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Short Communication

One-dimensional thin-layer chromatographic separation of phospholipids and lysophospholipids from tissue lipid extracts

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ABSTRACT

A modified one-dimensional thin-layer chromatographic procedure is presented for the separation from tissues of five phospholipids (phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine and sphingomyelin) and three lysophospholipids (lysophosphatidylserine, lysophosphatidylethanolamine and lysophosphatidylcholine). This is achieved by simple involvement of 0.4% ammonium sulphate in silica gel H and of acetone in a developing solvent as chloroform–methanol–acetic acid–acetone–water (40:25:7:4:2, v/v). The procedure is simple and the separation is reproducible. The weakness of this method is the partial degradation of phosphatidylethanolamine to lysophosphatidylethanolamine, but a method to prevent this degradation is also presented.

INTRODUCTION

One-dimensional thin-layer chromatography (TLC) is one of the most frequently used methods in lipid research, mainly because of its simplicity, rapidity and high resolving power [1–4]. Previously, the methods of Skipski *et al.* [5] and Parker and Peterson [6] for separation of the major phospholipids occurring in mammalian cells have been proven to be fast and reproducible. Nevertheless, several authors [7–15] have encountered difficulties, as we did, in separating phosphatidylserine (PS) and phosphatidylinositol (PI) by these methods. Kaulen [12] used a chromatographic plate impregnated with 0.167% ammonium sulphate together with his developing solvent, chloroform–methanol–acetic acid–water (50:25:8:1), and the separation was reported to be good, but the method would not give a clear-cut separation of PS and PI and the procedure was furthermore rather time-consuming. The separation of PS and PI became essential in our laboratory, since the influence of lipid infusions, such as Intralipid, and an inositol phosphate, D-myoinositol-1,2,6-triphosphate (PP56), on cell lipid metabolism was to be studied systematically. After testing a number of different chromato-

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graphic plates and developing solvents, we achieved a satisfactory separation of these phospholipids and others and three lysophospholipids.

EXPERIMENTAL

Analytical-reagent-grade chemicals and solvents were used. The following solvents were obtained from Merck (Darmstadt, Germany): chloroform, methanol, acetic acid, acetone. Silica gel H also came from the same company. The following substances were used as references and obtained from Sigma (St. Louis, MO, USA): *l*-α-phosphatidylethanolamine (PE), *l*-α-phosphatidylserine (PS); *l*-α-phosphatidylinositol (PI); *l*-α-lecithin (PC)/sphingomyelin (SM); phosphatidic acid (PA); cardiolipin; *l*-α-lysophosphatidylcholine (lysoPC); *l*-α-lysophosphatidylethanolamine (lysoPE); *l*-α-lysophosphatidylserine (lysoPS); and 5(6)-cholesten-3β-ol. 2,6-Di-terbutyl-*p*-cresol (BHT) was purchased from BDH (Poole, UK).

Preparation of extracts from red blood cells (RBCs)

The RBC lipids were extracted by the Rose and Oklander extraction method [16], using methanol–chloroform (11:7, v/v) omitting the haemolytic procedure.

Preparation of TLC plates

A 50-g aliquot of silica gel H was mixed with 125 ml of 0.4% ammonium sulphate. The mixture was applied as a 0.5-mm-thick uniform slurry using an adjustable spreader on 20 cm × 20 cm glass plates. The plates were dried in air at room temperature for 2 h and activated at 110°C for 20 min before use. The temperature of the plate and the moisture content of the environment do not affect the separation. The 0.4% ammonium sulphate solution could be stored at room temperature for several months. Plates impregnated with ammonium chloride, copper sulphate and sodium sulphate were prepared in an identical way.

Chromatography

The lipid extract was evaporated to dryness under nitrogen, redissolved in 50 μl of chloroform and applied to the TLC plate with a microsyringe, giving a band 4–5 cm long, 1.5 cm above and parallel to the lower edge of the plate. The application of a band as thin as possible improves the separation of lipids, especially when the lipid content of the extract is high. In our hands, 2.0–2.4 mg of total lipids were applied at the origin. The lipid extract container was washed three more times with 20–25 μl of chloroform and the washings applied. The plate was then developed in a tank containing chloroform–methanol–acetic acid–acetone–water (40:25:7:4:2, v/v), with 0.005% BHT to prevent lipid oxidation. Prior saturation of the tank with the solvents appears to be unnecessary. A total amount of solvent of 40 ml is enough for developing one plate. It takes about 1.5 h for the developing solvent to migrate to 1.5 cm from the upper edge of the plate. After drying the plate at room temperature for 10 min, the individual phospholipids were visualized in iodine vapour. During the chromatography, several standard lipids were also applied to the plate as references.

RESULTS AND DISCUSSION

One remaining problem in phospholipid TLC has been the incomplete separation of acidic phospholipids, especially of PS and PI. Skipski *et al.* [5] first pointed out that a "basic pH" plate (such as a plate impregnated with 1 m*M* sodium carbonate) would give a better separation of PS and PI than a "neutral" plate (impregnated with water only). However, this plate does not consistently give satisfactory separation of these two phospholipids [7–15] and, as a result, several other different silica gel plates together with corresponding developing solvents have been proposed, including plates impregnated with 3% ammonium phosphate [9], 0.167% ammonium sulphate [12], 1 m*M* EDTA [13] and 7.5% magnesium acetate [17]. Separation of PS and PI might be achieved, but the proposed procedures are complicated and time-consuming. Moreover,

these plates were unable to separate three lysophospholipids from five phospholipids at the same time.

The idea of using ammonium sulphate to impregnate silica gel H came from Mangold and Kammereck [18], and was also used by Kaulen [12]. Kaulen [12] reported a good separation using a silica gel H plate impregnated with 0.167% ammonium sulphate (the ratio of silica gel to ammonium sulphate was 100:0.4, w/w) and a solvent of chloroform–methanol–acetic acid–water (50:25:8:1, v/v), but we found that the separation of PS and PI was obtained with "tails", which overlap other phospholipid bands.

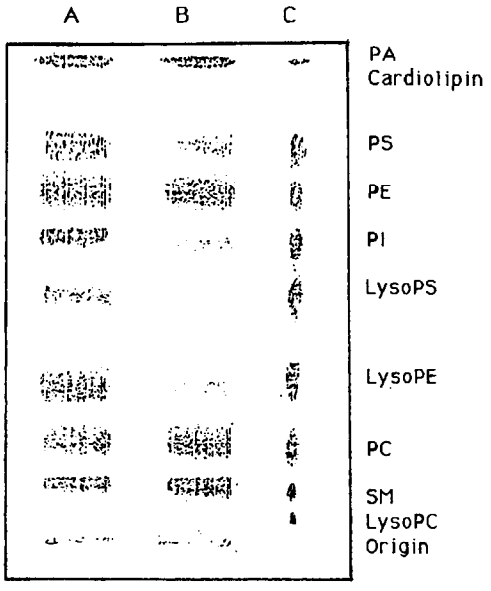

Fig. 1. Thin-layer chromatogram of human RBC lipids. The plate was prepared by mixing silica gel H with 0.4% ammonium sulphate, activated at 110°C for 20 min before use and developed in chloroform–methanol–acetic acid–acetone–water (40:25:7:4:2, v/v). Detection method: iodine vapour. Lanes: A = red blood cell (RBC) lipid extract with standard PI, lysoPS and lysoPE; B = RBC lipid extract; C = standard phospholipids (PA, cardiolipin, PE, PC and SM, 20 μg; PS, 30 μg; PI, 25 μg; lysoPS, lysoPE and lysoPC, 40 μg). Pigment and neutral lipids (triglycerides, cholesterol, cholesterol ester, fatty acids) migrate with PA and cardiolipin. Gangliosides remain at the origin. LysoPC is a weak band in lanes A and B. Abbreviations: PA = phosphatidic acid; PS = phosphatidylserine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PC = phosphatidylcholine; SM = sphingomyelin; lysoPS = lysophosphatidylserine; lysoPE = lysophosphatidylethanolamine; lysoPC = lysophosphatidylcholine.

This study shows that a quite satisfactory, constant and reproducible separation of PS and PI can be achieved by utilizing a silica gel H plate impregnated with 0.4% ammonium sulphate and a developing solvent of chloroform–methanol–acetic acid–acetone–water (40:25:7:4:2, v/v) (Fig. 1). This method proved, in our hands, to be useful for separating five phospholipids and three lysophospholipids extracted from human and rat red blood cells, rat liver, kidney, brain, aorta and small and large intestinal tissues, although lysoPS was not always present in the plate. The R_F values of PS, PE, PI, lysoPS, lysoPE, PC, SM and lysoPC were 83.3 ± 2.7, 74.2 ± 4.5, 57.7 ± 6.2, 47.0 ± 4.4, 34.8 ± 3.4, 21.2 ± 2.1, 12.1 ± 1.7, 9.0 ± 1.6, respectively (n = 9), by utilizing corresponding standard phospholipids. PA and cardiolipin migrated together with pigment and neutral lipids (triglycerides, cholesterol, cholesterol ester, fatty acids) in the front of the plate, while all gangliosides remained at the origin.

During the development of our chromatographic system we tested plates impregnated with varying concentrations of ammonium sulphate (0.04–0.25%), copper sulphate (0.105–0.84%), sodium sulphate (0.2–0.8%) and ammonium chloride (0.08–0.64%) in the silica gel, plates without salts and commercial DC plates using the chosen developing solvent, chloroform–methanol–acetic acid–acetone–water (40:25:7:4:2, v/v). It was found that only the TLC system recommended here yielded good separations of PS and PI among phospholipids and lysophospholipids. With 0.06% and 0.167% ammonium sulphate in the silica gel, PS and PI migrated with long "tails". This was true for the copper sulphate plate, the plate without salts, the commercial plate and even more so for plates impregnated with sodium sulphate and ammonium chloride in the tested concentrations. However, we noted that with ammonium salts in silica gel, the R_F values of PS and PI tended to increase, while copper and sodium salts made them decrease. Apparently, this phenomenon is salt-dependent although the mechanism is not clear.

The 0.4% ammonium sulphate-impregnated silica gel H plate was also combined with the fol-

lowing developing solvents: chloroform–methanol–acetic acid–water (50:25:8:1, 25:15:4:2 or 40:25:7:2, v/v); however, none of the combinations were as good at separating phospholipids and lysophospholipids as our recommendation. The addition of acetone in our developing solvent appears to eliminate the long "tail" of PS.

In our chromatograms, were detected two "new" bands migrating between PI and PC. They were identified as lysoPS and lysoPE, co-migrating with the standard lysoPS and lysoPE, respectively. When standard PE was run on sulphate-containing plates, lysoPE appeared in addition to PE so that the lysoPE band may be due to a partial degradation of PE during the separation. LysoPE occurred only on plates containing sulphate salts, indicating that the formation of lysoPE is sulphate-dependent. One possible explanation of the lyso formation is that the sulphate ions in the gel are capable of cleaving an acyl chain from the PE molecule. Furthermore, it was shown that the degradation of PE to lysoPE could be prevented by applying 20–50 μl of acetic acid to the sulphate-containing plate at the site of lipid application before sampling. The plate was then dried and reactivated before use and this procedure completely prevented the formation of lysoPE without influencing the favourable separation of PS and PI. However, the lysoPE content is less than 3% of total PE content. The detailed mechanism for this phenomenon is not yet revealed. The degradation of PS into lysoPS could not be verified in our hands.

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