Journal of Chromatography, 226 (1981) 484—487
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1035

Note

Improved thin-layer chromatographic assay for monitoring lecithin/sphingomyelin ratios in amniotic fluid

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(First received May 7th, 1981; revised manuscript received July 9th, 1981)

Numerous methods have been proposed for assessing fetal lung maturity by analysis of the amniotic fluid. The lecithin/sphingomyelin (L/S) ratio is the test most widely used as being specific for that purpose, and one-dimensional thin-layer chromatography (TLC) seems to be the method most often used.

A number of reagents, chromogenic as well as fluorogenic, are available for the visualization. Sulfuric acid [1], phosphomolybdic acid [2], and ammonium molybdate perchloric acid—HCl reagent [3] seem to be the most frequently used reagents in the chromogenic mode, whereas 2,7-dichlorofluorescein [4], and rhodamine B [5] are used in the fluorogenic mode.

This paper presents the first report using high-performance thin-layer chromatoplates (HPTLC plates) for the separation of the phospholipids and 8-anilino-1-naphthalene sulfonic acid (ANSA) as the visualization reagent [6], a combination which seems to yield a chromatographic separation and a sensitivity superior to most of the modifications of this assay that have recently been published [7, 8].

EXPERIMENTAL

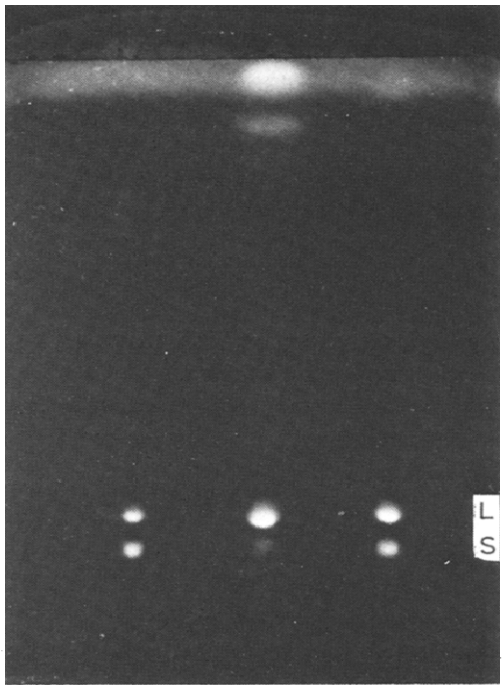
The phospholipid standards, LS-10 with L/S = 1 and LS-20 with L/S = 2, and the detection reagent 8-anilino-1-naphthalene sulfonic acid, ammonium salt, practical grade (Cat. No. A 3125) were from Sigma (St. Louis, MO, U.S.A.). For all experiments we used HPTLC plates from E. Merck, Darmstadt, G.F.R. (Cat. No. 5644). The plates were spotted by a 50-μl luer-tipped Hamilton syringe mounted in a Hamilton repeating dispenser and equipped with a 10-mm tuberculin needle. The plates were scanned with a spectrodensitometer equipped with a recorder and integrator (Schoeffel Instruments, Model SD 3000) in the fluorescence mode.

Amniotic fluid samples obtained by transabdominal amniocentesis are centrifuged within half an hour for 10 min at 2000 *g* to remove cells and sediment. A 1-ml aliquot of the supernatant is pipetted into a 10-ml disposable test-tube containing 3 ml of chloroform—methanol (2 : 1, v/v). The mixture is shaken vigorously for 3 min, and the aqueous and organic phases are separated by centrifugation for 5 min at 2000 *g*; the aqueous (upper) layer is discarded and the organic layer transferred to a 5-ml Pyrex beaker and the chloroform—methanol mixture is removed by placing the beaker in a 60°C water-bath under a stream of air from a hair-dryer. After drying the residue is re-dissolved in 100 μl of chloroform—methanol (1:1, v/v) and 5 μl are spotted on a 5 × 10 cm HPTLC plate. One microliter of standard LS-10 or LS-20 is also spotted on the plate, which is developed in chloroform—methanol—water (65 : 25 : 4, v/v) to a solvent height of 6 cm from the origin, in a saturated tank. The plate is then removed from the tank and dried under a stream of hot air until solvent removal is complete. To visualize the spots of phospholipids the plate is dipped in a 0.2% aqueous solution of the ANSA reagent. To remove excess reagent the plate is placed face downwards on lint-free tissue paper. When the plate is inspected in UV light at 365 nm the spots will appear yellow—green on a dark, uncolored background which is only faintly fluorescent (Fig. 1). It should be emphasized that this high contrast is only achieved if all of the developing solvent has been removed from the plate and the plate is examined and scanned while still wet with the ANSA reagent. If the plate is allowed to dry out subsequently during the scanning procedure, some contrast is lost and the accuracy may decrease. Therefore it must be strongly pointed out that only three spots, for example two standards and one sample, should be spotted on the same plate. The plate is scanned in the direction opposite to development. The spectrodensitometer is set with the excitation wavelength at 365 nm. The speed of the densitometer and the recorder is set at 10 mm/min. The recorder tracing of the plate (Fig. 1) is shown in Fig. 2.

RESULTS AND DISCUSSION

To determine the precision of the method two samples of amniotic fluid with L/S ratios of 1.6 and 14.9 were each analyzed ten times within one day. The results are shown in Table I. As can be seen, the coefficient of variation rises proportionally to the increase in the L/S ratio. This is due to the sharp decrease in the sphingomyelin fraction during the last few weeks of gestation. When using ANSA reagent the detection limits for lecithin and sphingomyelin are about 4 μg/ml and 1 μg/ml of amniotic fluid [6], and the superiority of this reagent over 2,7-dichlorofluorescein and rhodamine B can be explained by the improved background of the plate after the dipping procedure. The HPTLC plates give excellent separation of the substances and usually nice round spots. The length of the chromatographic run is only 6 cm (15—20 min) and the staining procedure can be performed within a few seconds; thus the main advantage of this TLC method is its speed and simplicity.

The whole analytical procedure, including the extraction, can be performed within 1 h. It should also be emphasized that the content of other lipids



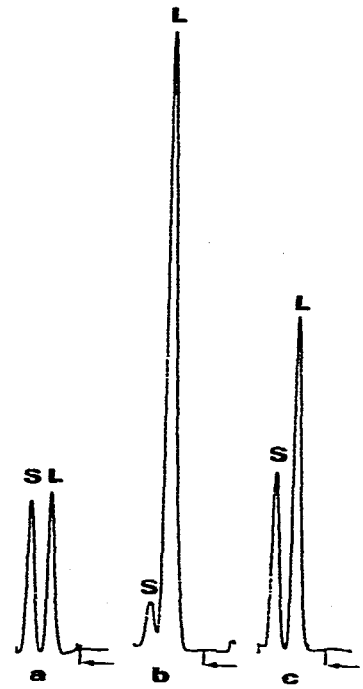


Fig. 1. HPTLC plate photographed in UV light at 365 nm. L = lecithin, S = sphingomyelin. (a) Sigma standard LS-10. (b) Extract of a "mature" (39th week of gestation) amniotic fluid; two spots of unidentified lipids are seen near the front. (c) Sigma standard LS-20.

Fig. 2. Recorder tracing of the chromatogram shown in Fig. 1. The arrows indicate the start of the scanning. L = lecithin, S = sphingomyelin.

TABLE I

THE ANALYTICAL VARIATION OF TWO STANDARDS AND TWO PATIENTS' SAMPLES ANALYZED TEN TIMES WITHIN ONE DAY

	Standard LS-10 L/S = 1.0	Amniotic fluid ("immature") L/S = 1.6	Standard LS-20 L/S = 2.0	Amniotic fluid ("mature") L/S = 14.9
Mean	0.98	1.58	1.94	14.85
S.D.	0.02	0.04	0.06	0.74
C.V. (%)	1.93	2.78	2.89	5.00

normally present in the amniotic fluid have not interfered with the results. Finally, it should be pointed out that this method, producing regular round spots, could also be used visually as a screening assay in laboratories where no scanning instrument is available.

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