Stabilization of bromthymol blue colour in lecithin-sphingomyelin ratio determinations in amniotic fluid

A. G. ANDREWS, I. HORACEK, AND F. R. BETHERAS

From The Royal Women's Hospital, Melbourne, Victoria, Australia

SYNOPSIS Amniotic fluid lecithin and sphingomyelin areas after extraction and chromatography are rendered permanently visible by the use of bromthymol blue dye buffered to a pH of 11.3.

Since the publication by Gluck *et al* (1971), estimation of amniotic fluid phospholipids has become an accepted method of determining fetal lung maturity. Most workers use the thin-layer chromatography method (TLC) with very little variation from the original method of Borer *et al* (1971).

Visualization of the phospholipid spots after TLC remains a problem, and a number of different techniques have been used (Olson and Graven, 1974).

The method based on the visualization of the phospholipid spots with bromthymol blue suffers from the disadvantage of rapid fading of the colour after initial development in ammonia, thus rendering densitometry difficult (Olson and Graven, 1974). A method is described in which, by using bromthymol blue in buffered alkaline solution, the phospholipid areas are stained immediately, without the need for subsequent colour development in ammonia, and retain their stain for a long period. Data demonstrating the stability of colour, reproducibility, and accuracy of the results and clinical correlation are presented.

Method

The chromatograms are run on plastic-backed silica gel thin-layer chromatography strips, 1.5 cm wide × 13.5 cm long (Eastman Kodak 13179 silica gel without fluorescent indicator).

The bromthymol blue dye is prepared by dissolving 0.05 g of the sodium salt (Bromthymol Blue, water soluble, indicator grade, manufactured by Aldrich Chemical Company Incorporated,

Milwaukee, Wisconsin) in 50 ml of distilled water. 20 ml of 1 M sodium hydroxide is added and the mixture is diluted to 120 ml with distilled water. Boric acid (1.10 g) and sodium bicarbonate (0.15 g) are added, the solution is mixed vigorously for 30 minutes, and the pH is checked and, if necessary, adjusted to 11.3 by the addition of 1 M sodium hydroxide. The dye thus prepared is stable for one month at room temperature.

The amniotic fluid is extracted by the method of Borer *et al* (1971). The dried acetone precipitate is taken up in 10 µl of chloroform and applied 1.5 cm from the end of the strip. The mobile phase is methanol 25 vol, ammonium hydroxide 4 vol, and chloroform 65 vol. After chromatography the strips are air dried and then immersed in bromthymol blue dye for 5 minutes; excess dye is blotted with tissue paper, and the strips are finally dried in an oven at 110°C for 3 minutes. The phospholipids appear dark blue on a pale blue background.

The strips are scanned on a Beckman R110 transmission densitometer, slit width 0.1 mm, setting 2 mm, and interference filter of 590 nm. The ratio is calculated from the area under the curve. The strips can be scanned at leisure as the colour is stable.

Stock standards are prepared by dissolving (β, γ)-dipalmitoyl-lecithin, 10 mg, in 100 ml of chloroform and sphingomyelin, 10 mg, in 100 ml of chloroform (General Biochemicals, supplied by Sigma). The stock solutions are stored at -10°C.

Results

Table I shows the stability of the developed chromatograms. There was no significant change in the lecithin/sphingomyelin ratio on repeat scans as long as 25 days later. No special precautions were taken

L/S ratio		Time (days elapsed)
1st scan	2nd scan	
0.8	0.7	20
1.2	1.1	20
2.6	2.5	13
3.0	3.1	25
4.6	4.7	10
7.6	7.6	17

Table I Stability of chromatograms stained with bromthymol blue (pH 11.3) showing L/S ratios over a wide range as determined immediately and up to 25 days later

on storage nor were the chromatograms treated in any way after the initial development.

Twenty estimations of L/S ratios on a pool of frozen amniotic fluid were performed in different assays on different days; the mean ratio ($\pm$ SD) for that pool was 2.6 ($\pm$ 0.05).

The linearity of the method was tested using mixtures of stock solutions of lecithin and sphingomyelin. These were mixed to give the appropriate ratio, evaporated to dryness, and then re-dissolved in chloroform to give a final concentration of 8 μ g per 10 μ l. The mixture was then subjected to TLC and

Figure L/S ratio determined using mixtures of stock lecithin/sphingomyelin solutions and subjected to TLC, staining, and finally densitometry. The concentration of total phospholipids was 8 μ g per 10 μ l. The dotted line is the theoretical curve for absolute linearity.

Outcome	Lecithin-sphingomyelin ratio	
	Less than 2	2 or greater
Normal (no idiopathic RDS)	7 (17%)	191 (98.5%)
Idiopathic respiratory distress syndrome (RDS)	27 (66%)	3 (1.5%) ¹
Neonatal death from hyaline membrane disease	7 (17%)	0
Total number of cases	41 (100%)	194 (100%)

¹Three cases of maternal diabetes mellitus

Table II Neonatal outcome according to lecithin/sphingomyelin ratio determined in amniotic fluid collected within one week before delivery of a live infant

staining as described above. The results are shown in the figure. The method was linear up to a ratio of 2.0. There was slight deviation from linearity at higher ratios but this does not cause any clinical problem as the deviation is small and the clinically significant ratio is 2.0 or less.

The method has been extensively applied clinically. The outcome of 235 high-risk pregnancies yielding living infants in which the L/S ratio was determined by this method in the amniotic fluid within a week before delivery is shown in table II.

Discussion

The method has been used clinically since 1972, during which time more than 2500 estimations have been performed in high-risk pregnancies. The method has proved simple and reproducible, and the clinical application has given results which correlate closely with reported series using other staining methods.

This procedure is better than the colour stabilization method of Blau (1975) because the final blue colour is obtained directly after staining without the need for an additional spray.

References

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