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SEPARATION OF POLAR LIPIDS BY COLUMN CHROMATOGRAPHY ON HYDROXYLAPATITE*

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SUMMARY

The separation of various lipids by column chromatography on hydroxylapatite was investigated. Selective elution of lipid classes was effected by varying the ratio of acetone to methanol in the eluant. Separations achieved compared favorably to those obtained with silicic acid or DEAE-cellulose.

Column chromatography on hydroxylapatite afforded complete recovery of polar lipids, judged by the 99–100% recovery of total phosphorus. Careful examination of eluted fractions did not indicate degradation of lipids during column development.

INTRODUCTION

Hydroxylapatite, $Ca_{10}(OH)_2(PO_4)_6$, an adsorbent introduced in 1956 by TISELIUS *et al.*[1], has been widely used for separation of proteins[2], lipoproteins[3] and polynucleotides[4] by CC. This adsorbent has been used also for the separation of polar lipids by TLC with 7% calcium sulfate as a binder[5].

In our work on the separation of immunologically-active polar lipids from cattle serum, we found that CC with DEAE-cellulose or silicic acid gives inadequate separation of certain individual polar lipid components. This led us to explore the use of CC on HTP to achieve improved separation of individual polar lipids. We report here details of the method of CC of polar lipids on HTP and the advantages gained from the application of this method.

METHODS

Reagents

All reagents used were analytical grade. Triolein was a gift from Dr. N. PAYZA, New York Medical College; L-α-cephalin dipalmitoyl (synth) and phosphatidyl-L-serine (according to Folch) from Mann Research Laboratories, Inc., New York;

* Abbreviations HTP = hydroxylapatite; DEAE-cellulose = diethylaminoethyl cellulose, TLC = thin-layer chromatography, CC = column chromatography; Fr = fraction

cerebrosides (beef brain), phosphatidylinositide (Fr I from beef brain), and sphingomyelin from General Biochemicals, Chagrin Falls, Ohio; L-α-lecithin and cardiolipin (extract bovine heart) from Pierce Chemical Company, Rockford, Ill.; hydroxylapatite Bio-gel HTP was from Bio-Rad Lab., Richmond, Calif. TLC plate Silica Gel HR was purchased from Analtech, Inc., Wilmington, Del.; bovine J^{cs} serum was supplied kindly by Dr. W. STONE, Madison, Wisc.

Extraction of lipids from serum

The powder obtained from lyophilizing 500 ml of dialyzed bovine serum was washed with 500 ml of acetone. The acetone-insoluble residue was extracted with 2 l of chloroform–methanol (2:1) at room temperature under a stream of nitrogen for 48 h using a magnetic stirrer[6]. The extract was clarified by filtration on a sintered glass funnel (grade M) and then was concentrated to near dryness under nitrogen. The concentrate was dissolved in 20 ml of diethyl ether, and the polar lipids were precipitated with an excess of acetone containing 1 ml of acetic acid. The precipitated polar lipids were collected by centrifugation at 2,000 r.p.m. at 5° for 20 min in the International Centrifuge Model PR-2. The supernatant which contained neutral lipids and pigments was discarded.

The precipitated polar lipids were dried to constant weight over P_2O_5 at 8° in a vacuum desiccator previously flushed with nitrogen.

Hydroxylapatite column chromatography

Column preparation. Twenty grams of Bio-gel HTP were washed three times with 150 ml of methanol, two times with 150 ml of acetone and two times with 100 ml of diethyl ether. After each washing, the supernatant was decanted. Residual ether was removed in a stream of nitrogen which was bubbled for 10 min through the adsorbent.

The ether-free adsorbent was activated in an oven at 120° for 18 h. Activated HTP was than cooled in a desiccator previously flushed with nitrogen, and then the adsorbent was suspended in diethyl ether. The slurry was poured into a glass column (1.2 × 35 cm) containing a fritted disc (grade M) which was covered with fine glass wool. The column bed was then washed with 100 ml of chloroform.

Chromatography of lipids. A 250-mg mixture of standard lipids dissolved in 7 ml of chloroform was applied to the column for chromatography of the standard lipids. A 300-mg sample of serum polar lipids dissolved in 10 ml of chloroform was applied to a separate identical column. The lipids were eluted successively from the columns under slight nitrogen pressure by the following solvents: (I) chloroform, 250 ml; (II) acetone–methanol (9:1), 250 ml; (III) acetone–methanol (7:3), 300 ml; (IV) acetone–methanol (5:5), 250 ml; (V) acetone-methanol (3:7), 250 ml; (VI) methanol, 300 ml; (VII) diethyl ether–ethanol–aqueous 0.04 *M* KOH (10:7:5), 650 ml (Solvent mixture VII previously was used to advantage for the chromatography of lipids on alumina[7].) Total phosphorus in eluted fractions was determined by the method of BARTLETT[8]. Recovery from the column was judged by total phosphorus recovery of serum lipids after subtraction of the amount of phosphorus eluted by these solvents from a blank column to which no sample was added.

Analytical methods. After acid-methanolysis, long-chain bases and sugars were analyzed by gas–liquid chromatography on SE-30 (ref. 9).

Serological assay. Hemolysis test and inhibition of hemolysis of cattle J^{cs} red cells were performed according to STONE AND IRWIN[10].

Isolation of lipids from Fr VII. Fractions of 35 ml were collected. After the eleventh fraction, the eluate became alkaline and was immediately brought to pH 8.3 (colorless to phenolphthalein) with glacial acetic acid. After complete elution with the total 650 ml of eluant, fractions were combined and evaporated to a small volume under nitrogen. To the concentrate was added 40 ml of aqueous 0.1 *M* KCl and the mixture was extracted twice with 200 ml of chloroform–methanol (2:1). Then the aqueous phase was further extracted two times with equal volumes of chloroform–methanol (4:1). The lipids were contained in the combined chloroform extracts.

Thin-layer chromatography. Silica Gel HR plates, 250-μ coating thickness, were activated at 130° for 2 h and cooled under nitrogen. Column fractions were evaporated to dryness under nitrogen, dissolved in chloroform–methanol (2:1) and then applied to the plate. Individual lipid standards also were applied to the plate as reference compounds. The chromatography tank was lined with equilibration paper and was equilibrated with developing mixture for 2 h before TLC. The mixture chloroform–methanol–water (65:25:4)[11] was used for TLC of the various lipids. After chromatography, individual lipids were visualized by either iodine vapors, ammonium bisulfate or ninhydrin reagent.

RESULTS

Hydroxylapatite column chromatography

The lipid fractions eluted from this column were monitored and identified by TLC. Solvent was changed when no further elution of lipids occurred. After concentration under reduced nitrogen pressure, column fractions and lipid standards were applied to TLC plates and developed with chloroform–methanol–water (65:25:4).

Standard lipid fractionation

Standard lipids after elution from the column were identified in each fraction by TLC (Fig. 1). On the basis of the information obtained by comparing the migration

TABLE I

ELUTION OF STANDARD LIPID MIXTURE FROM HYDROXYLAPATITE

Volumes of solvents are for a column 35 × 1.2 (I.D.) cm prepared with 20 g of HTP; a 250-mg sample of standard lipids was applied.

Fraction No	*Eluent*	*Volume (ml)*	*Lipids eluted (monitored by TLC)*
I	Chloroform	250	Triglycerides (neutral lipids)
II	Acetone–methanol (9·1)	250	Cardiolipin
III	Acetone–methanol (7 3)	300	Lecithin
IV	Acetone–methanol (5.5)	250	Sphingomyelin
V	Acetone–methanol (3:7)	250	Upper spot of cerebrosides mostly
VI	Methanol	300	Phosphatidylethanolamine, cerebrosides
VII	Diethyl ether–ethanol–aqueous 0.04 *N* KOH (10:7·5)	650	Phosphatidylserine, phosphatidylinositol

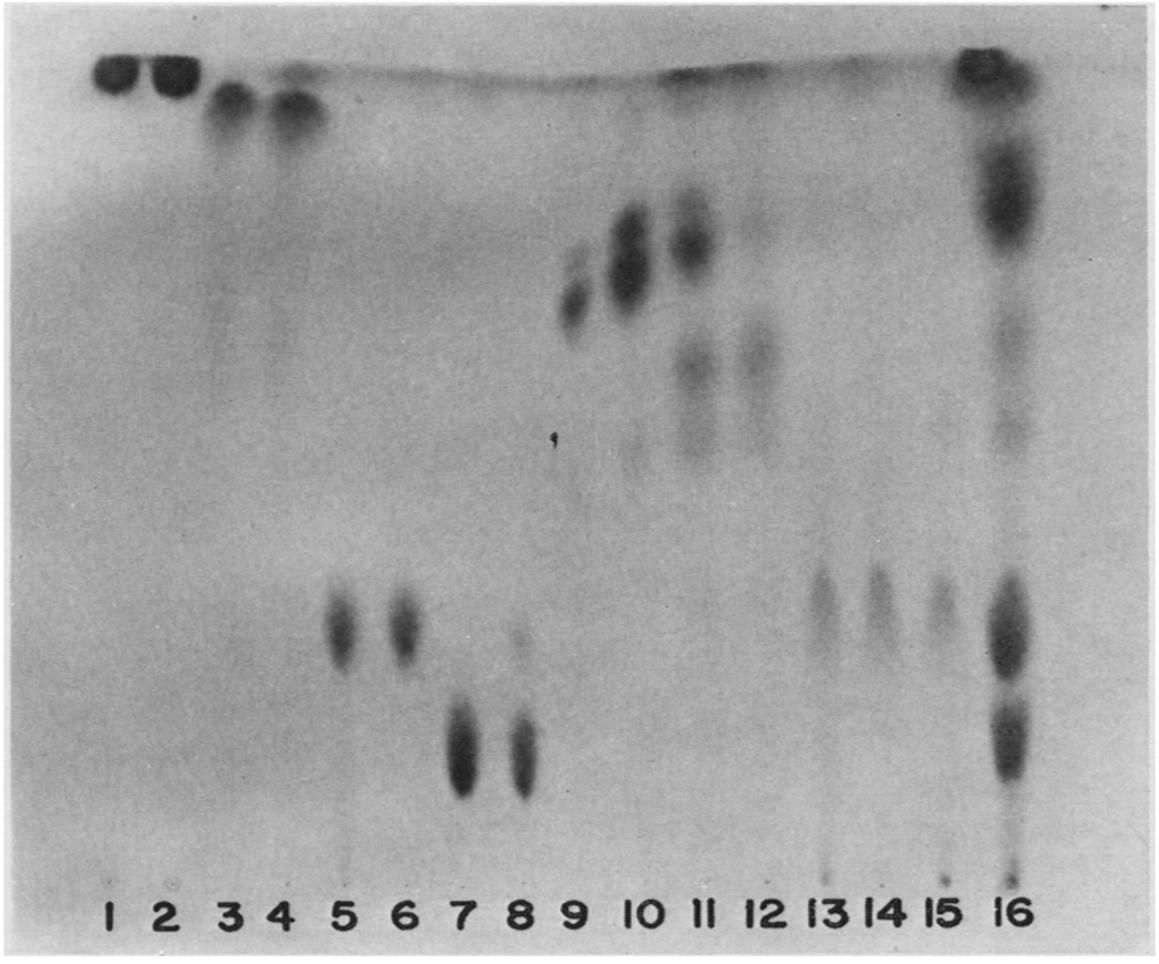

Fig. 1. Thin-layer chromatogram of lipid standards and fractions of standard lipid mixture eluted from a HTP column. Adsorbent. Silica Gel HR. Solvent· chloroform–methanol–water (65:25:4). Visualization: ammonium bisulfate spray. 1 = triolein; 2 = Fr I; 3 = cardiolipin, 4 = Fr II; 5 = lecithin; 6 = Fr III; 7 = sphingomyelin; 8 = Fr IV, 9 = Fr V; 10 = cerebrosides; 11 = Fr VI; 12 = phosphatidylethanolamine; 13 = phosphatidylserine, 14 = Fr VII, 15 = phosphatidylinositol; 16 = mixture of 1, 3, 5, 7, 10, 12, 13, 15.

of the components of each fraction with those of standard lipids, the generalizations in Table I were made.

A 300-mg sample of polar lipids extracted from serum was fractionated on an identical hydroxylapatite column to test the usefulness of the method for resolving complex mixtures of lipids. A recovery of 99–100% of the serum lipids placed on the column was obtained, based on total P determinations. The components in each eluted fraction were identified by TLC using authentic lipid standards as reference compounds. The resulting separation is shown in Fig. 2.

Fr II (spot 5) contained only traces of lipids not visible on this plate. Analysis of Fr II showed the presence of a small amount of hexoside ceramides. The lipids in Fr V (spot 10) contained only traces of sphingosine, but Fr V contained considerable amounts of long-chain bases which differed from sphingosine by showing a greater retention on SE-30. Spots 11 and 12 contained mainly phosphatidylethanolamine, which in this TLC system always gave two spots. Fr VII (spot 14) which was adjusted

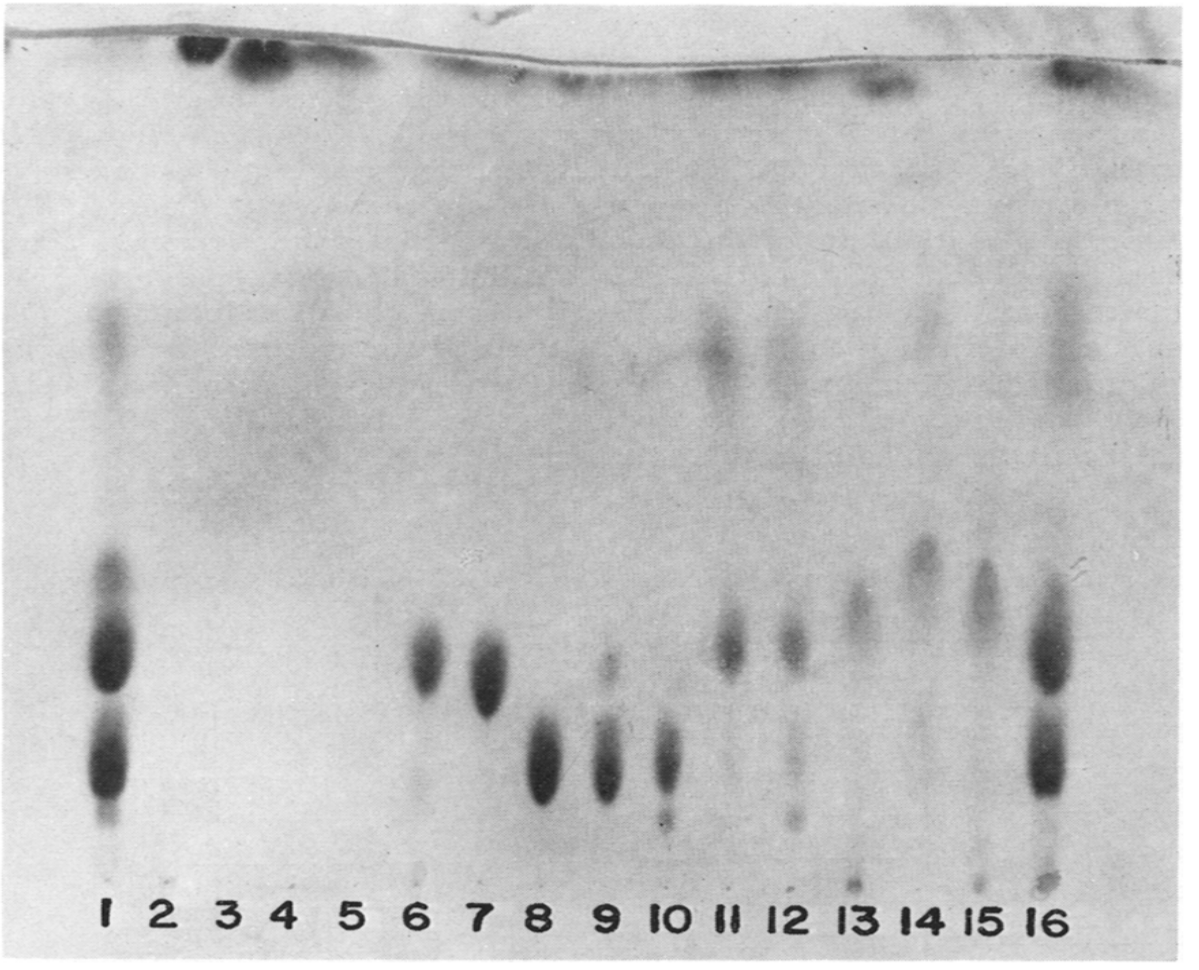

Fig. 2. Thin-layer chromatogram of lipids separated from bovine J^{cs} serum by HTP column chromatography. Adsorbent, Silica Gel HR. Solvent chloroform–methanol–water (65 25 4). Visualization ammonium bisulfate spray. 1 = bovine serum lipids, 2 = triolein, 3 = Fr I; 4 = cardiolipin; 5 = Fr II, 6 = lecithin, 7 = Fr III, 8 = sphingomyelin, 9 = Fr IV, 10 = Fr V, 11 = phosphatidylethanolamine, 12 = Fr VI, 13 = phosphatidylinositol, 14 = Fr VII; 15 = phosphatidylserine, 16 = mixture of 2, 4, 6, 8, 11, 13, 15

with acid till colorless to the phenolphthalein indicator showed the phenolphthalein spot near the solvent front.

The immunologically active bovine J hapten was eluted with Fr VII from HTP.

The sheep brain cerebroside standard gives two spots by TLC. This probably results from the different fatty acids present in these cerebrosides[12]. An attempt was made to resolve further these cerebrosides by chromatography on a HTP column with mixtures of acetone and methanol. Careful selection of the ratio of acetone to methanol afforded separation of the cerebrosides into three groups: (1) cerebrosides corresponding mostly to the upper spot on TLC (Fig. 1, cerebroside spot), eluted from column by acetone–methanol (3:7); (2) cerebrosides corresponding to both spots, eluted from column by acetone–methanol (1:9); (3) cerebrosides corresponding to the lower spot of the thin-layer chromatogram, eluted from column by methanol.

DISCUSSION

Chromatography on columns of silicic acid[13] and DEAE-cellulose[14,15] is commonly used as the initial step in separation of various lipid classes from crude polar lipids. However, the fractionation of phospholipids by silicic acid CC does not resolve phosphatidylserine from phosphatidylethanolamine. Furthermore, overlapping of certain lipids such as lecithin and sphingomyelin in the eluted fractions is difficult to avoid. On the other hand, CC of lipids on DEAE-cellulose does not separate lecithin from sphingomyelin and cerebrosides, nor cardiolipin from phosphatidylinositol.

Column chromatography on HTP may be used advantageously for resolving mixtures containing these compounds. This can be seen readily by referring to Table II, where the resolving ability of HTP is compared with the resolving abilities of DEAE-cellulose and silicic acid, as given in the literature[13,14].

TABLE II

COMPARISON OF THE RESOLVING POWER OF SILICIC ACID, DEAE-CELLULOSE AND HYDROXYLAPATITE COLUMNS[a]

Compound	Silicic acid column (13)				DEAE-cellulose column (14)							Hydroxylapatite column						
	1	2	3	4	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Lecithin			■		■									■				
Phosphatidylethanolamine		■	■			■											■	
Phosphatidylinositol			■								■							■
Phosphatidylserine		■						■										■
Cerebroside					■											■	■	
Cardiolipin											■		■					
Sphingomyelin				■	■										■			

[a] Blackened square denotes elution of component; numbers denote fractions.

The commonly employed eluting mixtures of chloroform and methanol fail to give good resolution of lipids on HTP columns. Mixtures of acetone and methanol, similar to those used by VANCE AND SWEELEY[9] for eluting hexoside ceramides from a silicic acid column, exhibited good resolving power on HTP. Selectivity was obtained by varying the ratio of acetone to methanol.

Occasionally, lecithin is incompletely desorbed from HTP by solvent mixture III, and acetone–methanol (6.5:3.5) may then be substituted to achieve the desired resolution of lecithin from sphingomyelin. Variations in the preparation of the gel and in the activation procedures may be responsible for the occasional aberrant behavior of lecithin on HTP columns.

Several methodological points warrant discussion. Methanol-soluble particles of HTP may appear in the column effluent. We recommend that the gel be washed with methanol and decanted at least three times. This treatment not only greatly reduces the amount of particles present in the effluent but also facilitates a fast flow rate from the column. We have been able to complete the elution of the various lipid

classes from a HTP column, operated under slight nitrogen pressure within 8–9 h.

TLC of the cerebrosides usually gives an elongate-dumbell shaped zone or two spots; fatty acid heterogeneity is believed to contribute to this chromatographic heterogeneity[12]. Hydroxylapatite CC affords partial resolution of these cerebrosides.

No degradation of the lipids occurred during chromatography on HTP as judged by the absence of new spots on TLC examination of the eluted fractions.

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