Three Methods Compared for Determining Phosphatidylglycerol in Amniotic Fluid

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Phosphatidylglycerol is one component of amniotic fluid that is unaffected by several of the reported interferences with conventional thin-layer chromatographic measurement of phospholipid. To compare a rapid immunological agglutination test, "Amniosat-FLM" with two-dimensional thin-layer chromatography, we determined phosphatidylglycerol by both methods in 41 amniotic fluid specimens obtained at 31 to 40 weeks of gestation. We also assayed phosphatidylglycerol in 14 of these specimens by an enzymic, colorimetric procedure. The agglutination test is rapid and simple but relatively insensitive; it yielded positive results for only seven of 23 specimens in which phosphatidylglycerol was detected by thin-layer chromatography. In 18 specimens in which no phosphatidylglycerol was detected by thin-layer chromatography, results by Amniosat-FLM were also negative; however, eight of these specimens, assayed by the enzymic method, had phosphatidylglycerol present. Apparently, the Amniosat-FLM detects phosphatidylglycerol only at concentrations exceeding 25 µmol/L, or more than 15% of the total phospholipid composition.

**Materials and Methods**

All specimens were centrifuged at 400 × g for 5 min. If analysis was delayed, the specimens were stored frozen at −20 °C and assayed within one month, conditions under which phospholipid reportedly is stable (9).

PG was determined by 2-D TLC as described by Hallman et al. (2). Amniosat-FLM, a kit supplied by Transidyne General Corp., Ann Arbor, MI 48103, employs the enzyme of sheep, catalyzes the reaction of phosphatidylglycerol, phosphatidylcholine, and phosphatidylethanolamine to phosphatidylglycerol, phosphatidylcholine, and phosphatidylethanolamine, respectively.

**Additional Keyphrases:** agglutination test, thin-layer chromatography, immunossay (Amniosat FLM), phospholipids, fetal status, enzyme methods, "kit" methods

Phosphatidylglycerol (PG), a component of pulmonary surfactant, appears late in gestation. Its presence in amniotic fluid reportedly confirms fetal lung maturity when the lecithin/sphingomyelin ratio is 2 or greater (1, 2), particularly in maternal diabetes mellitus (3, 4) and other complicated pregnancies (5). Determination of PG is unaffected by blood (1), by light staining with meconium (6), or by vaginal contaminants (7), because these substances do not contain PG.

For determination of PG the most commonly used method (2) involves two-dimensional thin-layer chromatography (2-D TLC), a time-consuming and costly procedure for which highly skilled technologists are required. Thus, the development of a rapid and simple, yet reliable, assay of PG would be useful in screening for fetal maturity. Two recently developed methods have been introduced that are much less arduous than TLC: an immunological kit assay, "Amniosat-FLM" (AFLM), involving antibody to L-phosphatidylglycerol, and an enzymic method that involves extraction but no TLC (8). We compared the ability of AFLM and TLC to detect PG in 41 specimens of amniotic fluid obtained from women between 31 and 40 weeks of gestation. For 14 of these specimens the volume sufficed to permit enzymic determination of PG also.

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resulting agglutination is categorized as negative or positive after both gross and microscopic (10X) examination. In an intermediate reaction, or weakly positive result, there is some clearing of background turbidity, but the agglutinates are smaller than in the positive controls. In the protocol accompanying the kit, the manufacturer warns that such results must be interpreted cautiously. Accordingly, we chose to tabulate weakly positive reactions as negative; only unequivocally positive reactions were recorded as positive.

Before performing the enzymatic analysis described by Artiss et al. (8), we also extracted the phospholipids from amniotic fluid with chloroform/methanol (2/1 by vol). In this method, glycerol hydrolyzed from PG by phospholipase D (EC 3.1.4.4) is then phosphorylated and oxidized to generate hydrogen peroxide; catalyzed by peroxidase (EC 1.11.1.7), the latter reacts with substrate to form a red compound. The absorbance at 510 nm is measured and compared with values on a standard curve.

Results and Discussion

Figure 1 shows the relationship between the concentrations of PG applied to the TLC plate and the integrated areas of the chromatographic spots determined by reflectance densitometry with a white-light source (2). A spot that is visually and densitometrically well-defined on a chromatographic plate developed in one dimension can be obtained with as little as 0.3 µg of PG; in two dimensions, 0.5 µg of PG was required. Additional quantities of PG produce a nonlinear additive amount of reflectance with the white-light scan at the upper end of the scale for both one-dimensional TLC (Figure 1) and 2-D TLC (data not shown), consistent with previous reports of the problems associated with trying to obtain quantitative data from TLC and densitometric analysis (11-21). Quantitative analytical responses can be obtained only by use of carefully controlled calibration devices involving identical treatment of samples and standards, with use of separate standards for each phospholipid.

For 18 specimens from which PG was absent as assessed by TLC, the AFLM assay was negative in every case (Figure 2). Eight of these 18 specimens, assayed enzymically, were determined to have PG concentrations of 12.8 to 18.0 µmol/L (Table 1). PG was detected by TLC in 23 samples, only seven of which were positive by AFLM (Figure 2). Six of these 23 specimens, assayed enzymically, gave the following results (Table 1): four that were negative by AFLM had PG values of 15.5 to 24.9 µmol/L (3.5-15% PG), and two that were positive by AFLM had PG values of 25.3 µmol/L (16% PG) and 65 µmol/L (15.8% PG). Four weakly positive AFLM results were distributed in specimens determined by 2-D TLC to contain 4 to 14% PG (Figure 2).

Results of amniotic fluid analysis were correlated with clinical outcome and 28 cases were excluded in which delivery occurred more than 72 h after amniocentesis (15 patients), corticosteroids were used antepartum (11 patients), or neonatal pneumonia was the cause of respiratory distress (two patients). Only 13 of the 41 cases were not excluded by these criteria. Each of these 13 had PG demonstrable by TLC and none showed evidence of respiratory distress syndrome. The AFLM results, with six enzymic values in parentheses, are as follows: five AFLM positive (25.3 and 65 µmol/L), five AFLM negative (15.5, 20.5, and 24.9 µmol/L), and three AFLM weakly positive (18.1 µmol/L). Because vigorous efforts to delay premature delivery and induce pulmonary maturity with corticosteroids are used at our hospital, none of the cases in which PG was absent by TLC were delivered within 72 h after amniocentesis or without antepartum administration of corticosteroids.

The AFLM assay is specific for negative specimens (i.e., no false positives); the 18 specimens with 0% PG by TLC were all negative by AFLM. However, AFLM was positive for only seven of 23 samples in which PG was detected by TLC (69% false negatives). Categorizing weakly positive reactions as positive only decreased the false-negative rate to 52% (12/23). Garite et al. (22), comparing results by a modification of the TLC method of Kulovich et al. (1) with

| Table 1. Phosphatidylglycerol Assayed by Three Methods in 14 Specimens of Amniotic Fluid |
|-----------------|-----------------|-----------------|
| TLC, % PG       | Amniostat       | Enzymic, µmol/L |
| 0               | 12.8            | 0               |
| 0               | 13.1            | 0               |
| 0               | 13.2            | 0               |
| 0               | 14.1            | 0               |
| 0               | 14.1            | 0               |
| 0               | 15.0            | 0               |
| 0               | 17.1            | 0               |
| 0               | 18.0            | 0               |
| 3.5             | 15.5            | 0               |
| 4               | +              | 18.1           |
| 4               | +              | 20.5           |
| 15              | 24.9           | 65.0           |
| 15.8            | +              | 25.3           |

* This specimen weakly positive.

Fig. 1. Relation between amounts of phosphatidylglycerol and the integrated areas of the resulting chromatographic spots as obtained by reflectance densitometry with a white-light source.
those by the AFLM kit, found 5/94 (5.3%) false positives and 12/99 (12%) false negatives by AFLM. However, the reagent and antibody concentrations in their study differed slightly from those in the commercial kit now available, so these results may not be directly comparable with those of other studies. Lockitch et al. (23) compared results for PG by the commercially available AFLM kit with those by 2-D TLC. In five of their samples both TLC and the AFLM gave trace-positive results, but the clearly positive and negative results reported showed a false-positive rate of 6.4% (3/48) and a false-negative rate of 17% (6/35) for the AFLM as compared with TLC. There is no apparent explanation for the low proportion of false negatives in these two studies as compared with our data.

The lower limit of sensitivity of the enzymic assay is less than 5 μmol/L (8). The stated concentration of PG in the two AFLM-positive controls is 2.6 and 5.1 μmol/L. However, AFLM assays failed to detect PG at concentrations <25 μmol/L as measured with the enzymic assay. A possible explanation for this apparent discrepancy may lie in the conformation of PG in the AFLM standard as compared with the amniotic-fluid extracts. The phase of micellar configuration—hexagonal, isotropic, or bilayer (24)—and any additional components associated with the PG in the AFLM standards other than phosphatidylcholine and cholesterol (e.g., lipoproteins) may differ in vivo from that for contrived standards or extracts of amniotic fluid. These differences in the conformation may decrease the number of antigenic determinants available as compared with the number of phospholipase-D-reactive sites and thus explain the variation in reactivity noted in Table 1.

Assay of various concentrations of PG in one-dimensional TLC demonstrated the lower limit of sensitivity to be 0.3 μg of PG per spot (Figure 1) or 0.39 μmol per liter of amniotic fluid. This theoretical value assumes 100% analytical recovery of PG after chloroform/methanol extraction and precipitation with acetone. However, some PG is lost during the extraction step (25, 26)—apparently more disaturated PG than total PG (25). The additional precipitation with cold acetone, performed only before the TLC procedure, may have further decreased the yield of PG sufficiently to explain why samples with no PG detectable by 2-D TLC had values by enzymic assay of 12.8 to 18.0 μmol/L (Table 1).

We conclude that the AFLM is a rapid but relatively insensitive test for PG in amniotic fluid. It appears to confirm the presence of PG only if the concentration of PG exceeds 25 μmol/L, or 15% of the total phospholipids.

References


