Rapid Thin-Layer Chromatographic Method for Assessing the Lecithin/Sphingomyelin Ratio in Amniotic Fluid

Submitters: Emily H. Coch, Gerald Kessler, and John S. Meyer, Department of Pathology and Laboratory Medicine, The Jewish Hospital of St. Louis, St. Louis, Mo. 63110

Evaluators: Paul T. Russell, Department of Obstetrics and Gynecology, University of Cincinnati Medical Center, Cincinnati, Ohio 54229

Donald T. Forman, Division of Clinical Biochemistry, Evanston Hospital, Evanston, Ill. 60201

Laszlo Sarkozi and Hahn N. Kovacs, Mount Sinai School of Medicine, New York, N. Y. 10029

Introduction

The method described here is essentially that of Gluck (1), for assessing the lecithin/sphingomyelin (L/S) ratio in amniotic fluid. It is modified to decrease assay time and allow easier estimation of the L/S ratio in laboratories that do not possess densitometric scanning equipment.

The ratio is a useful and practical index of fetal pulmonary maturity (1–6). Lecithin is a principal component of pulmonary surfactant, a material that coats the air sacs of the lungs and lowers surface tension, thereby allowing the lungs to maintain sufficient residual air volume upon expiration to prevent alveolar collapse. Synthesis of this pulmonary surfactant by the fetus first becomes significant (and it first appears in amniotic fluid) sometime during the 32nd to 36th week of gestation. Infants born after this time usually will have no difficulty with breathing. If pregnancy is terminated prior to this time, however, there is a high risk of development of the respiratory distress syndrome, which involves the collapse of pulmonary alveoli despite intense inspiratory effort and possible formation of hyaline membranes (1).

The lecithins involved are mainly dipalmitoyl lecithin and α-palmitoyl, β-myristyl lecithin, but the rapid increase in surface-active lecithin observed between 32–36 weeks of gestation is mainly an increase in dipalmitoyl lecithin (1). The changing concentrations of lecithin in the amniotic fluid are measured and are most conveniently expressed as a ratio of lecithin to another phospholipid, sphingomyelin, which is present in much more nearly constant concentrations [until about the 35th week of gestation, when it gradually decreases (1)].

Gluck et al. (1) described a method for extracting lecithin and sphingomyelin from amniotic fluid, concentrating the “surface-active” lecithin and sphingomyelin by cold acetone precipitation, separating the two phospholipids by thin-layer chromatography on glass plates coated with silica gel, and then making them visible by an acid-spray, heat-char technique (1). They have demonstrated, by densitometric quantitation of the lecithin and sphingomyelin in more than 300 amniotic fluid specimens, that the L/S ratio is 1 or less before 32 weeks gestation, and that the ratio increases between the 32nd and 36th week, corresponding to the sharp surge in fetal synthesis of pulmonary surfactant. By this procedure, an L/S ratio of 2 or greater indicates fetal pulmonary maturity, with minimal risk of respiratory distress syndrome. These criteria have been corroborated in other laboratories (3, 4, 6).

In the procedure we describe here, the phospholipid fraction is directly extracted and concentrated, but no precipitation with cold acetone is used and the two phospholipids are separated more quickly by use of a different thin-layer chromatographic support medium. The visualization technique described here
yields higher values for the L/S ratio for specimens from immature and mature subjects than does Gluck’s procedure, does not require a densitometer, and it differentiates those lecithins peculiar to amniotic fluid from those peculiar to blood (and thus can indicate when there is significant contamination of amniotic fluid with blood).

**Principle**

Phospholipids are extracted from amniotic fluid into chloroform and concentrated by evaporation. In the concentrated extract, lecithin is separated from sphingomyelin by thin-layer chromatography on glass-fiber sheets impregnated with silica gel. Phospholipid standards are run concurrently. After chromatography, the phospholipids are made visible by the independent use of two different reagents. One reagent, containing bismuth subnitrate, is relatively specific for lecithin and sphingomyelin, because it produces an orange color with compounds containing a choline residue. Thus dipalmitoyl lecithin and sphingomyelin appear as bright yellow-orange spots (7). In the second technique, the developed chromatogram is exposed to iodine vapor, whereupon all phospholipids appear as yellow to brown spots (8). The latter procedure is less specific but is about threefold as sensitive as the former. It is nondestructive; that is to say, the part of the chromatogram exposed to iodine vapor can also later be used for other detection methods, if desired.

The size of the lecithin spot is compared visually to that of the sphingomyelin spot (or the amounts can be semi-quantitated by measuring the areas with a grid). The ratios are reported as whole numbers and are interpreted as described below.

**Materials**

**Reagents**


*Ammonium hydroxide*, reagent grade (Fisher Scientific Co.)


*Bismuth subnitrate*, powder (Matheson, Coleman and Bell, Norwood, Ohio 45212).

*Potassium iodide*, certified A.C.S. (Fisher Scientific Co.).

*Iodine crystals*, U.S.P. sublimed (Fisher Scientific Co.).

*Bismuth spray reagent*:

Solution A: 3.48 mol/liter acetic acid. Dilute 200 ml of glacial acetic acid (17.4 mol/liter) to one liter with distilled water.

Solution B: 1.7 g bismuth subnitrate in 100 ml of solution A. Store in a brown bottle. The compound requires about 3–4 h to dissolve.

Solution C: 40 g of KI in 100 ml of water (this must be stored in a brown bottle). If the solution is not colorless, discard it and prepare a fresh solution.

**Final working spray (to be stored in a brown bottle):**

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 ml</td>
<td>solution A</td>
</tr>
<tr>
<td>20 ml</td>
<td>solution B</td>
</tr>
<tr>
<td>5 ml</td>
<td>solution C</td>
</tr>
</tbody>
</table>

*Phospholipid stock standard* (General Biochemicals, Chagrin Falls, Ohio 44022). L-α-Lecithin (dipalmityl, synthetic), sphingomyelin, phosphatidylethanolamine, and lysolecithin. Store at −20 °C.

**Thin-layer chromatography working standards:**

Store at −20 °C.

Mix No. 1: lysolecithin and phosphatidylethanolamine, each at 2.0 g/liter of chloroform.

Mix No. 2: L/S ratio, 2:1; lecithin and sphingomyelin at 4.0 g/liter and 2.0 g/liter of chloroform, respectively.

Mix No. 3: L/S ratio, 5:1; lecithin and sphingomyelin at 10.0 and 2.0 g/liter of chloroform, respectively.

**Apparatus**

*Thin-layer chromatography chamber*; Chromatography Kit (Eastman Kodak Co., Rochester, N. Y. 14650).

*Thin-layer chromatography sheets*: 20 × 20 cm silica gel impregnated glass fiber sheets (ITLC-SG) (Gelman Instrument Co., Ann Arbor, Mich. 48106).

*Gelman spotting guide* (Gelman Instrument Co.).


*Extraction tubes*, Pyrex, 25 × 150 mm, with Teflon-lined screw tops.

*Chromatography spraying vessel* (Scientific Products, St. Louis, Mo. 63043).

*Evaporating dish*, Pyrex, 80 × 45 mm.

**Collection and Handling of Specimens**

When received, amniotic fluid is centrifuged for 10 min at 800–1100 × g (2300 rpm in a Sorval GLC-1 centrifuge with Type HL4 head or at about 1600 rpm in an International Model V with Type 240 head). Specimens are not assayed if the specimen appears visibly bloody, with a hematocrit greater than 1.5% (see below), or if meconium is present, because other forms of lecithin are present in these contaminants (1, 2, 5, 13, 16, 17).

Not: Centrifugation of material for 10 min increases the temperature of the material by less than 5 °C above ambient.

The supernatant fluid is removed by pipet and used immediately for the L/S assay and any other chemical determinations (25). The sedimented pellet may be used for staining with Nile Blue Sulfate (9, 25). If the L/S assay cannot be performed the same day, the supernatant material is frozen at −20 °C. If the assay is only delayed for 1–2 h, the supernate is refrigerated.
Procedure

Mix 5 ml of the amniotic fluid supernate with 5 ml of methanol in one of the extraction tubes. When less material is available, 3 or 4 ml can be assayed, but all reagent volumes are reduced proportionately. For the most meaningful interpretations, however, 5 ml should be extracted.

Add 10 ml of chloroform, cap tightly, and shake for 5 min to extract. Centrifuge at 600 X g (approximately 1700 rpm with the International Model V with type 240 head) for 5 min.

Remove and discard the aqueous (upper) layer. Filter the lower (chloroform) phase through Whatman No. 1 paper into an evaporating dish. Alternatively, filter through Whatman No. 1 PS (siliconetreated phase-separating paper) or through anhydrous magnesium sulfate (6) to remove the maximum amount of water before the evaporation.

Concentrate the material to about 0.5 ml by using a gentle stream of air flowing over the solvent surface. (The material may be allowed to go to dryness, but exposure to room temperature should be limited to 5–10 min.) This evaporation step requires 20 to 30 min.

Note: Some workers prefer to use a stream of nitrogen rather than air (1, 2, 4) or to use a rotary-type evaporator (at low pressure and elevated temperature) (6). These alternatives may be used if the temperature is not allowed to rise above 60 °C. Use of a stream of air as described above keeps the temperature of the evaporating chloroform extract below 4 °C, and there appears to be no selective loss of phospholipid over the time period as assessed by relative recoveries of standard L/S mixes carried through this procedure.

Rinse the dish with 1 to 2 ml of chloroform and transfer the rinsings to a 13 X 100 mm tube. Further evaporate to about 50 μl (i.e., 1–2 drops).

The concentrated extract can be stored at −20 °C at this point if the chromatography cannot be done the same day.

Note: If it is desired to include a precipitation step with cold acetone, as recommended by Gluck (1), evaporate the chloroform extract to 0.1–0.2 ml in a conical-tip glass-stoppered centrifuge tube. Add 10 ml of ice-cold acetone dropwise with gentle mixing of tube. Stopper the tube and chill it at −20 °C for 1 h. Centrifuge at 600 x g for 5 min (do not allow the temperature of material in tube to exceed 8 °C). Immediately and carefully draw off the supernate, which can be concentrated by evaporation for thin-layer chromatographic analysis. The precipitated material is redissolved in 2 ml of chloroform and concentrated by evaporation to about 50 μl for thin-layer chromatographic analysis. We do not find this precipitation necessary, as will be discussed below.

Place a 20 X 20 cm chromatographic sheet on the Gelman spotting guide and mark off 11 points. These points are approximately 2 cm apart and 2.5 cm from the bottom edge of sheet.

At points 1 and 11, apply 5 μl of standard mix No. 1 (lyssolecithin and phosphatidylethanolamine, 1:1). At points 3 and 7, apply 5 μl of mix No. 2 (L/S = 2). At points 5 and 9, apply 5 μl of mix No. 3 (L/S = 5).

There is sufficient space on the sheet for duplicate applications of samples from two patients, or of the acetone precipitate and acetone supernate of the same patient, if desired. Divide one sample equally between points 2 and 8 and a second sample between points 4 and 10. Leave point No. 6 empty. Apply 5 μl at a time to ensure spot diameters of less than 4 mm. Use the entire extract.

Prepare the solvent freshly each time: 170 ml of chloroform, 20 ml of absolute methanol, and 3 ml of concentrated ammonium hydroxide.

Assemble the Eastman Kodak chromatography easel and trough. Pour the solvent into the trough up to the etched line. Place the thin-layer chromatography sheet into the Eastman Kodak chromatography chamber, clip the sides, and set the chamber into the trough. Allow development to proceed for 15 min.

Remove the sheet and allow it to air dry for 5 min. Carefully divide lengthwise along the center (point No. 6).

Place one half of the sheet into a closed chamber containing iodine crystals for at least 5 min. Yellow to brown spots will be observed. Remove the sheet and immediately circle the spots lightly with a pencil. (If the spots should fade too quickly, they will appear again upon re-exposure to iodine vapor.)

Spray the other half of the sheet thoroughly with the bismuth spray until the standards appear as whitish spots against an orange background, and allow it to air dry for several minutes.

Place the bismuth-stained sheet in a pan containing solution A (3.48 mol/liter acetic acid) and swirl the pan gently until the background is again white. The lecithin and sphingomyelin standards will appear as yellow-orange spots. Remove the sheet from the pan and circle the spots lightly in pencil. They are better seen by holding the sheet up to a light. Observations should be made as soon as possible after destaining, because the background color gradually (within 15 min) reappears.

Visually compare the size of lecithin and sphingomyelin spots, and report the results as listed under "Interpretation."

When the sheets are dry, wrap completely in cellulose sheeting ("Saran Wrap") and tape to a sheet containing all pertinent information.

After the iodine-stained spots have been allowed to fade, the iodine-stained half of the sheet may be used for several other types of visualization techniques, including 8-anilino-1-naphthalene sulfonic acid (ANS), phosphomolybdic acid–stannous chloride, or acid spray and heat char as described below.

Other techniques that may be used to make the spots visible are:

(a) ANS: Spray the developed and dried chromatogram with a 0.25 g/liter aqueous solution of ANS.
or iodine of which appear.

(c) Acid spray or dip, heat char: Spray the chromatogram either with 9 mol/liter \(\text{H}_2\text{SO}_4\) (1) or with aqueous ammonium sulfate (300 g/liter) acidified with 12 ml of concentrated sulfuric acid per deciliter (6). Heat for 5 min over a hot plate set on high heat (do not allow sheet to touch surface of plate). An alternative spray consists of phosphoric acid (18 g/ml) containing cupric sulfate pentahydrate (8 g/ml), which reportedly gives charred spots within 15 min at a lower temperature (100 °C) (Evaluator D. T. F.).

(d) Phosphomolybdic acid–stannous chloride: Dissolve 1 g of phosphomolybdc acid in 50 ml of chloroform and 50 ml of methanol. Spray the sheet thoroughly. Overspray with a solution containing 1 g of stannous chloride per deciliter of dilute (2 mol/liter) HCl. Heat the sheet over a hot plate until blue spots appear.

Note: The following improvement has been reported for use with thin-layer chromatography plates (22). It has not been tried by the Submitters on Gelman ITLC sheets. The following solutions are needed: Solution A. 4 g of molybdenum trioxide is added to enough 25 mol/liter \(\text{H}_2\text{SO}_4\) to make a final volume of 1 dl. Boil the mixture gently until all trioxide salt is dissolved. Solution B. Add 175 mg of powdered molybdenum to about 50 ml of Solution A. Boil gently for 15 min and cool. Carefully mix solutions A and B and dilute to 1 dl with distilled water. This reagent is stable at room temperature for at least six months in an amber-colored bottle. It reportedly can be sprayed on thin-layer chromatography plates and blue spots will develop at room temperature immediately.

Interpretation

Immature (high risk of respiratory distress syndrome): L/S ratio, 2 or less.

Lecithin and sphingomyelin appear as faint traces or as approximately equal but small spots by bismuth stain. By iodine stain, L/S ratio is 2 or less.

Transitional (some risk of respiratory distress syndrome but survival probable): L/S ratio between 2 and 5.

Definite but small lecithin spot and only a trace or no sphingomyelin by bismuth (size of lecithin usually less than that in the 2:1 standard). By iodine staining, the ratio of spot sizes is greater than 2 but less than 5.

Mature (minimal risk of respiratory distress syndrome): L/S ratio, 5 or higher.

Definite lecithin and no sphingomyelin by bismuth (size of lecithin usually greater than the lecithin spot of the 2:1 standard). By iodine staining, the ratio is 5 or greater. (No sphingomyelin may be seen, even with iodine staining, in many cases.)

Discussion

This method includes several modifications of Gluck’s original assay (1), all designed to reduce assay time and facilitate interpretation in the absence of densitometric scanning equipment.

Use of glass-fiber sheets impregnated with silica gel rather than coated glass plates or Mylar sheets decreases solvent development time from at least 1 h to only 15 min and makes it easier to spray and then destain by immersion. The Eastman Kodak chromatography chamber, with which the sandwich plate technique is used, accommodates the Gelman ITLC sheets easily and obviates the need to pre-equilibrate the chamber with solvent. The sheets are easy to divide for different visualization techniques after solvent development and easy to store. We observe clean separation of lecithin from sphingomyelin and clean separation of both from other phospholipids that might be present in amniotic fluid (11). Representative \(R_F\) values are: phosphatidyl ethanolamine 0.72; lecithin, 0.67; sphingomyelin 0.48; lysolecithin, 0.32; phosphatidyl serine, 0.30; and phosphatidylinositol (or phosphatidylinositol), 0.23. (The latter three standards were obtained from General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio 44022; Cat. No. 100130, 100120, and 800120, respectively.) Both cholesterol and triglyceride (triolein) migrate with or just behind the solvent front in this system. These lipids are present in the “surface-active” fraction of rabbit lung washings (23).

Evaluation of the L/S ratio is based on the use of a reagent that makes visible both dipalmitoyl lecithin and sphingomyelin as bright yellow-orange spots against a white background. This modified Dragen-dorrh stain (7), containing bismuth subnitrate and potassium iodide, reacts with compounds possessing a choline residue. Because sphingomyelin and lecithin each contain one choline moiety per molecule and are of similar molecular weight, equal concentrations will display spots of similar size and color intensity. Lysolecithin is also made visible, but the spot is a darker shade of orange and is well resolved from lecithin and sphingomyelin; in our experience, it is only seen in amniotic fluids from term or near-term infants (such fluids contain large amounts of lecithin) or in specimens contaminated with blood. The bismuth subnitrate spray can detect sphingomyelin at a concentration of 1 mg/ml (dipalmitoyl lecithin stains slightly more than does sphingomyelin with this stain, and therefore its detection limits are a little lower). From analyses of 103 amniotic fluids, we conclude that the concentration of sphingomyelin in amniotic fluid is usually less than 2 mg/ml when compared with extracted L/S standard mixes of known concentration.

Most specimens from mature fetuses do not show any sphingomyelin by this stain, and many specimens from immature fetuses show only the faintest traces of both lecithin and sphingomyelin. Therefore an alternative, more sensitive stain is also needed.

CLINICAL CHEMISTRY, Vol. 20, No. 10, 1974 1371
that will always make visible the lecithin and sphingomyelin in early amniotic fluid specimens, i.e., that can detect concentrations of 0.5 mg/dl or less. Iodine vapor, although less specific (8), has the necessary sensitivity. Although pure dipalmitoyl lecithin does not stain with the same color intensity as does sphingomyelin when exposed to iodine vapor, we observed that dipalmitoyl lecithin added to amniotic fluid previously shown to have only traces of lecithin and sphingomyelin did exhibit a color intensity similar to that of sphingomyelin; we have no explanation for this phenomenon. At this time we have performed over 200 analyses of the kind described here, and in over 95% of these assays the ratio estimated on the basis of the bismuth stain correlated with that observed by iodine vapor according to the scheme listed under “Interpretation.” The only cases in which the results of the two techniques did not agree at all involved bloody taps, and the significance of blood contaminatin will be discussed briefly below. The iodine vapor stain is of greatest use in confirming an interpretation of immaturity or transitional maturity: both lecithin and sphingomyelin spots may be very small when made visible by the bismuth stain, but both lecithin and sphingomyelin always appear with iodine vapor in both these categories. Specimens for which a substantial lecithin spot, but no sphingomyelin, was revealed by bismuth (larger than the lecithin standards) are easily interpreted as mature and display large lecithin spots with only faint trace, if any, of sphingomyelin by iodine vapor.

The recoveries of lecithin and sphingomyelin were found to be equivalent by extracting various dipalmitoyl L/S standard mixes (12). The concentration of sphingomyelin was 2 mg/dl and the concentration of lecithin ranged from 2 to 12 mg/dl. Relative recoveries were similar when sphingomyelin was decreased to 1 mg/dl.

The cold-acetone precipitation step recommended by Gluck to eliminate “non-surface-active” lecithins (1) is routinely omitted, but instructions for the precipitation have been included so that the reader may make his own comparison. The interpretation of 10 different amniotic fluid specimens in this laboratory remained the same whether or not this precipitation step was included [eight of these were interpreted as “mature” and two as “immature” (12)]. There was a loss in sensitivity noted, however (i.e., smaller thin-layer chromatographic spots for the specimens carried through the precipitation step). In addition, a 1:1 and a 4:1 L/S standard mix were extracted with and without the precipitation step. There was no difference in the results obtained, but again the sensitivity was lower after precipitation. When the acetone supernates were assayed, both dipalmitoyl lecithin and sphingomyelin could be observed. Sarkszi et al. (6) and Forman et al., who utilize the same extraction and almost the same thin-layer chromatography and visualization techniques as Gluck, also have reported that the precipitation with cold acetone is unnecessary and does not alter the interpretation. It is critical that great care be exercised to ensure that the relative recoveries of dipalmitoyl lecithin and sphingomyelin are the same, if this step is used (26).

Although the bismuth subnitrate and iodine vapor techniques do not readily lend themselves to quantitation by densitometry, because the spots fade on the I2 sheet and gradual background color develops on the bismuth-sprayed sheet, the clinical followup information available indicates that the L/S assay as described (i.e., involving visual determination only) reliably indicate fetal pulmonary maturity. Of 86 patients for whom follow-up information was available, the last L/S ratio was measured within 24 h of delivery on 28. Of these 28 patients, the interpretation was “immature” (L/S < 2) for two; both infants developed respiratory distress syndrome, one “moderate” and one “severe” with immediate death. In addition, there were three “transitional” cases; two of these (each L/S = 3) survived with no evidence of respiratory distress syndrome and one (L/S = 4) developed “mild” respiratory distress syndrome but survived; the latter case involved moderate to severe Rh isoimmunization. Of the 23 cases for whom the interpretation was “mature” (results obtained within 24 h of delivery), two exhibited “mild” respiratory distress syndrome, but both survived (25). One of these cases (L/S = 5) involved a diabetic mother; the second mother underwent a cesarean section. A frozen aliquot of the second specimen (L/S = 10) was sent to Dr. Gluck’s laboratory; the value they obtained was 3.2, which according to their criteria (1) was also interpreted as mature. In summary, of the total of 71 cases interpreted as “mature,” two showed “mild” respiratory distress syndrome and both survived (the interval between the last assessment of L/S ratio and delivery ranged from 0 to 29 days). Of a total of four “transitional” results, all survived, with one case of respiratory distress syndrome (intervals were 24-48 h). Of a total of 11 “immature” results there were five cases of respiratory distress syndrome, four of them “severe,” and three deaths (intervals were 1, 3, 3, 23, and 60 days).

There have been other cases cited in the literature in which respiratory distress syndrome developed after a “mature” interpretation was made (13). A prognosis of “maturity” based on the L/S ratio does not invariably assure that respiratory distress syndrome will not develop, especially in pregnancies involving maternal toxemia and diabetes mellitus. Conversely, an L/S interpreted as “immature” within 48 h of birth is not an infallible indicator that respiratory distress syndrome will develop (2-4, 14).

The L/S range indicated in the interpretation scheme is considerably wider than that of Gluck et al.

---


3 We would like to thank Dr. Louis Gluck and Mrs. Marie Kulovich for performing this analysis for us.
(1), who report an L/S of less than 1.5 as immature, 1.5–1.8 as transitional, and 2 or greater as mature. The variation in range is mainly due to the difference in visualization techniques. The bismuth, \( I_2 \), and ANS reagents give L/S ratios that more closely correlate to the actual weight ratio of the two phospholipids. The acid spray or dip-and-heat-char technique emphasizes sphingomyelin much more than lecithin. Sarkozi et al. (6) have illustrated that, with the latter procedure, the densitometric L/S ratio increases at about only a half to a third the rate of the weight ratio. Gluck and Kulovich (15) state that an L/S of 2 obtained by their densitometric method actually reflects a molar ratio of L/S concentrations of about 4. The difference in visualization techniques also explains why sphingomyelin is rarely seen in mature specimens, even with iodine-vapor staining, whereas a sphingomyelin spot is usually noticeable with the heat-char technique.

Dipalmitoyl lecithin is routinely used as the standard. If some other lecithin is used, its behavior with respect to sphingomyelin in the various visualization techniques must be evaluated. Egg lecithin, which contains the unsaturated fatty acid, oleic, and possibly its saturated counterpart, stearic, behaves differently with the bismuth stain and the acid spray and heat-char technique. Whereas dipalmitoyl lecithin, upon spraying with the bismuth reagent, appears bright yellow-orange, similar to an equivalent amount of sphingomyelin, the spot produced with an equal amount of egg lecithin is distinctly browner and much fainter. With the acid spray and heat-char technique, dipalmitoyl lecithin does not char as intensely as does sphingomyelin and egg lecithin; 5 \( \mu g \) of egg lecithin chars identically with 5 \( \mu g \) of sphingomyelin, but both char much more darkly than 5 \( \mu g \) of dipalmitoyl lecithin. In addition, dipalmitoyl lecithin is relatively insoluble in cold acetone, but egg lecithin is soluble. Kits are supplied commercially (Supelco, Bellefonte, Pa. 16823) that contain individual vials with various ratios of L/S specified (1:1, 1.5: 1, and 2:1). These kits are intended for use with the acid-treated, heat-char technique (1, 6). The lecithin in these vials behaves similarly to egg lecithin with the bismuth reagent, the acid-spray, heat-char technique, and with respect to its solubility in cold acetone (12). The 1:1 L/S mixture, for example, will give two spots of equal size and intensity when charred after acid treatment (1, 6), but cannot be used as a standard for the bismuth spray or to test the recoverability of “lecithin” by the cold acetone precipitation technique. Therefore, such kits should only be used with the specific technique against which they were standardized.

An additional advantage of the bismuth stain is that it distinguishes dipalmitoyl lecithin from the lecithins in serum and erythrocytes and will therefore indicate if there is significant contamination of the sample with blood. The latter class of lecithins behaves similarly to egg lecithin; i.e., when sprayed with the bismuth reagent, they stain a distinctly darker shade of orange (almost brown). Iodine vapor will not distinguish the various forms of lecithin.

Extraction of standard mixes of known L/S ratio, each titrated with increasing amounts of serum, demonstrated that serum in excess of 0.1 ml per 5 ml of amniotic fluid may distort the L/S ratio (12). (We have also used plasma and hemolyzed erythrocytes, and the results were similar.) The extent or direction of this distortion is not easy to predict. In general, if the specimen is contaminated with blood, the iodine vapor appears to give a larger ratio than does the bismuth spray. Both lecithin and sphingomyelin are carried over from serum; therefore, whenever a sphingomyelin spot equal to or greater than the sphingomyelin standard spot is seen and a dark brown color in the lecithin area also appears, blood contamination of the specimen is to be suspected and an interpretation cannot be made. Precipitation with cold acetone will not compensate for blood contamination; although serum lecithin will not be precipitated, the sphingomyelin component will be recovered in the precipitate. A hematocrit greater than about 1.5% in amniotic fluid usually significantly distorts the L/S ratio. Other workers have also commented on the effects of serum contamination (2, 5, 13, 16).

The iodine vapor stain, in addition to being easy to read and sensitive to concentrations of 0.2 mg of sphingomyelin or 0.5 mg dipalmitoyl lecithin per deciliter of amniotic fluid, is essentially a nondestructive stain. After the iodine-stained spots fade, the same sheet can be used for visualization by other techniques described at the end of the Procedure section.

1. Acid spray or dip, and heat char (1, 6): As already described, this technique gives blacker and somewhat larger spots for sphingomyelin than for equivalent concentrations of dipalmitoyl lecithin and lower ratios for a given L/S mix than the method presented above. Quantitation by densitometry would be the method of choice for obtaining a useable L/S ratio. Sphingomyelin is detectable at amniotic fluid concentrations well below 0.2 mg per deciliter. Dipalmitoyl lecithin is detectable at about 0.5 mg per 100 ml.

2. ANS spray (10): Lipids fluoresce a bright blue under ultraviolet light immediately after spraying. This method makes visible equal quantities of lecithin and sphingomyelin and detects them at concentrations of 0.2 mg/dl. The fluorescence begins to fade perceptibly within 12 h.

3. Rhodamine B spray (8): Lipids fluoresce a bright orange against a pink background under ultraviolet light about 60 min after spraying. The fluorescence is stable for at least 2 weeks and concentrations of sphingomyelin and lecithin as small as 0.2 mg/dl can be detected.

4. Phosphomolybdate–stannous chloride: Phospholipids appear as dark-blue spots against a lighter blue background. About 0.2–0.5 mg/dl of both phos-
pholipids can be detected, but the spot edges are often not discernible from the blue background.

Attempts to evaluate a bromthymol blue--NH₃ stain (2, 8) produced only nonspecific blue background.

Evaluators L. S. and H. N. K. analyzed 40 specimens by the method presented in this chapter and by their own modified method (6). The results are presented in Table 1.

Method A involves visual evaluation of spots made visible with bismuth or iodine, with L/S of 2 or less taken as "immature," between 2 and 5 as "transitional," and 5 or greater as "mature." In method B there is densitometric evaluation of acid-treated, heat-charred spots; an L/S ratio of less than 1.5 is considered as "immature," 1.5–1.9 as "transitional," and 2.0 or more as "mature."

Of 13 specimens interpreted as "mature" by method A (L/S range 5–9), all were also "mature" by method B (L/S range 2.2–6.5).

Of six specimens interpreted as transitional by method A (L/S range 3–4), two were transitional (L/S 1.3–1.8) and four were mature (L/S range 2.0–2.7) by method B. An additional specimen was placed in the "transitional" range of method A because of the small size of the lecithin spot by bismuth staining, even though the iodine ratio was 6:1; this specimen gave a "mature" value by method B (L/S 2.1).

Of the 20 specimens analyzed as "immature" by method A (L/S range 1–2), 13 were "immature" by method B (L/S range 0.5–1.4), six were "transitional" by method B (L/S range 1.5–1.8), and one was "mature" by method B (L/S 2.1).

The results show fairly good correlation in the "mature" and "immature" ranges, with considerable overlap in the "transitional" range. There was only one specimen that was "immature" by one method and "mature" by the other (No. 1821).

The overlap may be due to the differences expected when comparing a method of visual estimation with densitometric evaluation. However, even with densitometrically obtained ratios, the reported deviation from the mean of up to ±25% or more can mean similar overlaps between adjacent ranges (15), i.e., an interpretation of immature and transitional maturity or of transitional maturity and maturity on replicate analyses of the same specimen.

The method described in this chapter involves only a modified thin-layer chromatographic analysis of the L/S ratio. Brief mention should be made of other approaches to the assessment of fetal pulmonary maturity including: absolute measurements of lecithin concentration by phosphorus analysis of spots eluted from chromatograms (18), gas-chromatographic measurements of amniotic fluid palmitic/stearic acid ratios (19), or of palmitic acid concentration alone (20), and formation of stable bubbles upon shaking serial dilutions of amniotic fluid with 95% ethanol (21, 27).

In summary, the method presented here constitutes a rapid means of visually assessing the stage of fetal pulmonary surfactant synthesis. The method is technically simple to perform and is a sufficiently sensitive index of the sharp surge in pulmonary surfactant synthesis to be of considerable value in cases in which premature induction of labor or elective cesarean section are desired, or as an index of gestational age when the expected date of confinement is unknown.

References

*No reprints of this paper will be available.*