Simultaneous Determination of Phosphatidylglycerol and the Lecithin/Sphingomyelin Ratio in Amniotic Fluid

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We describe a single-dimension thin-layer chromatographic method by which one can simultaneously determine the lecithin/sphingomyelin ratio and the proportion of phosphatidylglycerol in amniotic fluid. The phospholipids are conveniently detected by an improved molybdenum-blue spray reagent, which immediately produces blue spots on a white background. The stained phospholipids are stable for at least 90 min after spraying, and densitometry results in symmetrical peaks that are easily quantitated.

Additional Keyphrases: thin-layer chromatography • fetal status • phospholipids

The ratio of lecithin to sphingomyelin (L/S) in amniotic fluid as a measure of fetal lung maturity is a valuable aid in predicting the occurrence of respiratory distress syndrome. Lecithin (L) and sphingomyelin (S) are the most frequently determined phospholipids, other minor phospholipids—phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol—have received relatively little attention.

Recently Hallman and Gluck (1) described the function of phosphatidylglycerol as a lung surfactant and the possibility of measuring it in amniotic fluid as an additional indicator of fetal lung maturity. Hallman et al. (2) have shown a direct correlation between amniotic fluid phosphatidylglycerol concentration and L/S ratios greater than 2.0. Accordingly, the determination of both the proportion of amniotic fluid phosphatidylglycerol and the L/S ratio may be helpful in evaluating prenatal lung maturity.

Gluck et al. (3) first reported use of the technique of thin-layer chromatography to determine the L/S ratio in amniotic fluid. More recently, Hallman et al. (2) described a two-dimensional thin-layer chromatographic method for determining the L/S ratio, along with phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglycerol concentrations. A similar two-dimensional thin-layer chromatographic method is currently used by Gluck and his co-workers (personal communication), but they detect the phospholipids by high-temperature charring, with subsequent quantitation by reflectance densitometry. Unfortunately, the high temperature (280 °C) required to char the phospholipids causes many commercial thin-layer chromatography plates to shatter, and so more expensive Pyrex plates must be used. Giridhar and Dalal (4) and Cusick (5) describe the use of molybdenum-blue to detect phospholipids, and both report a progressively intensifying blue background color; Cusick (5) also reported that the L/S ratio varied with time.

This paper describes a single-dimensional thin-layer chromatographic method by which the L/S ratio and phosphatidylglycerol concentrations can be simultaneously determined in amniotic fluid. The phospholipids are made visible by an improved molybdenum-blue spray reagent that produces bright-blue phospholipid spots without causing background discoloration. The phospholipid color is stable and the L/S ratio remains constant for at least 90 min after spraying.

Materials and Methods

Reagents: All solvents and reagents used were AR grade. The following phospholipid standards were obtained from Supelco Inc., Supelco Park, Bellefonte, Pa. 16823: Phosphatidylcholine (3-sn-phosphatidylcholine) from egg yolk. Sphingomyelin (2-acylaminooctadecyl-1-phosphocholine-3-diol) from bovine brain. Phosphatidylserine (3-sn-phosphatidylserine) from bovine brain. Phosphatidylethanolamine (3-sn-phosphatidylethanolamine) from egg yolk. Phosphatidylinositol [1-(3-sn-phosphatidylinositol)] from aplant source. Phosphatidylglycerol[1-(3-sn-phosphatidyl)-(sn-glycerol)] from a bacterial source.

Working L/S standards (L/S ratio 1.0, 1.5, 2.0) were obtained from Sigma Chemical Co., St. Louis, Mo. 63173. These standards were purchased as purified phosphatidylcholine from egg yolk, and sphingomyelin from bovine brain. The purity of the standards were verified by chromatographing each standard in five solvent systems of different polarity. Only one phospholipid spot was detected in each solvent system. The solvent system for thin-layer chromatography consisted of chloroform/methanol/58% ammonia hydroxide (65/30/3 by vol). The molybdenum-blue spray reagent was prepared by dissolving 4 g of molybdenum trioxide in 100 ml of 125 mol/liter sulfuric acid, with gentle heating, then adding 125 mg of powdered molybdenum and gently heating until dissolved. After cooling, this solution was added to 200 ml of distilled water. This reagent is stable for at least two months at room temperature, if kept in an amber-colored bottle.

Equipment: We used 10 x 20 cm precoated Silica Gel G (0.25 mm coat thickness) thin-layer plates from Analtech Inc., Newark, Del. 19711, and a R112 transmission densitometer (Beckman Instruments, Fullerton, Calif. 92634) set at 600 nm.

Method: Centrifuge the amniotic fluid for 10 min at 2000 rpm (800 x g). Mix 3.0 ml of supernatant amniotic fluid and 3.0 ml of methanol in a centrifuge tube, add 6.0 ml of chloroform, and shake the mixture on a mechanical shaker for 5 min. Centrifuge, and aspirate the top layer. Filter the chloroform
Fig. 1. Thin-layer chromatogram of amniotic fluid (A) and working L/S standard (B)

The phospholipids are abbreviated as follows: phosphatidylinerine (PS), phosphatidylinositol (PI), sphingomyelin (S), lecithin (phosphatidylcholine) (L), phosphatidylethanolamine (PE), phosphatidylglycerol (PG)

through Whatman No. 1 filter paper, rinse the extraction tube with 2.0 ml of chloroform, and filter the rinse chloroform. Evaporate the pooled chloroform at 60 °C, chill the tube in ice, add 1.0 ml of cold acetone, mix, and again chill the tube in ice for 10 min. Centrifuge the tube at 3000 rpm (1200 × g) for 5 min at 4 °C. Carefully decant the supernatant acetone and evaporate any residual acetone by heating at 60 °C. For chromatography reconstitute the residue in 50 μl of chloroform. The supernatant acetone that was removed just above also may be saved and evaporated, reconstituted in chloroform, and spotted. This represents the acetone-soluble lecithin described by Gluck et al. (6).

Thin-layer chromatography: Apply 30-μl and 10-μl aliquots of the reconstituted extract(s) to the chromatographic plate. Similarly apply 5 μl of each Sigma working standard and develop the plate to 12 cm from the application point. After air drying, spray the plate evenly with the modified molybdenum-blue reagent until the plate just begins to dampen. Cover the sprayed plate with a clear glass plate, tape the edges, and allow the plate to remain at room temperature for 15 min to develop maximum color.

Results

Standards: The working Sigma standards are weight ratios of lecithin and sphingomyelin. Following molybdenum-blue staining, the densitometric area ratio of these standards were 15–20% greater than the weight ratios. Therefore, all unknown L/S ratios were corrected to the average standard L/S area ratio.

Linearity: When increasing weight ratios of lecithin to sphingomyelin were plotted against the densitometer area ratios the resulting curve was linear from a L/S ratio of 0.5 to 5.

Accuracy: A commercially available amniotic fluid control (Amniotic Fluid Check Sample Kit, Supelco Inc.) containing three samples each with different L/S ratios was assayed in duplicate. The numerical L/S ratios are not supplied by Supelco, however, they are labeled as immature, transitional, and mature. Duplicate L/S ratios obtained using the described method were: for immature 0.9, 1.0; for transitional 1.6, 1.5; for mature 2.3, 2.4. These values are in agreement with Gluck's definitions (6).

Phospholipid color stability: The stained phospholipids reached maximum density in 10 min and remain stable for at least 90 min after spraying with the modified molybdenum-blue reagent.

Precision: Within-day precision of the L/S ratio was evaluated by processing 10 replicate aliquots of pooled amniotic fluid. The mean L/S ratio was 3.2 ± 0.2 (1 SD) and the CV was 7.3%. Day-to-day precision of the L/S ratio and percentage of the trace represented by phosphatidylglycerol was evaluated on 18 consecutive days. The mean L/S ratio was 3.9 ± 0.4 (1 SD) and the CV was 10.7%. The mean phosphatidylglycerol was 2.5% ± 0.3% (1 SD) and the CV was 13.5%.

RP of phospholipids: A representative thin-layer chromatographic separation and densitometric tracings of amniotic fluid and a working L/S standard are shown in Figures 1, 2, and 3.

Amniotic fluid studies: Eight amniotic fluid analyses resulted in L/S ratios between 2.0–2.8, and phosphatidylglycerol percentages between 1–6.6%. No respiratory distress developed in any of these newborns. Two additional amniotic fluids had L/S ratios of 1.2 and 1.3. Phosphatidylglycerol was absent from both samples. Respiratory distress syndrome subsequently developed in both of these newborns.
Discussion

It seems probable that the determination of the proportion of phosphatidylglycerol in amniotic fluid, in addition to the L/S ratio, will be useful in monitoring fetal lung maturity. In order to have an assay for these phospholipids that is as simple and as reproducible as possible, we developed a single-dimensional thin-layer chromatographic technique, which utilized an improved molybdenum-blue spray reagent. This method is now being used routinely in our laboratory, with good results, but it is still a rather lengthy procedure. We are presently investigating the possibility of achieving the separation and quantitation of these compounds by liquid chromatography.

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References