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Note

Improved analysis of species of phospholipids using argentation thin-layer chromatography

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Thin-layer chromatographic (TLC) separation of compounds based upon their degree of unsaturation using silver nitrate-impregnated silica gel is a standard and widely used technique[1–3]. Reversed-phase high-performance liquid chromatography (HPLC) has recently largely supplanted this technique for the separation of closely related lipid compounds and is based on hydrophobicity differences of compounds of interest[4,5]. Argentation-based chromatography has the important feature that separations are based primarily upon the number and arrangement of double bonds in the compounds of interest rather than on hydrophobicity although the degree of unsaturation affects the latter.

Separation of molecular species within phospholipid classes has been approached using a variety of modalities. In an extensive paper by Renkonen in 1968[6] the dimethyl esters of phosphatidic acids (DMPAs) derived from choline phospholipids of iterest were separated using argentation TLC. Gas chromatographic analysis of the fatty acid methyl esters formed by the methanolysis of lipids eluted from the silver nitrate TLC plate permitted deduction of the molecular species present. This author demonstrated that DMPA compounds with one double bond could be separated from those with a total of two, three, or more double bonds using a single solvent development. A different solvent system was required to distinguish compounds with four, five, or six or more double bonds but this system loses resolution of the more saturated species and displayed marked smearing[6]. Separation of the major molecular species of phospholipids has been reported[5,7,8] but requires costly HPLC equipment and the long development times used make multiple analyses cumbersome. In this report a technique is described that results in the separation on a single chromatogram of phospholipid derivatives with none, one, two, three, four, five, or six or more double bonds using plates that are much less costly and more convenient to work with than commercially available silver nitrate-impregnated TLC plates. The phosphatidic acid core of phospholipids is preserved (rather than employing other techniques that assess diacylglycerol derivatives[9,10] in order to use this technique in the analysis of *in vivo* ^{32}P-labeled lipids formed in a variety of important cellular metabolic events[11–13].

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EXPERIMENTAL

Phospholipids (Serdary, Port Huron, MI, U.S.A.) were converted to phosphatidic acid (PA) prior to chromatographic analysis by the action of phospholipase D (cabbage; Sigma, St. Louis, MO, U.S.A.) for phosphatidylcholine (PC), phosphatidylserine and phosphatidylethanolamine[14]. Conversion of the resultant PA (10–50 μg dissolved in 50 μl chloroform) to DMPA was accomplished by incubation with 50 μl of 0.15 *M* ethereal diazomethane[15] for 10 min in a sealed vial at room temperature as described previously[6].

Phosphatidylinositol (PI) was converted to the presumed 2,3,4,5,6-pentaacetyl derivative by acetylation using 150 μl of acetic anhydride–pyridine (1:2) at 22°C for 1 h. After adding methanol, the reaction mixture was dried and the acidic hydroxyl group of the phosphodiester linkage was methylated using diazomethane (50 μl of 0.15 *M* diazomethane in diethyl ether as described above) to form acetylated methyl PI (AMPI).

The DMPA and derivatized PI were separated from underivatized or partially derivatized substrate by TLC on silica gel plates (Polygram Sil G 20 × 20 cm; Brinkmann, Westbury, NY, U.S.A.) developed in ethyl acetate. Fig. 1 shows that this TLC resulted in good separation of DMPA from underivatized PA (remaining at the origin) with essentially no secondary product formation. Previous work[6] demonstrated that once PA is singly methylated it is rapidly converted to DMPA. Monomethyl PA has an R_F in this solvent system that is approximately one half that of DMPA (data not shown). Nearly quantitative conversion of PI to AMPI was observed with only a faint band of labeled material below AMPI. This minor product likely represents an incompletely acetylated derivative since experiments with briefer acetylation demonstrated greater labeling of this band and other still lower R_F products (data not shown). The radiolabeled product band was localized by staining adjacent lanes containing unlabeled DMPA or AMPI with iodine (after cutting the lane from the plate), removed by scraping and the lipid eluted by two extractions with chloroform–methanol (2:1).

Silver nitrate plates were prepared by lowering a 20 × 20 cm plastic backed silica gel plate slowly (over 30 s) into a solution of 2.5%, 5% or 10% (w/v) aqueous silver nitrate (Aldrich, Milwaukee, WI, U.S.A.) to 2 cm from the top of the plate. The plate was removed slowly and dried at room temperature for at least 1 h and then activated by heating to 50°, 80° and 110°C in a stepwise fashion for 20 min at the first two temperatures and 40 min at the last. The plates were cooled and reactivated at 110°C for 30 min within 6 h of their use. By weighing the plates it was deduced that retention of silver nitrate on the plates was 0.20, 0.38 and 0.77 g per plate for 2.5%, 5.0% and 10% silver nitrate solutions, respectively, giving rise to densities of 0.53, 1.00 and 2.03 mg/cm^2. Because preliminary results showed that 5% and 10% treated plates performed identically, but 2.5% layers had reduced resolution (data not shown), 5% layers were used in all remaining experiments as has been suggested by others[2]. The cost of commercially prepared 10% silver nitrate plates is US$ 6.80 each for a 20 × 20 cm glass backed plate (Redicoats; Supelco, Bellefonte, PA, U.S.A.) while the cost of the 5% plate described above is US$ 1.45 (US$ 1.15 for the plate and US$ 0.30 for the siler nitrate).

DMPA or AMPI was streaked 1.5 cm from the bottom of the silver nitrate

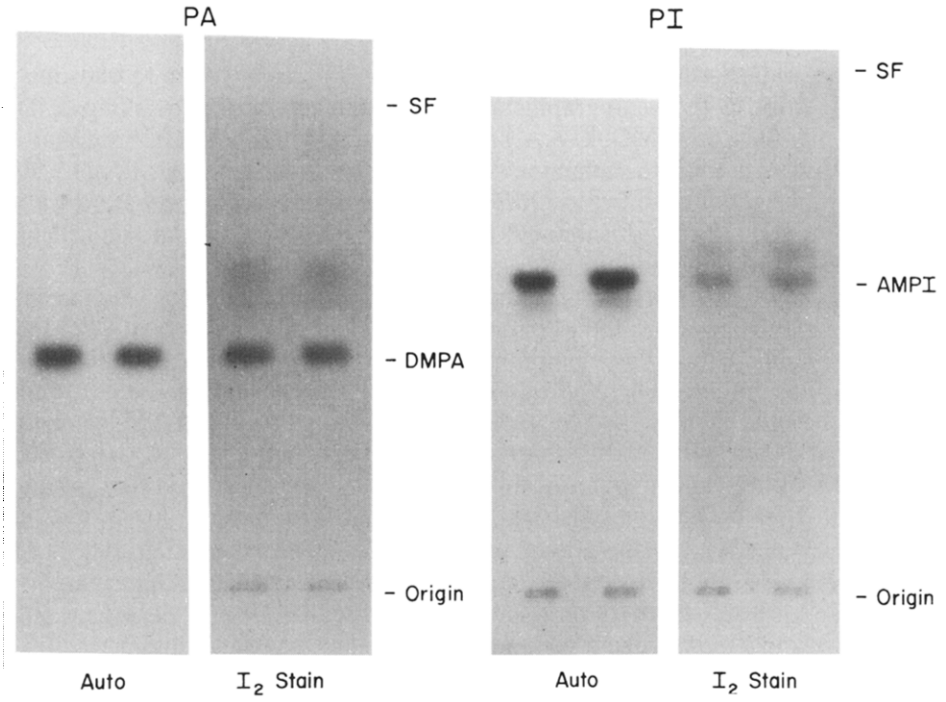

Fig. 1. TLC separation of more hydrophobic derivatives of PA and PI from underivatized substrate. ^{32}P-labeled PA was methylated and ^{32}P-labeled PI was acetylated and methylated as described in Experimental and duplicate samples of reaction products spotted at the origin of untreated silica gel plates. Development was accomplished using ethyl acetate and the final solvent front indicated as SF. Detection by autoradiography of labeled compounds (Auto) and by iodine staining (I_2 stain) are illustrated for each lipid.

plate. Separation of species containing highly unsaturated fatty acids from the origin and each other was accomplished by developing to 5 cm above the origin with chloroform–methanol (96:4). The chromatogram was dried for 10 min then redeveloped to 16 cm above the origin with chloroform–methanol (98.5:1.5 for DMPA and 98.75:1.25 for AMPI) to resolve more saturated compounds from each other and the former solvent front (the highly unsaturated materials are nearly stationary in this second development).

1,2-Diacylglycerol (DAG) species produced from egg lecithin (Serdary) by the action of phospholipase C (*Cl. welchii*, Sigma)[16] as well as single molecular species of DAG (Serdary) were converted to [^{32}P]PA in the presence of [γ-^{32}P]ATP (ICN, Irvine, CA, U.S.A.) by *E. coli* DAG kinase[17]. These labeled PA moieties were converted to DMPA as described above. An autoradiograph of the silver nitrate TLC separation is shown in the left-hand panel of Fig. 2. The right-hand panel of Fig. 2 demonstrates an autoradiograph of the separation accomplished when derivatized PI from ^{32}Pi-labeled mast cells was developed in its slightly different solvent system. Sharp bands were produced with minimal smearing. The lower R_F observed for the band containing four double bonds derived from egg PC compared to the dilinoleoyl DMPA is due to the greater binding of a compound with a single more highly un-

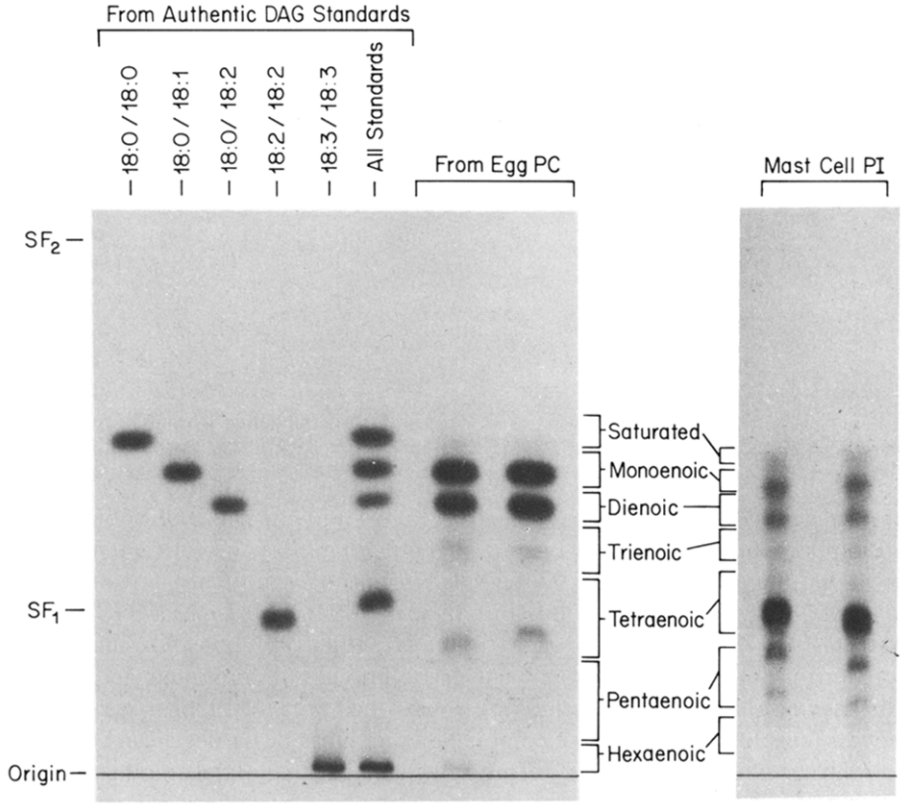

Fig. 2. Autoradiograph of argentation TLC analysis of defined species of PA, egg PC and mast cell PI. Left-hand panel: egg lecithin was hydrolyzed by *Cl. welchii* phospholipase C and the resultant diacylglycerol converted to ^{32}P-labeled PA. Authentic DAG species were similarly converted to their [^{32}P]PA derivatives. After treatment with diazomethane the ^{32}P-labeled DMPA product was isolated by TLC, extracted from the plate and spotted at the origin of a plate previously dipped in 5% silver nitrate and activated as described in Experimental. Sequential development was accomplished using chloroform–methanol mixtures (96:4 to 5 cm above origin followed by 98.5:1.5 to 16 cm above origin). Right-hand panel: [^{32}P]PI was acetylated and methylated as described in Experimental and subclasses separated by argentation TLC as described above except that chloroform–methanol (98.75:1.25) was used in the second development. 18:0 = stearic acid; 18:1 = oleic acid; 18:2 = linoleic acid; 18:3 = linolenic acid.

saturated fatty acid in the natural lipid (16:0/20:4 or 18:0/20:4) compared to a compound with two less unsaturated fatty acids (18:2/18:2). This phenomenon has been observed previously[5]. The complexity of the fatty acid pattern seen for egg lecithin-derived DAG was not fully appreciated by Renkonen's method[6] due to substantial smearing. Radioactive areas of interest were cut out and radioactivity quantitated by scintillation counting (in 3 ml ACS-II; Amersham, Arlington Heights, IL, U.S.A.). Data from the analysis of molecular species of egg lecithin obtained in this manner compare favorably to those reported by others[5,6,18], as illustrated in Table I, confirming that the derivatizations and extractions used in the current method are not selective with respect to the fatty acids represented in a given phospholipid molecule.

TABLE I

MOLECULAR SPECIES ANALYSIS OF EGG LECITHIN

Molecular species	*Composition (%)*			
	*Current study**	*Ref. 6***	*Ref. 8****	*Ref. 18****
Saturated	2.5 ± 0.4	NR§	0.5	1.0
Monoenoic	47.3 ± 0.9	42.2	53.4	53.6
Dienoic	35.9 ± 1.9	35.2	30.2	33.7
Trienoic	6.7 ± 0.7	3.3	3.0	2.2
Tetraenoic	5.5 ± 0.2	8.0	6.4	5.7
Pentaenoic	1.5 ± 0.5	1.6	1.4	0.6
Hexaenoic	1.5 ± 0.5	6.5	2.6	3.7

* Egg lecithin (Serdary) was converted to DAG using phospholipase C, labeled by radioactive phosphorylation and methylated to yield [^{32}P]DMPA as described in Experimetal. Analysis of radioactivity after argentation TLC of [^{32}P]DMPA was performed in three different experiments (mean ± S.E.M.).

** Analysis by argentation TLC.

*** Analysis by HPLC.

§ Not reported.

DISCUSSION

The use of a dipping chamber is not novel for impregnating TLC plates with silver nitrate[2]. Using plates with the combined advantages of harder adsorbent surfaces as well as plastic backing is an improvement because: (1) the reduction of abrasive loss of softer layers reduces analytic errors and markedly improves ease of handling and storage; (2) these plates are much less costly than commercially prepared silver nitrate plates; (3) using a dipping chamber avoids the need to coat plates by the investigator with the corrosion problems attendant to the use of silver nitrate[1]; and (4) radioactive areas identified by autoradiography can be quantitated by simply cutting plastic backed plates rather than scraping glass plates.

Using a double solvent development improves the resolution of complex mixtures of these hydrophobic derivatives of phospholipids possessing a variety of combinations of saturated and unsaturated fatty acids. This approach permits a complex sample containing 0–6 double bonds to be analyzed in a single chromatograph rather than splitting it for two separate analyses as required by Renkonen's method[6]. A variety of techniques have utilized the diacylglycerol core for molecular species analysis[9,10] but the loss of the phosphate group precludes analysis of ^{32}P-labeled cellular lipids. The current method permits a more detailed analysis of the type of fatty acids represented in the ^{32}P-labeled intermediates in the "PI cycle" for example.

Unlabeled cellular phospholipids can also be analyzed with sensitivity in the picomole range by converting a purified phospholipid class to diacylglycerol using phospholipase C followed by ^{32}P-derivatization and argentation TLC as was done in the current study using egg lecithin. The results obtained (Table I) show that this type of analysis generates data similar to that obtained independently using different techniques.

From the point of view of cost, ease, reproducibility and time of analysis this

technique surpasses those previously described for the TLC resolution of species of a given phospholipid class according to degree of unsaturation. HPLC separation of molecular species of phospholipids has been reported by a number of authors[5,7,8] but requires costly equipment and involves extensive analytic times (30–90 min per sample), thereby markedly reducing the number of possible analyses compared to the TLC separations described here. Because of the ability to convert all phospholipids to DMPA or other appropriate non-polar derivatives, this technique will facilitate more sophisticated assessments of the metabolism of individual subclasses currently lost in the complexity of the many molecular species of a given phospholipid class.

One issue not addressed by the technique described is descrimination based on the type of bond at the 1 position of glycerol (ester *vs.* alkenyl ether *vs.* ether), but mast cell ^{32}P-labeled PI and egg lecithin are greater than 98% diacyl lipids[5,19]. If desired, separation of compounds with different types of linkages can preceed argentation TLC using previously described techniques[6,20].

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