Journal of Chromatography, 381 (1986) 233—240
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3225

SEPARATION OF PHOSPHOLIPIDS USING A DIETHYLAMINOETHYL-SILICA GEL COLUMN AND THIN-LAYER CHROMATOGRAPHY

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(First received January 15th, 1986; revised manuscript received April 28th, 1986)

SUMMARY

Phospholipids derived from egg yolk are readily separated by DEAE-silica gel column chromatography using stepwise gradient elution. The overall recovery of phospholipids from the column is 85—95% at a loading capacity of 120 mg of lipids per 10 g of DEAE-silica gel The complete separation of eight phospholipids (phosphatidylcholine, sphingomyelin, lysophosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine and lysophosphatidylserine; 5 μg each) is also achieved by one-dimensional DEAE-silica gel thin-layer chromatography with the solvent system chloroform—methanol—water—pyridine—58% ammonia solution (130:55:8:4:4, v/v).

INTRODUCTION

Separation of phospholipids has been achieved by the use of relatively limited number of sorbents, viz., cellulose, silicic acid, alumina and their derivatives. Recent successes with chemically bonded sorbents in liquid chromatography prompted us to develop a modified silica gel that takes advantages of the merits of silica gel, cellulose and DEAE-cellulose. This sorbent, DEAE-silica gel, retains the mechanical strength of unmodified silica gel, but reduces considerably the reactive silanol groups and adsorptive properties of silica gel by chemically bonding both DEAE and free hydroxyl groups to the silica gel surface. Hence DEAE-silica gel possesses the protective effect of hydroxyl groups abundant in cellulose and the weak anion-exchange capacity similar to that of DEAE-cellulose in addition to its strong mechanical microbial resistance. Kundu and Roy [1] have reported the quantitative isolation of gangliosides and neutral glycosphingolipids by DEAE-silica gel column chromatography.

0378-4347/86/$03.50

This paper describes the application of DEAE-silica gel for the separation of phospholipids by both column and thin-layer chromatography (TLC).

EXPERIMENTAL

Materials

Authentic lipid standards, rat brain phospholipids, Hiflosil (60—200 and 200—350 mesh) silica gel, Adsorbosil (200—350 mesh) silica gel, specific spray reagents and silica gel TLC plates were obtained from Applied Science Labs. (State College, PA, U.S.A.). DE-52 DEAE-cellulose was purchased from Whatman (Clifton, NJ, U.S.A.). Enhance, a spray surface autoradiography enhancer, and the radioactive lipids were obtained from DuPont, NEN Products (Boston, MA, U.S.A.). The crude egg yolk phospholipids and rat liver microsomes were prepared as described previously [2, 3] and extracted by the procedure of Folch et al. [4]. 2-Diethylaminoethanol and γ-glycidoxypropyltrimethoxysilane were purchased from Eastman (Rochester, NY, U.S.A.) and Petrach Systems (Levittown, PA, U.S.A.), respectively.

Analytical methods

Lipids were chromatographed on TLC plates with the following solvent systems: chloroform—methanol—water (65:25:4), chloroform—methanol—58% ammonia solution (65:35:5), chloroform—methanol—acetic acid—water (25:15:4:2) and chloroform—methanol—water—pyridine—58% ammonia solution (130:55:8:4:4). Specific spray reagents, ninhydrin for amino groups, molybdenum blue for the detection of phospholipids at room temperature and Dragendorff reagent for choline groups, were used to identify various lipids [2]. Radioactive lipids were rendered visible by autoradiography of TLC plates at −8°C overnight with DuPont Cronex X-ray film after spraying the plates with Enhance. To semiquantitate radioactivity, the TLC plates were scanned with a Packard Model A 7221 radioactive TLC scanner [5]. Radioactivity was also quantitatively measured by liquid scintillation counting [6]. The lipid phosphorus was determined by the procedure of Hess and Den [7].

Preparation of DEAE-silica gel

DEAE-silica gel (chloride form) is now commercially available as NuGel P-DE from Diagnostic Specialties (Methuchen, NJ, U.S.A.).

The DEAE-silica gel used in this study was prepared by a modified method of Kundu and Roy [1] for a large production scale as described below. Dry 300 g of either Hiflosil or Adsorbosil silica gel in a 5-l three-necked, round-bottomed flask with 24/40 joints at 150—160°C overnight. Fit the round-bottomed flask with an overhead stirrer to the center neck. Attach a pressure-equalizing funnel to the left-hand neck of the flask and a condenser to the right-hand neck. Connect a drying tube filled with Drierite (10—20 mesh) to the top of the condenser and mount the entire set-up on a 5-l heating mantle. Add 1.8 l of dry toluene to the flask with stirring, then add 90 ml of γ-glycidoxypropyltrimethoxysilane in 110 ml of dry toluene through the funnel with constant stirring. At the end of addition, remove the funnel and attach a thermometer. Heat the reaction mixture with a variac transformer so that the reaction temperature reaches 100—150°C in 40—60 min. Maintain the tempera-

ture for an additional 1 h, then add 90 ml of 2-diethylaminoethanol in 110 ml of dry toluene. Maintain the reaction temperature at 95—103°C for 16—18 h with stirring. Filter the resulting DEAE-silica gel on a Büchner funnel and was wash with 1.5 l of toluene followed by 1 l of methanol. Convert the DEAE-silica gel into the acetate form by stirring with 1.5 l of 0.2 *M* aqueous sodium acetate for 4 h, then filter the DEAE-silica gel on a Büchner funnel and wash with distilled water until neutral. Finally, rinse the DEAE-silica gel with 0.6 l of methanol and dry under vacuum or at 80°C overnight. The ion-exchange capacity of DEAE-silica gel is determined by titrating the gel with 0.1 *M* perchloric acid and in *p*-dioxane [8]. The ion-exchange capacities of typical DEAE-Hiflosil and DEAE-Adsorbosil are found to be 0.15—0.30 and 0.35—0.50 mmol/g dry weight, respectively.

DEAE-Hiflosil column chromatography

DEAE-Hiflosil columns are packed and regenerated as described by Kundu and Roy [1], except that conversion of the chloride to the acetate form of DEAE-Hiflosil is omitted. A typical slurry packing of DEAE-Hiflosil is described below. Suspend DEAE-Hiflosil (10 g) in 50 ml of chloroform—methanol—0.8 *M* sodium acetate (30:60:8) in a beaker and stir for a few minutes. Pack a column (20 cm × 1 cm I.D.) which is provided with a small glass-wool plug at the bottom. Wash the column successively with 50 ml of chloroform—methanol—water (30:60:8), 50 ml of chloroform—methanol (2:1) and 50 ml of chloroform under a slightly positive pressure of nitrogen to remove any undesirable impurities. Apply lipids dissolved in chloroform at a concentration of 120 mg or less per 10 g of DEAE-Hiflosil. Elute the lipids with five to ten column volumes of solvent mixtures specified under Results. The flow-rates vary from 0.4 to 1.5 ml/min. The column can be used for a minimum of four runs without a significant loss of resolving power after regeneration by washing with chloroform—methanol—0.8 *M* sodium acetate (30:60:8) followed by chloroform—methanol—water (30:60:8).

DEAE-Adsorbosil TLC

The DEAE-Adsorbosil TLC plates (20 × 20 cm; 250 μm coating) are prepared by the method of Stahl [9] in the presence and absence of 3% calcium sulfate that has been passed through a 200-mesh sieve before use. TLC of phospholipids (4—50 μg) is carried out at room temperature with chloroform—methanol—water—pyridine—59% ammonia solution (130:55:8:4:4) [10] as the solvent system in glass tanks lined with filter-paper and equilibrated for at least 1 h before developing the chromatograms. The compounds are detected by spraying the plates with molybdenum blue or ninhydrin or by autoradiography.

RESULTS

DEAE-Hiflosil column chromatography

Fig. 1 shows a typical separation of egg yolk lipids eluted from a DEAE-Hiflosil column (60—200 mesh) with increasing concentration of methanol in chloroform. A portion of eluent containing 2—100 μg lipids was spotted on

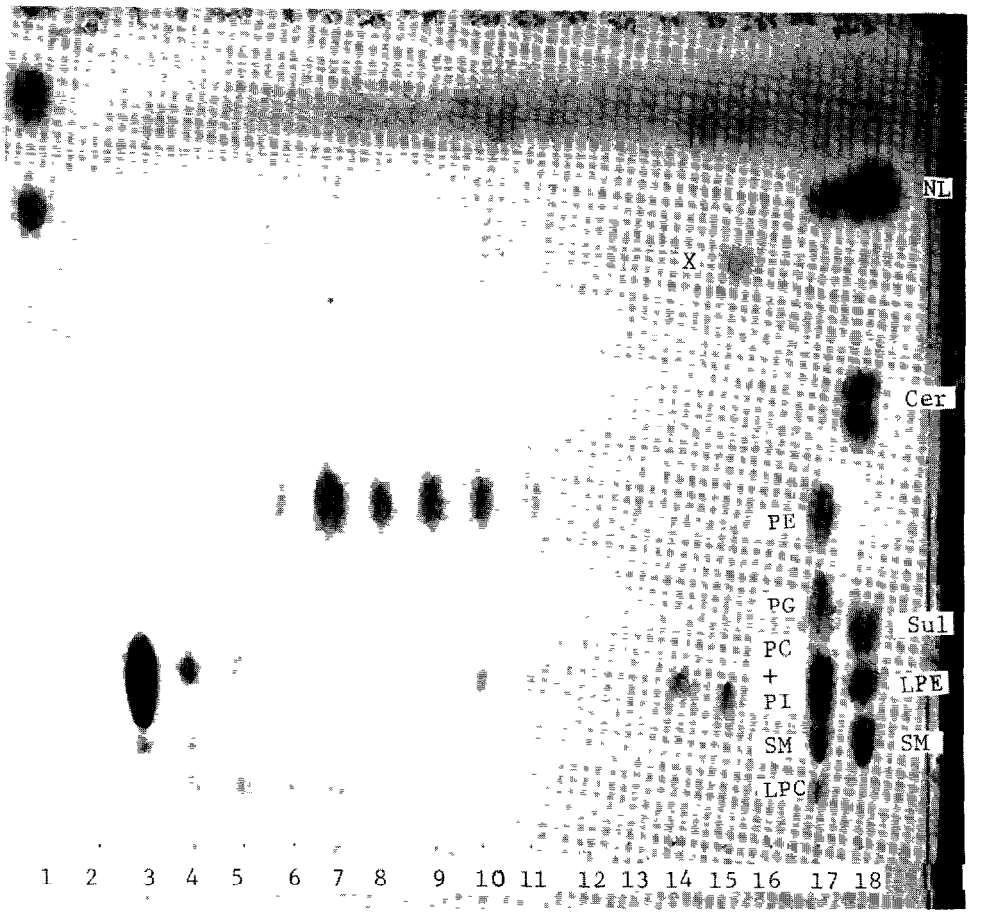


Fig. 1. TLC of fractions of egg yolk lipids eluted from a DEAE-silica gel column. TLC plate: silica gel H Prekote. Solvent: chloroform—methanol—water (65.25:4). The spots were detected with 50% aqueous sulfuric acid. Abbreviations for lipid standards: NL = neutral lipids; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PC = phosphatidylcholine; PI = phosphatidylinositol; SM = sphingomyelin; LPC = lysophosphatidylcholine; Cer = cerebrosides; Sul = sulfatide; LPE = lysophosphatidylethanolamine; X = uncharacterized. See for other details under Results. Lipid standards (10 μg each) and aliquots (2—100 μg) of egg yolk lipid fractions were spotted on each lane.

each lane. Neutral lipids are eluted with chloroform (lanes 1 and 2). Neutral phospholipids, phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SM), are eluted with 5% methanol in chloroform (lanes 3—5). Phosphatidylethanolamine (PE) starts to be eluted with 10% methanol in chloroform with a trace amount of LPC (lane 6). Continued elution with 10% methanol in chloroform results in fractions containing fairly pure PE (lane 9). A mixture of PE and lysophosphatidylethanolamine (LPE) is eluted with 20—40% methanol in chloroform (lanes 10 and 11). PE and LPE are strongly retained on this column because the amino group of both lipids binds to the DEAE-acetate of the column. The elution order of PC and PE observed with the DEAE-silica gel column is the reverse of that observed with the ordinary silica gel column.

TABLE I

ELUTION OF PHOSPHOLIPIDS WITH DEAE-SILICA GEL (ACETATE FORM) COLUMNS

Sequence	Column dimensions	Sample size (mg)	Flow-rate (ml/min)	Eluting solvent	Elution volume (ml)	Lipids eluted
1	26 × 2 cm I D (45 g), 60–200 mesh	200	1.7	(1) $CHCl_3$	250	Neutral lipids
			1.5	(2) $CHCl_3$–CH_3OH (95:5)	250	PC, LPC, SM
			1 5	(3) $CHCl_3$–CH_3OH (9·1)	250	PE, LPC (traces)
			1 5	(4) $CHCl_3$–CH_3OH (8·2)	250	PE, LPE
			1 5	(5) $CHCl_3$–CH_3OH (6 4)	250	PE, LPE
			1.4	(6) $CHCl_3$–CH_3OH–H_2O (30:60 8)	250	LPE
			1 2	(7) $CHCl_3$–CH_3OH–0 8 *M* CH_3CO_2Na (30.60 8)	250	PI, PS
2	27 × 2 cm I.D. (50 g), 200–350 mesh	300–1000	0 8	(1) $CHCl_3$- CH_3OH (9 1)	300	PE
			0.7	(2) $CHCl_3$–CH_3OH (7:3)	300	None
			0.5	(3) CH_3OH	300	PI (trace)
			0.5	(4) $CHCl_3$–CH_3OH–0 8 *M* CH_3CO_2Na (30.60·8)	300	PI
3	15 × 1 cm I D (5 g), 200–350 mesh	10	0.7	(1) $CHCl_3$–CH_3OH (8·2)	50	PC, LPC, SM
			0.6	(2) $CHCl_3$–CH_3OH–H_2O (80·20·2)	50	None
			0 5	(3) $CHCl_3$–CH_3OH–conc NH_3 (80.20.1)	50	PI
			0 5	(4) $CHCl_3$–CH_3OH–conc. NH_3 (80·20·2)	50	PI + PS (trace)
			0 5	(5) $CHCl_3$- CH_3OH–conc. NH_3 (80:20.2) + 50 m*M* $CH_3CO_2NH_4$	50	PS + PI (trace)

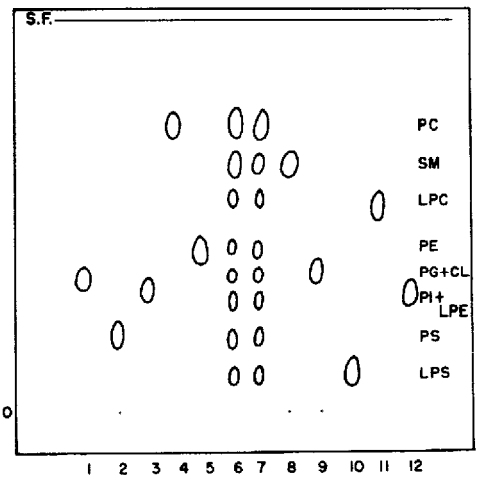
The majority of an acidic phospholipid, phosphatidylinositol (PI), is eluted with chloroform–methanol–0.8 *M* sodium acetate (30:60:8) (lane 15). The authentic phosphatidylserine (PS) is also eluted with this solvent. The egg yolk lipids used in this experiment contained no detectable amounts of PS. However, Rhodes and Lea [11] reported the presence of trace amounts of PS, which consisted of less than 0.2% of total phospholipids, in egg yolk lipids.

The elution pattern of egg yolk phospholipids on the DEAE-silica gel column clearly demonstrates that neutral phospholipids are eluted faster than acidic phospholipids on this column. Thus, separations by DEAE-silica gel column chromatography have been achieved mainly through ion-exchange reactions and hydrogen bond equilibria. Typical elution of phospholipids by DEAE-silica gel column chromatography is summarized in Table I. Elution sequence 1 can be employed for the general separation of phospholipids. PE is not completely eluted with 40% methanol in chloroform in sequence 1. This incomplete elution is usually due to the presence of divalent metal ions in the biological sample, which bind PE tightly. Free fatty acids are eluted with either 1% acetic acid in chloroform or a chloroform–methanol mixture (95:5 or 9:1) that contains 1% or more of acetic acid (data not shown). Without acetic acid, free fatty acids are not eluted from a DEAE-silica gel column. The average recovery of phospholipids ranges from 85 to 95% when a mixture of tritium-labeled PC, PE and PI is applied to a DEAE-silica gel column, eluted by sequence 1 and assayed by liquid scintillation counting. The entire separation using the sequence 1 can be completed within two to three days. Sequence 2 is useful for the isolation of pure PE from crude PE sample contaminated

with PI. Sequence 3 can be conveniently utilized for separating PI from PS. The complete separation of these acidic phospholipids is a challenging task when either an alumina or silica gel column is used.

DEAE-Adsorbosil TLC

We have attempted to separate eight major phospholipids by one-dimensional TLC by use of DEAE-Adsorbosil plates and a basic solvent system, chloroform—methanol—water—pyridine—58% ammonia solution (130:55:8:4:4). Fig. 2 shows the complete resolution of eight phospholipid standards (4—10 μg each). The mixture of phospholipids in lanes 6 and 7 consists of PC, SM, LPC, PE, phosphatidylglycerol (PG), PI, PS and lysophosphatidylserine (LPS) in descending order from the solvent front to the origin. The R_F values are PC 0.80, SM 0.72, LPC 0.64, PE 0.52, PG 0.42, PI 0.36, PS 0.26 and LPS 0.11. Cardiolipin (CL) migrates with PG and LPE is not separated from PI. However, these overlapping phospholipids are readily separated by TLC on silica gel H plates with chloroform—methanol—acetic acid—water (25:15:4:2). When 3% calcium sulfate-impregnated DEAE-Adsorbosil plates are used, the R_F values are PC 0.71, SM 0.62, LPC 0.54, PE 0.49, PG 0.42, PI 0.30 and PS 0.24. As calcium sulfate-impregnated DEAE-Adsorbosil resulted in a poorer resolution of phospholipids than DEAE-Adsorbosil, the latter was selected for separating individual phospholipids of tissues and subcellular organelles. Fig. 3 illustrates the application of the



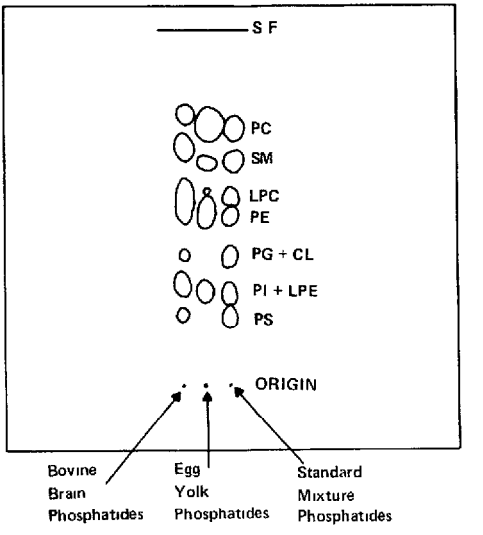


Fig 2. Separation of reference phospholipids on DEAE-Adsorbosil. Solvent: chloroform—methanol—water—pyridine—concentrated ammonia solution (130:55:8:4:4). The spots were detected with molybdenum blue spray reagent . Abbreviations for lipid standards: CL = cardiolipin; PS = phosphatidylserine; LPS = lysophosphatidylserine; others as in Fig. 1. Lane 1, CL; lane 2, PS; lane 3, PI; lane 4, PC; lane 5, PE; lanes 6 and 7, a mixture of PC, SM, LPC, PE, PG, PI, PS and LPS; lane 8, SM; lane 9, PG; lane 10, LPS; lane 11, LPC; lane 12, LPE. Lipid standards (4—10 μg each) were spotted on each lane.

Fig. 3. Separation of bovine brain and egg yolk phosphatides on DEAE-Adsorbosil. Abbreviations and other details as in Fig. 2.

DEAE-Adsorbosil for the separation of brain and egg yolk phosphatides. The brain phosphatides are found to contain three major phospholipids, PC, SM and PE, and three minor phospholipids, PI, LPE and PS. Egg yolk phosphatides contain two major phospholipids, PC and PE, and four minor phospholipids, SM, LPC, PI and LPE. Identification of the overlapping phospholipids, PI and LPE, is achieved by the simultaneous development of these phosphatides on silica gel H plates with the neutral and acidic solvent systems described earlier. Rat liver microsomes contained significant amounts of PS in addition to PC, LPC, SM, PE and PI, but CL was not detected in the microsomes (data not shown). The quantitation of phospholipids on DEAE-Adsorbosil plates was carried out by either radiometric [5, 6] or phosphorus [7] assays.

DISCUSSION

The stepwise gradient elution procedures for DEAE-silica gel column chromatography described here make the isolation of individual phospholipids in a variety of biological samples simple and convenient. In general, separations on a DEAE-silica gel column can be made with solvent systems similar to those reported for DEAE-cellulose and a DEAE-silica gel column can be regenerated by the same sequence of solvents described for DEAE-cellulose [12, 13]. However, DEAE-silica gel column chromatography possesses the following advantages over alumina, DEAE-cellulose or silica gel column chromatography.

(1) DEAE-silica gel is rapidly equilibrated and regenerated.

(2) DEAE-silica gel columns maintain highly uniform packing with little swelling or floating during gradient elution and washing with various solvents, owing to their mechanical strength.

(3) The total elution time of a DEAE-silica gel column is less than half that of a DEAE-cellulose column.

(4) DEAE-silica gel is less susceptible to microbial attack than DEAE-cellulose and requires no special treatment for storage.

(5) Partial hydrolysis of phospholipids, which often occurs on a basic alumina or silica gel column [14], is not observed with DEAE-silica gel columns.

The DEAE-silica gel column has one notable limitation, viz., a lower ion-exchange capacity (0.15—0.45 mmol/g dry weight) than DEAE-cellulose (0.8—0.95 mmol/g dry weight) [13]. However, the repeated application of a DEAE-silica gel column for the separation of phospholipids from egg yolk and rat liver demonstrates its usefulness and the lower ion-exchange capacity appears not to be a serious drawback.

A single one-dimensional TLC step on silica gel G or H plates often gives incomplete separation of phospholipids from biological samples and results in inaccurate identification of individual phospholipids. For example, PI and PS streak and migrate together and PC and LPE overlap in neutral solvent systems (unpublished observations). In order to achieve better resolution of phospholipids, borate-impregnated TLC plates [15] or five-component solvent systems [16, 17] have been successfully utilized. We report here the use of DEAE-silica gel-coated TLC plates, which permits the rapid and complete separation of eight phospholipids (PC, SM, LPC, PE, PG, PI, PS and LPS) by single one-

dimensional TLC. However, the PI and LPE pair and the CL and PG pair have to be resolved by other means. DEAE-silica gel TLC has two additional drawbacks. First, it has lower ion-exchange capacity and hence suitable loads have to be determined empirically for each sample. Overloading must be avoided for the good resolution of phospholipids. Maximum sample loads for common lipids ranged from 40 to 60 μg per lane in our experiments. Second, the number of detection methods that can be employed with lipids is limited as the DEAE group readily reacts with conventional spray reagents such as dilute sulfuric acid or alcoholic phosphomolybdate solution, resulting in very dark background colors on heating the plates. Iodine vapor also reacts with DEAE-silica gel plates to give a brown background and is not suitable for the detection of lipids with these plates. However, the use of radioactive lipids and autoradiography [5] completely eliminates the detection problem and provides excellent sensitivity.

A combination of silicic acid and DEAE-silica gel columns or combinations of DEAE-silica gel columns and silica gel TLC or silicic acid columns and DEAE-silica gel TLC can be useful in preparing pure phospholipid components. The excellent properties of DEAE-silica gel should find wide applications for the rapid identification, purification and quantitation of phospholipids in biological samples.

ACKNOWLEDGEMENTS

We thank Drs. D.G. Ahern, H. Burrell, P. Pei and S. Ramachandran for helfpful discussions and A. Gay for the preparation of the manuscript.

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