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PHOSPHOLIPID AND NEUTRAL LIPID SEPARATION BY ONE-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A simple and rapid method for separation of six major phospholipids and four major neutral lipids from cell extracts by one-dimensional preadsorbant thin-layer chromatography was developed. Due to the inert characteristics of the preadsorbent layer (Celite) no separation occurs until the sample reaches the preadsorbent (Celite)—silica gel junction. The compounds were applied to the preadsorbent (Celite) area (625 mm^2) in 10-μl aliquots (total volume of 0.15 ml). By this method, samples can be applied rapidly in large volumes without respotting any area of the preadsorbent layer. The time required to apply one sample was reduced considerably (2 min) compared to conventional methods (10 min). Since all the compounds move with the solvent front as a sharp, narrow band to the preadsorbent (Celite)—silica gel boundary, excellent separation was achieved when up to 650 μg of lipid material was applied on each lane (25 mm wide). Thus, this method is suitable for the separation of relatively large amounts of radiolabeled and non-radiolabeled lipids and free fatty acids from extracts of biological fluids, tissues, or cells maintained in monolayer culture.

INTRODUCTION

In investigations of the metabolism of phospholipids, neutral lipids and free fatty acids, a method(s) is required for the separation of a number of compounds with similar mobility in various chromatographic systems. Thin-layer chromatography (TLC) on silica gel is used widely for the separation and identification of lipids extracted from tissue fragments, cells maintained in monolayer culture or biological fluids [1]. However, many of the separation procedures presently employed are tedious and require chromatography in more than one solvent system [1, 2]. Often the capacity of the thin-layer

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system is small and localization of the separated lipids must be accomplished by processes that destroy the original compounds. Recently Touchstone et al. [3] described a method that was suitable for the separation of six major phospholipids by one-dimensional TLC. However, the total amount of lipids that can be separated using this TLC system is small (0.2 μg) and therefore charring of the chromatograms is necessary to visualize the separated lipids. The charring process is a distinct disadvantage since it may bring about destruction of the original lipid and thereby preclude further purification and identification of the compound. In addition, the quantification of radiolabeled lipids is hampered by charring since this process causes severe quenching of β-emitting radioisotopes [4].

According to conventional methods for TLC, samples are applied to the silica gel in a successive series of small aliquots (5—10 μl) overlaid at the origin. This process is very time consuming since the solvent must be removed completely between each application of sample. In the present investigation we developed a method for the complete separation of six major phospholipids as well as the separation of the phospholipids from free fatty acids and neutral lipids. In addition a method for the separation of four major neutral lipids from free fatty acids and phospholipids is described. We have employed these methods to monitor the incorporation of radiolabeled fatty acids into the various lipids of cells maintained in monolayer culture. By use of this method up to 650 μg total lipid can be applied on each TLC lane (25 mm wide) without the necessity of respotting any area of the origin, thereby reducing considerably the time required for application of the sample.

EXPERIMENTAL

Standards

Phospholipid and neutral lipid standards were obtained from Serdary Research Laboratories (London, Canada). The standard-mix solution used routinely for our experiments for phospholipid TLC was prepared in chloroform and consisted of L-3-phosphatidylcholine dioleoyl, 0.5 mg ml^{-1}; phosphatidylserine, pig brain, 1.5 mg ml^{-1}; phosphatidylinositol, pig liver, 1.5 mg ml^{-1}; DL-phosphatidylethanolamine dioleoyl, 1.5 mg ml^{-1}; oleic acid, 1 mg ml^{-1}; triolein, 0.5 mg ml^{-1}. A 100-μl volume of this mixture was applied to each TLC lane. The standard-mix solution for neutral lipid TLC was prepared in hexane and contained 1-monoolein, 0.3 mg ml^{-1}; 1,3-diolein, 2 mg ml^{-1}; oleic acid, 0.3 mg ml^{-1}; triolein, 0.3 mg ml^{-1}; cholesteryl ester, 0.3 mg ml^{-1}. A 50-μl volume of the mixture was applied to each lane of the TLC plate.

Chromatography

Silica gel G preadsorbent thin-layer plates were purchased from Analtech (Newark, DE, U.S.A.), and were used without an activation or washing procedure. The important feature of the preadsorbent TLC plate is the sharp demarcation between the inert preadsorbent (Celite) and the silica gel layer that facilitates high resolution of the compounds to be separated. The silica gel and preadsorbent (Celite) zone on each plate were scored into seven lanes

(25 mm each). Samples were applied with a microselectapette (Clay Adams, Parsipanny, NJ, U.S.A.) on an area (625 mm^2) at a spot 5 mm above the lower edge and 5 mm below the preadsorbent (Celite)—silica gel limit of the plate.

Solvents

All solvents were the best analytical grade available from scientific supply houses. The following solvent systems were used: system A (phospholipid TLC), chloroform—ethanol—water—triethylamine (30:34:8:35); system B (neutral lipid TLC), *n*-heptane—diethyl ether—acetic acid (75:25:4).

Procedure

In a typical experiment human endometrial stromal cells were maintained in monolayer culture in the presence of [^{14}C]arachidonic acid (Amersham, Arlington Heights, IL, U.S.A.). Cells were extracted and washed according to the method of Folch et al. [5]. After concentration of the extract to 2 ml by evaporation under nitrogen, aliquots for phospholipid (10%) and neutral lipid (10%) TLC were transferred into 75 × 12 mm tubes and the appropriate standard-mix solution was added. The extracts were reduced to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 0.15 ml chloroform—methanol (2:1), and spotted on the preadsorbent (Celite) area of the chromatogram under an atmosphere of nitrogen by use of a device supplied by Sindco (Miami, FL, U.S.A.). After equilibration with the solvent system to be used in a plate conditioning apparatus (Analtech) for 15 min, plates were lowered into the solvent and developed to a height of 16 cm above the preadsorbent (Celite)—silica gel boundary. After removal from the chamber, the solvent was removed under a stream of air or nitrogen and the chromatogram was exposed to iodine vapor to visualize standards. After evaporation of the iodine, the silica gel was scraped from the areas of the chromatogram that corresponded to the various lipids. The lipids were eluted from the silica gel with 6 ml chloroform—methanol (2:1). The extracts were evaporated under a stream of nitrogen at room temperature and radioactivity was assayed in 16 ml Liquiscint (National Diagnostics, Somerville, NJ, U.S.A.) by liquid scintillation spectrometry.

RESULTS

The chromatograms that are illustrative of the separation of phospholipids, neutral lipids and free fatty acid are shown in Figs. 1 and 2. The standards were applied on TLC lanes either separately or as the standard-mix in 0.15 ml chloroform—methanol (2:1). When solvent system A was employed (phospholipid TLC), six major phospholipids were separated. In addition free fatty acids and neutral lipids (monoacylglycerols, diacylglycerols, triacylglycerols, cholesteryl esters) that migrate near the solvent front were separated from the phospholipids. With solvent system B (neutral lipid TLC) the separation of four major neutral lipids, free fatty acids and phospholipids was achieved. By use of this solvent system (B), the phospholipids migrate only to the preadsorbent (Celite)—silica gel boundary. The mobilities of the various compounds tested, expressed as R_F values, are given in Table I. The preadsorbent (Celite)—silica

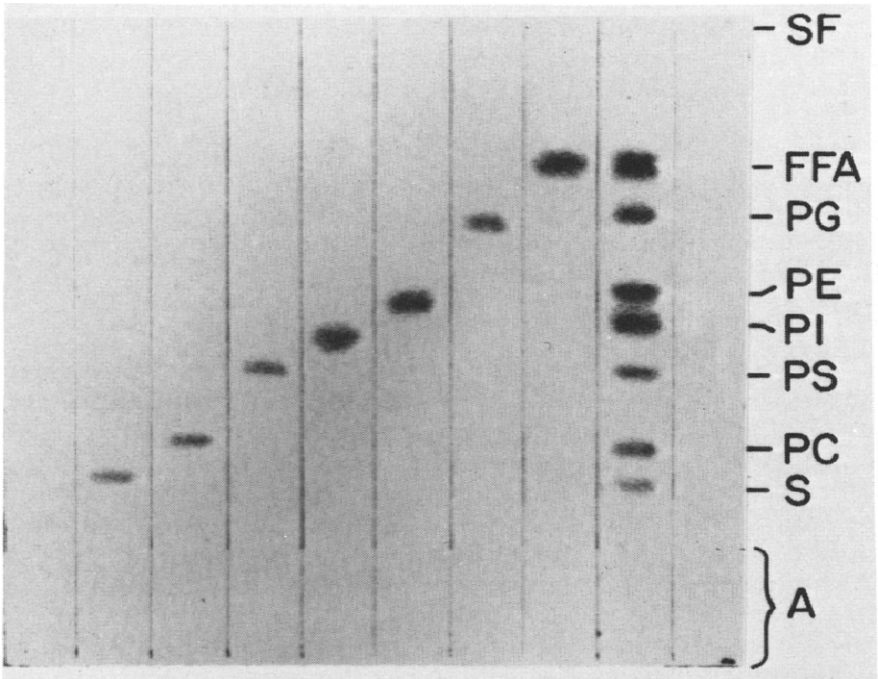

Fig. 1. Phospholipid TLC chromatogram (solvent system A) after iodine staining. The compounds were applied either separately or as a mixture. A = preadsorbent (Celite) layer; S = sphingomyelin (100 μg); PC = phosphatidylcholine (50 μg); PS = phosphatidylserine (150 μg); PI = phosphatidylinositol (150 μg); PE = phosphatidylethanolamine (150 μg); PG = phosphatidylglycerol (100 μg); FFA = free fatty acid (100 μg); SF = solvent front. For purpose of illustration neutral lipids were not included.

TABLE I

R_F VALUES OF SEPARATED COMPOUNDS IN SOLVENT SYSTEM A (PHOSPHOLIPID TLC) AND SOLVENT SYSTEM B (NEUTRAL LIPID TLC)

Compound	Solvent system	
	A	B
Sphingomyelin*	0.13	0
Lysophosphatidylcholine*	0.14	0
Phosphatidylcholine	0.21	0
Lysophosphatidylethanolamine*	0.32	0
Phosphatidylserine	0.37	0
Phosphatidylinositol	0.42	0
Phosphatidylethanolamine	0.49	0
Phosphatidylglycerol*	0.62	0
Free fatty acid	0.73	0.59
Monoacylglycerol	0.96	0.03
Diacylglycerol	0.97	0.31
Cholesterol*	0.97	0.35
Triacylglycerol	0.98	0.68
Cholesteryl ester	0.97	0.91

*These compounds were not included routinely in our experiments.

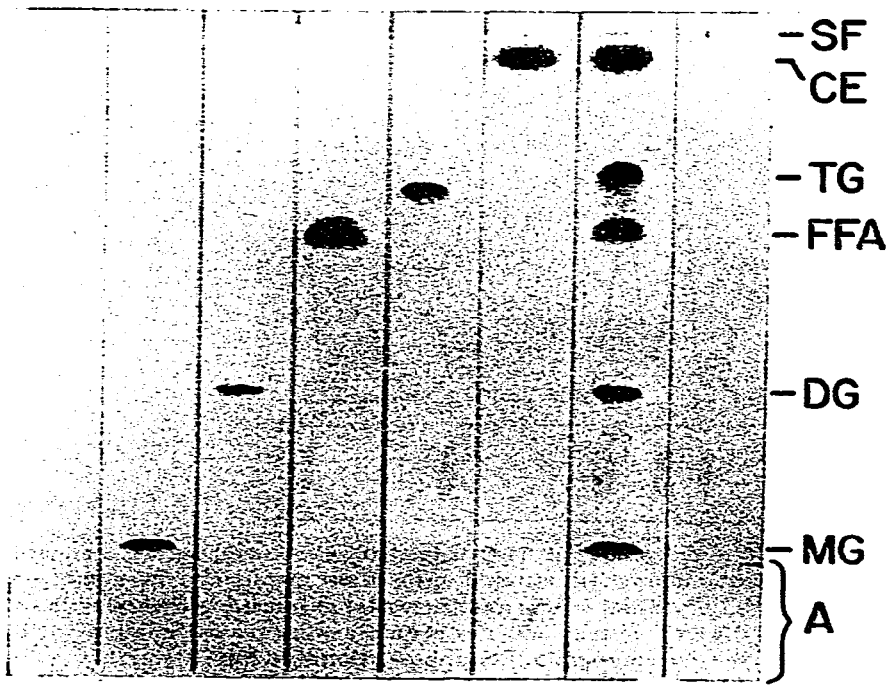

Fig. 2. Neutral lipid TLC chromatogram (solvent system B) after iodine staining. The compounds are applied either separately or as the mixture. A = preadsorbent (Celite) layer; MG = monoacylglycerol (15 μg); DG = diacylglycerol (100 μg); FFA = free fatty acid (15 μg); TG = triacylglycerol (15 μg); CE = cholesteryl ester (10 μg); SF = solvent front.

gel boundary is taken as the origin to estimate the R_F value. Importantly it can be seen that the separation characteristics were not altered by the application of the compounds as a mixture.

As an example of an application of the methods described, human endometrial stromal cells maintained in monolayer culture were incubated with [^{14}C]arachidonic acid for 24 h. At the end of the incubation period lipids were extracted from the cells as described. The total lipid content of the cell extract was estimated to be approximately 50 μg, as determined previously by phosphorus assay according to the methods described by Rouser [6]. Thus, it can be calculated that the total amount of lipid material on each lane (standard plus samples) of a phospholipid chromatogram was approximately 650 μg. A radiometric scan (Berthold Instruments LB2760, G.F.R.) of a TLC lane of a chromatogram on which lipids of endometrial stromal cells were separated with solvent system A after incubation with [^{14}C]arachidonic acid for 24 h is presented in Fig. 3. Standards were chromatographed in parallel lanes and visualized after exposure to iodine vapor. Sharp peaks of radioactivity are apparent, indicative of the incorporation of [^{14}C]arachidonic acid into compounds that comigrate with the standards. It should be noted that sphingomyelin, phosphatidylglycerol, lysoglycerophospholipids and cholesterol were not included routinely in our experiments.

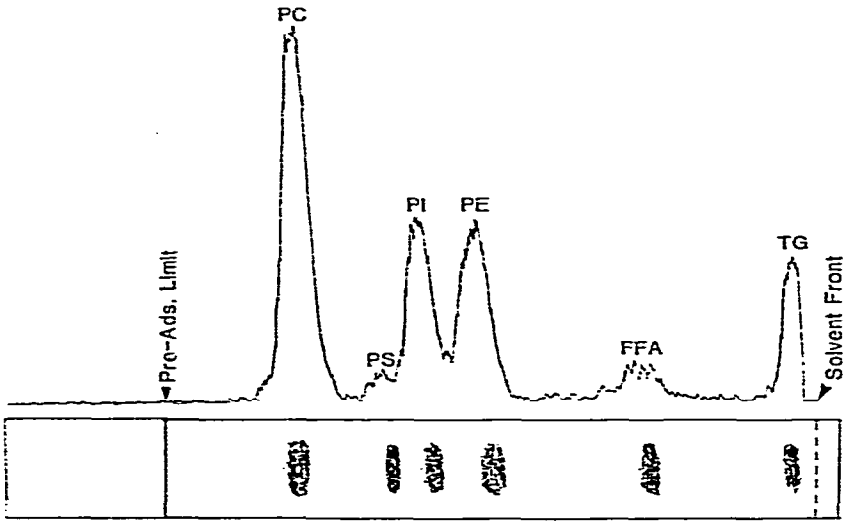

Fig. 3. Radiometric scan of a phospholipid TLC lane with the corresponding standards after iodine staining. Human endometrial stromal cells were maintained in monolayer culture in the presence of [^{14}C]arachidonic acid and thereafter extracted as described in the text. An aliquot (10%) of the cell extract and the standard-mixture was applied to the preadsorbent (Celite) area of the chromatogram. For abbreviations see Figs. 1 and 2.

DISCUSSION

The conditions of isolation of major phospholipids and neutral lipids have been described in detail [1]. However the separation of phospholipids by conventional one-dimensional TLC is limited by the fact that phosphatidylglycerol and phosphatidylethanolamine have very similar R_F values. In addition phosphatidylserine and phosphatidylinositol often do not migrate as sharp bands; rather during chromatography these compounds tend to drag or tail [7, 8]. Recently Touchstone et al. [3] described a method suitable for the separation of six major phospholipids by one-dimensional TLC. These investigators included in the solvent system triethylamine, which has a high selectivity for these critical pairs [9]. The phospholipid TLC solvent system used in the present investigation was devised from the findings of Touchstone and coworkers. However, in many of the methods described previously, including that of Touchstone et al. [3], the quantity of lipid material that can be separated is small, and, therefore, charring of the chromatograms may be necessary for visualization of the compounds. One disadvantage of this process is that the lipids may be altered structurally such that further purification and identification of the original lipid species is precluded. In addition, charring causes severe quenching of β-emitting radioisotopes in liquid scintillation spectrometry [4]. In these experiments we routinely used iodine vapor for visualization of the lipids. The staining process is reversible due to oxidation of the iodine molecules. The silica gel corresponding to standards can be scraped from the chromatogram and the lipids can be eluted quantitatively with organic solvents. If the fatty acid composition of the separated lipids is of interest, the TLC chromatograms can be sprayed with dichlorofluorescein (0.02%) in methanol

and the compounds then can be localized under ultraviolet light. The structure of the fatty acids will be retained and gas chromatography or high-performance liquid chromatography may be employed to identify the fatty acids after hydrolysis and methylation.

The separation of radiolabeled phospholipids, neutral lipids and fatty acids from extracts of cells in culture incubated with radiolabeled precursors is difficult. The quantity of each separated compound must be sufficient to assay β-radiation accurately, but not so great that the TLC plates are overloaded with lipid material. Moreover, according to conventional TLC methods, the sample is applied to the plate at the origin as a single band. This process is extremely time consuming, particularly when the application of large sample volumes is necessary. Therefore, we applied the compounds in a volume of 0.15 ml on the entire preadsorbent (Celite) area (625 mm^2). It was not necessary to respot the origin area and the time required for the application of a sample (2 min) was greatly reduced compared to conventional methods (10 min). A relatively large amount of lipid material can be applied since all the material moves with the solvent front through the inert Celite as a sharp, narrow band to the preadsorbent (Celite)—silica gel junction. The rapidity of sample application together with the achievement of complete separation of a number of lipids are supportive of the proposition that this method is well suited for the separation of relatively large amounts of endogenous or radiolabeled lipids and fatty acids from extracts of biological fluids, tissues, or cells maintained in monolayer culture.

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