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Short Communications

The Chromatographic Separation of Phospholipids on Alumina with Solvents Containing Ammonium Salts

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Although several methods have been described for the use of alumina for phospholipid separations (see Sheltawy & Dawson, 1969), they suffer mainly from two disadvantages. First, most procedures have employed commercially supplied alumina of Brockmann grade II. This adsorbent gains considerable amounts of moisture on storage, and this may have caused various investigators to describe different conditions for the elution of a certain phospholipid [compare Long & Owens (1966) and Dawson (1958)]. Secondly, extremes of pH were sometimes employed to elute certain acidic phospholipids (phosphatidylserine; Long *et al.*, 1962). Even then, the behaviour of some acidic phospholipids (e.g. cardiolipin) on alumina was not described.

In this communication we standardize the conditions for the elution of phospholipids from freshly prepared alumina of Brockmann grade IV, and describe the novel use of ammonium salts for the fractionation of most of the acidic phospholipids under mild conditions.

Materials and Methods

The phospholipid mixture used in the present work was extracted (Folch *et al.*, 1957) from lamb liver obtained freshly from the local slaughterhouse. The combined chloroform–methanol extracts were purified by adding chloroform and 40mM-$CaCl_2$ so that the final composition of the mixture was chloroform–methanol–40mM-$CaCl_2$ (4:2:3, by vol.). The high concentration of water in this system produces a lower phase with maximum chloroform content. This condition leads to quantitative recovery of all the phospholipids, including the acidic and lyso compounds (Sheltawy & Dawson, 1969). The lower phase was further washed with 0.5vol. of synthetic upper phase [methanol–40mM-$CaCl_2$ (2:3, v/v)]. All phospholipids were therefore in the calcium form. The yield of phospholipids varied slightly from preparation to preparation, but on average 1.03mg of phospholipid P/g fresh wt. of liver was extracted. The mixture had the following composition when analysed by t.l.c.: phosphatidylcholine (lecithin), 47%; phosphatidylethanolamine+cardiolipin, 35%; phosphatidylserine+sphingomyelin, 10%; phosphatidylinositol, 8%.

'Neutral' alumina for chromatography was supplied by M. Woelm, Eschwege, Germany. To prepare Brockmann grade IV, the commercially supplied adsorbent was activated at 110°C overnight, 10ml of distilled water/100g of adsorbent was added and the mixture was shaken in a closed container for 2h.

Silica gel H (E. Merck A.-G., Darmstadt, Germany) was washed and adjusted to pH7 with solid Na_2CO_3 as described by Sheltawy & Dawson (1969). The slurry was prepared in 1% (w/v) potassium oxalate as described by Gonzalez-Sastre & Folch (1968). The solvent system chloroform–methanol–water–15M-ammonia (65:50:4:11, by vol.) was used.

The phosphorus content of the various fractions eluted from the columns was measured by the method of Bartlett (1959). Phospholipids were identified by the successive-hydrolysis procedure of Dawson (Sheltawy & Dawson, 1969).

Results

When Brockmann grade II alumina was freshly prepared by activating the commercially supplied adsorbent at 110°C overnight and adding 3ml of distilled water/100g, the phospholipids were tightly bound and the elution pattern was quite different from that described by previous authors (Long & Owens, 1966). We have found that it was only when Brockmann grade IV was prepared (see above) that elution of phospholipids was possible.

Columns of alumina typically made of 100g of adsorbent (diameter/length ratio 1:5) were prepared in chloroform–methanol–water (66:33:3.2, by vol.). The lipid sample (0.5mg of P/g of adsorbent) was introduced in the same solvent and elution was continued (4 column volumes) to remove the simple lipids and phosphatidylcholine in a sharp peak. To elute sphingomyelin and lysophosphatidylcholine 5 column volumes of chloroform–methanol–water (25:25:4, by vol.) were used, and to elute ethanolamine phospholipids 9 column volumes of chloroform–methanol–water (10:10:3, by vol.) were used. The pattern of elution of phospholipids under these conditions is illustrated in Fig. 1(*a*).

The solvents used to elute the acidic phospholipids were then applied to the column. These contained

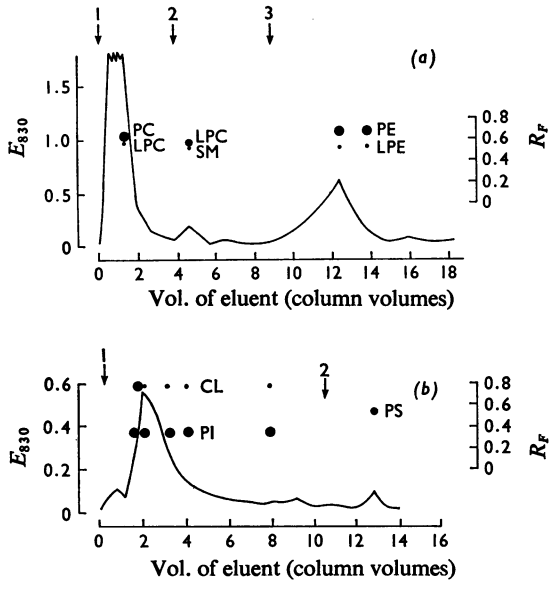

Fig. 1. *Elution pattern of simple lipids and phospholipids from columns of Brockmann grade IV alumina*

The dimensions of the columns are described in the text. Portions of each fraction (0.2ml) were used to determine the phosphorus content by the method of Bartlett (1959), and the E_{830} values obtained are indicated by the left-hand scale. Numbered arrows indicate the solvent sequence: in (*a*), 1, chloroform–methanol–water (66:33:3.2, by vol.); 2, chloroform–methanol–water (25:25:4, by vol.); 3, chloroform–methanol–water (10:10:3, by vol.); in (*b*), 1, chloroform–ethanol–70mM-NH_4NO_3 (18:25:7, by vol.); 2, chloroform–ethanol–70mM-ammonium acetate (18:25:7, by vol.). The right-hand scale indicates the R_F values of spots on t.l.c. examination of the fractions shown. The elution patterns for phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) are shown in (*a*), and those of phosphatidylinositol (PI), phosphatidylserine (PS) and cardiolipin (CL) are shown in (*b*).

salts but no added acid or alkali, mainly to produce the mildest possible conditions for elution. The column was eluted with 10 column volumes of chloroform–ethanol–70mM-NH_4NO_3 (18:25:7, by vol.). This solvent had a measured pH of 5.1. The peak eluted contained phosphatidylinositol and cardiolipin. Finally, phosphatidylserine was eluted with 4 column volumes of chloroform–ethanol–70mM-ammonium acetate (18:25:7, by vol.). This solvent had a measured pH of 7.8.

The following comments on the composition of the solvents are appropriate at this juncture: (1) addition of ammonia to the above-mentioned solvents did not alter the elution patterns demonstrated in Fig. 1(*b*), only perhaps the sharpness of the cardiolipin fractions was increased; (2) the high concentration of ethanol in the solvents used to elute the acidic phospholipids was essential; decreasing the proportion of ethanol led to a considerable diminution in the elution capacity of the solvents.

For large-scale preparations 600–1000g of alumina Brockmann grade IV was used in columns of diameter/length ratio 1:2–3. The same loading ratio and sequence of solvents as for the small-scale experiments was maintained. The final volumes of solvents used were as described above and illustrated in Figs. 1(*a*) and 1(*b*). The whole chromatographic procedure usually lasted for 10h from the point of sample introduction to the collection of phosphatidylserine. Fractions of the latter phospholipid were pure when examined either by t.l.c. or by examination of their successive hydrolysis products (Sheltawy & Dawson, 1969). Salts were removed from the pooled phospholipid fractions by adjusting the composition of the solvent system to chloroform–ethanol–water (4:2:3, by vol.). The lower phase contained the pure phospholipids and the upper phase the salts.

It is difficult to measure the true degree of hydrolysis on the columns in terms of the total recovery of a phospholipid, since the values obtained will include low recoveries due to autoxidation. All the phosphatidylinositol introduced to the columns was usually recovered, and during a 10h run only 8% of this phospholipid was in the form of the lyso derivative. Incomplete recovery of phosphatidylserine (60–70%) was, however, experienced, but only 21% of this was in the form of the lyso derivative.

Discussion

The use of alumina as a chromatographic adsorbent for phospholipid separations has been restricted during recent years mainly because of its alkaline nature (Sheltawy & Dawson, 1969). The rate of hydrolysis taking place on its surface varies with the phospholipid and the chromatographic conditions employed. For example, 14% of applied phosphatidylinositol was lost during a 6h elution with a chloroform–methanol–water solvent system (Long & Owens, 1966). Moreover, with an eluting solvent containing KOH, the breakdown of phosphatidylserine has been estimated as 85%/12h at room temperature (Long *et al.*, 1962).

The principle demonstrated in this communication that acidic phospholipids can be fractionated on alumina simply by including ammonium salts in the eluting solvents has two advantages. First, by eluting phospholipids as sharp peaks in small volumes (for

comparison with previous values see Sheltawy & Dawson, 1969) the duration of contact with the adsorbent is minimized. Secondly, by avoiding extremes of pH (13.8 with the KOH-containing solvent mentioned above) the breakdown of phospholipids is also minimized. The rate of breakdown of acidic phospholipids obtained in the present work is less than that quoted by previous workers (see above).

The ability of different salts to elute specific phospholipids must presumably be related to their degree of ionization and consequently to the final pH of the solvent system. Ammonium acetate provided a pH higher than that provided by ammonium nitrate (7.8 and 5.1 respectively). The latter salt was therefore used to elute phosphatidylinositol+cardiolipin and the former to elute phosphatidylserine.

The conditions described here are particularly useful for the large-scale preparation of acidic phospholipids. The phosphatidylserine fractions contained no contaminating phospholipids, and the peak containing phosphatidylinositol and cardiolipin is easily fractionated to individual components on much smaller columns of silicic acid (for methods see Sheltawy & Dawson, 1969).

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