Isolation of Beef Brain Phosphonolipids by Thin Layer Chromatography: Their Identification and Silicic Acid Column Chromatographic Separation

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The isolation and identification of the phosphonolipids from beef brain – right lobe, left lobe and cerebellum – is herein reported. The phosphonolipids were isolated by preparative thin layer chromatography (TLC) using the solvent system methanol/water (2:1, v/v) and identified preliminarily by TLC. The isolated phosphonolipids were subjected to silicic acid column chromatographic separation and the thus separated phosphonolipids were identified by TLC, nitrogen-phosphorus determinations and IR spectroscopy.

Introduction

In this continuing project concerned with the isolation and identification of phosphonolipids from animal and plant tissues, the isolation and identification of the phosphonolipids from whole sheep and goat brains was reported in a previous paper [1].

In order to investigate the matter more thoroughly it was decided to subdivide the brain into the three major parts and determine the inherent phosphonolipids in each part separately.

Consequently, in this present communication is reported the isolation of the phosphonolipids from beef brain right and left lobes and cerebellum by preparative TLC in methanol/water (2:1, v/v) [2]. Initial assignments were accomplished by TLC using the solvent system chloroform/methanol/water (65:25:4, v/v/v) and following this the isolated phosphonolipids were fractionated on a silicic acid column.

The individual phosphonolipids thus separated were subsequently identified by TLC, nitrogen-phosphorus determinations and IR spectroscopy.

Experimental

Materials and Methods

Solvents used were pro-analysis or AR grade and were distilled before use. Silica gel G was purchased from Merck, Darmstadt, GFR, and silicic acid for column chromatography from SIGMA Chemical Company, St. Louis, Missouri, USA.

The beef brain sample, which weighed 296.18 g, was obtained from a 15-month old animal following slaughter. The three samples, right and left lobes and cerebellum, were homogenized separately in chloroform/methanol (2:1, v/v) with a Sorvall homogenizer.

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, v/v) as solvent (system A) with the run normally taking approx. 80 min for full development. The solvent system, chloroform/methanol/water (65:25:4, v/v/v) (system B) was also used for identification purposes and for the quantitative isolation of the respective phosphonolipids.

Visualisation was effected with iodine, ammonium molybdate, ninhydrin, α-naphthol-sulphuric acid sprays and the Stillway-Harmon procedure [3]. Total phosphorus and phosphono-phosphorus were determined by the procedure of Kapoulas [4] and total nitrogen and lipid nitrogen by the procedures of Kjeldahl and Lea-Rhodes [5].

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

A glass column 1.6 × 35 cm was used for the chromatographic separation of the isolated phosphonolipids.

Procedure

The lipids from the homogenized brain samples were extracted according to the procedure of Bligh-Dyer [6] and the solvents were evaporated under vacuum at 35 °C. The residue was redissolved in 150 ml of chloroform and rapidly extracted several times with water containing 5% NaCl. The chloroform layer was filtered through anhydrous sodium sulphate and again evaporated to dryness. The total lipids were, in each individual case, extracted exhaustively with acetone to constant, phospholipid weight and were dried in a vacuum desiccator over phosphorus pentoxide for 24 h at 7 °C.

The total lipids were then dissolved in chloroform/methanol (2:1, V/V) and subjected to preparative TLC in solvent system A. The band whose R_F ranged from 0.80–0.98 was scraped off and the phosphonolipids were re-extracted from the silica gel using chloroform. The phospholipids were similarly

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re-extracted from the origin using chloroform/methanol (2:1, v/v).

Following this the phosphonolipids were checked for purity by rechromatographing a small sample in solvent system A, when no phosphorus or other lipid could be detected at the origin.

The phosphonolipids were then subjected to TLC analysis to identify preliminarily the components present. They were chromatographed on glass plates coated with silica gel G to 0.25 mm thickness, in system B. The chromatograms were additionally tested for carbohydrates (α-naphtho/sulphuric acid spray) and amino-acids (ninhydrin spray) and the absence of both classes of compounds was confirmed.

After initial identification the phosphonolipids were fractionated on a silicic acid column as described in previous experiments [7].

Silicic acid, 11.00 g, was used for the three separate chromatographic experiments; the columns were loaded to a height of 11.2 cm with a total volume of 26.5 ml, in each case. The flow rate was maintained at 1.6–1.8 ml per minute.

Results

The accompanying Table I provides information regarding the amounts of phospholipids and phosphonolipids present in the three separate brain samples. No attempt was made to identify the phospholipids present in the respective fraction, as this did not constitute the object of this project.

The IR spectra for the three phosphonolipid fractions – right lobe, left lobe and cerebellum – are shown in Figs. 1, 2 and 3, respectively. Frequencies characteristic for phosphonolipids were observed at 1010, 1040, 1076 and 1120 cm^{-1}.

Table I. Amounts and percentages of phospholipids and phosphonolipids obtained from the respective beef brain samples.

	Right lobe	Left lobe	Cerebellum
Weight of beef brain	109.97 g	133.81 g	52.40 g
Weight of total phospholipids isolated	5.56 g	7.14 g	6.44 g
% Phospholipids in initial whole brain sample	1.90	2.41	2.17
Weight of phosphonolipids after preparative TLC	0.185 g	0.207 g	0.184 g
% Phosphonolipids in total phospholipids	3.33	2.90	2.86
% Phosphonolipids in initial brain sample	0.168	0.160	0.350
% Phosphonolipids in initial whole brain sample	0.062	0.069	0.062

The following phosphonolipids were identified in the respective brain samples:

Beef brain left and right lobes:
phosphono analogue of phosphatidyl serine, $R_F = 0.13/0.13$
phosphono analogue of lecithin, $R_F = 0.40/0.43$
phosphono analogue of cephalin, $R_F = 0.76/0.74$
ceramide amnioethyl phosphonate, $R_F = 0.84/0.84$
phosphono analogue of sphingomyelin, $R_F = 0.96/0.96$

Beef brain cerebellum:
phosphono analogue of phosphatidyl serine, $R_F = 0.14$
phosphono analogue of lecithin, $R_F = 0.42$
phosphono analogue of cephalin, $R_F = 0.74$
ceramide aminoethyl phosphonate, $R_F = 0.86$
phosphono analogue of sphingomyelin, $R_F = 0.92$

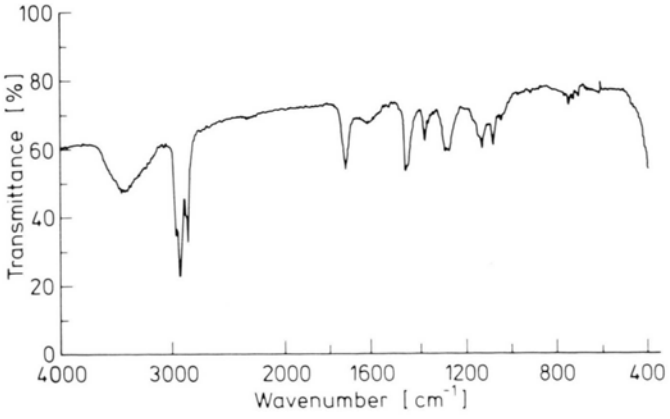

Fig. 1. IR spectrum of the total phosphonolipids isolated from beef brain right lobe.

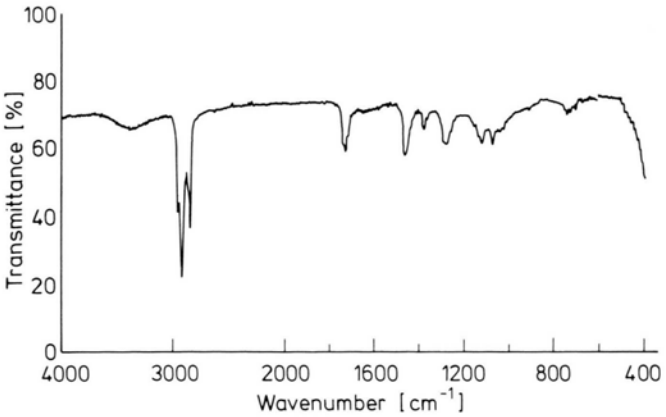

Fig. 2. IR spectrum of the total phosphonolipids isolated from beef brain left lobe.

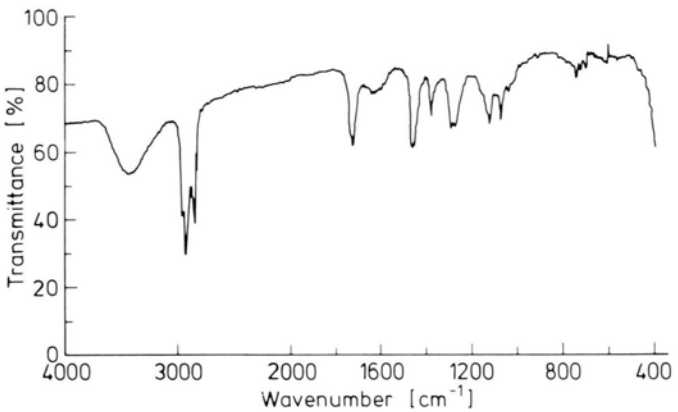

Fig. 3. IR spectrum of the total phosphonolipids isolated from beef brain cerebellum.

Table II provides information regarding the relative abundance of the various phosphonolipids in the fractions isolated. The fractionation pattern for the phosponolipids for the three separate cases collectively is as shown in Fig. 4.

Information regarding the elution of the column is given in Table III and the composition of the fractions obtained by the column chromatography of the natural phosphonolipids is contained in Table IV. During the course of these investigations, no amino-

Table II. Analytical data for the isolated phosphonolipids after column chromatographic separation and fractionation.
R–L: Right lobe. L–L: Left lobe. C: Cerebellum.

Phosphonolipid	TLC-R_F in system B			% N	% P	% abundance		
	R–L	L–L	C			R–L	L–L	C
Phosphono-serine	0.13	0.13	0.14	1.90	4.04	4.00		4.30
Phosphono-lecithin	0.40	0.43	0.42	2.10	4.26	33.4		33.8
Phosphono-cephalin	0.76	0.74	0.74	2.60	4.43	16.5		16.0
CAEP	0.84	0.82	0.86	4.08	4.96	18.1		16.9
Phosphono-sphingomyelin	0.96	0.94	0.92	4.01	4.10	27.6		28.4

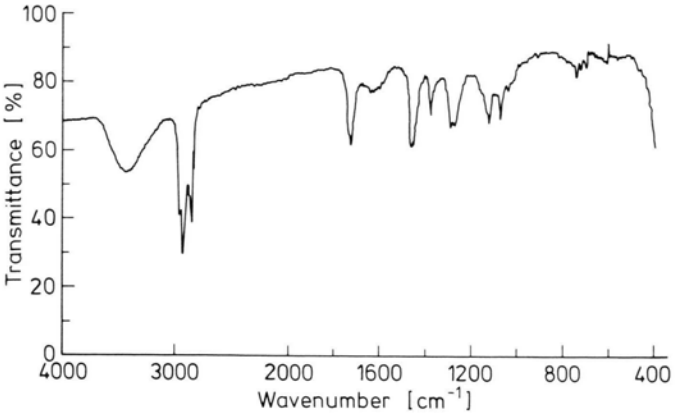

Fig. 4. General elution pattern of the natural phosphonolipids isolated from the three brain samples: beef brain right lobe, beef brain left lobe and beef brain cerebellum. Solvents used were various percentages of methanol in chloroform. The composition of the fractions is indicated in Table II. The phosphonolipids were applied to the column in 2.5 ml of chloroform.

acids or sugars were detected on the chromatograms examined.

Table III. Elution of the chromatographic columns. Column, 35 cm × 1.6 cm I.D., loaded with 11 g of silicic acid to a height of 11.2 cm and a total volume of 26.5 ml (approx. values for the three brain samples). Flow rate, 1.6–1.8 ml per minute. Fractions of approximately 4.5 ml were collected.

% Methanol in chloroform	Column volumes	Total ml of solvent	Fractions collected
5	3	75	1–18
20	5	130	19–46
40	7	180	47–80
80	5	110	81–102

Discussion

The presence of phosphonolipids in whole sheep and goat brains has been demonstrated and reported previously [1]. The possibility of the existence of unidentified phosphonolipids was pointed out to account for the differences in the phosphorus values observed.

To investigate the matter further, it was decided to use beef brain, which, because of its size, could be subdivided into the three major parts required for these experiments. By this means, the nature, number and amount of the various phosphonolipids in the three brain samples could be readily determined. The isolated phosphonolipids were submitted to chromatographic analyses and nitrogen-phos-

Table IV. Composition of fractions obtained by chromatography on silicic acid of natural phosphonolipids isolated from beef brain right lobe, left lobe and cerebellum samples. To the three columns were applied 40 mg of total phosphonolipids from the respective brain samples. The total recovery for beef brain right lobe was 39.6 mg (99.0%); for beef brain left lobe 39.8 mg (99.5%) and for beef brain cerebellum 39.85 mg (99.6%).

Solvent	Fractions collected	TLC R_F system A	R_F system B	IR spectral data component identified
5% methanol in chloroform	2–10	0.94	0.96	phosphono-sphingomyelin
20% methanol in chloroform	18–24	–	0.13	phosphono-phosphatidyl serine
	26–36	0.86	0.76	phosphono-cephalin
	38–45	0.78	0.84	CAEP
40% methanol in chloroform	57–68	0.80	0.40	phosphono-lecithin
80% methanol in chloroform	–	–	–	–

phorus determinations; 99.4 per cent of the phosphono phosphorus could be accounted for. The possibility of the presence of other phosphonolipids in the total phospholipid extract cannot be ruled out. The phosphonolipid fraction contained no interfering phospholipids; the fraction was not contaminated with either amino-acids or sugars.

The IR spectra of the total phosphonolipids from the three brain samples bear a close resemblence to each other. From the above experimental data, it was concluded that the whole animal brain could be employed for the determination of the inherent phosphonolipids. An interesting observation is that cerebellum contains slightly higher amounts of total lipids, compared to the two lobes. By the joint application of thin layer and silicic acid column chromatography, the beef brain phosphonolipids have been isolated, fractionated and characterized.

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