Modification of the Dittmer–Lester reagent for the detection of phospholipid derivatives on thin-layer chromatograms

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Summary A simple modification of the Dittmer–Lester reagent is described that allows the detection of phospholipid derivatives at very low concentrations on silica gel and reversed-phase thin-layer plates. This modification, which involves the addition of acetic acid to the mixture, permits the observation of sharp blue spots on a white background. The specificity and sensitivity of the spray are discussed.—**Ryu, E. K., and M. MacCoss.** Modification of the Dittmer–Lester reagent for the detection of phospholipid derivatives on thin-layer chromatograms. *J. Lipid Res.* 1979. **20:** 561–563.

Supplementary key words acetic acid · silica gel · reversed phase

The Hanes–Isherwood spray (1) for the detection on paper chromatograms of phosphorus-containing compounds has proven highly useful in phospholipid research. However, this acidic ammonium molybdate spray is not satisfactory when thin-layer silica gel plates are used because of the extremely slow development of color. To overcome this problem, Dittmer and Lester (2) have modified the molybdenum blue reagent of Tzintzadze (3). Similarly, Vaskovsky and Kostetsky (4) have described a spray reagent based on the reagent of Lucena-Conde and Prat (5). A phospholipid-specific spray developed by Jatzkewitz and Mehl (6) utilizes a reagent which, for its preparation, requires several steps and considerable manipulation. In addition, Goswami and Frey (7) have developed a spray utilizing metallic copper and ammonium molybdate in sulfuric acid, and Kundu et al. (8) have described a modified Dittmer–Lester reagent.

In our laboratory (9), we recently had occasion to use silica gel and reversed-phase thin-layer chromatography for the separation of various nucleoside phospholipid derivatives. On the reversed-phase plates, the Dittmer–Lester spray proved ineffective due to insufficient "wetting" of the hydrophobic surface of the plate by the aqueous spray reagent. We report here a simple modification of the Dittmer–Lester spray which gives excellent staining (blue coloration) of phospholipid derivatives on reversed-phase and silica gel chromatograms. In addition, the spray is sensitive and stable (over 6 months at room temperature), and also eliminates the problem of the development of background blue color which is apparent with the Dittmer–Lester spray.

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Materials and Methods

The following phospholipids were purchased from Sigma Chemical Co., St. Louis, MO, and were dissolved in the solvents indicated in parentheses: L-α-dipalmitoylphosphatidylcholine (compound *1*) (CH_3OH); L-α-lysophosphatidylcholine (compound *2*) (CH_3OH); cardiolipin disodium salt (compound *3*) (CH_3OH); sphingomyelin (compound *4*) ($CHCl_3$), L-α-dipalmitoylphosphatidic acid disodium salt (compound *5*) ($CHCl_3$–CH_3OH–H_2O 2:3:1); and DL-α-dipalmitoylphosphatidylethanolamine (compound *6*) ($CHCl_3$–CH_3OH 7:2 containing a small amount of *N* HCl). CDP-DL-dipalmitin (compound *7*) ($CHCl_3$–CH_3OH 1:1) was purchased from P-L Biochemicals, Inc., Milwaukee, WI, and ara-CDP-L-dipalmitin (compound *8*) ($CHCl_3$–CH_3OH 1:1) was synthesized as described previously (9). Samples (2-μl aliquots) of concentrations 0.05 g/l, 0.5 g/l, and 2.5 g/l were spotted (3-mm diameter spots) on precoated Merck silica gel 60, F-254, and Whatman KC_{18} reversed-phase plates. Spot intensities on reversed-phase plates were quantitated using a MacBeth TD-501 transmission densitometer.

The spray reagents were prepared as follows.

Solution I. A mixture of molybdic anhydride (8 g) in 70% H_2SO_4 (200 ml) was boiled gently with magnetic stirring until a solution was obtained. This was cooled to room temperature.

Solution II. To 100 ml of solution I was added powdered molybdenum (0.4 g) and the mixture was boiled for 1 hr before being cooled to room temperature.

Solutions I and II were mixed as described by Dittmer and Lester (2) and filtered through a sintered-glass funnel (medium porosity). The filtrate (Solution III) was used as described below.

For silica gel plates, solution III (1 vol) was diluted with water (2 vol), and this solution was mixed with glacial acetic acid in the ratio 4:1 (v/v). For reversed-phase plates, solution III (1 vol) was diluted with 30% aqueous acetic acid (v/v) (2 vol). In both cases, the solutions were allowed to stand at room temperature for 3–4 days before use. Prepared in this fashion, the reagents were stable for more than 6 months. Addition of various amounts of acetic acid was tried in each case and the formulations described above gave the best results. It should be noted that in both cases the final acetic acid concentration (v/v) is 20%; the

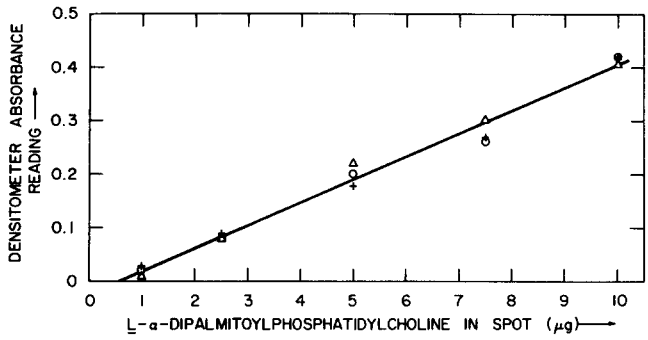

Fig. 1. Plot of densitometer absorbance reading against amount of L-α-dipalmitoylphosphatidylcholine present in spot. Two microliter aliquots of solutions in methanol containing 0.5 g/l, 1.25 g/l, 2.5 g/l, 3.75 g/l, and 5.0 g/l were spotted (2-mm diameter) on a reversed-phase plate and dried with a stream of air. At 30–50 min after spraying, the spot intensities were measured using a densitometer, subtracting blank readings from adjacent positions on the plate. Each aliquot was spotted in triplicate (+, O, and Δ).

major difference between the two sprays is the concentration of solution III. For the silica gel plates, this final concentration is 27% (v/v) and for the reversed-phase plates it is 33% (v/v).

For detection purposes, the plates were sprayed uniformly with the appropriate reagent until lightly damp. The problems of poor wetting of the surface and the spots encountered with the Dittmer–Lester spray on the reversed-phase plates were not apparent when using this modified spray containing acetic acid. Phospholipids gave sharp blue spots almost immediately. The intensity increased on standing in the atmosphere for approximately 10 min or after heating on a hot plate (60–70°C) for less than 10 sec. (An initial pale blue coloration of the background disappeared to give a uniform white background after approximately 3 min standing). Excessive application of the spray reagent can cause a colored (blue-green) background, but this can be easily removed from silica gel plates by dipping the plates in distilled water. No significant decrease in the spot intensity could be observed after this wash.

Sensitivity and specificity

Samples of compounds *1–8* in known concentrations were applied to the plates (see Materials and Methods), and the limits of visual detection were noted after ca. 3 min. On the silica gel plates, compound *3* could be detected at 1.0 μg and compounds *1*, *2*, and *4–8* were observable at the 0.5 μg level. The sensitivy on reversed-phase plates was somewhat less, the limit for compounds *1* and *4–8* being 1 μg and the limit for compounds *2* and *3* being 5 μg. However, on reversed-phase plates the color appeared to be more stable than on the silica gel plates and could be quantitated using a densitometer.

For all the compounds, except *3*, the spots were still readily visible after 24 hr. Compound *3* showed marked fading after 5–6 hr.

While all the phospholipid derivatives *1–8* gave a positive reaction on both reversed-phase and silica gel, no blue staining could be detected for phosphoric acid, cholesterol, ethyl nonanoate, palmitic acid, oleic acid, sodium dodecyl sulfate, or glycerol. Cytidine 5′-monophosphate (disodium salt) (50 μg) and cytidine 5′-monophosphomorpholidate (50 μg) gave weak, blue-gray spots on silica gel and strong, bright blue spots when 10 μg was applied to reversed-phase plates. Disodium hydrogen phosphate (50 μg) gave no staining on silica gel but gave a weak blue coloration on reversed-phase plates.

Quantitation

Quantitation of lipids using photodensitometry is a well-established practice, and it can utilize either transmitted or reflected light (10). The uniformity and reproducibility of the spray reagent described here was shown by application of known concentrations (0–10 μg, each spot in triplicate) of compound *1* on the reversed-phase plate and measurement with a densitometer of the spot intensities after 30–50 min. The straight line that was obtained (**Fig. 1**) shows that this method could be used for rapid quantitative estimation of particular phospholipids on reversed-phase chromatograms. The intercept of the line on the abscissa axis is a measure of the lowest levels of detection with the densitometer and, in this instance, the limit of detection can be seen to be ca. 0.3 μg/mm² (ca. 1 μg in a spot 2 mm in diameter). In addition, this procedure appears to permit quantitation of phosphorus at a level much lower than any previous method (11). It should be noted, however, that sample loadings of over 10 μg produced greater inaccuracies.

The intensities of the spots from Fig. 1 were monitored for 20 hr. Although no appreciable change was detected during this period, the scatter on the straight line became more marked after 1–2 hr. In addition, the background blue color became more apparent after ca. 1 day if too much reagent was applied.

In summary, the modified Dittmer–Lester spray reagent described here is extremely sensitive for the detection of phospholipids. It has the particular advantage of being useful for reversed-phase thin-layer plates as well as for silica gel plates and the coloration is sufficiently stable and uniform to allow direct quantitation using a densitometer.

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