Automated Enzymatic Measurement of Lecithin, Sphingomyelin, and Phosphatidylglycerol in Amniotic Fluid

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We describe methods for automated enzymatic measurement of lecithin, sphingomyelin, and phosphatidylglycerol in amniotic fluid. Phospholipase C (EC 3.1.4.3) and sphingomyelin phosphodiesterase (EC 3.1.4.12) are reacted with lecithin and sphingomyelin, respectively, to liberate phosphocholine. Phosphocholine is then reacted with alkaline phosphatase, choline oxidase, peroxidase, and 4-aminoantipyrine to form a colored complex, for which the absorbance at 500 nm is measured with a centrifugal analyzer. Phosphatidylglycerol is hydrolyzed by phospholipase D (EC 3.1.4.4) to form glycerol, which is subsequently reacted with ATP and NAD+ in the presence of glycerol kinase and glycerol-3-phosphate dehydrogenase to yield NADH. The absorbance of the NADH formed is measured at 340 nm. These methods provide a simple, rapid, and accurate alternative to thin-layer chromatography for determination of phospholipids in amniotic fluid for assessment of fetal lung maturity.

Additional Keyphrases: fetal status · thin-layer chromatography compared · centrifugal analyzer

Determination of the ratio of lecithin to sphingomyelin concentration (L/S ratio) in amniotic fluid is of value in assessing fetal lung maturity (1).1 Evidence suggests that hyaline membrane disease results from insufficient production of surface-active phospholipids in the fetal lungs. Phospholipids are produced and stored within the cells lining the alveoli and are then secreted into the lumen, where they coat the alveolar cells, thus decreasing surface tension and preventing alveolar collapse (2). For patients with uncomplicated pregnancies, an L/S ratio ≥2.0 is considered to be a reliable indicator of fetal lung maturity (3). In pregnancies complicated by diabetes, hypertension, premature rupture of membranes, or intra-uterine growth retardation, conclusions drawn from the L/S ratio may be unreliable (4). Data from several laboratories indicate that from 7.4 to 27.0% of infants of diabetic mothers develop respiratory distress syndrome despite "mature" L/S ratios (5-10). In addition, "immature" L/S ratios ≤2.0 often correlate poorly with fetal lung immaturity (11-15).

Several investigators (3, 5, 6, 11-13, 16) suggest that phosphatidylglycerol (PG) is also an important indicator of fetal lung maturity. Hallman et al. (17) reported that PG was undetectable in lung aspirates of infants with respiratory distress syndrome, but subsequently appeared as their recovery progressed. Obladen et al. found that respiratory distress syndrome did not occur when PG was present in amniotic fluid, even when the L/S ratio was ≤2.0 (18). In pregnancies complicated by maternal diabetes mellitus, the PG content in amniotic fluid may be more predictive of fetal lung maturity than is the L/S ratio (19).

Phospholipids in amniotic fluid have been measured by several techniques. Most methods require extraction of the amniotic fluid with a chloroform:methanol-mixture similar to that described by Folch et al. (20). The extract is then concentrated, transferred to a chromatography plate, and separated by thin-layer chromatography (TLC). Individual phospholipids may then be measured by various techniques, such as digestion of the organic material followed by phosphate determination (21), and staining (22) or charring (1) of the phospholipids followed by densitometry (23) or planimetry (24). Although the concentrations of lecithin, sphingomyelin, and phosphatidylglycerol have been determined by TLC techniques, the reproducibility of these methods has been criticized (25-30). In attempts to circumvent some of the problems associated with the chromatographic methods, various enzymatic assays have been developed for measuring lecithin (31-35), sphingomyelin (28, 33), and PG (4) by either spectrophotometric (31-35) or radiochemical (4, 28) techniques.

Here we report methods for simultaneously measuring lecithin, sphingomyelin, and PG by automated enzymatic techniques. We compare data obtained by the enzymatic methods with those by a commercially available TLC procedure and present a limited clinical evaluation. Use of automated enzymatic assays for lecithin, sphingomyelin, and PG may provide the clinical laboratory with objective quantitative methods for quick and simple determinations of amniotic fluid phospholipid "profiles."

Materials and Methods

Materials

Instrumentation. We used a Multistat III microcentrifugal analyzer and a Multistat III centrifugal loader (Instrumentation Laboratory, Inc., Lexington, MA 02173).

Thin-layer chromatography. Commercially available thin-layer chromatography reagents were used for determination of the L/S ratio (Helena Laboratories, Beaumont, TX 77704).

Samples. Amniotic fluid samples were obtained by amniocentesis and from vaginal pools. The samples were promptly centrifuged at 500 × g for 8 min, to remove cells and debris, then stored at -70 °C until assays were performed.

Reagents and phospholipid standards. Phospholipase C (from Bacillus cereus; phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3), sphingomyelin phosphodiesterase (from B. cereus; sphingomyelin cholinephosphohydrolase, EC 3.1.4.12), alkaline phosphatase (from calf intestine; orthophosphoric-monooester phosphohydrolase, EC 3.1.3.1), and phospholipase D (from Streptomyces chromofuscus; phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) were all purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250.

Peroxidase (from horseradish; hydrogen peroxide oxidore-
ductase, EC 1.11.1.7), choline oxidase (from Alcaligenes species; choline:oxygen 1-oxidoreductase, EC 1.1.3.17), glycero-
or kinase (from Escherichia coli; ATP:glycerol-3-phospho-
transferase, EC 2.7.1.30), 3,3-dimethylartenic acid, 4-amino-
antipyrine, barbitral, 5,5'-diethylbarbituric acid, L-α-phos-
phatidylcholine, dipalmitoyl (synthetic), sphingomyelin,
 trom bovine brain), adenosine 5'-triphosphate (from equine
 muscle), β-nicotinamide adenine dinucleotide, and L-α-
phosphatidyl-DL-glycerol were obtained from Sigma Che-
 mical Co., St. Louis, MO 63178.

Triton X-100, Tris buffer, phenol, magnesium chloride, hy-
drate sulfate, glycyne, sodium EDTA, and calcium
 chloride were all purchased from Fisher Scientific Co.,
 Pittsburgh, PA 15219.

Glycerol-3-phosphate dehydrogenase (from rabbit muscle; s
 glycerol-3-phosphate:NAD+ 2-oxidoreductase, EC 1.1.1.8) was obtained from Calbiochem–Behring, La Jolla,
 CA 92037.

Reagent Preparation

**Lecithin standards.** Dissolve 100 mg of L-α-dipalmitoyl 
phosphatidylcholine in 100 mL of 50 g/L aqueous Triton X-
100. Dilute the stock solution in distilled water to give 
concentrations of 10, 20, 30, 60, 100, 200, 300, 400, and 500 
mg/L for working standards. Stored at 4 °C, these standards 
were stable for at least a month.

**Sphingomyelin standards.** Dissolve 100 mg of sphingo-
myelin in 100 mL of 50 g/L aqueous Triton X-100. Dilute the 
stock solution with distilled water to give concentrations of 
10, 20, 30, 60, 100, 200, 300, 400, and 500 mg/L for working 
standards. These standards were stable for at least a month 
at 4 °C.

**Phosphatidylglycerol standards.** Dissolve 20 mg of PG in 
100 mL of 1 g/L aqueous Triton X-100. Dilute this stock 
solution with 1 g/L aqueous Triton X-100 to give concentra-
tions of 10, 20, 30, 60, 100, and 200 mg/L for working 
standards. These standards were stable for at least a month 
at 4 °C.

**Lecithin reagents.** Phospholipase C (4000 kU/L) was diluted 
in dimethylglycerol–sodium hydroxide buffer (each 0.1 
 mol/L, pH 7.5) to yield a final concentration of 400 kU/L.
 Alkaline phosphatase (1500 kU/L) was reconstituted in 1.0 
 mL of this same buffer. Phospholipase C was stable for 
at least three months at 4 °C, alkaline phosphatase for at least 
one month.

**Sphingomyelin reagents.** Sphingomyelin phosphodiester-
a (sphingomyelinase) was used at a concentration of 100 
 kU/L. The reaction diluent was barbitral (0.1 mol/L)–ma-
nesium chloride (0.1 mol/L) buffer, pH 8.5. Alkaline phos-
phatase (1500 kU/L) was reconstituted in 1.0 mL of the dimeth-
ylglycerol–sodium hydroxide buffer. Sphingomyelinase 
was stable for at least three months at 4 °C.

**Chromogen reagent for lecithin and sphingomyelin.** We 
prepared chromogen solution by adding 30 mg of 4-amino-
antipyrine, 20 mg of phenol, 300 U of choline oxidase, and 
900 U of peroxidase to 90.0 mL of Tris HCl buffer (50 
mmol/L, pH 8.0). After solution of all these was complete, we 
added additional Tris HCl buffer to the chromogen solution 
scant to bring the final volume to 100 mL. This reagent was 
stable for at least two weeks at 4 °C.

**Phosphatidylglycerol reagents.** Reagents for the PG assay 
were divided into two separate sets, reagents A and B.

Reagent A consists of phospholipase D (5 kU/L), calcium 
chloride dihydrate (10 mmol/L), magnesium chloride (5 
mmol/L), ATP (750 μmol/L), and glycerol kinase (0.5 kU/L);
these reagents were dissolved in Tris HCl buffer (50 
mmol/L, pH 7.6). To conserve reagents, we prepared only 20 
 mL of reagent, which we stored in 1.0-mL aliquots at −20 °C 
until ready for use. Reagent A was stable for at least a month.

Reagent B was prepared by dissolving 1.3 g of hydrazine 
sulfate (0.4 mol/L), 1.88 of glycine (1.0 mol/L), and 0.5 g of 
Na2 EDTA · 2H2O in 12.8 mL of 2 mol/L NaOH. The 
hydrazine/glycine buffer was diluted to 25 mL with distilled 
water and the pH was adjusted to 9.5. NAD+ (72 mg, 4.1 
mmol/L), and glycerol-3-phosphate dehydrogenase (500 U) 
were dissolved in this 25 mL of buffer. Reagent B was 
freely prepared daily and was stable for 24 h when stored at 
4 °C.

**Methods**

The enzymatic reactions we used for the assays of lecithin, 
sphingomyelin, and PG are summarized as follows:

**Lecithin (phosphatidylcholine)**

\[
\text{Phosphatidylcholine} + H_2O \xrightarrow{\text{phospholipase}} C_1,2-diacylglycerol + \text{phosphocholine}
\]

**Phosphocholine + H_2O \xrightarrow{\text{alk phosphatase}} \text{choline} + P_i**

**Choline + 2O_2 + H_2O \xrightarrow{\text{choline oxidase}} \text{betaine} + 2H_2O_2**

\[2\text{H}_2\text{O}_2 + \text{phenol} + 4\text{-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{immuno-quinone complex (λ_{max} = 500 nm)}\]

**Sphingomyelin (sphingosylphosphorylcholine)**

\[\text{Sphingosylphosphorylcholine} + H_2O \xrightarrow{\text{sphingomyelinase}} N\text{-acyl-
sphingosine} + \text{phosphocholine}\]

Subsequent reactions involving phosphocholine are the 
same as in the lecithin assay above.

**Phosphatidylglycerol**

\[\text{PG} \xrightarrow{\text{phospholipase D}} \text{phosphatidic acid} + \text{glycerol}\]

\[\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol 3-phosphate} + \text{ADP}\]

\[\text{Glycerol 3-phosphate} + \text{NAD}^+ \xrightarrow{\text{glycerol 3-phosphate dehydrogenase, pH 9.5}} \text{glycerone phosphate} + \text{NADH (λ_{max} = 340 nm)}\]

**Assay procedures.** The procedures for lecithin, sphingo-
myelin, and PG were divided into two phases. In phase 1 of 
the lecithin assay, the automated loader added 38 μL of 
ammoniatic fluid to 2 μL of phospholipase C reagent and 
diluted this with 95 μL of dimethylglycerol–NaOH buffer 
in a cuvette rotor. In phase 1 of the sphingomyelin assay, 38 
μL of ammoniatic fluid, 2 μL of sphingomyelinase reagent, 
and 95 μL of barbitral–MgCl2 buffer were loaded into a cuvette 
rotor. For both assays, these reaction mixtures were then 
incubated for 15 min at 37 °C in the centrifugal analyzer.

The phase 2 procedure is identical for both the lecithin 
and sphingomyelin assays: 25 μL of chromogen reagent, 2 
μL of alkaline phosphatase solution, and 30 μL of distilled 
water were added to the reaction mixture in the cuvette 
rotor and incubated at 37 °C for 15 min. Absorances were
then measured bichromatically (500/690 nm) vs a reagent blank 3 s and 15 min after the phase 2 reagents were added. The initial (3-s) absorbances were subtracted from the 15-min absorbance so that each amniotic fluid sample would serve as its own blank and minimize nonspecific absorbances. Also, the absorbances at 500 nm were adjusted by subtracting the corresponding absorbance reading at 690 nm. Concentrations of lecithin and sphingomyelin were then calculated by the analyzer by comparison with a calibration curve based on values for known concentrations of lecithin and sphingomyelin: 10, 20, 30, 60, 100, 200, and 300 mg/L each.

In the PG assay the loader module added 38 µL of amniotic fluid and 95 µL of reagent A to a cuvette rotor; the sample and reagent were mixed, then incubated at 37 °C for 15 min. Then 50 µL of reagent B was added and incubated for an additional 15 min at 37 °C. Absorbances at 340 and at 690 nm were recorded after 3 s and after 15 min. The absorbance data were corrected bichromatically as described above, but all readings were made against a reagent blank. PG concentrations were calculated by comparison with a calibration curve for PG standards of 10, 20, 30, 60, 100, and 200 mg/L.

Results

Lecithin and Sphingomyelin Assays

Linearity studies obtained by using lecithin and sphingomyelin standards at concentrations between 10 and 500 mg/L indicated that results for both assays are linearly related to concentration for concentrations up to 300 mg/L (Figure 1). This easily encompasses the expected normal ranges of lecithin and sphingomyelin in amniotic fluid, at the 12th week of gestation, and at term (39 weeks of gestation)—i.e., between 2.8 and 86.0 mg/L, and between 4.2 and 11.0 mg/L, respectively (36).

We assessed the accuracy of the methods by measuring the analytical recovery of various amounts of lecithin and sphingomyelin added to amniotic fluid. For added lecithin concentrations of 10, 25, 50, and 100 mg/L, we recovered 89, 93, 100, and 97%, respectively (mean 94.8%). For sphingomyelin added in concentrations of 10, 20, 30, and 60 mg/L, subsequent recoveries were 81, 94, 100, and 110% (mean 96.3%).

Table 1 gives results of our precision studies.

In enzyme specificity studies, we assessed the specificity of phospholipase C for lecithin by using sphingomyelin standards as samples to determine cross reactivity; similarly, we used lecithin standards to evaluate the specificity of sphingomyelinase for sphingomyelin. As Table 2 illustrates, sphingomyelin in concentrations usually present in amniotic fluid did not cross react with phospholipase C; it cross reacted only slightly at very high concentrations (1 g/L). Lecithin did not cross react significantly with sphingomyelinase, even at 3 g/L.

Phosphatidylglycerol Assay

In studies of the linearity of the PG assay we used PG standards and various dilutions of an amniotic fluid specimen (Figure 2). Results varied linearly with PG concentrations up to 200 mg/L; also, linearity was maintained in the dilution study. Table 3 summarizes precision studies done on two separate amniotic fluid pools.

In the specificity studies, we analyzed aliquots of lecithin and sphingomyelin standards (in concentrations of 20 to 200 mg/L) by the enzymatic PG method. Lecithin at 200 mg/L cross reacted slightly (<10 mg/L). Because this was not observed at any other lecithin concentration and was within the range of analytical variation of the method, we did not consider it significant. Sphingomyelin did not cross react at any concentration studied.

Clinical Studies

Thirty-seven amniotic fluid samples submitted to the clinical chemistry laboratory for evaluation of fetal lung maturity by a conventional TLC method for determining L/S ratio were also assayed by the enzymatic methods for
Table 1. Precision of Lecithin and Sphingomyelin Measurements in Amniotic Fluid

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean, mg/L</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run precision</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithin (n = 16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool I</td>
<td>26.1</td>
<td>0.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Pool II</td>
<td>116.0</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Sphingomyelin (n = 16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool I</td>
<td>19.0</td>
<td>1.9</td>
<td>10.0</td>
</tr>
<tr>
<td>Pool II</td>
<td>84.0</td>
<td>6.0</td>
<td>7.2</td>
</tr>
<tr>
<td><strong>Between-run precision</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecithin (n = 20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool I</td>
<td>28.1</td>
<td>2.1</td>
<td>7.5</td>
</tr>
<tr>
<td>Pool II</td>
<td>112.2</td>
<td>3.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Sphingomyelin (n = 20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool I</td>
<td>18.8</td>
<td>2.8</td>
<td>14.9</td>
</tr>
<tr>
<td>Pool II</td>
<td>66.0</td>
<td>7.7</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Table 2. Specificity of Phospholipase C and Sphingomyelinase

<table>
<thead>
<tr>
<th>Added sphingomyelin, mg/L</th>
<th>Measured lecithin, mg/L</th>
<th>Cross reactivity, %</th>
</tr>
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<tbody>
<tr>
<td>30</td>
<td>2.5</td>
<td>8.3</td>
</tr>
<tr>
<td>60</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>1000</td>
<td>66.6</td>
<td>6.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Added lecithin, mg/L</th>
<th>Measured sphingomyelin, mg/L</th>
<th>Cross reactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.4</td>
<td>7.0</td>
</tr>
<tr>
<td>60</td>
<td>1.6</td>
<td>2.7</td>
</tr>
<tr>
<td>3000</td>
<td>7.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

lecithin, sphingomyelin, and PG. The mean L/S ratio by TLC (Table 4) did not differ significantly from the mean ratio calculated from the quantitative enzymatic measurements of lecithin and sphingomyelin (paired t-test: t = 1.66, p > 0.10).

Figure 3 shows correlations found between the enzymatically determined L/S ratio and lecithin and PG concentrations and the L/S ratio by TLC. The lecithin concentration correlated significantly with the L/S ratio by TLC (r = 0.82, p < 0.001). The PG concentration also correlated significantly with the L/S ratio by TLC (r = 0.86, p < 0.001). The enzymatic L/S ratio correlated to a lesser degree than lecithin but was also statistically significant (r = 0.56, p < 0.001). Sphingomyelin concentration did not correlate with the L/S ratio by TLC (r = 0.27, p > 0.05).

Review of the medical records of the 37 patients from whom amniotic fluid was obtained revealed that 16 patients delivered within three days of amniotic fluid sampling. The remaining 21 patients delivered more than three days after amniotic fluid collection, thereby precluding correlation of phospholipid analysis with development of respiratory distress syndrome. There was no evidence of hyaline membrane disease in any of the 16 infants delivered within 72 h of amniotic fluid sampling. In this group the mean L/S ratio by TLC was 4.6 (range 1.5 to 11.7) compared with a mean enzymatic L/S ratio of 6.3 (range 3.0 to 13.9). The enzymatic lecithin concentration averaged 78 mg/L (range 18 to 180 mg/L) and the mean PG concentration was 74 mg/L (range 19 to 183 mg/L).

Discussion

The methods routinely used in most clinical laboratories for phospholipid analysis in amniotic fluids are tedious, time consuming, and subject to large analytical variation (37). The present enzymatic methods offer several advantages over conventional methodology: simultaneous measurement of lecithin, sphingomyelin, and PG; relative ease of performance; speed of obtaining results (~1 h); and less-subjective results than those obtained by TLC. Linearity, recovery, and precision data indicate that the methods were linear and reproducible in the range where clinical samples would be expected. However, at low concentrations of PG the assay CV may be substantial. At the phospholipid concentrations...
normally present in amniotic fluid, cross reactivity is insignificant.

In clinical studies, lecithin and PG concentrations determined with the enzymatic assay had the best correlation with the L/S ratio by TLC. The mean enzymatic L/S ratio (6.1), however, tended to be higher than the mean L/S ratio by TLC (4.4), an observation consistent with reports by other investigators (33, 36, 38). We had no amniotic fluid samples from cases with hyaline membrane disease, so we cannot define specific "cutoff" values to predict fetal lung maturity. Other investigators have suggested cutoff values of 35 to 55 mg/L for lecithin concentration (34, 35, 39, 40) and 7.4 mg/L for phosphatidylglycerol concentration (4) as predictors of fetal lung maturity.

In summary, we have developed automated enzymatic methods for measurement of lecithin, sphingomyelin, and PG in amniotic fluid. These assays are linear and reproducible in the ranges found in amniotic fluids. Advantages over conventional TLC techniques include improved turnaround time, quantitative results, and smaller sample size. Results obtained for amniotic fluids with the enzymatic methods were significantly correlated with the TLC-determined L/S ratio. Additionally, our enzymatically determined phospholipid data compared favorably with those reported by other investigators. We conclude that the enzymatic measurement of individual phospholipids in amniotic fluid offers potential for evaluating fetal lung maturity. Further studies to evaluate the clinical relevance of these methods and to establish reference intervals are currently in progress.

We wish to express our gratitude to Mr. William L. Wellers for his technical assistance in performing the phosphatidylglycerol analyses.

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