A novel spray reagent for phospholipid detection

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Summary Ammonium pentachlorooxomolybdate in a solution of 7–9 N H_2SO_4 was found to be an excellent spray reagent for the specific detection of phospholipids on thin-layer chromatograms. It is better than the usual molybdenum blue reagent due to its greater sensitivity and its ability to eliminate the problem of the development of background blue color on prolonged exposure.

Supplementary key words ammonium pentachlorooxomolybdate · molybdenum blue · thin-layer chromatography

The most widely used method for the specific detection of phospholipids on thin-layer chromatograms is that developed by Dittmer and Lester (1). This procedure employs the use of the so-called "molybdenum blue reagent", prepared by dissolving molybdenum trioxide and metallic molybdenum in conc. H_2SO_4 followed by dilution with water. However, the blue spots obtained for compounds containing phosphate esters are masked by the background blue color that appears within several hours, thus presenting difficulties in recording or photographing chromatograms. The present report describes a spray reagent that is not only specific for phospholipids but also eliminates the problem of the development of background blue color. It also appears to be more sensitive than the molybdenum blue reagent (1).

Materials and methods

Mouse brain and goat kidney phospholipids were extracted with chloroform–methanol 2:1 (v/v) (2). Gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1})[2] were prepared from beef brain according to the modified procedure of Ledeen, Yu, and Eng (3). Hematosides were isolated from beef adrenal medulla (4, 5); ceramide trihexoside and globoside-1 were from human erythrocytes (6, 7). Phosphatidylethanolamine, phosphatidylinositol and brain cerebroside were purchased from Supelco Inc. (Bellefonte, PA); phosphatidylserine and lysolecithin were from Nutritional Biochemicals Corporation (Cleveland, OH) and beef cardiolipin, cholesterol and its esters, desmosterol, and fatty acids and their methyl esters were from Applied Science Laboratories, Inc. (State College, PA). Sphingomyelin, egg lecithin, and phosphatidic acid were gifts from Dr. B. N. Ghosh, Biochemistry Department, and Mr. J. K. Dutta, Bose Institute, Calcutta; Tay-Sachs ganglioside and ceramide dihexoside were from Dr. R. W. Ledeen; Forssman glycolipid was from Dr. M. Naiki and sulfatide was from Dr. R. K. Yu. Ammonium paramolybdate (S. Merck, India) and hydroiodic acid (E. Merck, Germany, sp gr 1.7, 55–58% HI) were used without further purification. Reagent grade chemicals and solvents were used throughout this work. Thin-layer plates (250 μm thick) were prepared from silica gel G and silica gel H (E. Merck, Germany), activated at 110°C for 1.5 hr, and stored in a vacuum desiccator over anhydrous $CaSO_4$ prior to use. The most useful solvent was found to be chloroform–methanol–7 N NH_4OH 65:27:5 (v/v). Other solvent mixtures such as chloroform–methanol–water 65:25:5 (v/v) and chloroform–methanol–acetic acid–water 25:15:3:1 (v/v) were also used for routine analysis.

Ammonium pentachlorooxomolybdate $(NH_4)_2[Mo^vOCl_5]$ is a well-characterized compound and can be prepared easily by reducing ammonium paramolybdate $(NH_4)_2[Mo^vO_4]$ with HI in a solution of HCl (8) as follows. A solution of 4 g of ammonium paramolybdate in 30 ml of conc. HCl is treated with 4 ml of conc. HI and the solution is carefully boiled on a hot plate with stirring to drive off the liberated iodine. The solution is evaporated to a pasty solid. This is redissolved in 30 ml of conc. HCl and evaporated again. Finally, the moist solid is treated with 15 ml of conc. HCl to give a green solution along with some green crystals. This is cooled in an ice bath and gaseous HCl is allowed to pass through it until saturated. Emerald green crystals of APCOM appear, which are collected by quick filtration[3] through a sintered-glass funnel and dried under vacuum over solid KOH (yield, 5 g). The solid is quite stable and can be stored for an indefinite period under anhydrous conditions.

Spray reagent

The spray reagent is easily prepared by dissolving 0.75 g of APCOM in 25 ml of 7–9 N H_2SO_4. If

Abbreviations: APCOM, ammonium pentachlorooxomolybdate; TLC, thin-layer chromatography; PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin.

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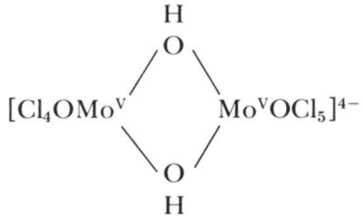
[2] The symbols for brain gangliosides are those of Svennerholm (11), which are correlated with the symbols of Korey and Gonatas (12) as follows: $G_{M1} = G_4$; $G_{D1a} = G_3$; $G_{D1b} = G_2$; $G_{T1} = G_1$.

[3] Unless filtration is rapid, the greenish crystals darken in color due to air and moisture.

necessary, the solution should be filtered through a sintered-glass funnel to remove any residue that may be present. The greenish-yellow spray reagent can be stored in the refrigerator for ready use for an indefinite period.

It is known that pentachlorooxomolybdates form para- and diamagnetic dimers through oxobridges in solutions of HCl (9, 10). Above 8 M HCl, only the emerald green anion $[Mo^{V}OCl_5]^{2-}$ exists and with increasing dilution, the dimers predominate giving dark colored solutions. Between 5–6 M HCl, it is mostly paramagnetic and dark in color whereas between 1–3 M HCl, it is all diamagnetic and reddish-brown in color. APCOM has been found unsuitable as a spray reagent in solutions of HCl, obviously due to the fact that Cl^- ion complexes with molybdenyl cation $[Mo^{V}O]^{3+}$ to form $[Mo^{V}OCl_5]^{2-}$, thus preventing the liberation of $[Mo^{V}O]^{3+}$, a species that takes part in the development of the blue color. We have also noted that below a concentration of 7 N H_2SO_4, APCOM is not satisfactory as a spray reagent owing to the development of blue color on the background of the TLC plate within a short time. The greenish-yellow solution of the spray reagent in 7–9 N H_2SO_4, probably containing an equilibrium mixture of $[Mo^{V}OCl_5]^{2-}$ and the paramagnetic dimer



along with a smaller amount of diamagnetic dimer $[Cl_4OMo^{V}OMo^{V}OCl_4]^{4-}$, seems to be ideal as a spray reagent for phospholipid detection. The appearance of blue spots is probably due to changes in oxidation state of Mo^{V} to a mean oxidation state between Mo^{V} and Mo^{VI} (9). The mechanism by which only phospholipids take part in oxidation is not yet understood.

Spraying of the TLC plate

After development in a suitable solvent system, the TLC plate is dried completely and then sprayed uniformly with the spray reagent until lightly damp. Phospholipids show up immediately, giving blue or sometimes greenish blue spots that change to blue completely after a few minutes, with the background remaining faintly yellow. The intensity of the blue spots increases on standing and becomes most intense after several hours. Fading starts after 24 hr but the background color remains unchanged. A representative TLC picture showing a standard mixture of phospholipids and phospholipid mixtures from mouse brain and goat kidney is presented in **Fig. 1**.

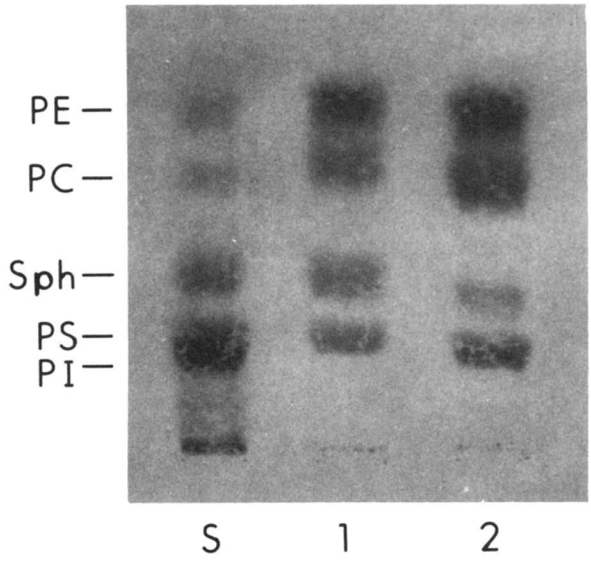


Fig. 1. TLC of phospholipids. TLC was carried out on a silica gel H plate with chloroform–methanol–7 N NH_4OH 65:27:5 (v/v) and the bands were visualized with APCOM spray reagent. All bands are blue. S, standard mixture of phospholipids; (1) phospholipids of mouse brain; (2) phospholipids of goat kidney.

Sensitivity

The sensitivity of the spray reagent was determined by using standard samples of cardiolipin, lecithin, lysolecithin, phosphatidylserine, phosphatidic acid, and sphingomyelin. From 1 to 10 μg of each sample (by weight) was spotted serially on TLC plates and developed. The plates were dried completely and then sprayed uniformly with the spray reagent. For all the above phospholipid standards, even 1 μg could be easily detected.

Specificity

The spray reagent has been found to be specific for all the phospholipid samples we have tested. This includes cardiolipin, lecithin, lysolecithin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, and sphingomyelin. Negative reaction was shown by inorganic phosphates, neutral glycosphingolipids (cerebroside, ceramide di- and trihexosides, globoside 1, Forssman glycolipid), gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1}, Tay-Sachs,[4] hematosides[5]), sulfatide, cholesterol and its esters, desmosterol, and fatty acids and their methyl esters.

[4] Tay-Sachs ganglioside is used to designate G_{M2} according to Svennerholm's nomenclature (11).

[5] The term hematoside is used to designate the subcategory of ganglioside that lacks hexosamine.

Discussion

The APCOM spray reagent is specific for phospholipids and is more sensitive than the molybdenum blue reagent of Dittmer and Lester (1) which is used as a standard spray reagent for phospholipid detection on thin-layer chromatograms. Moreover, the APCOM reagent totally eliminates the problem of the development of blue color in the background, even on standing for 48 hr. Thus, APCOM should find wide applications for phospholipid detection on thin-layer chromatograms.

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