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# MINIATURE TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC SEPARATION OF LECITHIN AND SPHINGOMYELIN*

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## SUMMARY

A miniature two-dimensional thin-layer chromatographic procedure employing silica gel impregnated glass-microfiber chromatography sheets (commercial product, ITLC-type SG sheets) has been developed for the separation of lecithin (L) and sphingomyelin (S) from a standard lipid mixture containing L, S, lysolecithin, phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine, phosphatidyl glycerol, and diphosphatidyl glycerol. The newly developed procedure eliminates possible interference from PI and PS. Complete separation of L and S was easily achieved with chromatographic solvent migration times of approximately 3 and 2 min for the first and second dimensions, respectively. The lipids were visualized by charring and fluorescent staining techniques. The procedure has been adapted for the separation of L and S from amniotic fluid samples.

## INTRODUCTION

Since Gluck et al. [1] described the thin-layer chromatographic (TLC) separation of amniotic fluid lecithin (L) and sphingomyelin (S) in 1971, the L/S ratio procedure has become the most popular chemical method for the estimation of fetal pulmonary maturity [2].

Phosphatidyl inositol (PI) and phosphatidyl serine (PS) have been shown to interfere in some TLC separation procedures for L and S [3—5]. Giudicelli et al. [6] have demonstrated that the acetone precipitation procedure does not remove PI and PS. However, they have removed these interferences by a two-di-

*Editorial remark. According to Editorial opinion the technique described herein, employing silica gel impregnated glass-microfiber chromatography sheets, may more appropriately be classified as a paper chromatography procedure.

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mensional TLC separation [6, 7]. Other researchers have removed these interferences by adsorption onto diethylaminoethyl cellulose [4, 5].

To significantly reduce the TLC solvent migration time, researchers have employed silica gel impregnated glass-microfiber sheets (commercial product, ITLC™-type SG sheets) [3, 5, 8—13]. The separation of lecithin and sphingomyelin has been reported to require less than 5 min [8]. A commercial kit employing ITLC-type SG sheets for the determination of L/S ratios in amniotic fluid has been marketed by Gelman (Ann Arbor, MI, U.S.A.) [14]. Phospholipid separation is achieved in approximately 3 min.

In this paper, lecithin and sphingomyelin have been separated from a phospholipid mixture by two-dimensional TLC on ITLC-type SG chromatography sheets. This new procedure has combined the separation advantages of the two-dimensional technique with the rapid separation achieved on ITLC sheets. The phospholipids were visualized by charring and fluorescent staining techniques [1, 5, 15].

## MATERIALS AND METHODS

Each of the following phospholipids were obtained in duplicate from Sigma (St. Louis, MO, U.S.A.) and from Supelco (Bellefonte, PA, U.S.A.): phosphatidyl glycerol (PG, egg yolk, from Sigma; PG, bacterial, from Supelco), diphosphatidyl glycerol (DPG, bovine), phosphatidyl inositol (plant), and phosphatidyl serine (bovine). Commercially prepared 1:1 L/S ratio standards, lecithin (Type III E, egg yolk), sphingomyelin (bovine brain), and phosphatidyl ethanolamine (PE, Type I, bovine brain; Type III, egg yolk) were purchased from Sigma. Synthetic lysolecithin (LL) was obtained from Calbiochem (San Diego, CA, U.S.A.).

The following Certified ACS grade chemicals were purchased from Fisher Scientific (Winnipeg, Canada): ammonium sulfate, chloroform, isopropanol, methanol and methylene chloride. Absolute ethanol and methanol were from the Standard Chemical Company (St. Boniface, Canada). Concentrated sulfuric acid and ammonium hydroxide (28—30% $NH_3$) were purchased from Canadian Industries (St. Boniface, Canada). The 2′,7′-dichlorofluorescein (DCF) was from Eastman Kodak (Rochester, NY, U.S.A.).

A 20 × 20 cm ITLC-type SG chromatography sheet was cut to size, 6.5 × 10.0 cm, to fit a Seprachrom chromatography chamber (Gelman, Ann Arbor, MI, U.S.A.). One hole was punched, 2 cm from the bottom and 2 cm from the right edge, with a Gem handpunch containing a 3-mm circular die (McGill, Marengo, IL, U.S.A.). Prior to use, the miniature sheets were heated in an oven at 100°C for 30 min and stored in a desiccator above calcium sulfate.

Chromatography solvent system I consisted of methylene chloride—ethanol—water (100:25:3, v/v). Solvent system II was composed of chloroform—methanol—ammonium hydroxide (170:20:3, v/v). Both solvent systems were prepared fresh weekly and stored in tightly-stoppered brown bottles at room temperature. Both solvent systems were shaken vigorously for one full minute just prior to use.

The ammonium sulfate—sulfuric acid charring reagent [15] was prepared by adding 20 g of ammonium sulfate and 4 ml of concentrated sulfuric acid to a

100-ml volumetric flask which was filled to volume with distilled water. The fluorescent visualization stain [5] was prepared by adding 1.5 mg of 2',7'-dichlorofluorescein to a 100-ml volumetric flask which was filled to volume with isopropanol.

Phospholipid standard No. 1 was prepared to contain LL, L, S and PG each at 3.0 mg/ml of chloroform—methanol (9:1, v/v). Standards Nos. 2—5, were each similarly prepared to contain LL, L, S and one of the following: DPG, PI, PS or PE, respectively. Standard No. 6 was similarly prepared to contain LL, L, S, DPG, PG, PE, PI and PS.

The standard application technique of Popowicz [16] was employed, as previously described [5]. One microliter of phospholipid mixture No. 1 was applied to a blank chromatography disc. The disc was air dried and inserted into the prepunched hole of a miniature chromatography sheet. Solvent system I was mixed vigorously for one full minute. Three milliliters were pipetted into a Seprachrom chromatography trough. The chromatography chamber was assembled and the solvent was allowed to migrate 60 mm above the origin. This required approximately 3 min. The chromatogram was air dried for 5 min. To permit chromatographic separation in the second dimension, the sheet was cut to size (6.5 × 6.5 cm). The second solvent system was allowed to migrate 40 mm above the origin. This required approximately 2 min. Following a 5-min air drying, the phospholipids were visualized by a DCF staining technique as previously described [5] or by spraying with 50% sulfuric acid and charring above a hot plate. Chromatographic separation and visualization of standards Nos. 1—6 were repeatedly performed as described above. $R_F$ values were calculated for each of the phospholipids tested.

A standard chromatography disc was prepared to contain LL, L, and S, each at 3 μg, and DPG, PG, PE, PI and PS, each at a concentration of 5 μg. Two-dimensional thin-layer chromatographic separation was performed as described above. Following a 5-min air drying, the chromatography sheet was immersed for 3 min in the ammonium sulfate—sulfuric acid charring reagent. The sheet was removed and placed in an upright position for approximately 5 min, to allow excess reagent to drain. The chromatogram was placed on a 350°C hot plate for 2 min. The charred phospholipids appeared as grey to black spots on a white background. The spots were observed most clearly when the chromatogram was illuminated from behind. The chromatogram was photographed with Kodak high-contrast copy film (HC710) rated at ASA 25, and developed for 4 min in diluted Dektol [stock Dektol—water (1:1, v/v), Kodak Canada, Toronto, Canada]. Photography was by Audio Visual Services, University of Regina, Regina, Canada.

The miniature two-dimensional glass-microfiber TLC separation technique was evaluated for the isolation of amniotic fluid L and S. Amniotic fluid sample preparation was as previously described [8, 9]. Chromatographic separation, visualization, and subsequent photography were performed as described above.

## RESULTS AND DISCUSSION

Solvent system I was evaluated for its ability to separate L and S from a mixture containing eight phospholipids. Similar to the observations of previous researchers [3—5], PI and PS were observed to interfere with the accurate determination of the L/S ratio. Both PI and PS formed elongated spots under the conditions employed in this study. At times, PI and PS were distributed through both the L and S spots on the chromatogram.

The separation of L and S from PI and PS was achieved in the second dimension with solvent system II. Minimal migration of PI and PS was observed to occur in the second dimension (Fig. 1). The $R_F$ values have been calculated for the first and second dimensions for each of the phospholipids studied (Table I).

TABLE I

THE $R_F$ VALUES OF PHOSPHOLIPIDS COMMON TO AMNIOTIC FLUID

Each value reported represents an average of at least ten test results.

| Phospholipid | First dimension $R_F$ | Second dimension $R_F$ |
|---|---|---|
| Lysolecithin | 0.23* | 0.28* |
| Sphingomyelin | 0.37* | 0.38* |
| Lecithin | 0.55* | 0.53* |
| Phosphatidyl serine | 0.71** | 0.20 |
| Phosphatidyl inositol | 0.57** | 0.17 |
| Phosphatidyl glycerol | 0.77* | 0.61* |
| Phosphatidyl ethanolamine | 0.81 | 0.59 |
| Diphosphatidyl glycerol | 0.95 | 0.79 |

*Each value reported represents an average of at least twenty-five results.

**Approximate $R_F$ results were calculated from diffusely distributed PI and PS spots (see Fig. 1). Refer to text for complete details.

It should be noted that the PI and PS values were inadvertently transposed in a preliminary abstract presentation [17]. Although standard mixtures of PI and PS were found to form elongated spots in the first dimension, more discrete and compact spots were observed when amniotic fluid samples were analyzed (Fig. 2). Ageing and decomposition of PI and PS in the standard mixtures may account for the observed variation. Another variation, labelled X in Fig. 1, was found to be either a contaminant or a decomposition product within a commercial PG preparation.

Previous publications [5, 8, 9] and the marketing of a commercial kit [14] attest to the fact that a rapid and reliable separation is achieved between L and S on ITLC-type SG chromatography sheets with solvent system I. The incorporation of solvent system II in the second dimension enhances the separation distance and eliminates interference from PI and PS.

The $R_F$ values for each of the phospholipids analysed have been observed to vary under the influence of a wide range of adverse experimental conditions, e.g. seasonal fluctuations in laboratory temperature, different lot numbers of ITLC sheets, and inter-laboratory differences. The latter is primarily attributed to variations in reagents, glassware, and laboratory personnel. However, in all

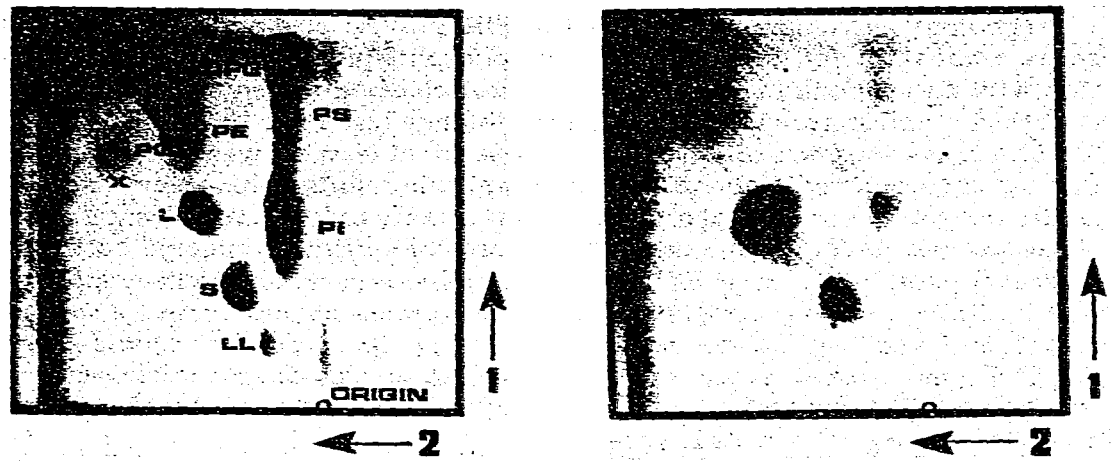


Fig. 1. Miniature two-dimensional thin-layer chromatogram depicting the separation of lecithin (L) and sphingomyelin (S) from a standard lipid mixture containing L, S, lysolecithin (LL), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and diphosphatidyl glycerol (DPG). Refer to text for complete details.

Fig. 2. Miniature two-dimensional thin-layer chromatogram depicting the separation of lecithin and sphingomyelin from other lipids contained in an amniotic fluid sample taken after fetal lung maturity. Refer to text for complete details.

cases it was observed that the relative positions of the phospholipids remained constant, and that complete separation of L and S was always achieved for both standards and patient samples. Representative chromatographic separations of L and S from amniotic fluid specimens collected before and after fetal lung maturation are depicted in Figs. 3 and 2, respectively. Maturity or immaturity was verified by comparison to another laboratory procedure [18] and by examination of the case histories.

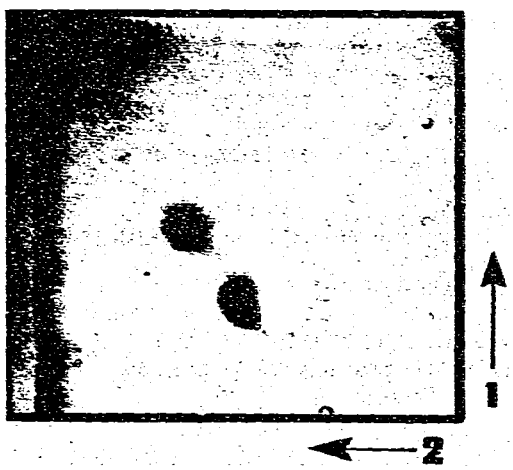


Fig. 3. Miniature two-dimensional thin-layer chromatogram depicting the separation of lecithin and sphingomyelin from other lipids contained in an amniotic fluid sample taken prior to fetal lung maturity. Refer to text for complete details.

Preliminary investigation employed a 50% sulfuric acid spray and charring above a hot plate for visualization of the phospholipids. Subsequent visualization with an ammonium sulfate—sulfuric acid charring reagent [15] was more convenient, and improved the charring consistency (see Figs. 1—3).

Solvent system I was observed to be stable for at least six weeks when stored in a tightly stoppered amber bottle. Reproducible results were achieved as long as the solvent was shaken vigorously for one minute before use. Solvent system II has been stable for up to 7 days, when stored in a tightly stoppered bottle.

The present two-dimensional chromatographic separation requires approximately 3 min for solvent migration in the first dimension and 2 min for solvent migration in the second dimension. The drying times are also rapid for the ITLC chromatograms, as the solvent evaporates quickly from the glass-microfiber sheets. Economy is achieved with the small size of the ITLC sheet and the minimal volumes of solvents required.

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