We assessed the analytical performance of an improved fluorescence polarization assay for use in evaluating fetal lung maturity and compared results with the lecithin/sphingomyelin ratio. During a three-month period 150 patients' samples were assayed by clinical laboratory personnel with both techniques. Values for the lecithin/sphingomyelin ratio correlated closely with net fluorescence polarization values \( r = -0.85 \), less closely with net fluorescence intensity \( r = 0.65 \). Background fluorescence intensity and polarization varied widely, indicating a need to correct measurements for endogenous fluorescence. Net fluorescence polarization values have a CV of 0.32% within-run, 1.07% between-day. A comparison of two amniotic fluid centrifugation procedures showed no significant difference in such values. For both methods, however, such values are slightly but significantly higher than those obtained for amniotic fluids without prior centrifugation. Short-term storage (<30 days) of uncentrifuged amniotic fluid samples at \(-20^\circ C\) does not significantly affect results.

A major complication of premature birth is the development of the neonatal respiratory distress syndrome, which results from insufficient lung surfactant. The advent of fetal lung maturity assays has provided the physician with information about the state of lung surfactant development in utero and has thus aided in decisions concerning elective premature delivery. These assays take advantage of the fact that lung fluids are expressed into the amniotic fluid. Fetal lung maturity assays typically involve quantitative measurement of chemical components or physical properties of the amniotic fluid.

The chemical components most commonly measured in amniotic fluid are phospholipids, e.g., lecithins and phosphatidyglycerols. These phospholipids increase in the amniotic fluid coincident with the maturation of the fetal lung. Assays for these phospholipids commonly require thin-layer chromatography of lipid extracts, a time-consuming and labor-intensive procedure. In addition, the procedures typically demonstrate relatively poor precision, are fairly expensive, and require specialized reagents and equipment.

In 1976 Shinitzky et al. introduced the use of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH)* as a technique for assessing fetal lung maturity. They demonstrated a trend of lower values for fluorescence polarization with increasing gestational age and increasing L/S ratio. Subsequent clinical trials of the fluorescence polarization technique have confirmed that the assay can be used to predict respiratory distress syndrome with an accuracy comparable to that of the L/S ratio (reviewed in (4)). A recent modification of the fluorescence polarization method (5) allows this assay to be performed with a common clinical laboratory instrument, the TDx Analyzer. In order to assess the analytical performance of this assay, we have compared it prospectively with the fetal lung maturity assay currently in use in our laboratories, the lecithin/sphingomyelin ratio (L/S ratio). We have also investigated several aspects of the assay that bear on its use as a routine clinical procedure: its precision, the effect of centrifuging or freezing specimens before analysis, and the contribution of background fluorescence to the measurement of total fluorescence. An initial clinical evaluation is reported in ref. 6.

Materials and Methods

**Specimen collection and handling.** During a three-month period, amniotic fluid specimens were obtained by transabdominal amniocentesis, for assessment of fetal lung maturity by use of the routine L/S ratio assay. Samples of amniotic fluid were routinely stored for less than 24 h at 4 °C before assay. In some cases (described in Results) we compared results for aliquots that had been stored at \(-20^\circ C\) for two to 32 days with those for fresh aliquots, to assess the effect of sample-freezing on fluorescence polarization measurements. Samples were routinely centrifuged (50 \times g, 20 min, 4 °C) before assay. Supernates were stored on ice until assayed. In some cases (as detailed in Results) aliquots of amniotic fluid were respectively subjected to three different centrifugation protocols before fluorescence polarization assay.

Controls were prepared as pools of amniotic fluids, aliquotted and stored at \(-20^\circ C\) until use. They were allowed to thaw at room temperature and analyzed without subsequent centrifugation.

**Equipment and reagents.** Fluorescence polarization measurements were performed, with reagents as described elsewhere (5), in a standard production model of the TDx Analyzer (Abbott Laboratories, Irving, TX 75015).

For thin-layer chromatography we used silica gel plates (5 \times 20 cm, "Baker-Flex"; J. T. Baker Chemical Co., Phillipsburg, NJ) and chromatograms were quantified with an integrating densitometer ("Microzone R-110"); Beckman Instruments, Fullerton, CA). Molybdenum blue reagent (Applied Science Labs., Deerfield, IL) was used to stain thin-layer plates.

Chloroform, methanol, and acetone were reagent grade and were glass-distilled before use.

**Fluorescence polarization.** This assay was performed as described in detail elsewhere (5). Specific conditions used in
obtaining the results reported here were as follows: temperature, 34.5–35.5 °C; incubation time, 6–7 min; 0.5 mL sample of amniotic fluid; 1.0 mL of TDX buffer; final concentration of NBD-PC, 0.5 mg/L. Background fluorescence polarization and intensity were measured before the fluorophore was added. After it was added and the mixture incubated, total fluorescence polarization and intensity were measured. Net intensity and polarization were calculated as described (5). The polarization is reported as a unitless ratio, the fluorescence intensity in arbitrary units. A 10 mL/L solution of Triton X-100 surfactant (Sigma Chemical Co., St. Louis, MO) in TDX buffer (Abbott Laboratories) was used as a polarization control.

L/S ratio. The L/S ratio was determined by a modification of the method of Gluck et al. (7): Control and patients’ samples were extracted with 4.5 volumes of chloroform/methanol (2/1 by vol) at 0 °C. The organic solvent layer was removed after centrifugation (10 min, 280 × g, room temperature) and evaporated under nitrogen at 37 °C. The residue was redissolved in two drops of chloroform and two drops of acetone (0 °C). Acetone (0.8 mL, 0 °C) was then added and sample was incubated for 25–60 min at 0 °C. Samples were then centrifuged (10 min, 800 × g, 4 °C) and the supernate was discarded. The precipitate was redissolved in 30 μL of chloroform and spotted on the pre-washed silica gel thin-layer chromatography plates, which were developed to 10 cm above the origin, air dried, stained by dipping in molybdenum blue reagent, blotted dry, and scanned at 550 nm with the densitometer.

The L/S ratio is calculated by dividing the integrated area for the lecithin spot by that for the sphingomyelin spot.

Results

Precision. The fluorescence polarization assay has a within-run CV of 0.32% for a control pool of amniotic fluid, and a within-run SD of approximately 0.001 for all the patients’ samples (Table 1). Corresponding values for the L/S ratio were 8% and 0.16.

The between-day CV for the polarization assay was about 1% for an amniotic fluid pool and for a 10 mL/L solution of Triton X-100 in TDX buffer (Table 1). In comparison, the CV for the L/S ratio method was 18% for the same specimen of pooled amniotic fluid.

Correlations of fluorescence polarization and L/S ratio. We measured fluorescence polarization in parallel with routine L/S ratio measurements for a total of 150 amniotic fluid samples from third-trimester patients. We stress that there is no a priori reason to expect a perfectly linear correlation between the two methods, because they are measuring different properties of the amniotic fluid. Nevertheless, the L/S ratio is a useful (although imperfect) proxy indicator of fetal lung maturity, and close correlation between the two methods therefore would indicate that fluorescence polarization is also likely to be a good predictor of the respiratory distress syndrome.

Net fluorescence polarization correlates well with the L/S ratio (r = 0.85, Figure 1A). This correlation is slightly closer than that between the L/S ratio and total fluorescence polarization (r = 0.82)—i.e., polarization not corrected for background fluorescence. The only contaminant that appears to affect the relationship between L/S ratio and total fluorescence polarization is meconium. This effect is not seen, however, in the relationship between L/S ratio and net fluorescence polarization.

Net fluorescence intensity is generally greater for more mature samples (r = 0.65), although the correlation is less close than for net polarization (Figure 1B). For the samples in this study, the only contaminant that appears to affect the relationship between L/S ratio and net fluorescence intensity is meconium, which significantly lowers net intensity, presumably by absorbing both excitation and emission light. [However, erythrocytes and abnormally high bilirubin concentrations lower the net intensity in experimental studies (5).]

Figure 2, A and B, illustrates the background fluorescence properties of amniotic fluid. Most samples of amniotic fluid have a low background fluorescence intensity relative to the net fluorescence intensity (<2000 vs 10 000 and 30 000 arbitrary units), but occasional samples give much higher values. Background polarization varies widely (Figure 2B); correlation for background is therefore desirable. One of the highest background fluorescence intensities we recorded was for a sample contaminated with meconium—but two other samples with no apparent contamination gave even higher background values.

Effect of centrifugation on fluorescence polarization measurements. Samples of amniotic fluid are often centrifuged before the L/S ratio is measured, to remove whole cells and gross debris, but there is evidence that too-vigorous centrifugation may falsely lower the L/S ratio [reviewed by Brown and Duck-Chang (2)]. We therefore assessed the effect of centrifugation on polarization values for patients’ amniotic fluid for three different protocols: (a) no centrifugation, (b) “low-speed” centrifugation (50 × g, 20 min, 4 °C), and (c) “high-speed” centrifugation (450 × g, 2 min, 4 °C). The “high-speed” protocol is intended to be used with the polarization assay, and is expected to give the same results because the product of g value and time is approximately equal to that of the “low-speed” protocol. We made pairwise comparisons of the protocols by linear regression with the Deming technique (8) to detect possible constant or proportional biases.

Centrifugation by either protocol increases net polarization, relative to that for uncentrifuged samples, by a constant amount (Table 2). However, the mean increase (approximately 0.005) is very small as compared with the full clinical range of polarization values: 0.340–0.180 (Figure 1A). As predicted, there is no difference between the “low-speed” and “high-speed” protocols. Thus, minor variations in centrifugation procedures should have little effect on the precision and standardization of the assay.

| Table 1. Precision of the Present Assay and of the L/S Ratio |
| Sample | Measurement | Mean | SD | CV, % | n |
| Within-run All patients | net polarization | — | 0.0010 | 448 |
| | L/S | 0.16 | 300 |
| Amniotic fluid pool | net polarization | 0.2406 | 0.0008 | 0.13 | 16 |
| Between-day Triton X-100, 10 mL/L | net polarization | 0.1783 | 0.0013 | 0.7 | 53 |
| Amniotic fluid pool | net polarization | 0.2413 | 0.0026 | 1.1 | 34 |
| | L/S | 2.67 | 17.7 | 75 |

*Net polarization is expressed as a unitless ratio. The within-run SD was calculated from the difference between n paired measurements, d: SD = \sqrt{\frac{1}{2} \sum d^2 / n}.
Fig. 1. A: L/S ratio vs net fluorescence polarization
L/S ratio and net fluorescence polarization were measured for a total of 150
patients' samples during three months. Linear regression of the first kind yields:
slope = -.032, y-intercept = .322 (r = -.66). Each run included a 10 ml/L
solution of Triton X-100 in TDX buffer; the mean net intensity for all runs was
28.277 arb. units (n = 53). 0 = no apparent contamination; 1 = bloody; 3 =
meconium in sample. The polarization units are net polarization x 1000
B: L/S ratio vs net fluorescence intensity
Same samples as in 1A. Linear regression of the first kind: slope = 3856, y-
intercept = 13.477 (r = .05). Symbols as in A

Fig. 2. A: L/S ratio vs background fluorescence polarization
Same samples as in 1A. Linear regression: slope = -.024, y-intercept = .331 (r
= -.31). Symbols as in Fig. 1A
B: L/S ratio vs background fluorescence intensity
Same samples as in Fig. 1A. Linear regression: slope = .64, y-intercept = 1298 (r
= .07). Symbols as in Fig. 1A

Table 2. Effect of Centrifugation on Net Fluorescence Polarization

<table>
<thead>
<tr>
<th>Comparison</th>
<th>n</th>
<th>Slope</th>
<th>SD</th>
<th>t</th>
<th>p</th>
<th>Mean</th>
<th>SD</th>
<th>t</th>
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<tbody>
<tr>
<td>None vs low-speed</td>
<td>48</td>
<td>.975</td>
<td>.015</td>
<td>1.65</td>
<td>.10</td>
<td>-.044</td>
<td>.0006</td>
<td>-7.61</td>
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<tr>
<td>None vs high-speed</td>
<td>47</td>
<td>.975</td>
<td>.017</td>
<td>1.51</td>
<td>.14</td>
<td>-.050</td>
<td>.0006</td>
<td>-7.59</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Low- vs high-speed</td>
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<td>.999</td>
<td>.016</td>
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<td>-.003</td>
<td>.0006</td>
<td>-0.60</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

*Slopes were calculated by linear regression of the second kind (8). Null hypotheses were: slopes are 1.0 and mean differences are 0.0. Statistical test used was
two-sided t-test. *Low-speed" centrifugation: 50 x g, 20 min; "high-speed" centrifugation: 450 x g, 2 min.
Effect of freezing amniotic fluid. We used 30 patients' samples to test the effect of short-term freezing on values for net fluorescence polarization. Polarization was measured on fresh samples after centrifugation at 50 × g for 20 min. Uncentrifuged samples were stored at -20 °C for two to 32 days before being thawed at room temperature; these samples were then centrifuged at 450 × g for 2 min before measurement. We saw no significant difference in regression slopes or means between the two sets of measurements (data not shown). Evidently, short-term freezing of uncentrifuged amniotic fluid specimens does not affect their net polarization values.

Discussion

In this study, we have used the L/S ratio as a proxy indicator of fetal lung maturity and the risk of respiratory distress syndrome. Our results demonstrate a strong negative correlation (r = -0.85) between the net fluorescence polarization of NBD-PC and the L/S ratio (Figure 1A). We believe that this correlation, although not strictly a method versus-method comparison, indicates the potential clinical value of the NBD-PC fluorescence polarization assay. In several previous studies, the DPH fluorescence polarization assay has been compared with L/S ratio measurements (3, 4, 9–16), and all of these studies showed a strong negative correlation between net fluorescence polarization and L/S ratio. Correlation coefficients ranged from -0.47 to -0.76 for these assays, all of which were based on the use of DPH as the fluorescent dye.

Net fluorescence intensity correlates fairly closely with the L/S ratio, although the correlation is not as strong as it is for net polarization (r = 0.65 vs -0.85). As seen in Figure 1B, several samples gave values for net fluorescence intensity that deviated sharply from the others. Moreover, fluorescence intensity measurements are more difficult to standardize than fluorescence polarization values; because they are affected by the additional variables of illumination intensity, detector response, fluorophore concentration, and quenching. Use of a fluorescence intensity standard (e.g., Triton X-100) would mitigate some but not all of these problems. Thus net fluorescence intensity values alone will probably be less reliable than net fluorescence polarization values in predicting fetal lung maturity, although they may be useful in combination with net fluorescence polarization in detecting certain interfering substances.

Background fluorescence intensity values do not correlate with L/S ratio at all (Figure 2B) and are typically <10% of the corresponding net fluorescence intensity values. The effect of such background fluorescence on total fluorescence polarization and intensity is, therefore, relatively small compared to the fluorescence of the exogenous fluorescent dye, NBD-PC. There are, however, numerous examples of amniotic fluids for which background fluorescence intensity values are substantially higher; for these samples background fluorescence makes a relatively large contribution to values for total fluorescence polarization and intensity. Such samples can be contaminated or seemingly uncontaminated (Figure 2B). In these cases the calculation of net fluorescence polarization and intensity values makes a significant correction for endogenous fluorescence. We conclude that background correction is desirable at the wavelengths used for the present assay, as it is at the different wavelengths used for the DPH assay (4). We are currently exploring the use of background fluorescence intensity values as a discriminant for certain kinds of contamination, such as bilirubin and meconium.

The fluorescence polarization technique used in this study yields a within-day CV of less than 0.5% and a between-day CV of 0.7–1.1% (Table 1). This performance is better than that of previous fluorescence polarization assays with DPH, which have yielded within-day CVs of 1.2–3.7% (with SD = 0.003–0.010) (4, 17, 18), and between-day CVs of 0.8–3.0% (with SD = 0.010) (4, 17, 19). The L/S ratio, however, is much less precise: we find a within-run CV of 7.9% and a between-day CV of 17.7%. This relatively poor precision has necessitated duplicate-sample analyses for reasonably accurate determination of the L/S ratio in our laboratory, which is not necessary with the present assay. The analytical precision of the present assay makes it potentially more valuable clinically than the L/S ratio assay, although it is not yet clear whether biological variation overwhelms analytical variation in determining the clinical performance of the fluorescence polarization assay.

The present assay also seems to be superior to the L/S ratio on a variety of technical points: There are many fewer steps for sample preparation and measurement. In addition, only a 0.5-mL sample is required for the polarization assay. The assay has a turn-around time of approximately 30 min, a significant improvement over the L/S ratio assay, which requires 4–5 h in our laboratory. Finally, the use of defined detergent (or oil) standards at a standard temperature in the fluorescence polarization assay should make possible the interlaboratory standardization of fetal lung maturity testing that is lacking in the current L/S ratio methodology. The present assay is also superior to the DPH fluorescence polarization assays in that it allows the use of an instrument (the TDx) that currently already is used in many clinical laboratories (5).

The true test of a new assay for fetal lung maturity, however, is its clinical performance, the ability to predict those pregnancies at risk for respiratory distress syndrome upon premature delivery. A limited retrospective clinical study is reported elsewhere (6). Maternal and infant chart review is being performed for all results from samples obtained within three days of delivery; the results will be the subject of a future report.

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References


