Critical Assessment of Phospholipid Measurement in Amniotic Fluid

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We assessed several methods of inorganic phosphate assay for their suitability in estimating phospholipids in digested extracts of amniotic fluids. The extraction and digestion procedures used for phospholipids from amniotic fluid were also examined critically. The effect of contamination by blood or obstetric cream has been examined. Accordingly, we suggest a method for measuring total phospholipids in amniotic fluids, and results of it are compared with the lecithin/sphingomyelin ratio measurement in some clinical situations.

Additional Keyphrases: lecithin/sphingomyelin ratio • respiratory distress syndrome • index of fetal viability • intermethod comparison

Gluck et al. (1-4) have shown that lecithin is the major active component of lung surfactant and that it is produced in sufficient quantities to support respiration only late in pregnancy. If, at the time of delivery, insufficient lung lecithin is being produced, there is a risk of respiratory distress that, in most medical centers, is responsible for a higher rate of neonatal mortality than any other single cause. Since this work was published, many workers have suggested a number of alternative methods of measuring the surfactant material present by analyses done on the amniotic fluid before delivery (5-12). Thus the risk of respiratory distress may be assessed and the delivery induced or postponed accordingly. Many of these methods involve the separation of lecithin by thin-layer chromatography, followed either by its absolute measurement in situ by densitometry, by colorimetry after removal from the plate, or by a relative measurement such as ascertaining the lecithin/sphingomyelin ratio. Sphingomyelin is a nonactive phospholipid, present in amniotic fluid in late pregnancy; its concentration changes very little over that period of gestation and thus it serves well as an index compound (2). The advantage of the latter approach is that analytical recovery need not be 100%. The disadvantage of any quantitative procedure based on the use of thin-layer chromatography is that it is a demanding technique that requires considerable experience before it can be used with reliability, and therefore it is not well suited to a busy routine laboratory. For that reason the present study was undertaken with the intention of establishing a colorimetric assay that could be done reliably by an experienced worker without specialized expertise.

Lecithin is the major fraction of amniotic fluid phospholipids and we therefore assumed that measurement of total phospholipids would validly reflect any significant change in lecithin concentration. A method was devised based on extracting lipid-soluble material and digesting this material to leave a residue of inorganic phosphate, which may then be assayed as inorganic phosphate. The extraction and digestion procedures were critically assessed until conditions were achieved under which reproducible results could be obtained. Several methods of inorganic phosphate analysis were investigated to find one with the sensitivity, stability, and reproducibility that we considered necessary for acceptably precise results under conditions of routine analysis. The procedure suggested by the above investigations was used to measure the total phospholipids in amniotic fluids taken...
at the time of induction of labor or cesarean section. The clinical significance of these results was examined in the context of respiratory distress syndrome and compared with the measurement of the lecithin/sphingomyelin ratio for the same amniotic fluids.

**Methods**

**Inorganic Phosphate Assays**

*Iron/trichloroacetic acid/thiourea method* (13). One reagent was prepared by dissolving trichloroacetic acid (50 g), thiourea (5 g), and ferrous ammonium sulfate hexahydrate (15 g) in water and diluting to 500 ml. A second reagent was prepared by slowly adding sulfuric acid (45 ml) to water (200 ml) with cooling, then adding ammonium molybdate (22 g) in water (200 ml) and diluting the solution to 500 ml. For assay, 5 ml of the first reagent was added to 1 ml of sample, and the molybdate reagent (0.5 ml) added after 10 min. Color was measured at 740 nm.

*Aminonaphthol sulfonic acid method* (14). Two reagents were prepared: a solution of 1,2,4-aminonaphthol sulfonic acid (British Drug Houses Ltd.) in water, 2.5 g/liter, and molybdate reagent, a 25 g/liter solution of ammonium molybdate in sulfuric acid (2.5 mol/liter). For assay, 1 ml of the molybdate reagent was added to 1 ml of sample, followed by 0.4 ml of the aminonaphthol sulfonic acid reagent, and the resulting color measured at 660 nm.

*Stannous chloride method* (15). Two reagents were prepared: for the molybdate reagent, 200 ml of sulfuric acid (5 mol/liter) was added slowly with cooling to 400 ml of water, and 200 ml of sodium molybdate in water (75 g/liter) was added. For the stannous chloride reagent, 10 g of ANALAR-grade stannous chloride was dissolved in 25 ml of hydrochloric acid (400 ml/liter); a working solution of this was prepared by a 200-fold dilution with water. Both solutions were prepared freshly each day. For the assay, 3.7 ml of water was added to 1 ml of sample, followed by 0.2 ml of the molybdate reagent and 0.1 ml of the stannous chloride reagent. Color was measured at 680 nm.

*Vanadate method* (16). The reagents were sulfuric acid (10 ml/liter), a solution of 1.25 g of ammonium vanadate in 400 ml of an equal-volume of nitric acid and water, and a solution of 50 g of ammonium molybdate in 400 ml of water. The last two solutions were mixed and the mixture diluted to 1 liter. For assay, 1 ml of sample, 1 ml of sulfuric acid, and 1.2 ml of vanadate reagent were mixed and the resulting color was measured at 403 nm.

*Malachite green method*. The Diagnostica kit (Roche Products Ltd.) was used; color developed according to their method was measured at 650 nm.

**Phosphate Standards**

A stock solution of phosphate was made by dissolving ANALAR-grade anhydrous potassium dihydrogen phosphate in water to give a concentration of 44 g/liter. This was diluted 100-fold for use, giving a standard of 100 mg (3.22 mmol) of inorganic phosphorus per liter.

**Lecithin Solution**

A stock solution of lecithin (Sigma Chemical Co.) was prepared in methanol to give a concentration of about 500 mg/liter.

**Amniotic Fluid Extraction**

Amniotic fluids (1–4 ml) were extracted with methanol/chloroform mixtures, and the organic phase was separated and evaporated on a water bath at 60 °C under a flow of nitrogen.

**Digestion of Extracts**

Sulfuric acid (0.5 ml of a 5 mol/liter solution) was added to the dried extracts and heated strongly until all organic material was destroyed. All charred material was removed by adding hydrogen peroxide (30%) followed by further heating until the solution was colorless and excess hydrogen peroxide had volatilized. After cooling, the extracts were neutralized by carefully adding 0.5 ml of sodium hydroxide (10 mol/liter). The whole extract was then used to estimate inorganic phosphate.

**Estimation of the Lecithin/Sphingomyelin Ratio**

*Staining reagent*. Molybdenum trioxide, 20 g, was added to concentrated sulfuric acid (350 ml) and this suspension added to 150 ml of water, slowly and with stirring, and heated gently until dissolved (Solution I). To 250 ml of Solution I, molybdenum metal powder (0.86 g) was added and the mixture was boiled gently for 15 min (Solution II). Equal volumes of Solutions I and II were mixed and two volumes of water was added.

**Method**. 1 ml of methanol and 2 ml of chloroform were added to 1 ml of amniotic fluid and mixed by inversion for 2 min in a stoppered tube. The tubes were centrifuged at 825 × g for 5 min, and the organic phase was transferred to a tapered tube and evaporated on a water bath at 60 °C under a stream of nitrogen, and cooled in a water/ice mixture. The residue was washed twice with 10 drops of cooled acetone, and finally dried at 60 °C under nitrogen. This residue was taken up in 5 μl of chloroform and transferred with a 1-μl Oxford pipette (Boehringer Corp. (London) Ltd., London W5 2TZ) to a Polygram Sil G (Camlab Ltd., Cambridge CB4 1TH) thin-layer plate that had been activated at 100 °C for 0.5 h. The plate was developed (development solvent: chloroform/methanol/water, 65/30/5 by vol), air dried, and spots made visible by immersing the plate in the staining reagent for a few minutes, followed by water immersion to remove the background. The stain is not permanent, and therefore the phospholipids must be quantitated as soon as the plate is dry, by measuring the width and breadth of each spot with a pair of geometrical dividers and calculating the product of these values.
Results

Inorganic Phosphate Assay

Color stability with time. The color developed by each method was measured at time intervals between 10 min and 3 h after the last reagent was added (Figure 1). Fresh reagents were used in each case. Color stability was demonstrated in each method over this time period.

Reagent stability with time. The color developed by each method was measured over a period of weeks, with the same reagents (Figure 2). In each case the color was measured 20 min after adding the last reagent. All reagents were shown to be stable over this time period.

Sensitivity and linearity. The color developed by each method for different concentrations of inorganic phosphate standard was measured and compared (Figure 3). Beer's law was obeyed in each case, and a considerable difference in sensitivity was seen among the methods.

Effect on color reaction of varying \([H^+]\). The effect of changes in the final acid concentration on the color development was examined by adding up to 0.5 ml of sulfuric acid (5 mol/liter) or sodium hydroxide (10 mol/liter) to the assay mixture and measuring the color developed. Dilution effects resulting from these additions were eliminated by adding water to give the same final volume in each reaction mixture. The results are shown in Figure 4.
Digestion Procedure

Three procedures for digestion were examined by doing repeated assays with a lecithin solution by heating specimens (a) singly with a Bunsen burner, (b) in batches of six on a Kjeldahl digester, or (c) batches of any reasonable number in an oven at 180 to 200 °C. Phosphate was determined by the vanadate method; the precision of each procedure is shown in Table 1.

Extraction of Amniotic Fluid

The results of extraction experiments are shown in Table 2. In the initial experiments amniotic fluid was centrifuged (825 × g), then extracted (1–4 ml) with three volumes of methanol/chloroform (1/2, by vol) to one of amniotic fluid, in separatory funnels with gentle shaking. The organic (lower) phase was evaporated in a water bath at 60 °C in a stream of nitrogen.

These experiments showed that the precision of phospholipid measurements with amniotic fluids was unacceptably worse than that of an extracted lecithin solution, suggesting that the extraction as well as the digestion procedure needed further investigation. The following points were considered in this context. Incomplete separation of these two phases resulting in loss of some of the organic phase and consequently loss of phospholipid, contamination, and finally, incomplete and hence nonreproducible extraction. The extraction mixture was centrifuged (1300 × g) for 15 min to improve phase separation, sodium sulfate was added to the organic phase to remove water, and finally, the composition of the extraction mixture was changed. The extraction mixture was changed to methanol/chloroform (1/1 by vol) used in the ratio of 4/1 of the amniotic fluid. These experiments were performed on uncentrifuged amniotic fluid and clearly showed an increase in color absorbance. When samples of amniotic fluid were extracted a second and third time with this extraction mixture, the mean yields were 103% and 112%, respectively, taking the mean yield for 10 single extractions as 100%. Procedure V was chosen for routine use. In all cases the vanadate method was used for phosphate determination.

Other Factors Affecting Phospholipid Assay in Amniotic Fluid

The presence of blood in amniotic fluid. Heparinized whole blood was added to amniotic fluid in various quantities, and the total phospholipids were estimated by the vanadate method (Procedure V). The lecithin/sphingomyelin ratio was also determined, and the packed cell volume was measured (Table 3).

The presence of obstetric cream in amniotic fluid. Amniotic fluid was assayed for total phospholipids by the vanadate method (Procedure V) before and after

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**Table 1. A Comparison of the “Within-Batch” Precision of Phosphate Analyses of a Solution of Lecithin Digested by Three Different Heating Procedures**

<table>
<thead>
<tr>
<th>Heating Procedure</th>
<th>Bunsen</th>
<th>Kjeldahl</th>
<th>Oven</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. specimens</td>
<td>9</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.4</td>
<td>8.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**Table 2. Precision of Different Extraction Procedures**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. repeats</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>CV, %</td>
<td>24.8</td>
<td>49.7</td>
<td>26.3</td>
<td>22.8</td>
<td>1.4</td>
<td>5.7</td>
<td>2.6</td>
</tr>
</tbody>
</table>

I: Centrifuged amniotic fluid, extraction mixture MeOH/CCI₃H (1/2, by vol), digested over a Bunsen burner
II: As I, plus centrifugation before phase separation
III: As I, with Kjeldahl digestion
IV: As I, plus addition sodium sulfate to organic phase
V: Uncentrifuged amniotic fluid, extraction mixture MeOH/CCI₃H (1/1, by vol), digestion in oven at 180–200 °C
VI: As V, but with two extractions
VII: As V, with three extractions

**Table 3. Effect of Whole Blood on Total Phospholipid Measurement and Lecithin/Sphingomyelin Ratio**

<table>
<thead>
<tr>
<th>Packed cell vol, ml/dl</th>
<th>Total phospholipid, μmol/liter</th>
<th>Lecithin/sphingomyelin ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>175</td>
<td>6.1</td>
</tr>
<tr>
<td>1</td>
<td>180</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>339</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>493</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>643</td>
<td>2.6</td>
</tr>
<tr>
<td>7</td>
<td>795</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Table 4. Effect of Storage Temperature and Time on Total Phospholipid Estimation**

<table>
<thead>
<tr>
<th>Storage time</th>
<th>Room temperature</th>
<th>Total phospholipids, μmol/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>180</td>
</tr>
<tr>
<td>8 h</td>
<td>4 °C</td>
<td>106</td>
</tr>
<tr>
<td>24 h</td>
<td>113</td>
<td>112</td>
</tr>
<tr>
<td>48 h</td>
<td>111</td>
<td>113</td>
</tr>
<tr>
<td>1 week</td>
<td>111</td>
<td>109</td>
</tr>
<tr>
<td>2 weeks</td>
<td>105</td>
<td>112</td>
</tr>
</tbody>
</table>

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the addition of a quantity of obstetric cream [1,6-di(4-chlorophenyl)diguanide] hexane diglucuronate]. The results showed the assay to be unaffected.

Storage and temperature. Samples of amniotic fluid were stored at room temperature, +4 °C and -20 °C, and assayed by the vanadate method (Procedure V) at time intervals up to two weeks (Table 4).

Centrifugation and filtration. Amniotic fluid was assayed by the vanadate method (Procedure V) and then reassayed after (a) centrifuging (825 X g) for 10 min, (b) centrifuging (250 X g) for 30 min, or (c) filtering through Whatman No. 1 filter paper. We observed an approximate 60% loss of phospholipid due to centrifugation under either condition, and a 90% loss because of filtration. The cellular content of a number of fresh amniotic fluids was examined microscopically, to establish the contribution of the cells to the above phospholipid loss.

Precision of Lecithin/Sphingomyelin Ratio Estimation

We assessed the precision of analysis of two different standard solutions, both containing lecithin and sphingomyelin in methanol, by 20 repeated separations according to the method described above. The solutions gave mean ratios of 1.12 and 3.40, with CV’s of 17% and 21%, respectively.

Analysis of Amniotic Fluid

More than 100 samples of amniotic fluid were collected from patients at the time of delivery, and the total phospholipid was estimated by the vanadate method (Procedure V) and the lecithin/sphingomyelin ratio measured. Of these patients, 70 had normal pregnancies and the neonate showed no sign of respiratory distress. These values were used to compute a reference range. The histogram in Figure 5 shows that the distribution of total phospholipid is non-gaussian, and the reference range was taken as being those values above the lower 5th percentile, i.e., those greater than 100 μmol/liter. By the same criterion, the reference value for the lecithin/sphingomyelin ratio is greater than 3.0. However, in this instance, the distribution is nearer to gaussian (Figure 5), giving a mean of 6.7 and an SD of 2.3, resulting in a reference range with 95% confidence limits of 2.1 to 11.0.

Figure 6 compares the total phospholipid and lecithin/sphingomyelin ratio values obtained for all the amniotic fluids examined.

Discussion

This study has aimed at establishing a procedure whereby total phospholipids may be assayed in amniotic fluid with acceptable precision and by a method that is quick, economical, easy to use in a routine laboratory (i.e., relatively inexperienced staff may be easily trained to produce accurate results), and finally a method that is equally applicable to a high or low workload. The method has also been compared with an alternative procedure in predicting the risk of respiratory distress syndrome.

Methodology was examined in four main areas: the relative merits of currently available methods of phosphate analysis when applied to this particular problem, the problems of amniotic fluid extraction and digestion, and the problems of specimen handling. Five methods of phosphate analysis were considered, three involving the reduction of phosphomolybdate to give a measurable molybdenum blue color. Of these, the method in which stannous chloride is used as reducing agent was rejected in the preliminary investigations because of difficulties in achieving a linear relationship between color and concentration. This could probably have been improved by preparing stannous chloride from tin and hydrochloric acid immediately before use. This necessity seemed to make the method impracticable. Of the other methods, that in which malachite green is used was the most sensitive (Figure 3). Reagent and color stability was good in all four methods (Figures 1 and 2). Sensitivity to changes in hydrogen ion concentration was examined, and the malachite green method was found to be most sensitive (Figure 4). A degree of insensitivity is important because after the digestion stage, strong acid is neutralized by a strong base, and small pipetting errors at this stage could produce a considerable change in hydrogen ion concentration. Before one of these methods was finally selected,
Treatment of specimens before extraction showed very high losses of phospholipid as a result of either filtration or centrifugation, even at relatively low centrifugal forces. We find that even those specimens containing gross amounts of particulate material are best processed with no prior treatment. Microscopic examination of fresh amniotic fluids indicated that most of the cells present were squamous or mesothelial, of maternal origin, although some fetal cells were present. Most cells did not stain for phospholipid, and the packed cell volume of almost all amniotic fluids did not exceed 1%. We would therefore suggest that the difference in phospholipid before and after centrifugation is not attributable to phospholipid in intact cells. Although the effect of filtration and centrifugation on lecithin/sphingomyelin ratio measurements was not investigated, it is less important because the measurement is relative, not absolute.

Table 3 indicates that the presence of blood in the specimens affects both total phospholipids and the lecithin/sphingomyelin ratio and that specimens with a packed cell volume greater than 1% are unlikely to produce reliable results. It also should be remembered that even if the erythrocytes are removed, the value for phospholipids in serum is relatively high. No significant loss of phospholipid caused by storage of specimens was detected even at room temperature (Table 4). This does not necessarily mean that no degradation has occurred, but merely that the organic phosphate moiety is sufficiently intact to be transferred to the organic phase during extraction. The presence of an obstetric cream did not affect phospholipid measurement.

Technically, estimation of total phospholipids has proved superior in our hands to measurement of the lecithin/sphingomyelin ratio. It is more precise, requires less manual dexterity, takes no more time to do, and is more readily applied to larger workloads. The clinical case is less well proven, mainly because few cases of respiratory distress syndrome were available to us during the time this survey was done. The histogram in Figure 5 shows a marked difference in the normal distribution for the two methods. For this reason the lower limit for the reference ranges was computed differently. The lower 5th percentile was taken for total phospholipids, and the mean minus two standard deviations for the lecithin/sphingomyelin ratio. These are the limits shown in Figure 6. The two cases examined that resulted in respiratory distress syndrome both give borderline results by both methods. Results for the patients with pre-eclamptic toxemia are well distributed among the normal for both methods. In the other cases of complicated pregnancies investigated, the total phospholipids have tended to be low while the lecithin/sphingomyelin ratios were well distributed. This could make the diagnosis of respiratory distress more difficult by the present method. The sharp cut-off point in the normal phospholipid distribution (Figure 5), however, could prove useful in differential diagnosis. It should
be noted that although measurements have been made on specimens before the time of delivery, these have not been included as they are not relevant to diagnosis. Neither have these results been related to gestation, as this test is not an indication of gestational age, but rather of fetal lung maturity.

We thank Miss T. Aviet and her staff for their invaluable help in collecting specimens, and also Dr. J. B. Holton, Southmeads Hospital, Bristol, who supplied some of the specimens. One of us (L.P.B.) is submitting some of this work as part of a thesis for the Fellowship of the Institute of Medical Laboratory Technicians.

References


