α-naphthol–sulphuric acid spray, and the absence of carbohydrates was confirmed.

After initial identification, the phosphonolipids were fractionated on a silicic acid column as described previously[1]. The amount of silicic acid used was 12 g (sheep brain) and 9.0 g (goat brain) and the column was loaded to a height of 7.5 cm (sheep brain) and 5.0 cm (goat brain) and a total volume of 31.0 ml (sheep brain) and 23.0 ml (goat brain), respectively. The flow-rate in both instances was maintained at 1.7–1.9 ml/min.

RESULTS

Table I indicates the amounts of phospholipids and phosphonolipids obtained from the whole brain samples. Chromatography of the phosphonolipid-free sheep and goat brain phospholipids (TLC in system B) provided evidence for the presence of the following phospholipids (with $R_F$ values for sheep and goat samples in that order): phosphatidylserine, $R_F$ 0.13 and 0.135; phosphatidylinositol, $R_F$ 0.23 and 0.23; sphingomyelin, $R_F$ 0.17 and 0.15; phosphatidylcholine, $R_F$ 0.33 and 0.33; cerebroside sulphate, $R_F$ 0.36 and 0.35; phosphatidylglycerol, $R_F$ 0.46 and 0.47; phosphatidylethanolamine, $R_F$ 0.65 and 0.67; cardiolipin, $R_F$ 0.73 and 0.71; and cerebrosides (total lipids), $R_F$ 0.75 and 0.78.

The IR spectra of the total whole brain phosphonolipids for sheep and goat are shown in Figs. 1 and 2, respectively.

TABLE I

AMOUNTS AND PERCENTAGES OF PHOSPHOLIPIDS AND PHOSPHONOLIPIDS OBTAINED FROM SHEEP AND GOAT BRAIN SAMPLES

| *Parameter* | *Sheep* | *Goat* |
|---|---|---|
| Weight of whole brain (g) | 179.9 | 105 |
| Weight of total phospholipids (g) | 11.37 | 6.50 |
| Phospholipids in initial brain sample (%) | 6.32 | 6.19 |
| Weight of phosphonolipids obtained after preparative TLC (g) | 0.041 | 0.021 |
| Phosphonolipids in total phospholipids (%) | 0.36 | 0.323 |
| Phosphonolipids in initial brain sample (%) | 0.0228 | 0.020 |

Chromatography of the isolated phosphonolipids in TLC system B provided evidence for the presence of the following phosphonolipids in whole sheep and goat brains, with $R_F$ values in that order: phosphonoserine, $R_F$ 0.17 and 0.15; phosphonolecithin, $R_F$ 0.32 and 0.30; phosphono analogue of phosphatidylglycerol, $R_F$ 0.48 and 0.46; phosphono-N,N-dimethylethanolamine, $R_F$ 0.56, and 0.55; phosphonoethanolamine, $R_F$ 0.72 and 0.70; ceramide aminoethylphosphate, $R_F$ 0.81 and 0.80; and phosphonosphingomyelin, $R_F$ 0.87 and 0.88.

The total phosphonolipids were then chromatographed on a silicic acid column, and were separated and fractionated as shown in Fig. 3.

Table II indicates the relative abundance of the various phosphonolipids in the fraction isolated. Table III provides information on the elution from the chro-

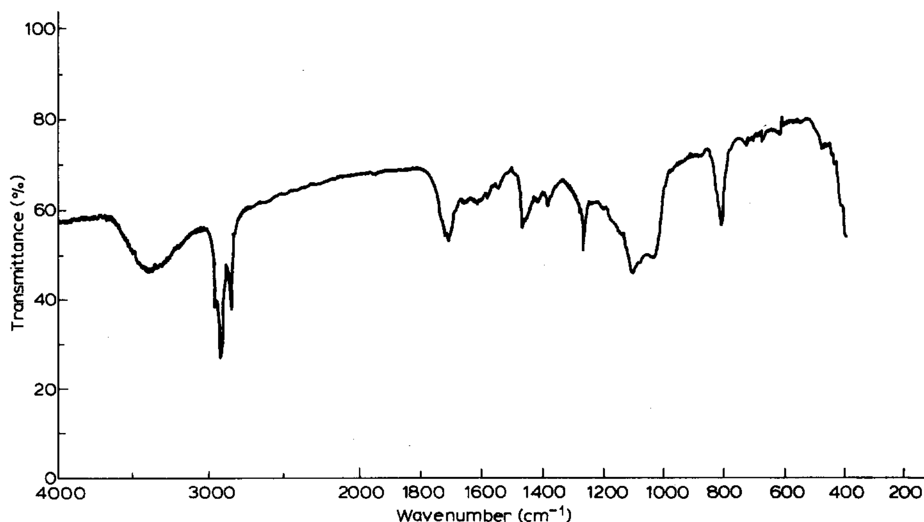


Fig. 1. IR spectrum of the total phosphonolipids isolated from sheep brain by preparative TLC.

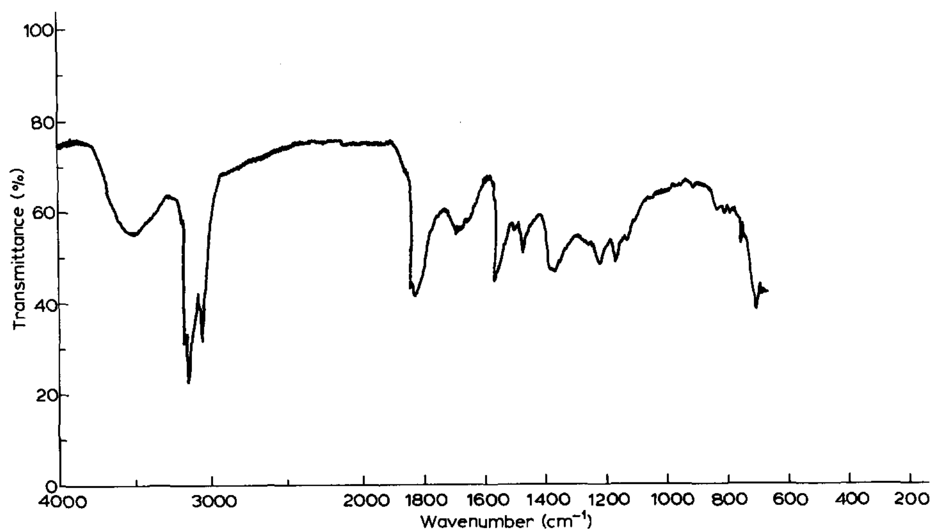


Fig. 2. IR spectrum of the total phosphonolipids isolated from goat brain by preparative TLC.

matographic column and the composition of the fractions obtained from column chromatography of the natural phosphonolipids is given in Table IV.

No amino acids or sugars were detected on the chromatograms.

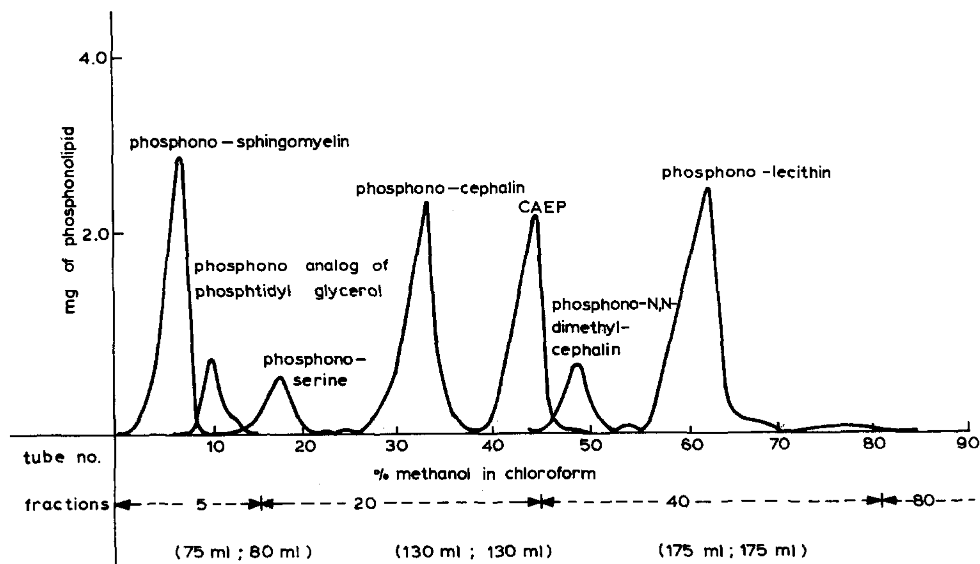


Fig. 3. Elution pattern of the natural phosphonolipids isolated from sheep brain. Solvents used were various percentages of methanol in chloroform. The composition of the fractions is indicated in Table II. The phosphonolipids were applied to the column in 3.0 ml of chloroform. The elution pattern of the goat brain phosphonolipids is similar to that exhibited by the sheep brain phosphonolipids.

TABLE II

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

| *Phosphono-lipid analogue of* | *$R_F$ (TLC system B)* | | *N (%)* | | *P (%)* | | *Abundance (%)* | |
|---|---|---|---|---|---|---|---|---|
| | *Sheep* | *Goat* | *Sheep* | *Goat* | *Sheep* | *Goat* | *Sheep* | *Goat* |
| Phosphatidylserine | 0.17 | 0.15 | 1.84 | 1.86 | 4.01 | 4.01 | 1.80 | 1.90 |
| Phosphatidylcholine | 0.32 | 0.30 | 2.03 | 2.06 | 4.36 | 4.40 | 27.9 | 26.9 |
| Phosphatidylglycerol | 0.488 | 0.46 | – | – | 2.78 | 2.80 | 2.84 | 2.96 |
| Phosphatidyl-N,N-dimethyl-ethanolamine | 0.55 | 0.56 | 1.88 | 1.91 | 4.60 | 4.62 | 1.90 | 1.96 |
| Phosphatidylethanolamine | 0.72 | 0.70 | 2.59 | 2.54 | 4.57 | 4.59 | 17.8 | 18.6 |
| Ceramide aminoethylphosphonate | 0.81 | 0.80 | 4.22 | 4.30 | 4.81 | 4.80 | 28.3 | 27.3 |
| Sphingomyelin | 0.87 | 0.88 | 3.82 | 3.86 | 4.37 | 4.38 | 28.9 | 29.1 |

TABLE III

ELUTION OF THE CHROMATOGRAPHIC COLUMNS

Column, 40 cm × 2.4 cm I.D., loaded with 12 g of silicic acid to a height of 7.5 cm and a total volume of 31.0 ml (sheep brain sample) or 9.0 g of silicic acid to a height of 5.0 cm and a total column volume of 23.0 ml (goat brain sample). Flow-rate, 1.7-1.9 ml/min. Fractions of approximately 5.0 ml were collected.

| *Methanol in chloroform (%)* | *Column volumes* | *Total volume of solvent (ml)* | | *Fractions collected* | |
|---|---|---|---|---|---|
| | | *Goat* | *Sheep* | *Goat* | *Sheep* |
| 5 | 3 | 75 | 80 | 1–14 | 1–16 |
| 20 | 5 | 130 | 130 | 15–44 | 17–45 |
| 40 | 7 | 175 | 175 | 45–80 | 46–81 |
| 80 | 5 | 130 | 130 | 81–106 | 82–108 |

## DISCUSSION

Phosphorus determinations were carried out on the phospholipid and phosphonolipid fractions separated by preparative TLC and were shown to be free from interfering phosphonolipids and phospholipids, respectively. Chromatography of the isolated phosphonolipids in solvent system B provided evidence for the presence of the phosphono analogues of phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatidyl-N,N-dimethylethanolamine, phosphatidylethanolamine, CAEP and sphingomyelin. The chromatograms were also examined for interfering amino acids and sugars by spraying with ninhydrin and α-naphthol–sulphuric acid reagents; none were detected.

The fractionation pattern of the isolated phosphonolipids proved to be of interest because of the presence of the phosphono analogs of phosphatidylserine, phosphatidylglycerol and phosphatidyl-N,N-dimethylethanolamine, which were initially

TABLE IV

COMPOSITION OF FRACTIONS OBTAINED FROM CHROMATOGRAPHY ON SILICIC ACID OF NATURAL PHOSPHONOLIPIDS ISOLATED FROM SHEEP AND GOAT BRAIN SAMPLES

To the two columns were applied 37 mg of sheep brain phosphonolipids or 20 mg of goat brain phosphonolipids. The total recovery was 36.93 mg (99.82%) for sheep brain phosphonolipids and 19.98 mg (99.90%) for goat brain phosphonolipids.

| *Solvent* | *Fractions* | | $R_F$ | | | | *Component identified from IR spectral data (sheep and goat)* |
|---|---|---|---|---|---|---|---|
| | *Sheep* | *Goat* | *System A* | | System B | | |
| | | | *Sheep* | *Goat* | *Sheep* | *Goat* | |
| 5% Methanol in chloroform | 2–7 | 3–9 | 0.95 | 0.96 | 0.87 | 0.88 | Phosphonosphingomyelin |
| | 8–12 | 8–14 | – | – | 0.48 | 0.46 | Phosphono analogue of phosphatidylglycerol |
| 20% Methanol in chloroform | 17–19 | 15–21 | | | 0.17 | 0.15 | Phosphono analogue of phosphatidylserine |
| | 26–37 | 23–35 | 0.86 | 0.84 | 0.72 | 0.70 | Phosphonocephalin |
| | 39–43 | 38–44 | 0.78 | 0.78 | 0.81 | 0.80 | Ceramide aminoethylphosphate |
| 40% Methanol in chloroform | 47–51 | 48–53 | | | 0.56 | 0.55 | Phosphono analogue of phosphatidyl-N,N-dimethylethanolamine |
| | 54–66 | 55–67 | 0.80 | 0.79 | 0.32 | 0.30 | Phosphonolecithin |
| 80% Methanol in chloroform | 82–108 | 81–106 | – | – | – | – | – |

identified by TLC in system B. No data where available on the silicic acid column chromatographic behaviour and elution pattern for these phosphonolipids. The phosphono analogue of phosphatidylglycerol is eluted with 5% methanol in chloroform, followed by the phosphono analogue of phosphatidylserine with 20% methanol in chloroform, then phosphonocephalin, the phosphono analogue of dimethylethanolamine is eluted with 40% methanol in chloroform and last phosphonolecithin.

No information is available in the literature on the silicic acid column chromatographic behaviour of the synthetic phosphonolipids mentioned above.

## REFERENCES

1 M. C. Moschidis, *J. Chromatogr.*, 268 (1983) 485, and references cited therein.
2 C. A. Demopoulos, M. C. Moschidis and L. G. Kritikou, *J. Chromatogr.*, 256 (1983) 378.
3 M. C. Moschidis, *J. Chromatogr.*, 294 (1984) 519.
4 L. W. Stillway and S. J. Harmon, *J. Lipid Res.*, 21 (1980) 1141.
5 M. Kates, in T. S. Work and E. Work (Editors), *Techniques of Lip idology —Isolation, Analysis and Identification of Lipids*, North-Holland, Amsterdam, 1972, pp. 347–353.