creasing the duration of sampling to 30 min. Finally, results by this method correlate well \( (r = 0.903) \) with results by the currently used method.

We suggest that this method is simpler, quicker, and more acceptable to the patient, whilst at the same time just as accurate as the method of Carlson and Rosner. The proposed method can also be used to study children, even newborns.

We acknowledge with thanks the expert technical help of Barbara Babcock, R.N., and Oleta Denz, R.N., CRC Unit; Patricia Zubor, Lipid Laboratory of the Children's Hospital of Buffalo; and Nell Sedransk, Ph.D., Division of Statistical Sciences, State University of New York at Buffalo.

This study was done while R.R. was a recipient of the Dr. Henry C. and Bertha H. Buswell Research Fellowship and was supported by the National Institutes of Health General Research Support Grant 5501 RR-05493-14 and Project 417 from the Maternal and Child Health Service, Department of Health, Education, and Welfare.

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CLIN. CHEM. 25/5, 793–796 (1979)

Assessment of Fetal Lung Maturity by Colorimetric Phospholipid Determination without Digestion

Howard G. J. Worth and Dennis J. Wright

A previously described method for the determination of phospholipid concentration in amniotic fluid without digestion has been modified to make it more suitable for use in a routine laboratory. Results compare well with those by the original procedure. Results from nearly 70 amniotic fluids, collected at delivery, were compared with lecithin/sphingomyelin (L/S) ratios determined on the same fluids. Statistical analysis of these data showed that for the prediction of lung immaturity, determination of total phospholipid concentration was at least as good as the L/S ratio.

The undoubted usefulness of measurement of the lecithin/sphingomyelin ratio (L/S ratio) in amniotic fluid in the assessment of fetal lung maturity is reflected by the large amount of literature that has followed the early work by Gluck and his colleagues (1). Disadvantages of L/S ratio measurement include the labor intensiveness of the procedure, its unsuitability for a routine laboratory, and its precision, which is only that of a semi-quantitative technique. These problems are overcome by measuring total phospholipid concentration colorimetrically by a method not involving a digestion step. We have previously described such a method in which phospholipids are complexed with molybdate through their quaternary nitrogen and then reduced to molybdenum blue (2). This method has been modified by carrying out the initial reaction and the reduction in a single system instead of a biphasic one. Results on more than 100 specimens have been compared with those obtained with the L/S ratio or those obtained by using our previously described method (2). These comparisons enable us to comment on the suitability of this method as a routine technique for the assessment of fetal lung maturity.

Materials and Methods

Reagents

*Dipalmitoyl phosphatidyl choline* (synthetic, 98% pure), Sigma Chemical Co., Poole, Dorset, U.K.

*Dodeca-molybdophosphoric acid*, BDH Chemicals Ltd., Poole, Dorset, U.K.

*Chloroform*, analytical grade.

*Methanol*, analytical grade.

*Stannous palmitate*, Sigma Chemical Co.

Determination of Lecithin by Reduction of Its Molybdophosphate Complex

In the procedure for lecithin determination described previously, molybdophosphoric acid in aqueous solution was reacted with lecithin in a chloroform solution, resulting in the transfer of molybdophosphate into the chloroform phase to form a lecithin–molybdophosphate complex, which was then reduced to molybdenum blue with a reducing agent prepared.
in aqueous solution (2). In our modified procedure, complexing and reduction are carried out in the organic phase.

A solution of dodeca-molybdophosphoric acid (2.7 mL) in chloroform/methanol (20/7, by vol) and 0.3 mL of lecithin in methanol was mixed mechanically for 15 s with 1 mL of water, then centrifuged at 1000 × g for 10 min. A 1-mL portion of the organic (lower) layer was mixed with 2.5 mL of a freshly prepared and filtered solution of stannous palmitate (1 g/L) in chloroform. The absorbance of the resulting molybdenum blue color was measured in 1-cm cuvets at 720 nm vs. a chloroform blank in a spectrophotometer (Pye Unicam, Cambridge, SP 1800). The absorbance was linearly proportional to the concentration of lecithin. The color is stable, so the time between development and measurement is not critical.

Effect of Molybdophosphate Concentration on Final Color Formation

Following the procedure described above, we used various concentrations of dodeca-molybdophosphoric acid up to 50 mmol/L with a lecithin standard at a concentration of 600 μmol/L to determine the effect of molybdophosphate concentration on the final absorbance at 720 nm. The absorbance increased linearly up to a molybdophosphate concentration of 50 mmol/L. At higher concentrations the reagent became biphasic and could be recombined only by increasing the proportion of methanol. We therefore recommend that the concentration of molybdophosphate not exceed 50 mmol/L.

Effect of Stannous Palmitate Concentration on Final Color Formation

We used solutions of various concentrations of stannous palmitate, up to 2.0 g/L, with a molybdophosphate reagent of 50 mmol/L and a lecithin standard of 600 μmol/L. The change in final absorbance at 720 nm with increasing concentration of stannous palmitate is shown in Figure 1. The reagent is unstable; it must be prepared daily and be filtered immediately before use. At stannous palmitate concentrations greater than 1.2 g/L, the molybdenum blue formed by the reaction does not remain in colloidal suspension but precipitates onto the wall of the assay tube. For this reason, a small change in the stannous palmitate concentration has considerable effect on the final absorbance, as illustrated in Figure 1. We now use stannous palmitate at a concentration of 1 g/L. This amount, coupled with molybdophosphate at 50 mmol/L, gives an absorbance of approximately 0.250 for a lecithin concentration of 400 μmol/L.

Use of Other Reducing Agents

We considered using reducing agents other than stannous palmitate, but it was difficult to find reagents that were both chloroform-soluble and sufficiently colorless not to affect the final color measurement. For the three we found, we compared the final color they developed with that for stannous palmitate (1 g/L), using a molybdophosphate reagent at a concentration of 50 mmol/L and a lecithin standard of 600 μmol/L (Table 1).

Measurement of Phospholipid in Amniotic Fluid by the Modified Molybdophosphate Method

Uncentrifuged amniotic fluid (1 mL), 2.7 mL of a solution of dodeca-molybdophosphoric acid (50 mmol/L) in chloroform/methanol (20/7, by vol), and methanol (0.3 mL) were mixed mechanically for 15 s, then centrifuged at 1000 × g for 10 min. A 1-mL portion of the organic (lower) layer was mixed with 2.5 mL of a freshly prepared and filtered solution of stannous palmitate (1 g/L) in chloroform. The absorbance was measured in 1-cm cuvets at 720 nm vs. a blank containing 1 mL of water instead of amniotic fluid. For calibration, we used a lecithin standard in methanol (0.3 mL), to which we added water (1 mL) and the molybdophosphoric acid reagent (2.7 mL).

For large batches of assays the molybdophosphate and stannous palmitate reagents were dispensed with automatic dispensers (Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, U.K.).

Results

Two groups of specimens were collected: (a) those received in the laboratory for the routine determination of L/S ratio (40 specimens) and (b) specimens collected either from patients undergoing elective lower-segment cesarian section or from patients at induction of labor, when membranes were ruptured artificially (68 specimens). The phospholipid concentration of the group a specimens was determined by the modified procedure and by the original method previously described (2). The correlation between the two procedures was highly significant (r = 0.83, p < 0.001). The L/S ratio and the phospholipid concentration (by the new technique) were determined on the specimens in group b. It was assumed that the data obtained from b reflected the situation at delivery and could therefore be subdivided into those data associated with

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>Absorbance at 720 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous palmitate a (60 mmol/L)</td>
<td>zero</td>
</tr>
<tr>
<td>1,4-Benzenediol (10 mmol/L)</td>
<td>0.07</td>
</tr>
<tr>
<td>1,4-Naphthalenediol (12 mmol/L)</td>
<td>0.20</td>
</tr>
<tr>
<td>Stannous palmitate (1 g/L)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

a Ferrous palmitate was prepared by shaking aqueous ferrous sulfate (60 mmol/L) with glacial acetic acid (1 mL) and palmitic acid (60 mmol/L) in chloroform and then using portions of the organic layer.
a normal outcome of pregnancy and those associated with respiratory distress syndrome and other fetal complications. We analyzed the data by computer (ICL 1903A) to calculate reference ranges for pregnancies with normal outcomes.

The between-batch precision of the modified method for phospholipid determination was calculated from data obtained by repeated analyses of portions of amniotic fluid collected at elective cesarian section and stored at −20 °C. The mean of 19 determinations was 95 μmol/L, with a CV of 9.7%.

Pregnancies with Normal Outcome

Statistical analysis showed that data on neither the L/S ratio nor total phospholipid concentration (PL) of specimens taken at delivery from patients who delivered a normal fetus gave a gaussian distribution. This was demonstrated by the Kolmogorov–Smirnov test (3). These data were transformed to give a gaussian distribution by the technique described by Flynn et al. (4). When the L/S ratio was transformed to \( \log_{10} (L/S) \) and total phospholipid concentration to \( \log_{10} (PL) \), the Kolmogorov–Smirnov test was satisfied and the parameters estimating skewness and kurtosis were at a minimum. The original and transformed data are compared in Figures 2 and 3. Using these transformed functions, we calculated a reference range at mean ±1.96 SD. On this basis the reference range for L/S ratio was 1.1 to 6.7; for total phospholipid concentration, 80 to 800 μmol/L.

Pregnancies with Abnormal Outcome

The L/S ratio and total phospholipid concentration were determined for 19 specimens of amniotic fluid from different patients whose pregnancies produced an abnormal fetus. These abnormalities were subdivided as follows:

Respiratory distress syndrome. In five patients, two of whom died, respiratory distress syndrome was diagnosed clinically. The L/S ratio was less than 1.1, except in one case where it was 1.8. Total phospholipid concentrations ranged from 25 to 60 μmol/L. In the cases of the two fatalities, the L/S ratio was 0.7 in both and the total phospholipid concentration was 50 and 60 μmol/L.

Hydramnios. Hydramnios was diagnosed in five patients. In four of these, lung maturity was predicted by both L/S ratio and total phospholipid concentration, the ranges being 2.7 to 3.7 and 100 to 410 μmol/L, respectively. One infant in the group was stillborn; the L/S ratio was 2.7, and the total phospholipid concentration was 100 μmol/L. The fifth infant, who died of multiple abnormalities, had an L/S ratio of 0.8 and a total phospholipid concentration of 25 μmol/L.

Rhesis isoimmunization. Four cases of rhesus isoimmunization had L/S ratios between 1.2 and 1.8 and total phospholipid concentrations of less than 80 μmol/L, except in one instance where the concentration was 120 μmol/L (L/S ratio, 1.2).

Stillbirths or neonatal death. In addition to the four fatalities mentioned above, we investigated five other cases. Two were stillbirths in whom lung immaturity was predicted by L/S ratio and total phospholipid concentration. The other three infants died soon after birth from nonrespiratory causes.

Contaminated Specimens

L/S ratio and total phospholipid concentration were measured on 24 specimens collected at delivery that were contaminated with blood or meconium or both. These data were transformed as described above and compared with the transformed data obtained from uncontaminated specimens by Student’s t-test. For the L/S ratio the mean values were 2.6 and 1.7 for uncontaminated and contaminated specimens, respectively, with \( p = 0.0001 \). For total phospholipid concentration the mean values were 264 and 284 μmol/L for uncontaminated and contaminated specimens, respectively.
contaminated and contaminated specimens, respectively, with $p = 0.0475$. The measurement of total phospholipid was therefore less susceptible to contamination than L/S ratio.

Discussion

The colorimetric measurement of total phospholipid concentration is analytically more precise than is determination of the L/S ratio and, with the elimination of the digestion step, the method is also quicker. The main purpose of the work reported here was to investigate whether the determination of total phospholipid concentration is as good as L/S ratio in predicting fetal lung maturity. The use of molybdophosphate described by us previously (2) had poor sensitivity. Because molybdenum blue was produced at the interface of two phases, it seemed likely that we could improve sensitivity by carrying out the necessary reduction in the organic phase. There was difficulty in finding a chloroform-soluble reducing agent that is colorless and has an appropriate redox potential. Stannous palmitate appears the most suitable, although it is not ideal because of the need to prepare the reagent immediately before use. However, it gives an acceptable sensitivity and precision to the technique.

The L/S ratio and total phospholipid concentration determined on amniotic fluids collected at delivery from patients who produced normal fetuses were used to calculate a reference range, the lower end of which gave a “cutoff” point for predicting fetal lung immaturity. Of the five cases of respiratory distress syndrome encountered during the course of the study, each had a phospholipid concentration below our calculated cutoff point of 50 μmol/L. One L/S ratio, at 1.8, was above our cutoff point of 1.1.

Most laboratories accept a cutoff point of 2.0 or 1.8 for L/S ratio, compared with which 1.1 seems low. However, their figures are usually taken out of context from the literature and not based on rigorous statistical analysis. Gluck (5) states that an L/S ratio of 2.0 or more is compatible with lung maturity, but that at 1.5 approximately 50% of fetuses will require respiratory assistance. Whitfield et al. (6), in a study of over 100 pregnancies, found no respiratory distress in cases where the L/S ratio was greater than 2.0, but in the range 1.5–2.0 25% developed respiratory distress syndrome, and below 1.5 this rose to 80%. These data are consistent with the reference range for L/S ratio we found. Of the normal pregnancies in our study, 27% had an L/S ratio below 2.0. Measurement of total phospholipids appears better to separate the two populations, although an extended prospective study is required to demonstrate this unequivocally. Only experience will show whether the 80 μmol/L concentration of phospholipid will be a useful cutoff value.

In cases of hydramnios, high phospholipid concentrations (>80 μmol/L) were obtained when the L/S ratio exceeded 2.0; i.e., the phospholipid concentration does not seem to be depressed as a result of an abnormally large fluid volume. Low phospholipid values and L/S ratios below 2.0 were obtained in rhesus isoimmunization. These findings are supported by previous workers (7), who found that the L/S ratio increases later in the pregnancy than was expected. The prediction of both measurements seemed to be least accurate in rhesus isoimmunization.

We conclude from this short-term trial that measurement of total phospholipid concentration in amniotic fluid is at least as good a predictor of fetal lung maturity as is L/S ratio. By use of a non-digestive procedure the method is made faster and more precise, with a result on a single specimen being obtainable within 30 min of amniocentesis.

We wish to thank Dr. M. O'Connor, Division of Perinatal Medicine, Clinical Research Centre, for collecting the specimens of amniotic fluid.

References