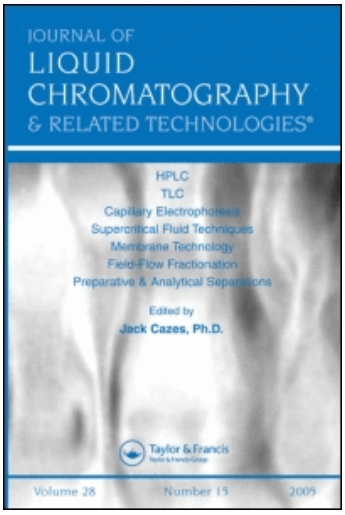

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Improved Method for Thin-Layer Chromatographic Resolution and Photodensitometric Assessment of the Major Classes of Phospholipids

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IMPROVED METHOD FOR THIN-LAYER CHROMATOGRAPHIC RESOLUTION AND PHOTODENSITOMETRIC ASSESSMENT OF THE MAJOR CLASSES OF PHOSPHOLIPIDS

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ABSTRACT

Thin-layer chromatography and photodensitometry were used to resolve and quantitate trace levels of the major phospholipid classes. A solvent system of chloroform/methanol/ethyl acetate/40% methylamine (20/20/20/10; v/v) resolved the phospholipids. After spraying with 10% $CuSO_4$ in 10% H_3PO4 and charring, photodenstiometric assesments were reliably achieved with as little as 100ng of phospholipid. To illustrate the ease and reliability of the methodology, analyses of various biological materials are presented.

INTRODUCTION

Photodensitometric assays for quantitation of lipids resolved by thin-layer chromatography (TLC) have proven useful for a variety of

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analyses (1-5). However, The routinely used photodensitometric techniques are relatively insensitive, and therefore applications are limited. When applied to phospholipids, photodensitometric assessments are often complicated by the failure of the TLC system to provide a consistant resolution of the phospholipids. We have overcome these limitations by coupling a sensitive detection protocol with a newly developed TLC system that resolves the major classes of phospholipids.

In this communication we report the development of a TLC system that employs inexpensive materials, and quickly resolves phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SPM), phosphatidylserine (PS), and phosphatidylinositol (PI). The phospholipids are quantitated by photodensitometry after application of a $CuSO_4/H_3PO_4$ spray reagent and subsequent charring. As little as 100 ng of phospholipid can be detected with this assay. We provide detailed protocols for this methodology and illustrate various applications of these methods for assessment of cellular, tissue and serum phospholipids.

MATERIALS AND METHODS

Materials

Silica gel G TLC plates were purchased from Analtech, Inc. (Newark, DE). Chloroform, methanol, and ethyl acetate were from J.T. Baker (Phillipsburg, NJ). Methylamine (40% in H_2O) was from Mallinckrodt (Paris, KY). $CuSO_4$ was from Sigma Chemical Co. and H_3PO_4 was from Fisher (Springfield, NJ). Phospholipids used as standards were from natural sources (brain or egg) and were purchased from Avanti Polar Lipids (Alabaster, AL) or Sigma.

Thin-Layer Chromatography

TLC plates were developed in paper lined tanks with a saturated tank atmosphere. Care was taken to apply all lipids to the TLC plate in spots having similar diameters of 3-4 mm. Resolution of the phospholipids was achieved using a solvent mixture of chloroform/methanol/ethyl acetate/40% methylamine (20/20/20/10; v/v/v/v).

Visualization and Photodensitometry Methods

A detailed description of the detection and quantification protocols has been published previously (6). Generally the methods are

as follows. Resolved phospholipids were visualized by heating the TLC plate at 180°C for 3 min, spraying the plate with 10% $CuSO_4$ in 10% H_3PO_4 (until the plate just becomes wet) and rapidly charring at 180°C for 20 min (6). The TLC plates were scanned (absorbance/reflectance mode) using a Camag TLC Scanner II set at 400 nm (deuterium lamp). Transduction of spot density to peak areas was achieved with a Varian 4290 integrator. Standard curves were best fit to lines defined by a second order polynominal (7-9). Cricket Graph software (copyright by Cricket Software; Malvern, PA) and an Apple MacIntosh SEII were used to generate standard curves and calculate coefficients of correlation.

Miscellaneous Methods

Culture of REF52 fibroblasts and A-10 vascular smooth muscle cells and collection of cellular lipid have been described (10-12). Cell number (cultures at approximately 80% of confluence) was determined using a Coulter Counter. Rats (mature male) were sacrificed by exposure to CO_2. Blood was immediately collected from the hepatic portal vein using a heparinized syringe and then centrifuged to pellet the cells. The serum was collected and the lipids were extracted (13). Rat thoracic aorta was surgically removed and washed in phosphate buffered

saline. The aortas were cut into 0.5 cm sections and the lipids were extracted as previously described (14).

RESULTS AND DISCUSSION

The resolution of total cellular phospholipids from REF52 fibroblast cells is illustrated in Figure 1; an adjacent lane illustrates the resolution of phospholipid standards. The R_f values of the phospholipids were: PE, 0.60; PC 0.50; SPM (doublet) 0.42 and 0.38; PS, 0.27; PI, 0.21. The R_f values observed for lyso PC and lyso PE were 0.46 and 0.25 respectively; phosphatidylglycerol and cardiolipin comigrated in a spot with an R_f of 0.54 (data not shown). Figure 2 illustrates a densitometric scan of phospholipids of REF52 cells and phospholipid standards resolved by the newly developed TLC system. The densitometric scan (Figure 2) was taken from a chromatogram developed with a solvent migration to 10 CM (~ 20 min run time). The short solvent migration distance minimizes band broadening and thus maximizes detection sensitivity (see below). Longer solvent migrations (18 CM; ~ 50 min run time) can be used when maximum distance between spots is required to aid subsequent isolation of individual phospholipids. Figure

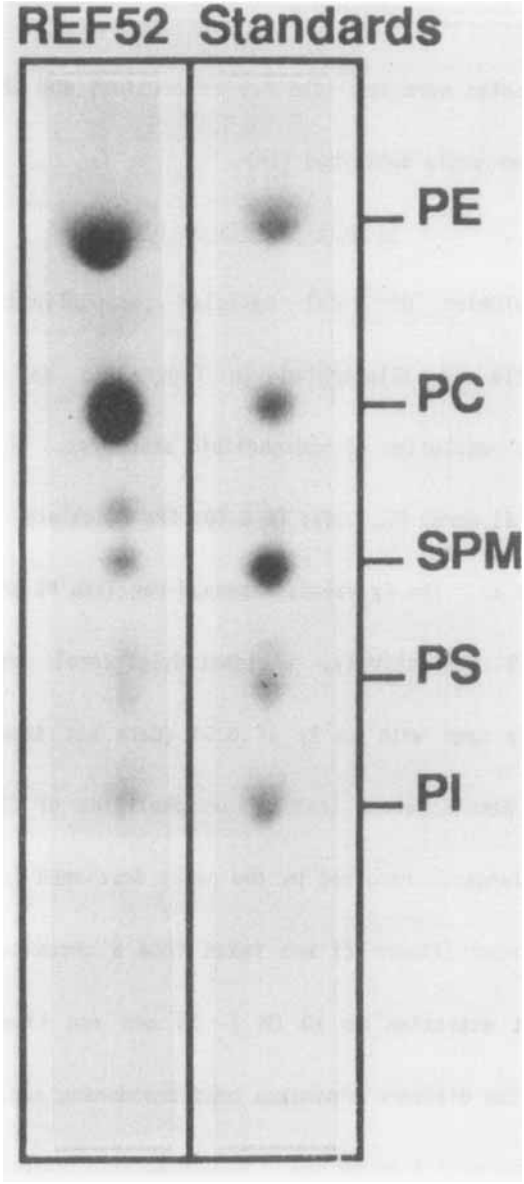

Figure 1. Resolution of Phospholipids from REF52 Cells and Standards by Thin-layer Chromatography. Total lipids from REF52 cells and phospholipid standards were applied to the TLC plate and resolved by a solvent mixture of chloroform/methanol/ethylacetate/40% methylamine (20/20/20/10; v/v/v/v/). The spots were visualized by spraying with 10% $CuSO_4$ in 10% H_3PO_4 and charring as described under "Materials and Methods". For the purpose of the illustration, the lower portion of the TLC plate containing charred phospholipids was photographed. R_f values for the lipids are listed in the text.

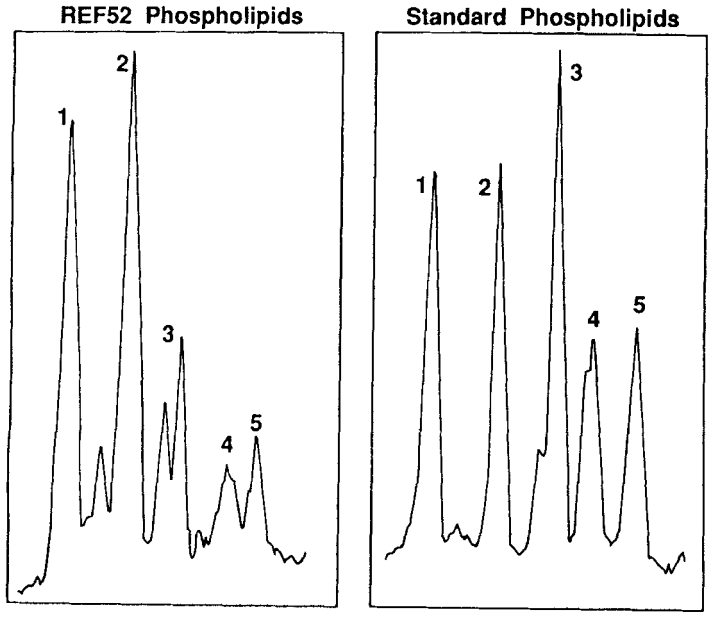

Figure 2. Photodensitometric Profile of Phospholipid Standards and Phospholipids from REF52 Cells Resolved by Thin-layer Chromatography. The lipids were resolved as described in Figure 1. The phospholipid spots were assessed by photodensitometric scans in the absorbance/reflectance mode at 400 nm. Peak numbers are as follows: 1,phosphatidylethanolamine; 2,phosphatidylcholine; 3,sphingomyelin; 4,phosphatidylserine; 5,phosphatidylinositol.

3 illustrates standard curves obtained after resolution (as described above) of known quantities of phospholipid standards. The curves characteristically deviate from linearity at higher mass quantities and for this reason more accurate estimates of unknowns are obtained when the curves are fit to lines defined by a second order polynomial (7-9). Correlation coefficients for the standard curves for each phospholipid

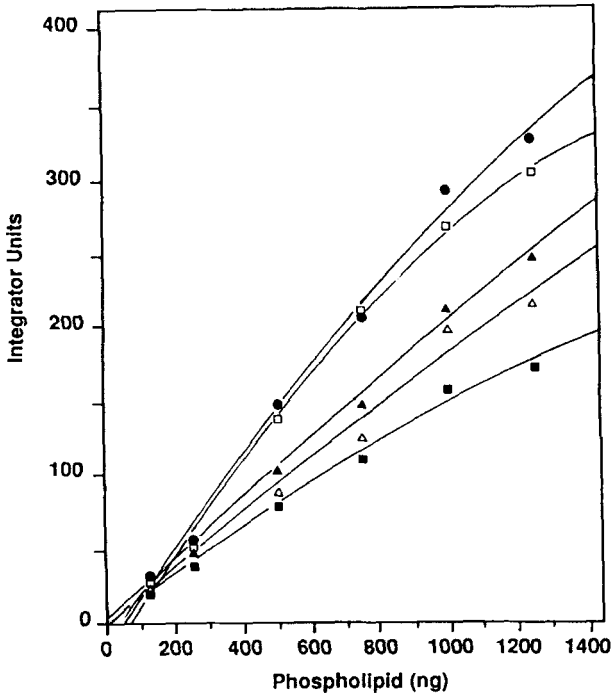

Figure 3. Standard Curves Established after Photodensitometric Assessment of Phospholipids Resolved by Thin-layer Chromatography. Known quantities of phospholipid standards were resolved and assayed as described in Figures 1 and 2. The standard curves were constructed after peak integration, using between 125 ng and 1.25 μg of the standard phospholipids.

were routinely above 0.990 (range = 0.963-0.999; 12 different experiments).

The variability between the different phospholipids, as regards the spot density derived from equivalent masses, is obvious (Figure 3). When testing different mixtures of standards, we found that SPM

Table 1. Assay of Phospholipids from Various Sources by Thin-layer Chromatography Coupled with Photodensitometry.

	Phospholipid Profiles (% of total)[1]		
Phospholipid	A-10 Cells	Rat Aorta	Rat Serum
PE	23	14	5
PC	46	55	67
SPM	13	19	12
PS	9	7	16
PI	10	4	ND[2]

Lipids were extracted, resolved by Thin-layer chromatography and assayed by photodensitometry as described under Materials and Methods.

[1] Data are the mean three different determinations (rat serum and rat aorta) or from a single determination (A-10 cells).

[2] ND = none detected

consistently gave a higher spot density than did the other phospholipids. SPM was also resolved into two spots, presumably the hydroxyacyl and acyl forms (4), in both cellular lipids and commercial standards. The variability in the magnitude of the signal derived from equivalent masses demonstrates the necessity of establishing standard curves for each phospholipid under investigation.

We have used this TLC system to assess the relative phospholipid composition of total lipids from various sources (Table 1). These data illustrate the utility of the method for determination of

phospholipid percent composition. Using similar analysis that examined only PC, we determined that rat aorta contains 8.6 mg PC/0.5 cm segment (mean of 4 determinations), A-10 cells contain 9.6 mg PC/60 mm dish (mean of 12 determinations using ~2 x 10^5 cells/dish), and REF52 cells contain 14.2 mg PC/60 mm dish (mean of 6 determinations using ~3 x 10^5 cells/dish). After the relative amount of PC in the total sample has been determined (as in Table 1), quantitative evaluation of only PC can then be used to estimate total phospholipid in the sample. With the detection limit of ~ 100 ng (Figure 3), only 2% or less of the total lipid of the samples described above is required for quantitation of PC. Thus, the vast majority of the lipid sample is left for other analyses.

The cupric sulfate charring methodology appears to be one of the most sensitive for lipid TLC (4, 15, 16). The detection limit of ~ 100 ng (reported here) is similar to that reported for lyso PC when using cupric sulfate charring (4). In addition, the cupric sulfate spray reagent has the added advantage of being insensitive to the location and number of carbon-carbon double bonds (6, 17) and being stable for weeks(4). In our hands the Analtech TLC plates are extremely inert with

regard to "background charring" of the silica gel; this aspect of the chromatography system enhances the sensitivity of the detection protocol.

A limitation of this system is that relatively small quantities of phospholipids overload the chromatographic system. When standards of more than 3 mg/phospholipid/lane were chromatographed, noticeable tailing of the spots occurred; this chromatographic aberration resulted in the poor resolution of the individual phospholipids. However due to the ease with which samples can be diluted, overloading the TLC plate is a relatively insignificant problem. Any limitation associated with the sample overloading is offset by the ability of the chromatographic/densitometric system to quantitate very small masses of phospholipid (Figure 3). In addition to minimizing sample manipulations, these protocols reduce analysis time and therefore increase sample throughput. A TLC plate can be loaded (~ 12 samples and 6 standards) and the lipids resolved and quantitated in approximately 2 hr. Running several TLC plates simultaneously will further increase analysis efficiency. These methods should be easily adapted to the analyses of

other lipids and therefore this chromatography/densitometry system should prove valuable to a variety of biochemistry and cell biology laboratories.

ACKNOWLEDGMENTS

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