Improved Procedure for Lecithin/Sphingomyelin Ratio in Amniotic Fluid Reduces False Predictions of Lung Immaturity

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Many laboratories have found that their procedure for determining lecithin/sphingomyelin (L/S) ratios gives an unacceptably high proportion of false predictions of fetal lung immaturity. We investigated each step in the procedure, in an attempt to improve the clinical performance of the test and to make the method more amenable to standardization between laboratories. L/S ratios were determined by the new procedure in amniotic fluid from 147 pregnancies, collected within two days of delivery of the infant. Four cases had an L/S ratio less than 2; all developed hyaline membrane disease. No other cases of hyaline membrane disease were encountered in this study. For 106 of these pregnancies, the L/S ratio was also determined by the procedure previously in use. This predicted lung immaturity for 16 infants, only four of whom developed hyaline membrane disease. All 12 cases incorrectly predicted as immature by the old procedure were correctly classified by the new procedure.

Additional Keyphrases: fetal status • respiratory distress syndrome • phospholipids

The determination of the lecithin/sphingomyelin (L/S)³ ratio of amniotic fluid (1) was the first method proposed for evaluation of fetal lung maturity, and it is still widely used for this purpose. It is now acknowledged that the test may give an unacceptable proportion of false predictions of lung immaturity (2); that is, many infants whose lungs would be judged immature on the basis of the L/S ratio do not develop hyaline membrane disease (HMD).

Another problem is the lack of standardization of the method between laboratories. Because there are several critical steps in determination of the L/S ratio, relatively minor differences in technique between laboratories, or even between operators within the same laboratory, may influence the results obtained (2). Many modified procedures have been described, often without clinical follow-up to establish whether the variations introduced have affected the cutoff values for clinical interpretation.

A major source of difficulty has recently been highlighted. In many thin-layer chromatographic (TLC) systems currently in use, two phospholipids normally present in amniotic fluid, phosphatidylyserine (PS) and phosphatidylinositol (PI), co-chromatograph with lecithin and (or) sphingomyelin, and thus affect the L/S ratio obtained (2, 3). The method previously in use in our laboratory suffered from this problem (3) and also gave an unacceptable number of false predictions of lung immaturity (4). On the basis of a detailed investigation of each step in the L/S ratio procedure we have developed a new method and compared its clinical performance with that of the procedure previously in use.

Materials and Methods

Materials

All chemicals were of analytical-reagent grade. Egg lecithin and bovine sphingomyelin were obtained from Applied Science Laboratories Inc., State College, PA 16801. PS and PI prepared from rat lung were used; their identity had previously been confirmed by co-chromatography with commercial standards in three different solvent systems.

Amniotic fluid was collected by amniocentesis as part of the routine management of complicated pregnancies, or at the time of cesarean section or induction of delivery. All samples were collected within two days of delivery of the infant and none was visibly contaminated with blood or meconium. The most common indications for amniocentesis were elective cesarean section (37%) or toxemia of pregnancy (22%). Ten percent of the pregnancies were complicated by diabetes mellitus. Cases where glucocorticoid had been administered to the mother before or after sampling were excluded, unless otherwise indicated, as were cases of gross fetal abnormality and multiple pregnancies.

Reagents

Standard mixture. A mixture of 2.5 mg of lecithin and 2.5 mg of sphingomyelin per milliliter was prepared, with chloroform/methanol (95/5, by vol) as diluent. The molar L/S ratio of the solution was accurately determined (5, 6); lecithin and sphingomyelin were separated by TLC on hand-made 0.5-mm-thick silica gel H (Merck, Darmstadt, F.R.G.) plates with a solvent of chloroform/methanol/acetic acid/water (100/60/16/8, by vol), appropriate regions of silica gel were scraped off, and the phospholipid phosphorus content was determined (5). The solution was distributed in 1-mL portions in air-tight vials and stored at −20 °C. Alternatively, if the standard phospholipids are chromatographically pure, separate solutions of lecithin and sphingomyelin (5 g/L) may be prepared and the total phosphorus concentration of each solution determined before equal volumes of the two solutions are mixed.

TLC plates. Plastic sheets, silica gel 60, 0.2 mm thick (no. 5748, Merck), cut to give plates 10 cm high and of various widths, depending on the number of samples to be run. Spotted solvent. Chloroform/methanol (3/1, by vol).

Chromatography solvent. Chloroform/methanol/aqueous ammonia, 280 g/kg (20/9.2/2, by vol), prepared daily.

Staining reagent. A solution of 39 g of cupric acetate monohydrate per liter of dilute (80 g/L) orthophosphoric acid, mixed with ethanol (4/1, by vol). This reagent is stable for at least one month.

Equipment

Centrifuge. Sorvall Model GLC-1 bench centrifuge.

Chromatography tank. Base 22 × 10 cm, height 22 cm; or base 12 × 7 cm, height 15 cm. Line the tank with Whatman no. 3 chromatography paper to a height of 10 cm.

Oven. Jacketed oven (Labmaster Products, North Ryde,

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³ Nonstandard abbreviations: L/S, lecithin/sphingomyelin; HMD, hyaline membrane disease; TLC, thin-layer chromatography (-ic); PS, phosphatidylserine; and PI, phosphatidylinositol.

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Sydney, Australia), set to the appropriate temperature (about 145 °C), as described below.

**Densitometer.** "Auto Scanner" (Helena Laboratories, Beaumont, TX 77704), with 1 mm X 3 mm slit and 525-nm light source.

**Procedure**

Centrifuge a 3-mL sample of well-mixed amniotic fluid for 5 min at 250 X g (1300 rpm, rmax = 13 cm) in a tapered plastic centrifuge tube as soon as possible after collection. Collect the supernatant fluid with a Pasteur pipette, leaving approximately 0.5 mL above the pellet. Vortex-mix 2 mL of the supernate with 2 mL of methanol for 10 s. Add 2 mL of chloroform, vortex-mix for 1 min, then centrifuge for 10 min at 950 X g to separate the phases. Transfer a portion of the lower chloroform layer (0.5 mL, unless otherwise specified) to a 15-mL tapered glass tube and evaporate under a stream of nitrogen, with gentle warming to 37-40 °C.

Add five drops of spotting solvent, vortex-mix for 10 s, and transfer the sample to the TLC plate as a 4-5 mm streak, 10 mm from the bottom of the plate and 10 mm from the edge of the plate, or 4-5 mm from adjacent samples. Use four more drops of spotting solvent to complete the transfer of the sample to the plate. We found 2 µL Microscope® (Drummond Scientific Co., Broomall, PA 19008), used without the rubber bulb, to be particularly suitable for this purpose. Also apply 4 µL of standard mixture to each plate. During sample application, blow cool air from a hair dryer onto the plate to facilitate solvent evaporation.

When the applied samples are completely dry (after about 5 min), place the plate in the chromatography tank (previously equilibrated with solvent for at least 1 h) and leave until the solvent has just reached the top of the plate. Remove the plate and let air dry, or dry with a hair dryer, until the odor of ammonia is no longer detectable (about 5 min). Keeping the plate vertical, dip it slowly into the staining reagent and leave it there for 2 min. Drain off excess stain by standing the plate on a blotter until the surface of the plate no longer appears glossy with moisture, then place it in an oven equilibrated at the appropriate temperature (see below) for 10 min.

After cooling, scan the plate with a densitometer. The L/S ratio is determined by cutting out and weighing the appropriate portions of the densitometer chart paper, corresponding to lecithin and sphingomyelin (Figure 1).

**Determining the correct oven temperature.** Before adopting the method as a routine clinical procedure, each laboratory must carry out the following preliminary check to establish the correct oven temperature for color development after staining.

Determine the L/S ratio of duplicate 4-µL samples of standard densitometrically as described above, with the oven equilibrated at 145 °C for color development. Repeat the procedure twice, allowing time for the oven to re-equilibrate between each batch of determinations. If the mean L/S ratio determined at this staining temperature is greater than the molar L/S ratio of the standard mixture, repeat the experiment, re-equilibrating the oven at 150 °C; if the L/S ratio is less than expected, lower the oven temperature. If necessary, adjust the oven temperature and repeat the procedure until the mean L/S ratio determined densitometrically is the same as the molar L/S ratio (within 5%).

**Clinical Assessment**

Criteria used for the diagnosis and classification of respiratory problems in the newborn have been described in detail elsewhere (7). All infants diagnosed as suffering from HMD showed the typical clinical course as well as the classical diffuse, fine granular pattern on roentgenographic film. Respiratory distress was regarded as insignificant when the condi-

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**Fig. 1.** Densitometric tracings of TLC plates, showing typical separations of lecithin (L), sphingomyelin (S), PS, and PI, marked to indicate the method of determining the area under the curve. A and B represent examples of tracings commonly encountered. C represents an atypical case containing an additional component (X)
250 × g showed that whole cells were virtually removed under these conditions.

Extraction of phospholipids. In many laboratories, one volume of amniotic fluid is extracted with an equal volume of methanol, then with two volumes of chloroform before determination of the L/S ratio (2). However, other laboratories (and we), in the routine procedure presented here, extract amniotic fluid with equal volumes of methanol and chloroform. When we compared the two extraction methods for six samples of amniotic fluid, there was no significant difference in the L/S ratio obtained (paired t-test, after log transformation of data); the mean L/S ratio was 3.5 (SD 1.6) by the first method and 3.4 (SD 1.4) by the second.

Acetone precipitation of the phospholipid was not included as part of the routine procedure, for reasons discussed in detail elsewhere (2).

Spotting solvent. To test the effect of spotting solvent composition, we evaporated replicate samples of a standard mixture containing 5 μg each of lecithin and sphingomyelin and transferred them to a TLC plate, using chloroform or various chloroform/methanol mixtures ranging from 19/1 to 2/1 by volume. The standard mixture was also applied directly to the TLC plate. The mean L/S ratio of the untreated standard mixture was 1.04. Significantly higher L/S ratios were obtained when chloroform alone or chloroform/methanol (19/1) was used as spotting solvent: 1.52 and 1.32, respectively (p < 0.001). All the other chloroform/methanol mixtures (from 6/1 to 2/1) gave L/S ratios comparable with that of the untreated standard mixture. About 70% of the phospholipid in the standard mixture was transferred to the TLC plate when chloroform/methanol (3/1) was used, as described in the routine procedure. For 11 different samples of amniotic fluid, the L/S ratio was significantly lower when chloroform/methanol (3/1) was used (mean = 2.4, SD 1.8) instead of chloroform alone (mean = 2.7, SD 1.8) (p < 0.001, paired t-test). When dried extracts were mixed with spotting solvent manually, instead of by vortex-mixing, even higher L/S ratios were obtained with chloroform alone. With chloroform/methanol (3/1), the results did not depend on the method of mixing.

Chromatography. With chloroform/methanol/ammonia as chromatography solvent, lecithin and sphingomyelin were well separated from each other and from PS and PI (Figures 1 and 2). Two-dimensional TLC showed that lecithin and sphingomyelin were also separated from other components of amniotic fluid. Sphingomyelin frequently ran as a double band, as has been noted by others (8). A small adjustment to the relative volume of aqueous ammonia in the solvent was sometimes necessary to achieve the separation shown in Figure 1, depending on the batch of reagent.

Recently there has been considerable interest in phosphatidyglycerol, a minor component of surfactant phospholipid, as a possible index of fetal lung maturity (2, 9, 10). The TLC procedure described in this paper is not suitable for quantitating phosphatidyglycerol. Two-dimensional TLC, with the ammonia-containing solvent in the first dimension and chloroform/methanol/water (65/25/4 by vol) in the second dimension, revealed that in our routine L/S ratio procedure phosphatidyglycerol migrates ahead of lecithin and co-migrates with phosphatidylethanolamine and another unidentified component of amniotic fluid.

Staining and color development. The staining reagent was adapted from that recommended by Fewster et al. (11). Including ethanol in the staining reagent facilitated the penetration of the reagent into the regions of the plate containing

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Raw Text Image:

Fig. 2. Thin-layer chromatography of standard preparations of phospholipids and of amniotic fluid samples with improved L/S ratio procedure

A, lecithin (upper spot) and sphingomyelin (lower spot); B, PS; C, PI; D, a mixture of A, B, and C; E and F, amniotic fluid extracts. For photographic purposes, the amounts of phospholipid applied to this plate were considerably greater than those routinely applied for L/S ratio determinations. Consequently, in the routine procedure, there is even better separation between sphingomyelin and the PS/PI band (cf. Fig. 1)

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Fig. 3. Effect of duration of heating on color development during staining of phospholipids on the TLC plate

Replicate samples were applied to TLC plates, chromatographed, dipped in staining reagent, and heated at 140–145 °C for the intervals indicated. Nine samples of four different mixtures of standard lecithin and sphingomyelin and nine different samples of amniotic fluid were treated in this way. The L/S ratios of these samples, determined by the routine procedure, ranged from 1.0 to 4.5 and from 1.9 to 7.1 for standard mixtures and amniotic fluid, respectively. For each sample, the area under the curve of the densitometric tracing obtained for lecithin (L) and sphingomyelin (S), or the ratio of these areas (L/S), is expressed as a percentage of the value obtained at 8 min. The data points represent the mean values ± 1 SEM. Each value differs significantly from the corresponding preceding value, except in the two cases indicated by asterisks.
large amounts of phospholipid. In the absence of ethanol, these regions stained unevenly. Exposure to the staining reagent for 2 min ensured uniform penetration.

The L/S ratio depends critically on the heating conditions used for developing the color after staining, because the rate of color development differs for lecithin and sphingomyelin (Figure 3). Routinely, and in these experiments (Figure 3), we always adjust the oven temperature so that heating for 10 min will produce a densitometric L/S ratio for the standard mixture equivalent to the molar L/S ratio determined by measuring phospholipid phosphorus. Although color development is incomplete under these conditions, the L/S ratio is relatively independent of heating time; an error of ±30 s in heating time would cause an error of less than 5% in the L/S ratio (Figure 3). Because color development is so very critical, all aspects of this procedure must be carefully controlled. For example, the position of the plate in the oven should be standardized and the time the oven door is kept open when the plate is placed inside should be minimized.

We recommend that a control chart be kept, indicating the L/S ratio obtained for the standard mixture from day to day. Variations within experimental error (±2 SD, see below) should be ignored. However, if an unexpectedly high or low result is obtained, the batch of assays should be repeated; in our experience, this is a rare occurrence. If a consistent upward or downward trend in the day-to-day L/S ratio becomes apparent, then the procedure for determining the correct oven temperature should be repeated. In any case, this temperature procedure should be repeated about every six months.

4 The L/S ratios reported for amniotic fluid extracts are defined empirically by the procedure and are not necessarily molar ratios; the intensity of the staining reaction is dependent on the degree of saturation of the fatty-acid side chains of the phospholipids.

We also investigated the effect on the L/S ratio of varying the delay between heating and scanning the TLC plate. Scanning a plate at 5, 10, and 15 min after heating, and again 24 h later, indicated that the L/S ratio of standard mixtures and samples of amniotic fluid representative of various stages of fetal lung maturity did not change during this period.

Quantitation. Standard curves for lecithin and sphingomyelin are shown in Figure 4. For samples containing up to about 10 μg of phospholipid, equivalent weights of lecithin and sphingomyelin stained to the same extent; but at greater amounts, sphingomyelin stained less intensely than lecithin. The staining response was linear with quantity to about 5 μg of phospholipid, and was approximately linear (within 10%) to about 7.5 μg of phospholipid (Figure 4). The use of only 0.5 mL of chloroform extract of amniotic fluid for each L/S ratio determination ensured that virtually all samples with L/S ratios in the range critical for clinical interpretation did not contain more than 7.5 μg of either lecithin or sphingomyelin. Very occasionally, 0.5 mL of chloroform extract contained too little phospholipid for accurate densitometric quantitation. In such cases, we repeated the determination with 1 mL of chloroform extract.

Three samples of amniotic fluid containing very high concentrations of phospholipid gave atypical results. The chloroform extract contained an additional component that chromatographed just ahead of sphingomyelin and tended to overlap with it (see compound X, Figure 1C). In these cases, the area under the curve for sphingomyelin was derived as shown in Figure 1C.

Reproducibility of the method. We determined the L/S ratio by this method for 24 replicate samples of pooled amniotic fluid (mean L/S ratio 2.4) in a single batch; the CV was 7%. The pairwise CV for 18 duplicate determinations (assayed over a six-month period) was 4% for a standard mixture applied directly to the TLC plate and 6% for amniotic fluid samples (mean L/S ratio 3.0, range 1.0 to 6.9).

L/S ratios were determined in triplicate for 4-μL portions of a standard mixture applied directly to the TLC plate and for 20-μL portions added to 2 mL of a 9 g/L solution of NaCl, then extracted and processed as for an amniotic fluid sample. This experiment was repeated six times to assess between-batch variation. The between-batch CV, calculated on the basis of the mean values within each batch, was 6% for the untreated standard mixture and 7% for the standard mixture processed through the whole L/S ratio procedure.

To determine whether other laboratories could reproduce the results obtained in this laboratory, we supplied details of the method and a series of mixtures of standard lecithin and sphingomyelin to three other laboratories (see Acknowledgments). After establishing the correct oven temperature as described above, and checking the standard curves as described in Figure 4, these laboratories were able to achieve densitometric ratios of 0.38 ± 0.05, 1.05 ± 0.02, and 3.07 ± 0.20 (overall mean ± SD based on the means of two to five determinations for each laboratory) for standard mixtures for which we had obtained molar L/S ratios of 0.33, 1.12, and 3.17, respectively. These results suggest that cutoff values for clinical interpretation established in this laboratory will be directly transferable to other laboratories, if the procedure is carried out exactly as described.

Clinical Evaluation

Table 1 summarizes the relationship between the L/S ratio and the incidence of respiratory problems in the newborn, for infants delivered within two days of collecting the amniotic fluid. All four cases of HMD encountered in this study were associated with L/S ratios of less than 2, whereas all 137 infants with L/S ratios of 3 or more, including 24 pre-term infants, were free from respiratory problems of this nature. Of
Table 1. Clinical Outcome in Cases Where Lung Immaturity Was Predicted by the Old Procedure (L/S <2)

<table>
<thead>
<tr>
<th>Case</th>
<th>Days to delivery</th>
<th>Gestational age, weeks</th>
<th>L/S ratio</th>
<th>Old method</th>
<th>New method</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>28</td>
<td>1.8</td>
<td>1.1</td>
<td>HMD, died</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>30</td>
<td>1.4</td>
<td>1.6</td>
<td>HMD</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>34</td>
<td>1.7</td>
<td>1.8</td>
<td>HMD, died</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>36</td>
<td>1.7</td>
<td>1.8</td>
<td>HMD</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>?40</td>
<td>0.9</td>
<td>2.3</td>
<td>TTN</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>37</td>
<td>1.0</td>
<td>3.1</td>
<td>No RD</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>?40</td>
<td>1.5</td>
<td>3.2</td>
<td>No RD</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>36</td>
<td>1.1</td>
<td>3.6</td>
<td>No RD</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>40</td>
<td>1.5</td>
<td>3.8</td>
<td>No RD</td>
<td></td>
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<tr>
<td>11</td>
<td>1</td>
<td>36</td>
<td>1.3</td>
<td>4.7</td>
<td>No RD</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>36½</td>
<td>1.7</td>
<td>5.0</td>
<td>No RD</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>36</td>
<td>1.9</td>
<td>5.2</td>
<td>TTN</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>38</td>
<td>1.5</td>
<td>5.9</td>
<td>No RD</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>?40</td>
<td>1.7</td>
<td>6.7</td>
<td>No RD</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>40+</td>
<td>1.7</td>
<td>7.2</td>
<td>No RD</td>
<td></td>
</tr>
</tbody>
</table>

* Time between collection of amniotic fluid and delivery of the infant.  
* These mothers were given an intravenous infusion of 1 g of hydrocortisone in 50 mL dextrose over a 12-h period, commencing 20 h, 19 h, and four days before delivery for cases 1, 2, and 3, respectively.  
* Showed all the clinical symptoms of HMD, but only minor changes on chest roentgenogram. TTN, transient tachypnea; No RD, no significant respiratory problems.

Table 2. L/S Ratio for Amniotic Fluid, and Respiratory Condition of the Infant on Delivery

<table>
<thead>
<tr>
<th>Condition on delivery</th>
<th>No. with L/S ratio</th>
<th>Total no.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>&lt;2.0</td>
<td>2.0–&lt;2.5</td>
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<tr>
<td>Hyaline membrane disease</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Transient tachypnea</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>No respiratory problems</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total no. of cases</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. &lt;37 weeks’ gestation</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

* Cases 1–4, Table 2.  
* Gestational age was based on pediatric assessment, or on certain dates consistent with the condition of the infant.

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References