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ION-EXCHANGE CHROMATOGRAPHY OF PHOSPHATIDES AND GLYCOLIPIDS ON QUATERNARY TRIETHYLAMMONIUM GLYCOPHASE COVALENTLY BOUND TO CONTROLLED PORE GLASS

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SUMMARY

Potential use of quaternary triethylammonium (QAE) Glycophase on controlled pore glass (CPG) for preparative ion-exchange chromatography of liver, brain and soybean lipids was investigated. We found that QAE-Glycophase–CPG bound and held polar and ionic lipids in chloroform. Phosphatidyl choline, sphingomyelin and cerebrosides were eluted with 4–6 column volumes of chloroform–methanol (9:1), phosphatidyl ethanolamine was eluted with 7–10 column volumes of chloroform–methanol (1.1), phosphatidyl serine was eluted with 5–7 column volumes of acetic acid and phosphatidyl inositol and sulfatides were eluted with 5–7 column volumes of 20 m*M* potassium acetate in chloroform–methanol (2 1)

Physical properties of QAE-Glycophase–CPG are more suitable for use in small-column (45 × 9 mm or less) low-pressure liquid chromatography than those of ion-exchange cellulose. QAE-Glycophase–CPG, in proper particle size, could probably function well in high-pressure, high-efficiency liquid chromatography.

INTRODUCTION

Fractionation of so-called "polar lipids"[1] (phosphatides and glycolipids) into compound classes has been the subject of intense investigation during the last decades[1,2]. Application of low- and high-pressure column (HPLC) and thin-layer chromatography (TLC) has lead to isolation, identification and quantitative measurement of many naturally occurring lipid species However, some of the most commonly used methods are time-consuming, require large volumes of solvents, and not readily adaptable to isolation of 5–10-mg amounts of pure lipids For example, the ion-exchange column chromatography procedure introduced in 1961 by Rouser *et al*[3] is an essential method for the preparative fractionation of acidic lipids from others of similar polarity because other preparative methods are not able to perform this particular task efficiently. Two-dimensional TLC and HPLC can separate acidic phosphatides and glycolipids from each other and from other lipids[1–12], but these procedures are not preparative ones.

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Although ion-exchange column chromatography of lipids is a key preparative procedure, the original methods of Rouser *et al.*[3] which use low-pressure columns packed with diethylaminoethyl (DEAE) or triethylaminoethyl (TEAE) cellulose, have not been improved. These methods are troublesome, time-consuming, and expensive due to the large volumes of solvents needed for elution of the samples. Most of the problems are caused by the physical properties of cellulose; even the microgranular ion-exchange cellulose preparations have relatively large particle sizes. Cellulose preparations are prone to channel formation (each newly packed column has to be tested[2]) and tend to swell or shrink in different solvents. Such materials perform best in columns of 2–4.5 cm diameter and they are difficult to use in smaller columns needed for fractionation of small amounts of lipids. Samples containing a few mg of lipids are very difficult to recover quantitatively, without degradation, from the large volume (400–500 ml) of solvents needed for elution of one fraction from a 20 × 2.5 cm DEAE-cellulose column[13]. Furthermore, cellulose cannot be used for preparation of HPLC columns since cellulose becomes tightly packed and crushed, and it eventually occludes the column under high pressures.

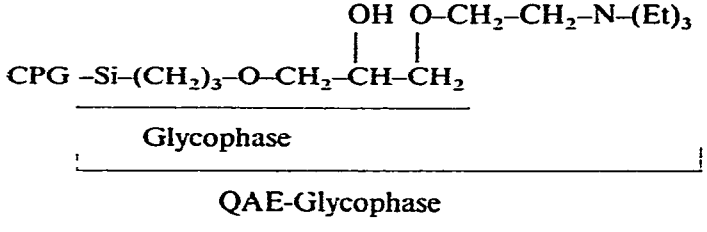
A new kind of ion-exchange support, quaternary triethylammonium chloride (QAE) Glycophase on controlled pore glass (CPG) has recently been introduced by Corning (Corning, NY, U.S.A.). QAE-Glycophase–CPG has anion-exchange groups similar to those of TEAE-cellulose, but instead of cellulose it has the more rigid Glycophase–CPG support which does not swell or shrink as cellulose does. Therefore, QAE-Glycophase–CPG has the potential to overcome many of the problems inherent to ion-exchange cellulose preparations for lipid chromatography.

The objective of this study was to determine if QAE-Glycophase–CPG chromatography could efficiently separate the four major glycerophosphatides of liver microsomal membranes[14], namely phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI). We report the application of QAE-Glycophase–CPG chromatography for partitioning the lipid mixtures of beef liver, beef brain and soybean extracts into five fractions. Depending on the source of a particular lipid extract, the major lipid classes that are recovered in the five fractions are as follows: I: neutral lipids (NL); II: cerebrosides (Ce), PC and sphingomyelin (Sph); III: PE; IV: PS; V: sulfatides (Su) and PI.

MATERIALS AND METHODS

Ion-exchange support

Controlled pore glass (CPG) is manufactured by Corning, distributed by Pierce (Rockford, IL, U.S.A.) and is available in various particle sizes and with various pore sizes. Glycophase–CPG is controlled pore glass covered with covalently



bound non-ionic carbohydrate monolayer, 18 Å thick, to minimize the effect of glass on the materials being separated. The strong basic anion exchanger QAE is bound covalently to the hydroxyl groups of Glycophase to form QAE-Glycophase–CPG. The distributor claims that QAE-Glycophase–CPG can be used from pH 1 to pH 8 continuously and over a broader range for short periods. After 4 h in 1 *M* potassium hydroxide solution about 75% of the ion-exchange capacity remains; 1 *M* hydrochloric acid affects the support to a lesser degree. High salt concentrations and non-aqueous solvents are claimed not to affect the material We used a QAE-Glycophase–CPG ion-exchange support which had particle size 120–200 mesh or 75–125 μm, pore diameter 250 Å, surface area 130 m^2/g and 0.05 mmol/g functional groups A 9 5-g quantity of this material forms a column with about 25 ml void volume (column volume)

Chemicals

Purified phosphatides and cholesterol standards were obtained from Sigma (St. Louis, MO. U S A.) All solvents, acids, bases, salts and other chemicals were reagent grade and were used without any further purification Soybeans, beef brain and beef liver were obtained from a local grocery store The lipids from these tissues were extracted and purified according to Folch *et al* [15]

Equipment for low-pressure liquid chromatography

A glass column (250 × 9 mm) equipped with an adjustable plunger, a 2-way Kel F slide valve and a PTFE slide sample injection valve (0 5-ml sample loop) and 0 8 mm I D. PTFE tubing (Rainin, Woburn, MA, U S A) was used During chromatography the solvents contacted only glass, PTFE or Kel F

TLC plates

Thin layers of silica gel (0 35 mm) were made from silica gel 60 H (E Merck, Darmstadt, G F R) on 20 × 20 cm glass plates

Procedure for column packing

Due to the fragile nature of QAE-Glycophase–CPG and to the small sizes of columns used, we found the following packing method to be most practical. The amount of ion-exchange support needed for the column volume selected (2 6 ml column volume/g of QAE-Glycophase–CPG) was weighed and poured into the column The adjustable plunger was inserted to the top of the ion-exchange support The bottom opening of the column was placed in a container filled with chloroform and suction was applied to the top of the column until the column was filled with chloroform. Both the bottom and top valves of the column were closed and the plunger was gradually withdrawn to create a partial vacuum in the column until small bubbles appeared among the ion-exchanger particles; then the plunger was released This process was repeated until no new bubbles appeared when the suction was applied. The plunger was then withdrawn to the end of the glass column filling the column with chloroform The column contents were mixed by inversion until a thin slurry of the ion exchanger formed. Then the column was placed vertically, which allowed the bubbles to rise to the head of the column and the ion exchange to settle to the bottom. The column was tapped gently on the benchtop, 5–7 times a minute

and was also tapped on the side at the level of packing. The entire procedure took about 15 min. Test chromatographic runs with Azulene always showed a very uniform column bed. The above procedure was successfully used to rebuild columns, without removing the ion exchange, when columns accidentally ran dry. Gas bubbles were removed from the top of column by lowering the plunger and forcing the bubbles out the sample injection valve

Activation and purification of the ion exchanger

The chloroform used for preparation of the column was removed by washing with 2 column volumes of chloroform–methanol (1:1) and the ion exchanger was converted to the hydroxyl form by washing it with 3–4 column volumes of 0.1 *M* potassium hydroxide in methanol. Exposure to potassium hydroxide was as brief as possible, washing with 4 column volumes was adequate at flow-rate of a 0.5 ml/min (for the 9-mm-diameter column). The excess potassium hydroxide was removed with 7 column volumes of methanol and the column was washed with 2 column volumes of chloroform–methanol (1:1) and 7 column volumes of chloroform before sample application. New columns were purified by passage of 7–10 column volumes of each solvent system through the column to remove any material that dissolved in the solvents. Reactivation of the column, with potassium hydroxide, was necessary after purification, and after the completion of elution sequences.

Identification of lipids

Lipid classes of lipid extracts were identified after TLC in neutral, basic and acidic solvent systems[1]. TLC plates were sprayed with sulfuric acids, or with reagents specific for phosphate containing lipids (molybdenum blue), for aminophosphatides (ninhydrin), choline-containing phosphatides (Dragendorff) and for glycolipids (δ-naphthol)[1], and R_F values compared to those of standards

RESULTS

Elution sequence

Table I indicates the lipid classes eluted by the solvent sequence shown, which is a modification of Rouser *et al.*[3]. The hydroxyl form of QAE-Glycophase–CPG bound and held anionic lipids in non-aqueous systems while, as expected, non-ionic, non-polar lipids (triacylglycerols, free and esterified cholesterol) passed through the ion-exchange Glycophase in chloroform (4–7 column volumes) Non-ionic polar lipids, *e.g.* cerebrosides, however, were retained as the hydrogen bonding through hydroxyl groups appeared to be strong enough to retain them in absence of polar solvents like methanol The zwitterion lipids (PC and Sph) and non-ionic polar lipids (cerebrosides) were eluted with non-ionic but polar solvents such as 10% methanol in chloroform (5–7 column volumes). Phosphatidyl ethanolamine was bound stronger but was eluted with relatively large volumes (7–10 column volumes) of methanol–chloroform (1:1) or with acetic acid–chloroform (2.98) (4–6 column volumes).

Acidic lipids were retained firmly and could not be eluted with non-ionic solvents. However, QAE-Glycophase bound the acidic lipids somewhat more gently than either DEAE- or TEAE-cellulose[3] Acetic acid–chloroform (1:1) eluted PS efficiently (5–7 column volumes) and 20 m*M* potassium acetate in chloroform–methanol, (2:1), eluted PI, (5–7 column volumes) just as easily.

TABLE I

ION-EXCHANGE CHROMATOGRAPHY OF PHOSPHATIDES AND GLYCOLIPIDS ON QAE-GLYCOPHASE-CPG

Column volume is about 2 6 ml/g ion exchange

Fractions	*Elution solvents*		*Elutable lipids*
	Composition	*Column volumes*	
I	Chloroform	5–7	Neutral lipids, non-polar lipids, triacylglycerols cholesterol
II	Chloroform–methanol (9 1)	6–8	Phosphatidyl choline, sphingomyelin cerebrosides and others
III	Chloroform–methanol (1 1)	7–10	Phosphatidyl ethanol-
	or chloroform–acetic acid (98 2)	4–6	amine and other polar lipids
IV	Chloroform–acetic acid (1 1)	6–8	Phosphatidyl serine and
	or acetic acid	4–6	other acidic lipids
Wash	Chloroform–methanol (2 1)	5–7	Excess acetic acid and unidentified material
V	20 m *M* potassium acetate in chloroform–methanol (2 1)	6–8	Phosphatidyl inositol and other acidic lipids

Column dimensions and elution speed

Since short columns with wide diameters allow higher elution rates, the separations achieved by various column lengths were evaluated Columns with a height to diameter ratio of 4–5 separated lipid mixtures consisting only of acidic compounds (PI, PS, Su) However separation of mixtures also containing zwitterions (PC, Sph, and PE) required columns with a height to diameter ratio of 9–10

Optimum flow-rate for a column of 9 mm diameter was 0 5 ml/min A sign of too fast elution rate was "tailing" of lipids into fractions that followed

Fraction of lipid mixtures

Fig 1a–c presents TLC chromatograms which illustrate how efficiently QAE-Glycophase–CPG chromatography separated the glycerophosphatide constituents of lipid extracts from soybeans, beef brain and beef liver, respectively

Fig 1a shows the separation achieved for 135 mg of soybean lipid extract which is a useful lipid mixture for testing chromatography column performance because it contains PI but not PS. Fraction I contained mostly triacylglycerols and did not contain any phospnatides Fraction II contained PC and some unidentified substances. Fraction III contained PE and other unidentified compounds near the solvent front. The fact that fraction IV did not contain any PE or PI shows that elution of PE was complete with the solvents of Fraction III and PI did not elute with Fraction IV. Fraction V contained PI and some other unidentified acidic lipids

Fig 1b shows the isolation of PS from 55 mg bovine brain lipids Fraction I contained only non-polar lipids Fraction II contained PC and cerebrosides Fraction III contained PE and only traces of other unidentified lipids. Fraction IV contained PS and only traces of other unidentified acidic lipids Fraction V contained PI and sulfatides Elution of each fraction was complete without any overlap

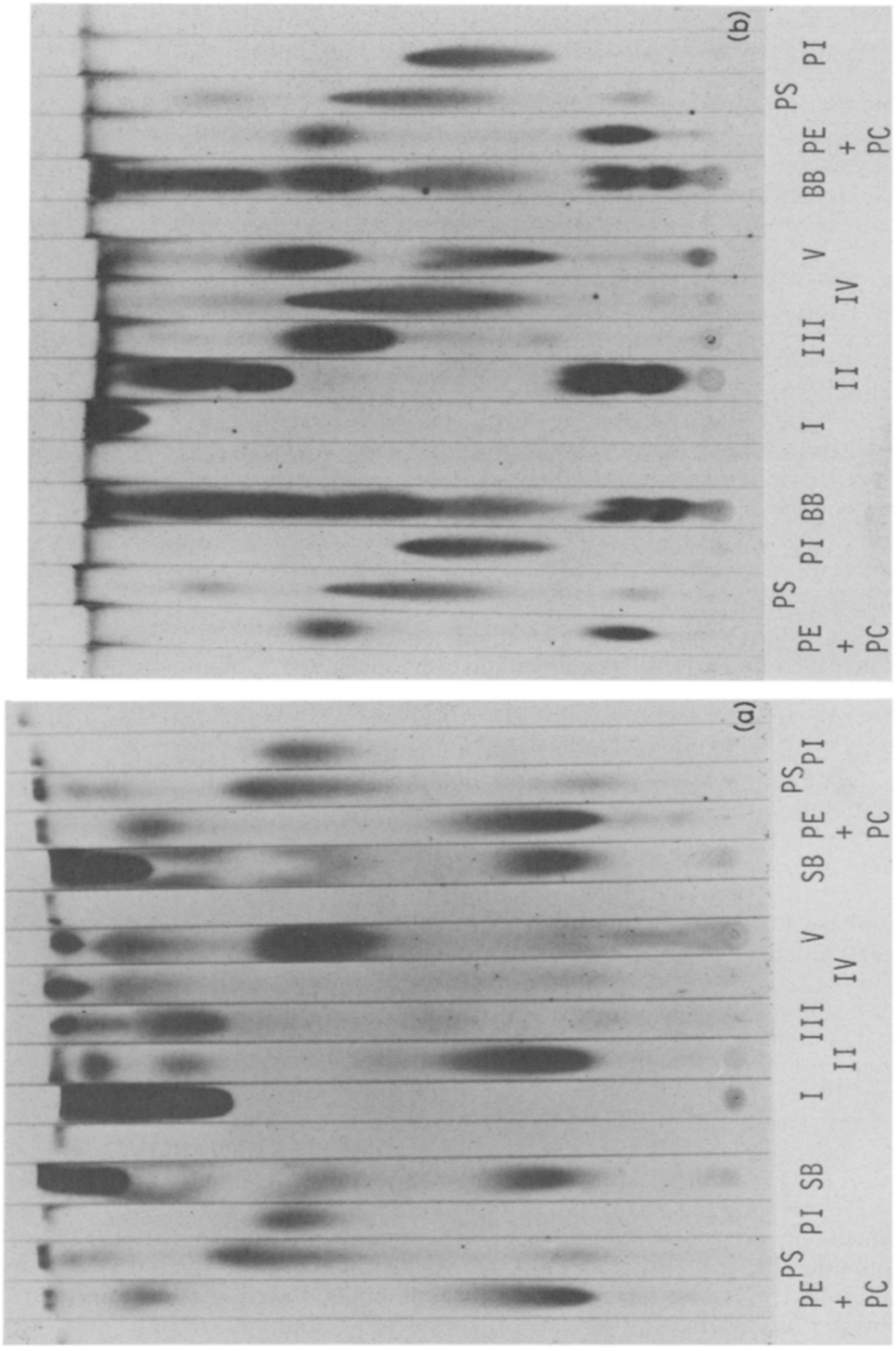

(a)
PE PS PI SB I III V SB PE PS PI
+ II IV +
PC PC
(b)
PS PE PI BB I III V BB PE PI
+ II IV +
PC PC

Fig. 1. Thin-layer chromatograms of QAE Glycophase C PG chromatography fractions I–V of lipids extracted from (a) soybeans (SB), (b) beef brain (BB) and (c) beef brain (BL). Sample sizes applied were 135 mg of soybean lipids, 85 mg of brain lipids and 35 mg of liver lipids. Column size and volumes also varied and were 45 × 9 mm (2.6 ml) for soybean lipids, and 85 × 9 mm (5.2 ml) for brain and liver lipids. All other conditions were constant. Phosphatide standards, PI = phosphatidylethanolamine, PS = phosphatidyl choline, PS = phosphatidyl serine, PI = phosphatidyl inositol. Eluting solvents, I = chloroform, II = chloroform–methanol (1 : 1), III = chloroform–methanol (1 : 1), IV = chloroform–acetic acid (1 : 1), V = 20 mM potassium acetate in chloroform–methanol (2 : 1). TLC plates were silica gel 60 (5 μm) and developing solvent was ethanol–methanol–acetic acid–water (33 : 14 : 3 : 1). TLC plates were sprayed with sulfuric acid

Fig. 1c shows the five fractions of 35 mg bovine liver lipids. Fraction I contained only non-polar lipids. Fraction II contained PC, Sp and trace amounts of a compound that appeared to be lysophosphatidyl choline (LPC). Fraction III contained PE and a large number of unidentified compounds both below and above PE. Fraction IV did not contain detectable amounts of PS, but it contained higher concentrations of those unknown compounds which already appeared in Fraction III. In addition, Fraction IV contained large amounts of substances which migrated to the solvent front on TLC. These could be fatty acids, bile acids and other acidic lipids which were neither phosphatides nor glycolipids. Fraction IV had a compound which had the TLC R_F value of PE, in acidic solvents, but did not seem to contain phosphate. Fraction V contained PI and other unidentified acidic compounds

Optimal sample size and column length

Our comparative fractionation of lipid extracts from soybeans, beef brain and beef liver indicated that the nature and complexity of the lipid mixtures were critical determinants of optimal sample size and column length. Larger amounts of lipids could be fractionated when the sample contained greater proportions of "neutral lipids" elutable with chloroform and a small proportion of acidic lipids (phosphatides, glycolipids, fatty acids, bile salts, bilirubin, etc.). Thus, with crude soybean extracts, which contain large amounts of triacylglycerols, 135 mg could be fractionated successfully on a 45 × 9 mm column With bovine brain lipids, which contained much less neutral lipids, separation of 55 mg required a column size of about 86 × 9 mm. Bovine liver extracts, which contain fatty acids, bile salts and other acidic materials in addition to phosphatides and glycolipids, were most difficult to fractionate Only 35 mg of whole liver extract could be fractionated, without overload, on a 86 × 9 mm column.

The optimum load had to be determined empirically for each mixture of lipids, but not necessarily for each column. The ratio of the squares of the column diameters or radii could be used to determine the conversion factor for columns of various diameters.

A reliable sign of column overload is the appearance of phosphatides in the chloroform fraction. If this occurred, then PE eluted with PC and the acidic phosphatides appeared together in solvents containing only two percent acetic acid If the chloroform fraction was free of phosphatides, then no other signs of overload were observed. A convenient way to detect phosphatides in the neutral lipid fraction was to evaporate the chloroform, redissolve the neutral lipids in 0.2–0.5 ml chloroform, place about 30–50 μl of the solution on a silica gel plate and spray it with molybdenum blue reagent to detect phospholipids directly without developing the thin-layer plate.

A great deal of effort and time could be saved by purposely low-loading the ion-exchange columns; good separation could still be obtained with columns of slightly altered and decreased capacity[3].

Reproducibility, stability and usefulness

Each new column functioned well without exceptions. Test runs were not necessary once the optimum load was previously determined for a particular lipid mixture. However, QAE-Glycophase does lose its activity gradually with normal use; a log of use should be maintained for each column and the limits of usefulness should

be determined for each set of solvent systems. After 23 cycles of reactivation, we did not observe any detectable decrease in binding capacity with the solvent systems used

In contast, as clearly shown by comparison of Fig. 1a–c, reproducible TLC is very difficult to carry out, especially in the humid environment of Galveston Island These plates were activated together, cooled and stored over phosphorous pentoxide in a desiccator and they were developed on the same relatively dry December morning. The variable migration rate of acidic glycerophosphatides, relative to each other and relative to other compounds, illustrates the importance and the significance of ion-exchange chromatography of lipid mixtures

The TLC plates of Figs 1a–c also show that successful separation and recovery of the phosphatides by one-dimensional TLC alone would not have been possible The five fractions eluted from QAE-Glycophase demonstrate clearly how very complex a mixture liver lipids are. Even the most contemporary, powerful separation techniques of HPLC[4–12] are not sufficiently selective to resolve such complex mixtures of phosphatides and glycolipids At least two methods, based on different principles of fractionation. are needed to resolve such mixtures into pure components Ion-exchange chromatography coupled with adsorption chromatography (TLC or HPLC) promises the needed capabilities

In conclusion, ion-exchange chromatography (alone) on QAE-Glycophase has advantages over adsorption TLC or HPLC (alone) for fractionation of lipid mixtures Glycophase is simple to de-gas and chromatography of lipids can be carried out in inert atmosphere (nitrogen) thus avoiding the potential damaging effects of oxygen Fractionation can be carried out without detectors because elution of the substances is an "all or none" process, automation of the system is relatively simple The physical properties of QAE-Glycophase–CPG allow the preparation of micro analytical (2 or 3 mm I D) or large preparative (2 or 3 cm I D) low-pressure columns and make it potentially suitable (in smaller particle size) for use in high-efficiency HPLC.

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