BACKGROUND:

Hyaline membrane disease (HMD) is a disorder primarily affecting premature infants and is a significant cause of morbidity and mortality in this population. The disorder is characterized by widespread atelectasis and an inability of the neonate to expand his or her lungs. Secondary hemorrhage, edema and desquamation of alveolar lining cells then occur which further compromise respiratory function.

Surfactant is a combination of several phospholipid compounds including phosphatidyl choline (lecithin), phosphatidyl glycerol, sphingomyelin and phosphatidyl ethanolamine. Approximate percentages for "mature" surfactant are shown in Table 1. These phospholipids are associated with several apoprotein moieties whose functions are only beginning to be understood. It appears, however, that these proteins are critical for proper surfactant function.

Table 1

<table>
<thead>
<tr>
<th>Chemical Composition of Pulmonary Surfactant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
</tr>
<tr>
<td>Phosphatidyl glycerol</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
</tr>
<tr>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>Acidic Phospholipids</td>
</tr>
</tbody>
</table>

During the last trimester of pregnancy (approximately week 35) the Type II pneumocytes begin synthesizing mature surfactant and excreting it into the alveolar spaces. Prior to this, the material produced by the alveolar cells is relatively lacking in lecithin and phosphatidyl glycerol and does not have the same surface tension lowering abilities. Prenatal fetal breathing movements and the ciliated epithelium of the respiratory passages promote the diffusion of surfactant into the amniotic fluid. This results in an alteration of the amniotic fluid phospholipid content and is the basis for the majority of fetal lung maturity assays. At birth the surfactant acts to lower the surface tension of the fluid lining the alveoli resulting in increased compliancy and decreasing the energy needed to keep the lungs inflated. Without surfactant the surface tension of the fluid resists alveolar inflation leading to atelectasis and subsequent HMD.

In the future infants at risk for HMD may be prophylactically treated with either animal or synthetic surfactant preparations at birth. Current therapies, however, are directed at infants who have developed HMD and primarily consist of intubation, mechanical ventilation with positive end expiratory pressure (PEEP) and high oxygen concentrations. Unfortunately, ventilatory therapies can cause further pulmonary injury such as pneumothorax, interstitial emphysema and oxygen toxicity. Prevention is therefore the best way to decrease the morbidity and mortality associated with HMD.

In this paper I will briefly review the available assays which have shown potential for predicting the risk of HMD.
ASSAYS:

Lecithin/Sphingomyelin Ratio by TLC:

Since its introduction by Gluck and associates in 1971 this test (along with its various modifications) has remained the 'gold standard' by which other assays have been judged. This method is based upon the finding that as the pulmonary surfactant matures there is a rapid rise in the lecithin content of the amniotic fluid. This is shown in Figure 1.

![Figure 1](attachment:image.png)

To circumvent the dependency of lecithin concentration measurements on amniotic fluid volume (ie. oligo-/polyhydramnios) and absolute analytic recovery of lecithin, the assay was developed to examine the ratio of lecithin (L) to sphingomyelin (S).

In their studies, Gluck et al. found that if the amniotic fluid had an L/S ratio of 2.0 or greater the fetus could be delivered with a negligible risk of developing HMD. The reported test performance from the literature is shown in Table 2. (Please note that a "positive test" is one in which the L/S ratio is greater than or equal to 2.0 and that this is predictive of fetal maturity.)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>by TLC</td>
<td>60%</td>
<td>99%</td>
<td>98%</td>
<td>50-60%</td>
</tr>
</tbody>
</table>

Note that the sensitivity of the test (ie. its ability to identify a mature infant) is relatively poor. This is true of essentially all current assays for fetal lung maturity and is most likely related to the
transit time needed for alveolar contents to diffuse into the amniotic fluid. This 'transit time' has been estimated to take several days. Therefore, a fetus who has started to produce 'mature' surfactant (and is therefore at low risk for HMD) may be labeled immature by assays using amniotic fluid as the test sample. This is supported by the finding that the L/S ratio is higher in samples aspirated close to the fetal mouth than in those from other sites.

Methodologically this assay is relatively straightforward: extract the phospholipids from an amniotic fluid sample, separate the component species by thin-layer chromatography, quantitate the relative amounts of lecithin (L) and sphingomyelin (S) and calculate the L/S ratio. In practice, however, the test is time-consuming and requires a fair degree of expertise to perform it in a consistent manner. This results in a long turn-around-time (4-5 hours) and makes it difficult for most laboratories to offer it on a twenty-four hour basis. Additionally, the test procedure is non-standardized and numerous modifications exist with regards to centrifugation time/speed, type of phospholipid precipitation used, TLC plate/gel type, method for spot development and even how the various components are quantitated. This variation in methodologies from that originally proposed by Gluck is of concern since many laboratories have adopted the 2.0 cutoff point for maturity without doing clinical correlation studies to determine if that ratio is appropriate for their methodology. In 1986 a CAP survey of 261 laboratories revealed a coefficient of variation in the L/S results of 27.5% which reflects the differences in the methods employed by various labs.

Finally, the L/S ratio is subject to several interferences. Contamination of the amniotic fluid sample with blood, meconium and/or vaginal secretions invalidates the test results as these materials contain lecithin. Approximately 15% of amniotic fluid specimens have such contamination (the percentage is probably higher in specimens from complicated pregnancies in which the test is most needed) and this is a serious disadvantage of this assay. It has also been shown that maternal disease such as diabetes mellitus affects the production of surfactant and that L/S ratios of 2.0 cannot be used to predict fetal maturity in these cases.

L/S Ratio by Enzymatic Colorimetry:

This is a relatively new technique which attempts to provide the same information as the thin-layer chromatography in a more convenient assay. The test is based upon the use of specific enzymes (phospholipase C or D, choline oxidase, horseradish peroxidase, etc.) to form a series of coupled reactions which break down choline-containing phospholipids into compounds that can be measured by colorimetric methods. One method is depicted in Figure 2.

Figure 2
As with TLC, an L/S ratio of 2.0 by this assay is generally accepted to indicate fetal maturity, however, clinical trials are needed to establish this for the several variations of this method which are available.

The advantage of this type of assay, apart from being faster and less demanding than TLC, is in the ability to automate such a procedure and potentially reduce the cost. The disadvantages are that these tests are non-standardized, they have not had extensive clinical evaluation and that they are subject to the same interferences from contamination and maternal disease as the TLC methodologies.

Phosphatidyl Glycerol by TLC:

Phosphatidyl glycerol (PG) is a component of mature surfactant that parallels the lecithin increase near term. This phospholipid, unlike lecithin, is produced only by Type II alveolar cells and therefore PG assays are generally not subject to interferences from sample contamination by blood, meconium or vaginal secretions. Additionally, PG levels do not appear to be altered in pregnancies complicated by maternal diabetes and have been shown to be a reliable indicator of fetal maturity in these cases. Initial studies indicated that a level of PG greater than or equal to 3% of the total phospholipids was a consistent indicator of fetal maturity. In practice, however, almost any detectable level of PG is associated with an extremely low risk of HMD.

The principles involved in detecting phosphatidyl glycerol by TLC are essentially the same as for lecithin and sphingomyelin. In most laboratories both the L/S ratio and %PG are determined simultaneously using either two-dimensional or unidimensional TLC techniques. Unfortunately, as with all TLC assays, turn-around-time and technical expertise issues make this an assay which is very costly to make available on an around-the-clock basis. Additionally, since TLC procedures identify compounds by their relative positions, any substance migrating into the same area as PG would give a falsely positive test result. Such 'pseudo-PG' compounds have been reported, however, this problem may be overcome with newer techniques which identify the phospholipid spots by using a molybdenum-blue spray which is specific for phosphorus-containing compounds. The analytic sensitivity of this technique is approximately 0.1-0.3 ug/ml and the reported test performance is listed in Table 3.

Table 3

<table>
<thead>
<tr>
<th></th>
<th>PG by TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>68-99%</td>
</tr>
<tr>
<td>Specificity</td>
<td>99%</td>
</tr>
<tr>
<td>Pos. Pred.</td>
<td>99%</td>
</tr>
<tr>
<td>Neg. Pred.</td>
<td>37-83%</td>
</tr>
</tbody>
</table>

PG by enzymatic colorimetry:

Except for an alteration in the enzymes utilized, this assay is essentially the same as for detection of lecithin and sphingomyelin. Advantages for this test are its ease of performance, speed, potential for automation and relative lack of potential interferences. The disadvantages are similar to those encountered with the L/S ratio by this method, namely, lack of standardization and paucity of available clinical data.

PG by immunoassay:

This is an agglutination assay which is based upon some of the unique characteristics of phospholipids. Rather than using an inert material (ie. latex) to show agglutination this assay utilizes a solution of lecithin and cholesterol which spontaneously aggregate in an aqueous environment to form micelles. When the sample of amniotic fluid is added any PG present is adsorbed onto the surface of these lipid
vesicles. An antibody to PG is then added yielding agglutination proportional to the amount of PG present. The test is available in kit form with positive and negative control wells against which the patient sample is read.

The advantages of this assay are that it is rapid (15 min), it is easy to perform and does not require sample preparation (ie. centrifugation). Additionally, as with the other PG assays this test is relatively unaffected by either sample contamination or maternal disease. The reported performance of this test is indicated in Table 4.

Table 4
PG Immunoassay

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>63-88%</td>
</tr>
<tr>
<td>Specificity</td>
<td>99%</td>
</tr>
<tr>
<td>Pos. Pred.</td>
<td>99%</td>
</tr>
<tr>
<td>Neg. Pred.</td>
<td>35-70%</td>
</tr>
</tbody>
</table>

The disadvantages of this assay are in its poor negative predictive value (ie: too many truly mature fetuses are 'immature' by this test) and that it is relatively expensive (approximately $45.00/test). The company that markets this test (Hana Biologies) may have overcome the first of these problems by offering a new "ultrasensitive" kit which should decrease the false negative rate. The kit is essentially the same, however, a trace control well has been substituted for one of the positive control wells. This well contains only 0.5 ug/ml PG and allows samples that only show a small degree of agglutination to be categorized. (In the former test 2 ug/ml (2+) and 4 ug/ml (4+) controls were used and specimens reacting less than 2+ were forced to be called negative). Clinical trials are now being conducted with this new kit to determine the outcome of infants where the test has been positive at the trace level only.

Shake Test:
The shake test, which was originally introduced by Clements et al, is the first of the functional assays for determining fetal maturity. In this family of tests the surfactant activity of the amniotic fluid sample is assessed rather than measuring a specific surfactant component.

Many biologic materials have surfactant like properties (proteins, fatty acids) in aqueous solution, however, ethanol when added in sufficient amounts decreases the surface tension lowering abilities of these compounds. The shake test takes advantage of the finding that mature pulmonary surfactant is the only substance that retains the ability to form stable foams when the volume fraction of ethanol in an aqueous solution exceeds 47%. This is diagramed in Figure 3 where surface tension is plotted against the volume fraction of ethanol.

The test procedure involves making 1:1, 1:1.3, 1:2, 1:4 and 1:5 dilutions of amniotic fluid in saline and adding equal volumes of 95% ethanol to each. This brings the final ethanol fraction in each sample to 47.5%. The tubes are then stoppered and shaken vigorously for 15 seconds and then allowed to stand untouched for 15 minutes at room temperature. The tubes are then examined for the presence of bubbles at the meniscus. A complete ring of bubbles in the 1:2 dilution sample indicates a positive test.

Additional information can be derived from the shake test by assigning + or - to the 1:1, 1:1.3 and 1:2 dilution tubes. The results can then be compared to the estimated gestational age of the fetus to predict the risk of HMD. A graph is shown in Figure 4 which compares the results to estimated risk of HMD at various gestational ages.
The advantages of the shake test are that it is rapid, inexpensive and actually measures the functional activity of the surfactant in the sample. Disadvantages to the test are that it is extremely technique-dependent and therefore requires an experienced person to both perform and read the test. The assay is also subject to interference by blood, meconium, or vaginal secretions in the sample and any oils, lubricants or detergent in or on the test tubes or stoppers. All of these materials can cause false positive results. Lastly, the results are affected by the amount of amniotic fluid in which the surfactant is distributed (i.e. oligo-/polyhydramnios). The reported performance of the shake test (without analysis with respect to gestational age) is given in Table 5.
Lumadex-FSI

The Lumadex-FSI (Foam Stability Index) is a commercially marketed kit which utilizes the principles of the shake test. Each kit consists of a cassette composed of plastic tubes attached side-by-side which contain premeasured volumes of ethanol and a small amount of blue dye. A positive control containing ethanol, dye and a surface active substance is included. Equal amounts of undiluted amniotic fluid are added to each tube yielding solutions with ethanol fractions ranging from 44-50%. The cassette is capped and shaken for 30 seconds and then allowed to stand undisturbed for one minute before being read. The 'foam stability index' is determined from the tube with the highest volume fraction of ethanol in which a complete ring of bubbles is seen at the meniscus. An index greater than or equal to 47 indicates fetal maturity. The test performance is shown in Table 6.

<table>
<thead>
<tr>
<th>Lumadex-FSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
</tr>
<tr>
<td>Specificity</td>
</tr>
<tr>
<td>Pos. Pred.</td>
</tr>
<tr>
<td>Neg. Pred.</td>
</tr>
</tbody>
</table>

This modification of the original shake test is an attempt to overcome some of the technical problems associated with that assay. The prefilled cassette eliminates errors due to making amniotic fluid dilutions, pipetting ethanol, variation in percentage of ethanol due to water accumulation in reagent bottles and glassware contamination. The test requires little prior training to perform and the blue dye enhances determination of the endpoint (both of which reduce technologist errors). The disadvantages to this test are in its cost ($40.00) and that it is still subject to interference from sample contamination by blood, meconium or vaginal secretions.

Fluorescence Polarization:

Fluorescence polarization technology has been utilized in the Abbott TDX discrete analyzer system. A complete explanation of the theory underlying this technique is beyond the scope of this review. In brief it is as follows. When polarized light is passed through a solution containing a fluorescent dye, the degree of polarization of the emitted fluorescent light is inversely proportional to the ability of the dye molecules to rotate within that solution. The amount of molecular rotation is dependent upon characteristics collectively termed the "microenvironment". In the amniotic fluid assay the test compound is 1,6 diphenyl-1,3,5 hexatriene, a lipophilic dye which is added to the sample and allowed to incubate for thirty minutes. The dye is taken up by the lipid particles (lamellar bodies) within the amniotic fluid and the microviscosity within these particles determines the rotation of the dye molecules. (The higher the microviscosity the lower the amount of rotation.) The microviscosity within the lamellar bodies is inversely proportional to the concentration of mature surfactant. Therefore, the degree of polarization of the emitted fluorescent light is itself inversely proportional to the amount of mature surfactant present. It has been found that an FP value < 0.360 is indicative of pulmonary maturation. A comparison of the fluorescent polarization values to the L/S ratio in a series of
uncomplicated pregnancies is shown in Figure 5.

Figure 5

Comparison of amniotic fluid fluorescence polarisation values and the lecithin sphingomyelin area ratio in 50 uncomplicated pregnancies.

Advantages to this assay are that it is simple to perform, relatively rapid (1/2-1 hour) and that it is reproducible. The disadvantages are the requirement for an expensive and sophisticated instrument to "read" the sample and that potential interferences have not been well examined in clinical trials. (Theoretically, this method would be subject to the same problems with sample contamination as the shake test and L/S ratio. The effects of maternal diseases are unknown.) The performance of this test on non-contaminated samples from uncomplicated pregnancies is shown in Table 7. As with the other assays the predictive value of a negative test result is poor.

Table 7

<table>
<thead>
<tr>
<th>Fluorescence Polarization</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>83%</td>
</tr>
<tr>
<td>Specificity</td>
<td>99%</td>
</tr>
<tr>
<td>Pos. Pred.</td>
<td>99%</td>
</tr>
<tr>
<td>Neg. Pred.</td>
<td>38%</td>
</tr>
</tbody>
</table>

Optical Density @ 650 nm:

The development of this test came from an empiric observation that mature amniotic fluid specimens were "cloudy" as compared to immature specimens. Initial studies by Sbarra, et al confirmed that the optical density of amniotic fluid measured at 450 nm increased at term. Measurement at 450 nm was problematical, however, due to the absorbance of several biologic molecules (eg. bilirubin) at this wavelength. This made the test uninterpretable in those cases where it was most needed. The absorbance at 650 nm is unaffected by these compounds and this wavelength was later adopted for use in the
Further work by the same researchers established that the optical density at 650 nm was proportional to the L/S ratio and that an OD > 0.15 was a good predictor of fetal lung maturity. A comparison of the L/S ratio to the optical density in a series of amniotic fluid samples is shown in Figure 6.

Figure 6

![Graph showing L/S ratio vs. optical density](image)

This work was later challenged by Plauche and co-workers who found that the optical density was related to the number of fetal squamous cells in the sample and that when these cells were removed by centrifugation the OD decreased to zero. This is shown graphically in Figure 7.

Figure 7

![Graph showing cell count vs. centrifugation time](image)

It was their argument that the test was a measurement of fetal squamous maturity instead of a direct measurement of surfactant. Therefore its use in predicting fetal pulmonary maturity should be
questioned. The original authors contend that surfactant is adsorbed onto the surface of the squamous cells and is therefore removed from the sample by centrifugation. They support this theory by showing that the lecithin content also decreases dramatically with prolonged centrifugation. The point of this controversy is perhaps irrelevant since the test appears to correlate well with the L/S ratio (Figure 6). Due to the lack of available clinical data comparing the OD at 650 nm to neonatal maturity, and interferences due to oligo/polyhydramnios and maternal diabetes the test has not gained widespread acceptance.

CONCLUSIONS:

Each of the tests discussed above has advantages and disadvantages with regard to cost, availability, turn-around-time, equipment/reagent requirements and clinical validation. Comparing the performance of these various assays reveals that all have high positive predictive values but unfortunately provide little information when negative. Therefore, the choice of one (or more) tests is dependent upon the nature of the environment in which it is going to be used.

If the test is to be utilized to establish fetal maturity before labor induction or elective cesarean section then factors such as turn-around-time and false negative rate may not be crucial in test selection. In these instances therapeutic intervention would only be contemplated when a "mature" test result is obtained and a rapid result is generally unnecessary. Such a setting might be found in community hospitals which generally handle only uncomplicated pregnancies. In this setting the use of thin-layer-chromatography (especially those providing both L/S ratio and % PG) may be optimal if the laboratory has experience with this methodology. Alternatively, such hospitals may explore the use of enzymatic assays or fluorescence polarization (if they have a TDX analyzer) to obviate the need for such a labor intensive method.

In a clinic or private office, where extensive laboratory facilities are unavailable, kit tests such as the Lumadex-FSI and Amniostat-FLM would probably be the only methods realistically useful. The high cost of these assays, however, should restrict their use in these settings to cases in which a rapid result is required and/or other testing facilities are unavailable.

In a larger hospital in which a significant number of complicated pregnancies are encountered the issues of availability, turn-around-time and negative predictive value may take precedence. In cases in which the fetus is at risk due to a hostile intrauterine environment the test is used to determine the earliest point at which the infant can be delivered with a low risk of developing HMD. At other times the assay results are used to decide whether premature labor should be allowed to continue or attempts should be made to stop labor with tocolytic agents and/or administer steroid preparations to stimulate pulmonary maturation. In both of these situations the need for a rapid, reproducible and sensitive assay is obvious. Unfortunately, such a test does not exist at this time.

The L/S ratio assays (ie. TLC) while reproducible and relatively sensitive are not rapid enough to provide an answer in an emergent situation. Interference from sample contamination and the poor positive predictive value in some types of maternal disease are other disadvantages to the theses tests. The shake test is rapid and sensitive, however, its reproducibility in inexperienced hands is questionable and this causes problems in making the test available around-the-clock. Although the Lumadex-FSI has been made more reproducible by eliminating several sources of potential error found in the shake test, it is several times more expensive to perform. Additionally, as with the L/S ratio these functional assays are also subject to interference by contaminants. The PG immunoassay is rapid and reproducible but has a poor negative predictive value in its present form (the "ultrasensitive" kit now being tested may be more sensitive) and as with other commercial assays it is expensive. The other methods described in this review are in general less attractive because of a lack of clinical experience with them.

Due to the problems with each individual assay, some authors have suggested the use of "panels" or "cascades" utilizing three to five tests. Such protocols are problematical, however, due to the inherent cost and logistic difficulty involved in providing multiple tests on an ongoing basis. It is also questionable that combinations involving more than two or three of these tests actually provide additional clinical information in a significant number of cases to warrant their adoption (in the worst case multiple results may even be confusing if they are not in agreement).
It is my opinion that in hospitals that see a significant number of complicated pregnancies a combination of at most three complimentary tests would be optimal at this time. An example would be the shake test and the PG immunoassay with or without a TLC method as backup for instances in which the first two tests disagree. (The use of TLC as a third method is debatable since it would only be useful in cases where time was available for it to be performed.) In this type of combination the slightly higher sensitivity of the functional assay would complement the ability of the PG assay to be used with contaminated specimens and in cases complicated by maternal diseases.

Until an assay is developed which overcomes the disadvantages outlined above hospitals must adopt a system which is best suited for their particular need. Potentially the new PG immunoassay may be such a test, however, this can only be assessed after extensive clinical trials.


