Phosphatidylglycerol in 261 Samples of Amniotic Fluid from Normal and Diabetic Pregnancies, as Measured by One-Dimensional Thin-Layer Chromatography

Michael Y. Tsai and Jo G. Marshall

We describe a one-dimensional thin-layer chromatographic method for separating lecithin, sphingomyelin, phosphatidylglycerol, and other phospholipids. The occurrence of phosphatidylglycerol in relation to the lecithin/sphingomyelin ratio is reported for 261 amniotic fluid samples. This compound does not consistently appear until the ratio exceeds about 3.1, and occurs less often in samples from diabetic patients than in those from normal patients. The respiratory distress syndrome did not occur when phosphatidylglycerol was present in amniotic fluid although the reverse was not necessarily true. Thus the presence of phosphatidylglycerol offers additional assurance of pulmonary maturity.

Additional Keyphrases: diagnostic aids · respiratory distress syndrome · fetal status · diabetes during pregnancy · phospholipids · lecithin/sphingomyelin ratio

Over the years, numerous methods have been proposed for assessing fetal lung maturity by analysis of the amniotic fluid. These include tests for creatinine concentration (1), bubble stability (2), the lecithin/sphingomyelin (L/S) ratio (3), and more recently, fluorescence polarization (4).1 The test most widely accepted as being specific for that purpose is the L/S ratio. Although a "mature" L/S ratio reliably indicates fetal lung maturity in most pregnancies, it is far from being satisfactory for predicting the occurrence of respiratory distress syndrome (RDS) in infants of diabetic mothers. According to recent studies, up to 28% of the neonates with insulin-dependent diabetic mothers develop RDS despite a mature L/S ratio (5, 6). Similar results have been obtained in our laboratory. For this reason, a more reliable method is needed for assessing fetal lung development, particularly in pregnancies in which there are associated alterations in the mother's metabolism.

Several recent studies have shown that phosphatidylglycerol (PG), a relatively minor lung phospholipid, is a potent surfactant with a greater hydrogen-bonding capacity than lecithin (7, 8) and may play a role in stabilizing the surfactant lipoprotein complex (9). Gluck and associates advocate measurement of this compound in amniotic fluid, and have suggested that fetal lung maturity should be evaluated through use of a phospholipid "lung profile" (10, 11). However, the two-dimensional TLC method recommended for measuring these phospholipids is cumbersome and insensitive.

Recently, a one-dimensional procedure for the simultaneous determination of the L/S ratio and PG has been reported (12). In the present paper, we describe a different one-dimensional procedure for complete phospholipid separation and measurement. The procedure has been applied to 261 samples and its usefulness for both normal and complicated pregnancies is documented here.

Materials and Methods

Clinical Samples

Amniotic fluids were obtained by abdominal tap between 28 and 42 weeks of gestation and were drawn from normal single-birth patients, as well as those with twin births, or with toxemia, Rh factor incompatibility, premature membrane rupture, placenta previa or abruptio placentae, diabetes, or other complications. Fluids were analyzed within an hour after drawing or were centrifuged promptly and the supernate frozen until analysis the next day. Samples containing meconium or more than 1% blood by volume were excluded from the study. Clinical data were not included unless delivery occurred within four days of the analysis. One infant of a diabetic mother was delivered 10 days after the determination of a mature L/S ratio, yet developed RDS, and this patient was included.

Criteria for the diagnosis of RDS included, all of the following: (a) obvious respiratory distress that requires oxygen, with symptoms including retractions and rapid, grunting respirations; (b) reticulogranular appearance in the chest roentgenograms; (c) negative cultures for β-streptococcus or lack of response to penicillin; and (d) a disease course lasting longer than 24 h. Cases of transient tachypnea were not included when it was possible to distinguish them from mild RDS.

Chemicals and Equipment

Synthetic dipalmitoyl lecithin, bovine brain sphingomyelin and phosphatidylserine (PS), egg-yolk phosphatidylglycerol and phosphatidylethanolamine (PE), and soybean phosphatidylinositol (PI) were purchased from Sigma Chemical Co. St. Louis, MO. All other chemicals were of reagent grade. Acetone was dried over sodium sulfate before use. Silica gel H plates with 5% ammonium sulfate (250 μm) were bought from Analtech, Neward, DE 19711, and a Quick Scan transmission densitometer (Helena, Beaumont, TX) was used with a 525-nm filter.

Method

The method for L/S ratio determination was essentially that of Gluck and Kulovich (13). We modified the method of dehydrating the chloroform layer and the solvent system for

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1 Division of Medical Genetics, Department of Laboratory Medicine and Pathology, Box 198, Mayo Memorial Building, University of Minnesota Hospitals, Minneapolis, MN 55455.

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TLC development. The amniotic fluid was centrifuged at 1000 x g for 5 min and two 3-mL samples of the supernate were removed. Three milliliters of methanol was added to each sample, shaken, and 6 mL of chloroform was added and mixed by inverting the tube several times. After centrifugation, the top and middle layers were aspirated. The chloroform was evaporated from one tube in a hot-water bath and the tube was stored on ice; this tube was used for the total L/S ratio. About 1 g of sodium sulfate was added to the other tube and mixed to remove water. The tube was centrifuged and the chloroform pipetted off, taking care to avoid floating water droplets. The chloroform was evaporated, the sample chilled on ice, and 0.85 mL of dry, cold acetone added. After 30 min on ice, the sample was centrifuged and the supernatant fluid poured off. The total and acetone-precipitated samples were each redissolved in 50 µL of chloroform. The TLC plates were activated by heating on a hot plate at 120 °C for at least 10 min. Both total and acetone-precipitated samples were spotted at two to four positions on the activated plates. Plates were developed in a solvent consisting of chloroform/methanol/water (67/25/3). Plates were allowed to dry, sprayed with sulfuric acid/water (equal volumes) and charred at 300 °C. The subsequently obtained densitometric values for the replicate total and acetone-precipitate spots were averaged. PG was considered to be present if a single, well-defined spot migrated in the same position as the standard. PG percentages were determined by adding the densitometer counts for acetone-precipitable lecithin, sphingomyelin, PS, PE, PI, and PG and dividing the total into the PG count. An acetone-precipitate value of 2.0, a total L/S ratio of 3.0, or both, was considered to indicate fetal lung maturity. The analysis can be completed in 2 h.

Accuracy Determination

Known amounts of dipalmitoyl lecithin, sphingomyelin, and PG were dissolved in a small amount of chloroform (standard solution). The same amounts were mixed with distilled water and sonicated (test solution). The standard solution was spotted directly on TLC plates; the test solutions were processed by the acetone-precipitate method described for amniotic fluids. Three standard solutions and five test solutions were analyzed for each L/S ratio tested. The L/S ratios were compared, and percentage analytical recoveries were calculated by comparing the densitometer counts for the standard and the extracted test solutions.

Results

Separation of Phospholipids

The one-dimensional TLC method routinely gives excellent separation of PG, PI, lecithin, and sphingomyelin. Only phosphatidylserine and phosphatidylethanolamine migrate together, although they are clearly separated from other phospholipids (Figure 1). Bilirubin, which is present in some samples, migrates with the solvent front, not with any of these phospholipids. We tested the effect of meconium; of maternal, fetal, and newborn blood; of plasma; and of erythrocyte hemolysates. All of these contain compounds that co-chromatograph with lecithin and sphingomyelin, so the L/S ratio of amniotic fluid contaminated by these substances cannot be accurately determined. In contrast, none of these, including three different meconium samples, contained compounds co-chromatographing with PG.

The solvent system we used in this method represents only a slight modification of that used in many laboratories for determination of the L/S ratio. In developing this procedure, however, we noted that small changes in the proportion of chloroform or water affect the mobility of certain phospholipids. Changes in the water content mostly affect the separation of PI from lecithin, so that an increase to more than five parts of water will cause these two compounds to co-chromatograph, and the L/S ratio will then be falsely high. On the other hand, decreasing the chloroform by a few percent may cause PG to move with the solvent front and co-chromatograph with neutral lipids. To ensure optimal separation, we prepare fresh solvents after two days or 10 samples, whichever comes first, and run a standard consisting of PG, PI, PS, lecithin, and sphingomyelin on each plate.

Good and consistent separations are achieved within a given lot of plates, but some minor adjustments in the solvent composition may be necessary with a new batch of plates. Typically, this involves changing the water content by 0.5 to 1.0 part or the chloroform by 2–5 parts. The ammonium sulfate in the silica gel causes better spot definition and improved resolution but does not char spots sufficiently darkly when the plate is heated. For this reason, the plates are sprayed with sulfuric acid before charring.

Precision

Three sets of pooled amniotic fluids were each analyzed 10 times within one day to determine the precision of the method. Both total and acetone-precipitate L/S ratios were determined. The mean total L/S ratios (±1 SD) were 5.5 ± 0.5, 2.8 ± 0.2, and 1.6 ± 0.1, and the coefficients of variation were 8.5, 7.7, and 7.5%, respectively. Mean acetone-precipitate L/S ratios were 5.0 ± 0.5, 2.4 ± 0.1, and 1.4 ± 0.2, yielding CVs of 9.2, 5.1, and 13%, respectively. Phosphatidylglycerol content was determined in the pool with the highest L/S ratio and was...
found to have a mean of 4.9 ± 0.3% in the total phospholipid sample and 4.8 ± 0.7% in the acetone-precipitate sample, yielding CVs of 6.7 and 15%, respectively.

One pooled amniotic fluid was analyzed seven times across four days. The mean total L/S ratio was 3.4 ± 0.4 and the mean acetone-precipitate ratio was 2.9 ± 0.3, giving CVs of 10.6 and 11.1%, respectively.

Accuracy

The accuracy of the method was determined by comparing the L/S ratios of standard solutions and test solutions after extraction and by recovery experiments. The L/S ratios of the test solutions tended to be slightly higher than the ratios of the standard solutions, as would be expected from the fact that lecithin showed a 10–15% higher analytical recovery than did sphingomyelin. The L/S ratios on the standard solutions averaged 1.3, 2.1, and 3.5, while the ratios on the test solutions were 1.4, 2.6, and 4.7, respectively. The CVs for replicate L/S ratio determinations were all less than 8%.

Densitometer Linearity

The densitometer was linear between L/S weight ratios of 4 and 28, which are equivalent to densitometer ratios of 1.3 and 4.9 when dipalmitoyl lecithin is used.

Clinical Correlations

By this method, the total L/S ratio and acetone-precipitable L/S ratio have been determined for a large number of samples from patients with both normal and complicated pregnancies. The correlation of the acetone-precipitable L/S ratio with PG occurrence is shown (Table 1). One of 47 samples with acetone-precipitate L/S ratios ranging from 1.1 to 2.0 showed PG, and the proportion did not rise significantly until the ratio reached 3.1. At an acetone-precipitate L/S of 4.1 or greater, 90% of the samples contain PG. Similar results were obtained when the occurrence of PG was compared to the total L/S ratio. PG was not detectable with total L/S ratios less than 3.1. Thirteen percent of the samples with ratios from 3.1 to 4.1 had PG and the proportion rose gradually with the ratio until 98% of the samples with total L/S ratios greater than 6.1 contained PG.

Phosphatidylglycerol seemed to be present later and not as consistently in samples from diabetic patients (Table 1). No PG was found in samples with acetone-precipitate L/S ratio less than 4.1, and only 67% of those with ratios greater than 4.1 contained PG. A similar situation occurred with total L/S ratio measurements. Phosphatidylglycerol did not occur at L/S ratios less than 6.0 and was present in only 58% of the samples with total ratios greater than 6.1. A chi-square analysis of the differences between samples from diabetic and nondiabetic patients indicates that the differences are significant at the p < 0.05 level for acetone-precipitate L/S ratios from 3.1 to 4.0 and at the p < 0.10 level for ratios greater than 4.1. These differences are even more significant when correlated with total L/S ratios. The amount of PG, measured as percent of total phospholipid, was also determined by densitometry in 30 samples. Samples in which the PG spot was well defined contained 2–15% PG. The amount generally rose with the L/S ratio in the range of 2–6, but the correlation was low and a plot of the values showed a great deal of scatter.

L/S ratios determined with and without acetone precipitation were compared. In our hands, the acetone-precipitate L/S ratio of a given sample averaged 80% of the total L/S ratio for that same sample, regardless of the numerical value of the ratio. A drop to 30 to 40% of the total generally indicated that water had not been removed completely from the chloroform extract. Serial determinations on 10 patients did not show any trend toward increasing proportions of precipitable lecithin. One advantage to the acetone-precipitate method is the elimination of occasional interfering compounds that cochromatograph with lecithin or sphingomyelin. These compounds char a different color than phospholipids, and are possible contaminants introduced during aminocentesis, but we have not been able to determine their identity or source.

During the course of this study, 12 cases of RDS developed in infants delivered within four days of the L/S ratio determination. None of the amniotic fluids from these patients showed PG.

Five cases of RDS occurred in non-diabetic patients. Four had immature L/S ratios. The fifth infant had an acetone-precipitate L/S ratio of 2.3 but a birth weight of only 980 g (Table 2). She was delivered shortly after her mother began hemorrhaging at 36–38 weeks due to placenta previa and abruptio. This is the only false mature ratio out of 117 infants delivered within four days of the L/S ratio determination, yielding a false positive rate of 0.9% for non-diabetic patients.

In comparison, there were seven cases of RDS in 22 diabetic

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**Table 1. Occurrence of PG in Samples with Different L/S Ratios**

<table>
<thead>
<tr>
<th>Acetone-precipitate L/S ratio</th>
<th>Samples from non-diabetic patients</th>
<th>Samples from diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total samples</td>
<td>Samples with PG present</td>
</tr>
<tr>
<td>0–1.0</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>1.1–2.0</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>2.1–3.0</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>3.1–4.0</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>&gt;4.1</td>
<td>94</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>223</td>
<td>100</td>
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</tbody>
</table>

**Table 2. Clinical Data on Patients with Respiratory Distress Syndrome and Mature L/S Ratios**

<table>
<thead>
<tr>
<th>Pregnancy complication</th>
<th>L/S ratio</th>
<th>Acetone precipitate</th>
<th>Total</th>
<th>PG</th>
<th>Weeks of gestation</th>
<th>Sex of infant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placenta previa and abruption; intrauterine growth retardation</td>
<td>3.3</td>
<td>5.5</td>
<td>NP</td>
<td>36–38</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Diabetes, Class F</td>
<td>2.2</td>
<td>2.6</td>
<td>NP</td>
<td>35</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Diabetes, Class F</td>
<td>3.3</td>
<td>7.8</td>
<td>NP</td>
<td>34</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Diabetes, Class B</td>
<td>3.1</td>
<td>4.8</td>
<td>NP</td>
<td>36</td>
<td>M</td>
<td></td>
</tr>
</tbody>
</table>

* Not present in more than 1% of the total phospholipids.

**Table 3. Occurrence of RDS in Diabetics with Mature L/S Ratios**

<table>
<thead>
<tr>
<th>White's classification</th>
<th>Total no. of patients</th>
<th>Cases of RDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>0</td>
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<tr>
<td>F</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Discussion

The one-dimensional TLC method described here gives fast, repeatable analysis of lecithin, sphingomyelin, and PG in amniotic fluid, and can be used to determine the content of PI and PS + PE if desired. In our laboratory, the method is used for the routine determination of total L/S ratio, acetone-precipitable L/S ratio, and PG on all amniotic samples submitted for analysis of fetal lung maturity. An attempt to compare our method with the recently published procedure of Gotelli et al. (12) failed. In our hands, their method allowed sphingomyelin to co-chromatograph with PS and sometimes with PI.

Although Gluck and Kulovich (13) claimed that acetone precipitation is needed to separate the surface-active from non-surface-active lecithin, considerable doubts have been raised as to the specificity of this procedure. Most notably, Roux et al. (14) found no chemical difference between acetone-precipitated and acetone-soluble lecithin in amniotic fluid. Our data confirm the experience of others that either the total (15, 16) or acetone-precipitable (13) ratio can be used, if each laboratory doing the test does a reasonable amount of study in correlating their data with the clinical outcome of the pregnancies. In our hands, the acetone-precipitate L/S ratio is a fairly constant percentage of the total ratio, regardless of the numerical value. Because the total ratio is almost always higher than the acetone-precipitate ratio, however, the value for maturity should be somewhat higher than for the acetone-precipitate. The clinical data collected in our laboratory seem to support the use of an acetone-precipitate value of 2.0 and (or) a total L/S of 3.0.

The L/S ratio as performed in this laboratory has been found highly reliable for non-diabetic pregnancies. However, up to 18% of diabetics with "mature" ratios may deliver infants who will develop mild to severe RDS. This data is in agreement with several other studies (5, 6) on the occurrence of RDS in infants of diabetic mothers with a mature L/S ratio.

The precise reason for this occurrence is still not clear. Studies of fetal lung-tissue cultures indicate that above-normal fetal insulin concentrations, which are often found with diabetic mothers, may antagonize the stimulatory effect of cortisol on lecithin synthesis (17). Our data demonstrating the delayed appearance of PG in diabetic patients indicate that a similar effect may occur with other phospholipids. In addition, fetal hyperinsulinism may increase the exchange of fetal lung fluid with fluid in the amniotic sac, resulting in the removal of phospholipid from the alveolar lining and an increase in the amniotic fluid L/S ratio (18). Whatever the mechanism may be, there is a clear need to develop a simple test for the more accurate prediction of RDS in infants of diabetic mothers.

The determination of PG has been proposed to fill this gap (10). Our preliminary data confirm the results of Gluck—no infant developed RDS when the amniotic fluid contained PG in excess of 1% of the total phospholipid. In contrast, RDS occurred in three infants out of 10 delivered by diabetic mothers with mature L/S ratios but no PG. The presence of PG, therefore, does seem to offer additional assurance of pulmonary maturity in diabetic patients.

Although the determination of PG seems to be a useful supplement to the L/S ratio in certain situations, it seems apparent that these parameters do not yet give a complete picture of lung development. The production of PG seems to be retarded in diabetics, yet most diabetic patients who lack PG do not have infants with RDS. The measurement of other compounds in amniotic fluid, such as insulin, (19), may eventually be used in conjunction with the L/S ratio and PG determination to provide the additional information necessary for accurate RDS prediction in these patients.

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References