Improved One-Dimensional Thin-Layer Chromatography of Phospholipids in Amniotic Fluid

Alexandros A. Pappas, Richard E. Mullins, and Richard H. Gadsden

With this extraction procedure and chromatographic method, six amniotic fluid phospholipids [phosphatidylcholine (lecithin), sphingomyelin, phosphatidylserinol, phosphatidylserine, phosphatidylethanolamine, and phosphatidylglycerol] are completely resolved in <70 min from the time the specimen is received. The mobile phase (chloroform/petroleum ether/methanol/acetic acid, 5/3/1.6/1, by vol) is used with commercially available 7.5 × 10 cm silica-gel plates. The phospholipids are made visible by immersing the plate in cupric acetate/phosphoric acid. Lecithin/sphingomyelin (L/S) ratios are determined densitometrically. Rf values are well reproducible for both samples and standards, as are L/S ratios, and the method is simple and inexpensive. The relation between L/S ratios (for 100 specimens of amniotic fluid measured by this method) and fetal status is consistent with other reports. We confirm phosphatidylglycerol to be a sensitive indicator of fetal lung maturity: when it was detectable in amniotic fluid, the newborn invariably showed no symptoms of the respiratory distress syndrome.

Additional Keyphrases: fetal status · respiratory distress syndrome · lecithin/sphingomyelin ratio

The lecithin/sphingomyelin (L/S)² ratio in amniotic fluid in uncomplicated pregnancies is a valuable clinical indicator of fetal lung maturity, and thus a reliable laboratory test for predicting RDS (1, 2). In pregnancies complicated by diabetes or toxemia the L/S ratio may increase independently of either pediatric gestational age or fetal lung maturity. Thus, RDS may occur in the presence of a “mature” L/S ratio (3, 4). Detection of PG in amniotic fluid appears to be useful in predicting fetal lung maturity in such complicated pregnancies (5, 6). Methods in which L, S, PG, PI, PS, and PE are clearly resolved help in deciding which phospholipids should be determined in amniotic fluid (7–9). Two-dimensional TLC methods (7, 10) so far described for determining the L/S ratio and for evaluating PS, PE, PI, and PG are cumbersome and time-consuming.

One-dimensional TLC methods for simultaneously determining the L/S ratio and PG in amniotic fluid have been described (6, 11), but PI and PS are unresolved in these procedures. Other methods have been described for evaluating the L/S ratio and concurrently separating PI, PS, and PG by one-dimensional TLC, involving either “stepwise” development (12), continuous development (13), or pretreatment of the chromatographic plate (14).

The method described by Mitnack et al. (12) takes more than 2 h and requires in-house preparation of two 20 × 20 cm TLC plates per analysis. The technique of Kolins et al. (13) involves use of commercially available 10 × 10 cm plates but still takes longer than 2 h, and L and S are inadequately resolved. Painter's method (14) reportedly is more rapid than others and makes use of commercially available plates, but the plates must be pretreated and the several satellite spots near PG and near S in patients' samples may complicate quantitation.

Here we describe a one-dimensional TLC method with which all the amniotic fluid phospholipids (S, L, PS, PI, PE, and PG) are resolved in 30 min on 7.5 × 10 cm commercially available silica-gel plates. The L/S ratio may be determined by transmission densitometry, with concurrent assessment of PI, PE, PS, and PG (15, 16).

Materials and Methods

Reagents and standards. All chemicals other than the phospholipids were of AR grade and were from Fisher Scientific Co., Fair Lawn, NJ 07140. The mobile phase is chloroform/petroleum ether (bp 36.3–54.5 °C)/methanol/glacial acetic acid (5/3/1.6/1, by vol). It is prepared daily, because longer exposure to the atmosphere may appreciably alter the composition of this anhydrous mixture. The indicating solution is a mixture of cupric acetate (300 g/L) and phosphoric acid (90 mL/L).

L-α-Lecithin (egg)/sphingomyelin (bovine brain) standard solution (LS-10), bovine brain phosphatidylserine, egg-yolk phosphatidylglycerol, phosphatidylethanolamine, and soybean phosphatidylserinol (all from Sigma Chemical Co., St. Louis, MO 63178) were used to prepare the phospholipid composite standard, which contains 1 mg of each phospholipid per milliliter of chloroform. L/S ratio controls (Helena Labs.,

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2 Nonstandard abbreviations: L/S, lecithin/sphingomyelin; RDS, respiratory distress syndrome; L, lecithin (3-sn-phosphatidylcholine); S, sphingomyelin; PI, 1-phosphatidylserinol; PS, 3-sn-phosphatidylserine; PE, phosphatidylethanolamine; PG, 1,3-bis(3-sn-phosphatidyl) glycerol; and TLC, thin-layer chromatography (LC).
Beaumont, TX 77704) had stated L/S ratios ranging from 0.8 to 4.0.

**Equipment.** We used precoated, channeled, 7.5 × 10 cm, 250-μm thick silica-gel plates; a micro-evaporation tray; a micro-evaporation hood; a TLC plate rack; a hot plate; and Quick Scan II and Quick Quant II (all from Helena Labs.).

**Procedure.** Amniotic fluid specimens, collected by trans-abdominal amniocentesis, are promptly transferred to conical, amber-colored plastic tubes and transported to the laboratory on ice.

Gently mix the specimen by inversion and centrifuge (100 × g, 5 min). Add 1 mL each of the supernate, methanol, and chloroform to a conical centrifuge tube, vortex-mix for 10 s, and centrifuge at 1500 × g for 10 min. Carefully aspirate the organic phase, transfer to a well of the microevaporation dish, and evaporate under the microevaporation hood. Redissolve the residue in 15 μL of chloroform and apply the solution to the TLC plates (the two outside channels of the plates are not used). To one channel of the plate apply 5 μL of the phospholipid composite standard and to another a reconstituted commercial L/S control that has been taken through the extraction procedure. Use the remaining channels for patients’ samples. Equilibrate with freshly prepared mobile phase for 30 min a chromatographic chamber containing a TLC plate rack and wick. Place the TLC plate in the chamber and allow it to develop for about 30 min, until the solvent front is 1 cm from the top. To make the phospholipid spots visible, immerse the plate in the cupric acetate/phosphoric acid indicating solution, blot the excess solution, and heat the plate on the hot plate for about 5 min.

Scan the developed chromatograph by transmission densitometry, using a 525-nm filter. Calculate the L/S ratio from

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**Table 1. Precision of Rf Values for Phospholipids in Amniotic Fluids and in the Composite Standard**

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Clinical samples (n = 100)</th>
<th>Standards (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Rf</td>
<td>SD</td>
</tr>
<tr>
<td>S</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>L</td>
<td>0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>PI</td>
<td>0.34</td>
<td>0.06</td>
</tr>
<tr>
<td>PS</td>
<td>0.51</td>
<td>0.08</td>
</tr>
<tr>
<td>PE</td>
<td>0.81</td>
<td>0.06</td>
</tr>
<tr>
<td>PG</td>
<td>0.90</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**Table 2. Day-to-Day Precision of L/S Ratios in Commercial Controls (n = 60 each)**

<table>
<thead>
<tr>
<th>Controls</th>
<th>x (± SD)</th>
<th>Range</th>
<th>Stated value</th>
<th>Stated range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.0 (0.2)</td>
<td>0.8–1.3</td>
<td>1.2</td>
<td>0.8–1.4</td>
</tr>
<tr>
<td>B</td>
<td>1.6 (0.3)</td>
<td>1.3–2.1</td>
<td>1.7</td>
<td>1.3–2.1</td>
</tr>
<tr>
<td>C</td>
<td>3.2 (0.5)</td>
<td>2.4–4.0</td>
<td>3.2</td>
<td>2.4–4.0</td>
</tr>
</tbody>
</table>

*Helena Laboratories stated values.*

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**Fig. 2.** Typical TLC plate for clinical samples 1 and 6, L/S control; 2, PS and PG; 3, PI, PE, and PG; 4 and 5, patient’s samples.

**Fig. 3.** L/S ratio as a function of gestational age (age at time of amniocentesis, calculated from estimated gestational age at birth).
the relative areas as integrated. Report the other four phospholipids as “present” or “absent.”

Results and Discussion

Figures 1 and 2 illustrate typical chromatograms of standards, controls, and a patient’s sample.

Table 1 lists the RT values of each phospholipid detected in 100 amniotic fluids, along with RT values derived from 40 replicate analyses of the phospholipid composite standard.

Lecithin and sphingomyelin were present in all of the clinical samples; the proportion of the other phospholipids varied from 10 to 98%.

These data indicate good RT reproducibility for the phospholipids in amniotic fluid. There is some statistical overlap for the accumulated run-to-run data for each phospholipid, but we saw no overlap of the individual phospholipids for any single clinical specimen, control, or chromatogram of the phospholipid composite standard.

Day-to-day precision (Table 2) was evaluated by use of various L/S ratios of commercial material assayed routinely along with patients’ samples.

The correlation between L/S ratio and gestational age (Figure 3) was determined by the estimated pediatric gestational age at birth, calculated back to the week the sample was obtained (17). The L/S ratio progressively increases during gestation. The three premature infants (lowest three values at 33 weeks’ gestation, Figure 3) developed RDS at birth. The amniotic fluid L/S ratio for these cases was <1.5, and no PG was detected. In no case did RDS develop in neonates who had detectable PG in their amniotic fluid. PG was invariably present in amniotic fluid from pregnancies at 38–40 weeks of gestation (essentially full term).

This pattern of increasing amniotic fluid L/S ratio vs pediatric gestational age correlates with and confirms the findings of many investigators. It is presented here primarily as method validation.

The simplicity and speed of the extraction and subsequent chromatography are definite advantages of this method when prompt clinical decisions regarding acute cases are required.

We thank Mrs. Mary Frances Huggins for her technical assistance in method development and for assaying clinical specimens.

References


