Journal of Chromatography, 89 (1974) 197–200

CHROM. 7055

MINIATURE THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF THE LECITHIN/SPHINGOMYELIN RATIO IN AMNIOTIC FLUID

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(Received August 8th, 1973)

SUMMARY

A miniature thin-layer chromatographic determination of the lecithin/sphingomyelin ratio is described. Rapid I.T.L.C. chromatography plates developed in miniature chromatography chambers reduce the chromatography time to 5 min. A sensitive, non-corrosive alcoholic Rhodamine B dye is used to detect microgram quantities of both lecithin and sphingomyelin. Determination of the lecithin/sphingomyelin ratio by this procedure requires less than 30 min.

INTRODUCTION

Measurement of the amniotic fluid lecithin/sphingomyelin ratio (L/S ratio) for the evaluation of fetal maturity is generally carried out on silica gel chromatography plates[1–9]. Previous researchers have used silica gel H[1–3], silica gel G (Kieselgel G)[4–6], silica gel F_{254} ("Silplate F-52")[7], and I.T.L.C. Type SG[8] for the thin-layer chromatographic (TLC) separation of lecithin and sphingomyelin. With the exception of the I.T.L.C. Type SG chromatography plates, chromatographic separation of lecithin and sphingomyelin generally requires 35 min, while completion of the L/S ratio determination is reported to require from 1 to 3 h.

The chromatographic separation time for lecithin and sphingomyelin has been reduced to 5 min by altering the chromatographic migrating solvent and by using a Seprachrom disposable chromatography chamber with an I.T.L.C. Type SG chromatography sheet. The L/S ratio procedure[8] was further shortened by spraying the developed chromatography sheets with Rhodamine B immediately after removal from the chromatography chambers. The moist chromatography plates were then subjected to a mild heating, which rapidly removes the organic solvents but does not remove the water fraction of the migrating solvent from the I.T.L.C. sheets. With these modifications the L/S ratio of an amniotic fluid can be determined within 30 min.

MATERIALS AND METHODS

Chromatography sheets were I.T.L.C. Type SG (20 × 20 cm) from Gelman

(Ann Arbor, Mich., U.S.A.). The sheets were cut to size (6.4 × 9.9 cm) with a scalpel and holes were punched 2.0 cm from the base of the sheet and at 2.0-cm intervals from the left edge. The holes were punched with a Gem hand-punch (with an oval-shaped die, 4 × 3 mm) from McGill (Marengo, Ill., U.S.A.). Both L-lecithin from egg-yolk Type III-E 1 g/10 ml hexane solution and sphingomyelin from bovine brain were from Sigma (St. Louis, Mo., U.S.A.). Standard solutions of lecithin and sphingomyelin were applied to blank discs with a Hamilton microliter syringe from Hamilton (Reno, Nev., U.S.A.).

Amniotic fluid samples were either determined immediately or frozen at −20°. Samples were centrifuged at 4500 *g* for 2–5 min and a 1.5-ml amniotic fluid aliquot was transferred to a 20-ml test tube. A volume of methanol (1.5 ml) was added and the solution was mixed on a Vortex mixer for 30 sec. Chloroform (3.0 ml) was added and the solution was mixed on a Vortex mixer for 90 sec. The emulsion which formed on mixing was separated by centrifuging the test tube at 4500 *g* for 2–5 min. The lower layer was transferred by a Pasteur pipette into 70-ml metal cups and evaporated at 65° in a Vapo-Vent (Hycel, Houston, Texas, U.S.A.) until dry. The lecithin and sphingomyelin residue was dissolved and concentrated by rinsing the sides of the metal cups with 0.1 ml of chloroform. Two blank I.T.L.C. discs were dropped into the solution and the metal cups were placed on an angle into the Vapo-Vent until dry. This concentrated the amniotic fluid lecithin and sphingomyelin residue onto the discs. A standard disc containing both lecithin and sphingomyelin was inserted into the first hole of the I.T.L.C. chromatography sheet, a disc containing the amniotic fluid extract was inserted into the second hole, and a dye marker disc containing Oil Red O dye[10] was placed into the third hole near the right edge of the chromatography sheet. The sheet was placed into a Seprachrom chromatography chamber which was obtained from Gelman. A volume of 3.0 ml of the chromatography solvent dichloromethane–ethanol–water (34:8:1) was added to the trough. To this solution, three drops of distilled water were added, and the resulting solution was mixed. The chromatography chamber was inserted part-way into the chromatography trough to allow chamber saturation (1.0 min), after which the chamber was depressed into the migrating solvent. After 5 min, the marker dye had migrated to the marked end-point (9.0 cm) and the chromatography sheet was removed and sprayed with Rhodamine B dye. The moist chromatogram was placed on top of half a dozen paper towels lying on a preheated 100° hot plate. After about 30 sec the chromatogram acquired a bluish hue. The sheets were then examined under UV (Ultra-Violet Products, San Gabriel, Calif., U.S.A.) and the pink lecithin and sphingomyelin spots which appeared on a blue background were outlined in pencil. (Care should be taken not to overheat the chromatogram.) Area measurements of the lecithin and sphingomyelin spots were carried out by multiplying the length of each spot by its midpoint width[1,8,9]. The L/S ratio was determined by dividing the area of the lecithin spot by the area of the sphingomyelin spot. Interpretation is as in the method of Gluck and Kulovich: 2.0 or greater as Mature, 1.5–1.9 as Transitional, 1.0–1.49 as Immature, and 1.0 as Markedly Immature.

RESULTS AND DISCUSSION

Chromatographic separation is dependent upon adding a specific amount of

water to the chromatography migrating solvent. The addition of three drops of water (administered by a Pasteur pipette) to the chromatography migrating solvent is demanded for optimum separation. In our laboratory, the addition of one drop proved insufficient while five drops were too many, resulting in a poorer separation.

The heating step was introduced to reduce the time required for evaporation of the organic solvent of the chromatography sheet after spraying with the alcoholic Rhodamine B stain. The heating should be mild and evenly distributed over the sheet without overheating. An overheated chromatogram evaporates the water phase of the migrating solvent, which results in a loss of contrast between the lecithin and sphingomyelin spots and the background of the chromatogram. Optimum conditions yield well defined pink lecithin and sphingomyelin spots on a blue background. An overheated chromatogram has bright pink lecithin and sphingomyelin spots on a pale pink background. As previously reported[8], the blue background can be regenerated by spraying the reverse of the chromatography sheet with a fine spray of distilled water. Overspraying with distilled water increases the size of the lecithin and sphingomyelin spots by diffusion.

The I.T.L.C. Type SG sheets are commercially available and do not require activation. The Seprachrom miniature disposable chromatography chambers are reusable. Although 1 min was allowed for solvent saturation of the Seprachrom chromatography chamber, elimination of the solvent saturation time did not appreciably affect the separation of the lecithin and sphingomyelin spots.

It was observed that chromatography spots of lecithin and sphingomyelin standards run on separate discs 2 cm apart do not separate as well as lecithin and sphingomyelin run on the same disc. It is thus suggested that lecithin and sphingomyelin standards be run on the same disc when comparison is made to the amniotic fluid lecithin and sphingomyelin spots. It is also advisable to run a lecithin and sphingomyelin standard disc with every I.T.L.C. sheet, because extra bands are present in amniotic fluid samples. The extra bands generally migrate close to the solvent front and do not interfere with the lecithin and sphingomyelin spots, which have R_F values of 0.62 and 0.37, respectively.

The migrating solvent and the standard discs (containing both lecithin and sphingomyelin) were stable over a 5-week period at room temperature. Standard discs of lecithin and sphingomyelin as well as discs containing amniotic fluid extracts were stable for months when stored in a freezer. All standards and solvents required can be prepared in advance and appropriately stored for future use in this rapid L/S ratio determination.

The separated lecithin and sphingomyelin on the I.T.L.C. type SG chromatography sheets can be visualized by a number of methods previously reported in the literature[1,4,5,7,9]. The L/S ratio can be calculated from area measurements[1,8,9] or from densitometer scans[1,4,7]. A 50% H_2SO_4 char on a preheated hot plate works well, although it is not as sensitive nor is it as reliable as the Rhodamine B detection system described in this paper. Bromothymol blue was found to be even less sensitive than the H_2SO_4 char and this dye was only useful at high concentrations of lecithin and sphingomyelin.

Modifications of the chromatography solvent allows for greater separation on the short I.T.L.C. Type SG chromatography sheets (6.4 × 9.9 cm). Employment of a Seprachrom miniature chromatography chamber reduces the chromatography

solvent volume to 3.0 ml, while also reducing the chromatography time. The chromatographic separation time for lecithin and sphingomyelin is less than 5 min, while the total time required for determining the L/S ratio of an amniotic fluid sample is less than 30 min.

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