Estimating Lecithin/Sphingomyelin Area Ratio in Amniotic Fluid

A MODIFIED METHOD FOR THE PREDICTION OF NEONATAL RESPIRATORY DISTRESS

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SUMMARY

A knowledge of the pulmonary maturity of the unborn fetus is an important factor in deciding when to induce birth. A lecithin/sphingomyelin area ratio is obtained by thin-layer chromatography. A molybdenum spray is used to make visible only the choline-containing phospholipids, without necessitating the use of heat. This is a quick and easy method which produces reliable results.

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The respiratory distress syndrome is a major cause of death in premature infants. Thus, a knowledge of the pulmonary maturity of the fetus would be a great asset in selecting the optimum time for induction of labour. Until recently, fetal lung maturity was very difficult, if not impossible, to predict. However, in 1971 Gluck *et al.*[1] successfully predicted the onset of neonatal respiratory distress by measuring the relative concentrations of the phospholipids lecithin and sphingomyelin in amniotic fluid. These phospholipids originate principally from the fetal lung, and since the lung secretions reach the amniotic fluid,[2,3] the increased accumulation of lecithin in the alveoli from about the 35th week of gestation is reflected in a sharply rising concentration of lecithin in the amniotic fluid.[1] As this terminal increase is not matched by a parallel increase in sphingomyelin, the latter can be used as a baseline and the ratio between these two surface-active phospholipids in the amniotic fluid calculated. This ratio provides a sensitive index of fetal lung maturity and of the likelihood of neonatal respiratory distress if delivery is induced.[1,4] Prior to 35 weeks of gestation the concentrations of lecithin and sphingomyelin are similar.

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Lecithin is the major component of surfactant, the surface-active layer which lines the alveoli, imparting stability to the alveolus by lowering the surface tension of its aqueous lining to almost zero.[5,6] When expiration occurs without the presence of this surface activity the pressure difference, as shown by (A), on the alveolus increases considerably, due to the high surface tension of the water-based alveolar lining and the decreasing radius of the alveolus:

$$Pi - Pe = \frac{2T}{r} \quad \ldots \text{(A)}$$

Pi = internal pressure
Pe = external pressure
T = surface tension
r = radius of alveolus

This causes alveolar collapse which is characteristic of the respiratory distress syndrome in which normal surface activity and thus alveolar stability are absent, leading to progressive atelectasis and hyaline membrane disease, sometimes resulting in death.

MATERIALS AND METHODS

Reagents

Developing solvent consists of chloroform : methanol : water :: 75 : 22 : 3 (v/v).

Silica gel plates: 30 g silica gel H (Merck, catalogue No. 7736) and 10 g silica gel G (Merck, catalogue No. 7731) are slurried in 90 ml water for 1½ min and the resulting mixture spread on 20 × 20 cm or 10 × 20 cm glass plates to a thickness of 250 μm. These quantities are sufficient for five 20 × 20 cm plates. When dry, the plates are activated at 110°C for 1 hour, and stored in a desiccator until needed.

Standards: Purified lecithin (dipalmitoyl) and sphingomyelin, purchased from General Biochemicals, were used for making up the two standards in the concentration of 5 mg/ml of chloroform. These standards are stable for several months when stored in a refrigerator.

Molybdenum spray reagent:[7] *Solution I*: To 100 ml 25N sulphuric acid 4,0 g molybdic acid is added and the mixture boiled gently until the molybdic acid has dissolved.

Solution II: To 50 ml solution I, 0,178 g powdered molybdenum is added and the mixture boiled gently for 15 min. The solution is allowed to cool and is then decanted from any residue that may be present.

Molybdenum spray: To 20 ml solution I, 20 ml of solution II and 80 ml water are added. This spray is stable for several months at room temperature.

Procedure

To 5 ml of uncentrifuged, unfiltered amniotic fluid, 5 ml methanol and 10 ml chloroform are added, and the resulting mixture is mechanically shaken for 5 min. This mixture is then centrifuged at 2 200 rpm for 4 min which results in its separation into two phases. The upper phase, which is the aqueous phase, is carefully removed and discarded, while the lower chloroform phase containing the phospholipids is filtered through Whatman No. 4 paper. The filtrate is heated to 80° - 85°C to evaporate off the chloroform, and the resulting dry lipid residue is then dissolved in 5 drops of chloroform (delivered with a 2-ml graduated pipette).

With the help of a spotting guide the sample is applied to the silica gel plate as a 5-μlitre spot at a point 2 cm from the base of the plate. Care must be taken to ensure that the application site is less than 3 mm in diameter. This can be achieved by applying a small volume of the sample at a time, and drying between each application with a warm-air dryer.

A 20 × 20 cm sheet of filter paper is then placed adjacent to each of the two faces of the chromatography tank, after which the solvent is poured into the tank and the filter paper moistened by gently tipping the tank to one side and then the other. The silica gel plate is then placed in the tank and allowed to stand until the solvent front has risen 12 cm above the application site of the amniotic fluid. This takes approximately 45 min. After the run, the plate is dried with a warm-air dryer. The phospholipids are then rendered visible by spraying the plate gently with the molybdenum spray reagent, at a distance of about 20 cm, until the lecithin and sphingomyelin spots become visible. This spray causes the choline-containing phospholipids, lecithin and sphingomyelin, to become visible, and they appear as blue spots against a white background. The lecithin appears as the upper of the two spots, and is generally larger in area and more intense than the lower sphingomyelin spot (Fig. 1). The areas of the lecithin and sphingomyelin spots are then measured by multiplying length by width (see Appendix) and the ratio:

$$\frac{\text{lecithin}}{\text{sphingomyelin}}$$

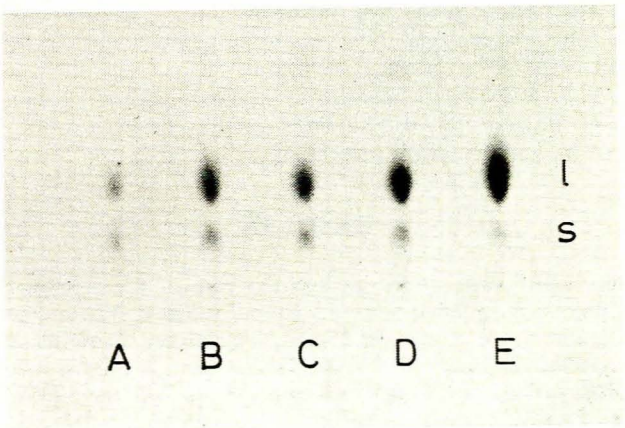

Fig. 1. Thin-layer chromatogram of amniotic fluid samples from 5 patients. LSAR values are A = 1,6; B = 2,3; C = 2,1; D = 2,6; and E = 4,7. Sample A was taken at 30 weeks, while samples B to E were taken at term. The relative increase in the area of lecithin (l) is clearly seen.

reported as the 'lecithin : sphingomyelin area ratio' (LSAR). Screw-type dividers facilitate accurate measurement of the spots. A few hours after spraying, these blue spots gradually begin to fade.

The LSAR estimation should not be performed on amniotic fluid samples contaminated with meconium or chlorhexidine antiseptic cream, as they have been proved to yield unreliable results.[8,9] Blood-stained fluids[9,10] and fluids contaminated with vaginal secretions should also be excluded.

The amniotic fluid sample should be assayed as soon as possible after collection, since an up-to-date LSAR result is obviously of far greater significance to the obstetrician than one of a sample taken a few days previously. Furthermore, there have been conflicting reports published as to the stability of lecithin in amniotic fluid on storage.[11-13]

Clinical Interpretation

Prior to 33 weeks of gestation the normal range of LSAR values is 0,3 - 2,0.[8,13] From the 33rd week onwards the LSAR begins to rise, corresponding to the increase in lecithin in the fetal lung. The rate of increase varies widely among individuals due to the widening of the normal range on approaching term. By 38 weeks LSAR values of at least 1,5 should be obtained, rising to between 2,0 and 9,0 at term.[8,13] Once the LSAR exceeds 2,0 a continued rise can be expected in normal pregnancies.

The LSAR values can be roughly divided into 3 groups:[8,13-15]

The high-risk group which has values of less than 1,5, indicating a dangerously immature fetal lung. Delivery at this time results in the almost invariable development of severe respiratory distress, often with hyaline membranes, and death.

The intermediate group with values ranging from 1,5 to 1,9, indicating a transitional lung in which there is some risk of respiratory distress, but in a milder form than in the case of the high-risk group.

The normal group with ratios of 2,0 or more, indicating a fully mature fetal lung where respiratory distress will not occur if the infant is delivered.

Thus it should be possible to eliminate neonatal respiratory distress by withholding elective delivery until a mature lung pattern is present.

RESULTS

The LSAR was determined in the case of 85 samples of amniotic fluid taken from 82 normal subjects. These determinations are shown in Fig. 2. This figure confirms the expected increase in LSAR values after the 34th week of gestation. The 3 pairs of points A-B, C-D and E-F are consecutive tests on 3 subjects. In each of these 3 cases, the LSAR value was considerably higher in the later determination.

In the case of 44 of these 85 determinations, delivery occurred within 72 hours of the amniotic fluid sample being removed and tested. Of these 44 cases 43 had

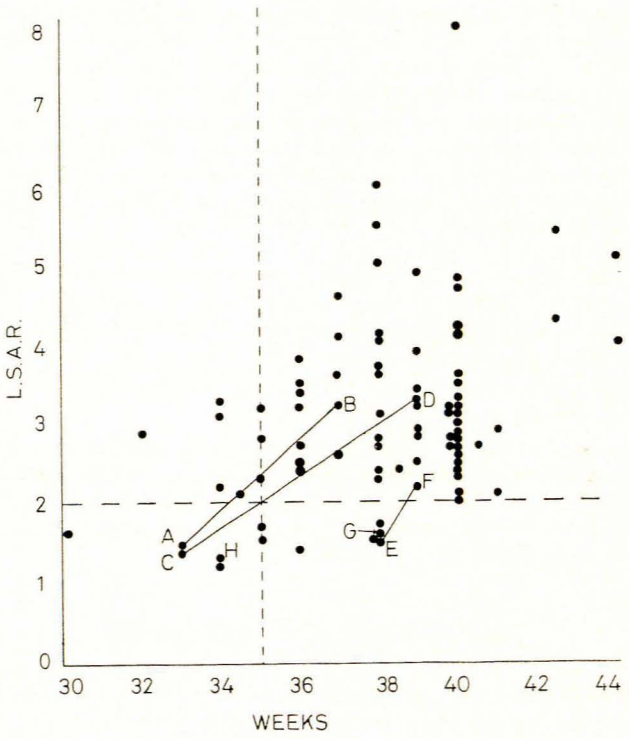

Fig. 2. Graph of LSAR values plotted against weeks of gestation in the case of 83 normal patients. Serial tests on individual patients are joined.

LSAR values above 2,0 and none of these infants developed respiratory distress even though some of them were premature. The remaining result (G) fell into the intermediate range, having an LSAR of 1,6 at about 38 weeks (gestational age uncertain). A Caesarean section was performed at this stage, and the infant developed respiratory distress but survived. In the case of the determination marked H the LSAR value at 34 weeks was 1,3 i.e. falling into the high-risk group. A Caesarean section was performed 2½ weeks later without requesting a repeat LSAR determination—the infant developed severe respiratory distress and died of hyaline membrane disease. The cause of death was confirmed histologically.

DISCUSSION

Increased-Risk Pregnancies

The LSAR estimation can prove to be an invaluable aid in the management of increased-risk pregnancies due to rhesus incompatibility, chronic placental insufficiency, diabetes, or other high-risk clinical conditions. In severe cases of rhesus incompatibility the amniotic fluid LSAR and bilirubin level[16] will prove a valuable guide to the treatment of the patient. If the bilirubin level indicates that the fetus is in jeopardy, and the LSAR shows a mature fetal lung, it would be best to deliver the infant immediately[8,10,15] rather than favour fetal blood trans-

fusion which carries a 6,4% risk[17] of traumatic fetal death. However, if a low LSAR is obtained, results would favour a fetal blood transfusion, thus allowing delivery to be delayed until the LSAR value is above 2,0, since much of the perinatal mortality in rhesus incompatibility is due to the combined effect of severe haemolysis and respiratory distress.

In cases of chronic placental insufficiency an accurate assessment of the degree of fetal pulmonary maturity will help in selecting the optimum time for induction of labour. The correct timing of Caesarean sections or induction of labour when gestational age is uncertain, can also be facilitated by up-to-date LSAR results. Uncomplicated Caesarean sections have an 8,6% incidence of premature infants, and this figure rises to as much as 16% in elective repeat Caesarean sections.[18] A normal LSAR result indicating a mature fetal lung would allow a Caesarean section to be performed in the knowledge that respiratory distress will not occur, even if the infant is premature. However, in acute complications such as placental abruption or fulminant pre-eclampsia, immediate delivery may be essential regardless of the risk of neonatal respiratory distress.

In pregnancies complicated with maternal diabetes results tend to indicate that the expected terminal rise in the LSAR value occasionally fails to occur,[19] or that there is a retardation of lung maturation.[20] In such cases the current level of the LSAR, and also the trend of the ratio determined by serial tests, could prove to be a deciding factor in determining the most suitable time for the induction of labour. Also, because of the increased incidence of stillbirths in diabetics after 37 weeks of gestation, labour could be induced as soon as full pulmonary maturity is established, thus reducing the risk involved in the unnecessary continuation of a potentially lethal intra-uterine state.

Current Methods

Bhagwanani *et al.*[11,21] measured the actual lecithin content of amniotic fluid as an index of fetal pulmonary maturity. Although more precise than measuring the lecithin/sphingomyelin area ratio, the method is much longer and more cumbersome, the modified version[21] still requiring 3 hours to perform. In addition, more specialised equipment is needed. A further disadvantage of this method, and also of the 'shake' test to be discussed later, is that results are dependent on the total amniotic fluid volume. Gadd[22] and Falconer *et al.*[23] found that the total liquor volume increased with gestational age up to 34 - 36 weeks, then reached a plateau, finally falling as term approached. These workers also found considerable variations above and below the mean liquor volume which would alter the lecithin concentration in the liquor in these cases. This would confuse the issue in a borderline case, since an accurate result would be dependent on a normal liquor volume.

On the whole, LSAR and lecithin results have been found to agree well,[21,23,24] with lecithin values above 3,5 mg/100 ml indicating a fully mature fetal lung.

By far the quickest of the 3 methods currently in use for estimating fetal lung maturity is the 'shake' test, a screening test devised by Clements *et al.*[25] This test is based on the ability of the pulmonary surfactant to generate stable foam in the presence of ethanol. The sample of amniotic fluid at various dilutions is shaken and examined for bubbles after 15 minutes. The obvious advantages of this method are that it is extremely quick and can be carried out by unskilled persons with a minimum of apparatus and chemicals, thus cutting down the cost. However, the proportions in this method are critical, clean glassware must be used, and care is needed in the interpretation of results. A clearly negative shake test at 1/1 dilution indicates a high risk of respiratory distress, whereas a clearly positive test at 1/2 dilutions signals a low risk.[25] The shake test can only be regarded as a semiquantitative test, and has been reported to give false positive and false negative results.[9,24,26] In the current work, LSAR values were determined in the case of 59 patients while the surfactant content of the amniotic fluid taken from these patients was determined simultaneously by other workers using the shake test. In 15% of the results the shake test gave results totally contradictory to those of the LSAR method. The graph in Fig. 3 indicates clearly the variation in results obtained by these two methods.

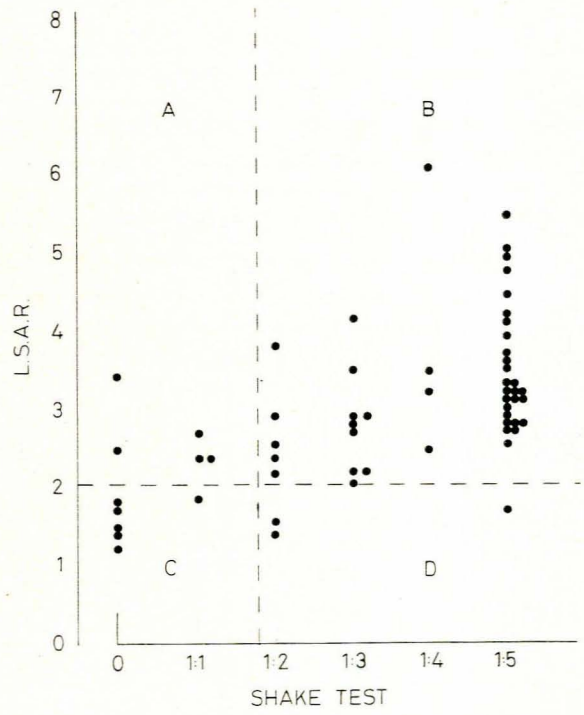

Fig. 3. Graph of LSAR values *v.* shake test values. The broken lines indicate the respective mature levels. All those points falling into rectangles A and D are total contradictions.

The LSAR method described, however, is accurate, quick, and simple to perform in any routine laboratory,

the entire procedure taking little more than an hour. Results are not affected by amniotic fluid volume, and the method does not require elaborate equipment.

The value of the LSAR method would be extended with the development of more effective methods for the inhibition of premature labour until the fetal lung is fully mature. Furthermore, a reliable method to stimulate production of surfactant pharmacologically would be of great use. Experimentally, both heroin and steroids have been administered to pregnant rabbits and sheep or injected directly into the amniotic fluid or fetus to accelerate the production of surfactant in the fetal lung.[27-30] Recently, steroids used in human pregnancy have been reported to accelerate fetal lung maturity.[31] This is further confirmed by the low incidence of respiratory distress syndrome in premature infants born to heroin-addicted mothers.[32]

APPENDIX

The area of an ellipse is:

$$\text{II ab} \quad \ldots (1)$$

where a and b are respectively the semimajor axis and the semiminor axis of the ellipse.

Since it is easier to measure 2a and 2b in practice, 2a and 2b are substituted in formula (1). The resulting area is then 4 times too large. However, since a ratio is required, the 4 II in both the numerator and denominator cancel out, leaving:

$$\frac{(a \times b) \text{ lecithin}}{(a \times b) \text{ sphingomyelin}} = \frac{(\text{length} \times \text{width}) \text{ lecithin}}{(\text{length} \times \text{width}) \text{ sphingomyelin}} \quad \ldots (2)$$

The above holds true only for perfect ellipses or circles, which is not always the case in the present work. However, with LSAR values in the range of 2,0 or less, the spots so closely resemble ellipses or circles (Fig. 1, A, B, C) that (2) can be used with a high degree of accuracy.

In general, only for LSAR values well above 2,0 does the lecithin spot vary considerably in shape from an ellipse (Fig. 1,E) and since all values above 2,0 are classified normal the inaccuracy in area measurement in this range is not critical.

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