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Note

Large scale purification of phosphatidylcholine from egg yolk phospholipids by column chromatography on hydroxylapatite

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Phosphatidylcholines have been purified from natural sources by column chromatography on silicic acid[1,2] or alumina[3,4], using a mixture of chloroform and methanol. These methods require high capacity columns, tedious elutions and a rechromatography of phosphatidylcholine to remove traces of lysophosphatidylcholine and sphingomyelin. The time and manipulation required can lead to the possible oxidation of unsaturated phosphatidylcholines[5]. Purification of phosphatidylcholine and phosphatidylethanolamine from crude egg phospholipids on radially compressed silica gel columns using a Waters Prep LC-500 has been described[6]. This method requires a large amount of solvent, and instrumentation not readily available in most laboratories.

Hydroxylapatite, $Ca_{10}(PO_4)_6(OH)_2$, a modified form of crystalline calcium phosphate developed by Tiselius *et al.*[7], has been used successfully for the separation of proteins[8], lipoproteins[9], and polynucleotides[10]. Slomiany and Horowitz[11] first reported the use of hydroxylapatite, prepared by the Tiselius method, as an adsorbent during the column chromatographic separation of polar lipids from cattle serum. This method resolved complex lipid mixtures, but it required careful preparation of the adsorbent to achieve good separations and reasonable flow-rates. Recent improvements in the preparation of hydroxylapatite have been reported[12–14]. Commercial products now make possible the use of hydroxylapatite with high crystal integrity and improved flow-rate, load capacity and resolution. This report describes the use of an improved hydroxylapatite for the rapid, large scale purification of phosphatidylcholine from crude egg yolk phospholipids.

This method is advantageous, as a single column and a short period of time is required for purification, thus avoiding the possible degradation of phosphatidylcholine. Extensive preparation of the hydroxylapatite before use is not required and chloroform, a potentially hazardous solvent, is avoided.

MATERIALS

Hydroxylapatite (High Resolution/dry), egg yolk phospholipids and phospholipid standards were obtained from Calbiochem (La Jolla, CA, U.S.A.). Methylene chloride, acetone and methanol were obtained from J. T. Baker (Phillipsburg, NJ,

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U.S.A.). Thin-layer chromatographic (TLC) plates coated with silica gel were obtained from E. Merck (Darmstadt, G.F.R.). They were developed in chloroform–methanol–water (65:25:4). Iodine and molybdate spray[15] were used to detect phospholipids on the plates, and the contents of each fraction were identified by direct chromatographic comparison with authentic standards. Phosphate determination were performed by the method of Martin and Doty[16].

EXPERIMENTAL AND RESULTS

Purification of phosphatidylcholine

Hydroxylapatite (300 g) was suspended in methylene chloride–acetone (1:1) and the slurry was poured into a 6 × 50 cm glass column fitted with a coarse fritted disc. The column was equilibrated with 2 l of methylene chloride–acetone (1:1). Crude egg phospholipids (10 g) dissolved in 50 ml of methylene chloride–acetone (1:1) were applied onto the column and carefully rinsed in with 100 ml of the same solvent. The column was eluted at a flow-rate of 30 ml/min, collecting 1-l fractions as indicated in Table I.

DISCUSSION

Rapid, large scale purification of phosphatidylcholine from egg yolk phospholipids can be achieved on hydroxylapatite without prewashing, activation or removal of fines, in sharp contrast to hydroxylapatite prepared by the Tiselius method[11]. Excellent flow-rates are observed with a simple gravity solvent feed system. Hydroxylapatite fines are not observed in any of the fractions.

TABLE I

ELUTION OF EGG PHOSPHOLIPIDS FROM HYDROXYLAPATITE

Acetone–methanol ratio of eluent (v/v)	*Total volume (l)*	*Fraction number*
9:1	2	1–2
8:2	1	3
7:3	10	4–13
6.5:3.5	3	14–16
6:4	2	17–18
5:5	2	19–20

Using a 30:1 ratio of hydroxylapatite to sample, pure phosphatidylcholine can be eluted with acetone–methanol (7:3) after brief elution of neutral lipids with acetone–methanol (9:1 and 8:2). Approximately 95% of the phosphatidylcholine in crude egg phospholipids was recovered without using chloroform, a potentially hazardous solvent used in the traditional purification methods. A single column chromatography provides pure phosphatidylcholine in a short period of time compared to silicic acid or alumina column chromatography.

We have also used this procedure to purify synthetic phosphatidylcholine prepared by the acylation of glycerophosphatidylcholine, and to isolate pure phospha-

tidylcholine from crude soy phospholipids. This procedure provides chromatographically pure phosphatidylcholine for further studies or conversions into intermediates.

Thin-layer chromatograms of each fraction were developed in chloroform–methanol–water (65:25:4) and visualized by iodine vapors or molybdate spray (Fig. 1). TLC indicated that fractions 1–3 contained neutral lipids. Fractions 4–11 contained pure phosphatidylcholine which were combined and the solvents were removed by evaporation. Upon drying, pure phosphatidylcholine (6.3 g) was isolated. The purity and identity was confirmed by comparison using TLC and high-performance liquid chromatography (HPLC)* with authentic standard. Phosphorus analysis based on mean moleculear weight of 772 for phosphatidylcholine showed: theoretical = 4.01%, found = 3.96%.

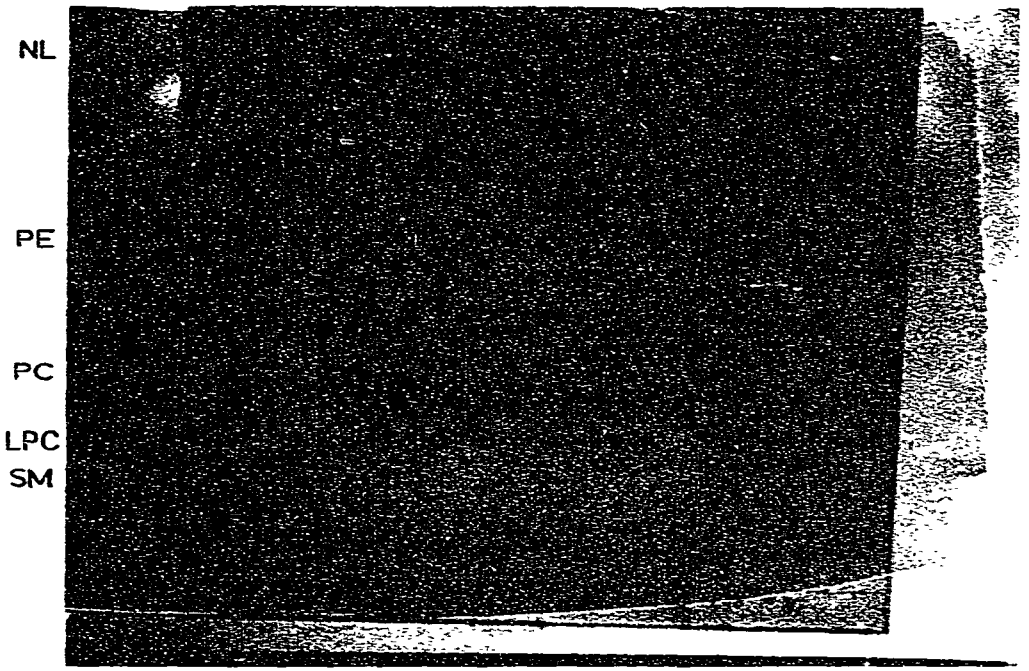

Fig. 1. Thin-layer chromatogram of fractions eluted from the chromatography of egg yolk phospholipids on hydroxylapatite as shown in Table I. The silica gel plate was developed in chloroform-methanol-water (65:25:4) and the spots visualized by exposure to iodine vapors. O = Egg yolk phospholipids; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin; LPC = lysophosphatidylcholine; NL = neutral lipids.

Evaporation of the solvent from fractions 12–17 (sphingomyelin with a trace of phosphatidylcholine) yielded 0.14 g; fractions 18–20 (sphingomyelin with a trace of lysophosphatidylcholine) yielded 0.20 g.

* HPLC analysis was performed on a Waters high-pressure liquid chromatographic system using a μPorasil column (Waters Assoc., Milford, MA, U.S.A.). The column was eluted with hexane–isopropanol–water (6:8:1, v/v) at a flow-rate of 1 ml min and detection at 206 nm.

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