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Note

Two-dimensional thin-layer chromatographic separation of phospholipid molecular species using plates with both reversed-phase and argentation zones

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Reversed-phase high-performance liquid chromatography (HPLC) has been used by a variety of investigators seeking to separate molecular species of glycerol-based lipids. This approach achieves fairly good separation of intact phospholipids[1,2], derivatives of 1,2-diglycerides[3–5] or derivatives of phosphatidic acid (PA)[6]. Several aspects of this technique limit application to studies of cellular lipid metabolism: (1) chromatographing each sample separately is time consuming since each sample requires 40–120 min, (2) HPLC equipment and solvents are costly and (3) adjacent peaks, incompletely resolved by HPLC, are often metabolically unrelated since very different fatty acids may have similar hydrophobicities[1–6].

Separating related subclasses of molecular species of diacyl forms of phospholipids using argentation thin-layer chromatography (TLC) of a derivative of PA was recently reported by this laboratory[7]. This technique permitted good separation of nine bands consisting of subclasses of molecular species based on the number and distribution of double bonds present in the esterified fatty acids. The compounds grouped within a single chromatographic subclass tended to have similar structures (16:0/20:4, 18:0/20:4 and 20:0/20:4 for example). Although this technique has proven valuable in assessing the validity of existing concepts of the origin of 1,2-diacylglycerol (DAG)[8], more complete resolution of molecular species would be highly desirable, particularly if the attractive characteristics of a TLC-based separation (ease of analysis of multiple samples and modest cost) could be retained.

The current studies describe a two-dimensional TLC analysis employing both argentation and reversed-phase chromatography on a single plate consisting of two distinct and differentially modified zones. Combining argentation and reversed-phase chromatography on a single TLC plate was initially described by Bergelson *et al.*[9] who used this technique for the separation of fatty acids with a variety of unusual isomeric forms. These studies did not use a bonded reversed-phase adsorbant, but rather impregnated the plates with dodecane, applied the sample, performed the reversed-phase separation, introduced silver nitrate into the plate and after drying it, then performed the argentation-based separation. Because argentation chromatography depends primarily on the degree of unsaturation of fatty acids, the application of both reversed-phase and argentation TLC on a single plate resulted in good resolu-

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tion of a sample containing a variety of fatty acid methyl esters. Unfortunately, our attempts to adapt this technique to the more challenging separation of molecular species of derivatives of DAG resulted in inadequate separation (data not shown). Additionally, the use of dodecane impregnation (as opposed to a bonded reversed-phase adsorbant) imposed serious limitations on the application of this approach because plates could not be fully prepared prior to TLC since the solvents for argentation TLC displace the impregnated dodecane while those for reversed-phase TLC similarly displace impregnated silver nitrate. Thus, so long as neither modification involved covalent coupling, impregnation with the second was necessary after development in the first dimension. As described subsequently, this limitation was overcome by designing a two-dimensional TLC plate that can be used "as is" for both argentation and reversed-phase TLC without further modification during the chromatographic development.

EXPERIMENTAL

Materials and their sources: unlabeled phospholipids (Serdary, Port Huron, MI, U.S.A.); *Clostridium welchii* phospholipase C (Sigma, St. Louis, MO, U.S.A.); authentic 1,2-diacylglycerols (Nuchek Prep, Elysian, MN, U.S.A.); [γ-^{32}P]ATP (ICN, Irvine, CA, U.S.A.); octadecyltrichlorosilane and silver nitrate (Aldrich, Milwaukee, WI, U.S.A.); XAR film for autoradiography (Kodak, Rochester, NY, U.S.A.); plastic backed silica gel TLC plates (Polygram Sil G 20 × 20 cm; Brinkmann, Westbury, NY, U.S.A.); organic solvents (Burdick & Jackson, Muskegon, MI, U.S.A.); all other chemicals (Sigma, St. Louis, MO, U.S.A.).

Phospholipids were converted to DAG using *Cl. Welchii* phospholipase C as described previously[10]. Authentic or phospholipid-derived DAG was labeled by radioactive phosphorylation using [γ-^{32}P]ATP and *Escherichia coli* DAG kinase as described in detail previously[7,8,11]. The resultant labeled phosphatidic acid ([^{32}P]PA) was converted to a more hydrophobic derivative (dimethyl-PA or DMPA) by methylation of its phosphoric acid hydroxyl groups using diazomethane and the DMPA purified by TLC[7,8].

TLC plates containing both argentation and reversed-phase zones were formed by derivatization of part of the silica gel plate with octadecylsilane (ODS) and impregnating the remainder with silver nitrate. Commercially prepared plates containing a 17-cm reversed-phase zone with a 3-cm band of silica gel can be purchased (KC18-F; Whatman, Clifton, NJ, U.S.A.) but despite considerable effort, separations on the reversed-phase portion of the commercially prepared plate were never adequate. The smearing and resultant poor resolution may in part have been due to insufficient reversed-phase derivatization of the silica gel (*vide infra*).

Fig. 1 illustrates our approach to derivatizing silica gel plates and the two-dimensional TLC employed in the subsequent studies. Silica gel plates were washed by development in chloroform–methanol (1:1) to 1 cm from the top, dried and heated to 110°C for 1 h. In a significant modification of a previous method[12], a portion of the silica gel plate was converted to a covalently linked ODS reversed-phase adsorbant by dipping the plate (with the solvent front of the chloroform–methanol wash on the left) into a dipping tank containing 5% (v/v) octadecyltrichlorosilane in dry toluene to a line 16 cm from the bottom. The plate was placed in a tank with a saturated

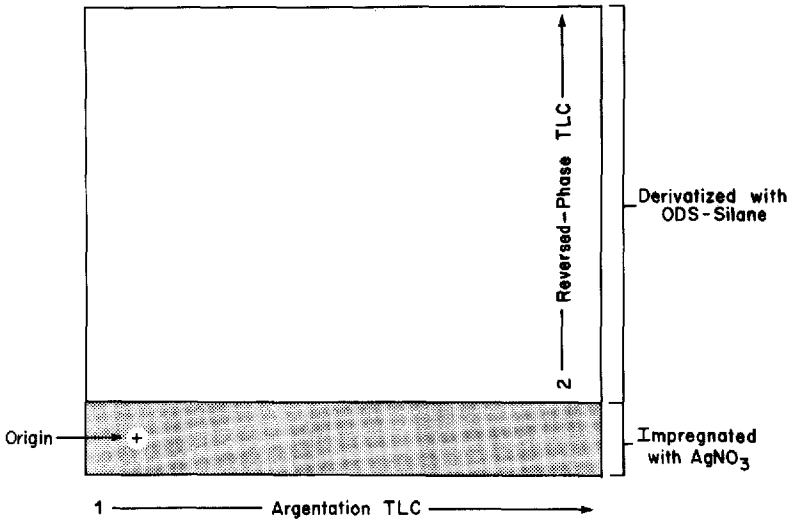

Fig. 1. Sequential use of both argentation and reversed-phase chromatography on a single TLC plate. The hatched area in this schematic representation represents the 4-cm portion of the plate impregnated with silver nitrate while the remainder is the portion covalently derivatized with ODS for reversed-phase chromatography as described in detail in the next. After activation, a sample was spotted at the origin. Sequential developments in the argentation TLC (arrow number 1) were accomplished using chloroform–methanol mixtures (95:5 to 5 cm above the origin followed by 98.75:1.25 to the top). After drying and turning the plate 90°, all compounds were chromatographed to the junction with the reversed-phase portion of the plate by developing in acetonitrile to 6 cm from the bottom. Reversed-phase TLC was then accomplished by developing twice using acetonitrile–tetrahydrofuran (4:1) (arrow number 2).

toluene atmosphere and after 5–10 min it was redipped in the octadecyltrichlorosilane solution, removed and allowed to react for 5–10 min more. The plates obtained by dipping only once did not produce sufficient reversed-phase separations due to band broadening (data not shown) probably as the result of inadequate reversed-phase derivatization. The plate was washed by successively dipping it into dry toluene and light petroleum (b.p. 35–60°C) and the remaining chlorosilane groups converted to methyl ethers by dipping the plate into dry methanol. After drying, the remaining 4 cm of underivatized silica gel was impregnated by dipping the plate (now with the solvent front from the chloroform–methanol wash on the right) into an aqueous solution of 5% (w/v) silver nitrate and activated as described in detail previously[7]. In experiments in which separated lipids were eluted and their fatty acid methyl esters examined by gas chromatography (GC), the derivatized plates were washed by sequential development with chloroform and diethyl ether.

Using these new plates, molecular species of [^{32}P]DMPA derivatives of DAG were separated by the sequential use of argentation TLC and reversed-phase TLC. As illustrated in Fig. 1, the lipid sample was applied in a spot 1.5 cm from each edge within the strip of the plate impregnated with silver nitrate and developed first with chloroform–methanol (95:5) to 5 cm above the origin and after brief drying, developed again in the same direction with chloroform–methanol (98.75:1.25) to the top of the plate. After drying, the plate was rotated 90° so that the silver nitrate impregnated strip was at the bottom and the labeled DMPA sample moved to the junction of the

reversed-phase zone by developing with acetonitrile to 6 cm from the bottom of the plate (2 cm above the silver nitrate and reversed-phase junction). Reversed-phase separation (based on the hydrophobicity of the relevant compounds) was accomplished by twice developing the chromatogram to the top of the plate using acetonitrile–tetrahydrofuran (4:1). After drying, the plates were autoradiographed for 3–8 h. In selected experiments in which separated lipids were subjected to further analysis, radioactive areas identified by autoradiography were removed from the plate and extracted using heptane–ethyl acetate (1:1) prior to alkaline methanolysis and GC of the fatty acid methyl esters[8].

Separation of molecular species of [^{32}P]DMPA formed by radioactive phosphorylation of DAG (derived from porcine liver phosphatidylcholine by phospholipase C hydrolysis) and methylation of the resultant [^{32}P]PA (using diazomethane) is illustrated in Fig. 2. All major and most minor molecular species of this lipid were resolved. The multiple forms of pentaenoic and hexaenoic molecular species were not characterized due to their modest representation in the lipids examined, but multiple

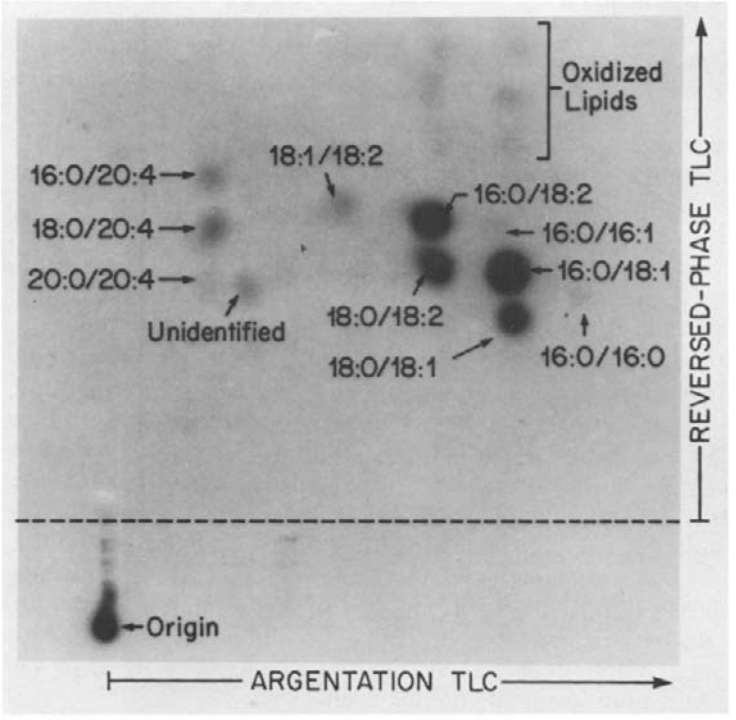

Fig. 2. Separation of molecular species of [^{32}P]DMPA. Porcine liver phosphatidylcholine was converted to DAG using *C. welchii* phospholipase C, purified and intensely radioactively phosphorylated using [γ-^{32}P]ATP and *E. Coli* DAG kinase[11]. The resultant [^{32}P]PA was purified and methylated (using diazomethane) to form [^{32}P]DMPA and 500 000 cpm of the latter was spotted at the origin, developed as illustrated in Fig. 1 and autoradiographed for 3 h. The junction of the argentation and reversed-phase phase zones is indicated by a dashed line and the relevant portion of the autoradiograph illustrated. Radioactive areas were removed and following elution, the lipids were either quantitated by liquid scintillation counting or characterized by GC of the fatty acid methyl esters formed by alkaline methanolysis. The identity of the labeled spots is indicated.

bands were observed when the authoradiographs were exposed for longer periods (data not shown). R_F values were quite reproducible from plate to plate and from day to day (± 5–10%; data not shown). The quantitative molecular species distribution of porcine liver phosphatidylcholine obtained using this method (Fig. 2) was similar to a previously published estimate that employed HPLC[2].

RESULTS AND DISCUSSION

These studies sought to develop an appropriate TLC matrix for two-dimensional TLC separation of structurally similar molecular species of radioactively labeled DMPA derived indirectly from phospholipids or DAG. Assessing the metabolic source and fate of DAG in mast cells by determining the molecular species "fingerprint" of DAG and comparing it to that of potential precursors and metabolic products is a major goal of our laboratory. Several previous studies using reversed-phase HPLC alone have had varying degrees of success in resolving molecular species of neutral glycerides or phospholipids[1–6]. In our experience, reversed-phase HPLC alone was not found to be sufficient for the separation of even the major molecular species of labeled DMPA derived from egg phosphatidylcholine[8] and additionally was found to be quite cumbersome. In that study, excellent separation of molecular species was accomplished by further subjecting HPLC-derived peaks to silver nitrate TLC[8] suggesting that complete molecular species analysis of DAG derivatives might be possible using argentation and reversed-phase TLC. The two-dimensional TLC described in the current study (using plates designed to take advantage of both reversed-phase and argentation chromatography) provide sufficiently good resolution of molecular species to generate a "fingerprint" that will permit more accurate prediction of the role of different pathways proposed to be involved in DAG formation and removal during cellular stimulation.

Compared to reversed-phase HPLC, this combined reversed-phase and argentation TLC separation of molecular species of a DMPA (which can be formed from either DAG or PA) has a number of advantages: (1) molecular species are well separated and compounds that are spatially close are structurally related so that an error in identifying a compound would be a conservative one (reversed-phase HPLC causes dissimilar compounds to often have very similar retention times[1–5]); (2) the current TLC-based method requires only simple techniques and relatively inexpensive materials and equipment; (3) multiple analyses require less time (6 h to run 25 samples in a single large TLC tank compared with 16–50 h for 25 sequential 40–120 min HPLC runs); (4) autoradiographic detection followed by liquid scintillation counting of labeled compounds is more sensitive than direct scintillation spectromery of HPLC effluent.

Certain aspects of the current technique limit its application. First, custom derivatization of the silica plates is labor intensive. Treating and activating 25–50 plates requires 4–6 h of attention during an 8–10 h period. Second, the 18:1/18:1 molecular species significantly overlaps the 18:0/18:2 spot as does the 16:1/18:1 with the 16:0/18:2 (determined by GC analysis of the latter) although the cellular presence of each of the former represent modest fractions of each of the latter. Third, the use of tritium labeled compounds is complicated by the fact tritiated lipids migrate significantly differently than do unlabeled compounds in argentation chromatographic

analysis[13]. Thus, identification of labeled areas by non-destructive staining of unlabeled standard compounds added to the samples prior to chromatography does not accurately identify the location of corresponding tritiated compounds which are best determined by fluorography. This differential migration of tritiated compounds can be used to advantage insofar as it permits enrichment of radioactive specific activity of tritiated synthetic products.

Chromatographic analysis of 1-alkyl and 1-alkenyl lipids is important in a variety of cells in which choline and ethanolamine phospholipids are enriched in ether-containing species. When 1-alkyl and 1-alkenyl molecular species were compared to their diacyl counterparts, a 2–8% change in R_F was observed for both the reversed-phase and argentation chromatographic steps (data not shown). As a result, utilizing the TLC described in this paper to resolve molecular species of phospholipids with a significant ether content requires prior separation of the labeled DMPA into 1-acyl, 1-alkyl and 1-alkenyl subclasses as has been described previously[14,15].

One refinement not routinely utilized is the use of parallel lanes containing authentic standards to enhance identification of relevant sample spots. By making a slightly wider silver nitrate impregnated band (5 cm), a lane for a sample containing authentic standards can be run in parallel with the principal sample (in the orientation of Fig. 1, standards were applied below the sample). This standard-containing lane is cut off prior to development in the second dimension. Appropriate authentic standards can similarly be employed to help identify compounds separated in the second dimension by spotting them to the right (orientation of Fig. 1) of the anticipated migration of the compound with the highest R_F value in the first dimension.

In addition to using these plates (containing both reversed-phase and argentation zones) for molecular species analysis of fatty acid containing lipids, this technique holds promise for a variety of other types of separations. Although no data exist in this regard, this method might prove to be quite useful for separating arachidonic acid metabolites, natural and synthetic steroids and the products of a variety of synthetic reactions in which the products have modest differences in hydrophobicity and degree of unsaturation.

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