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A RAPID, SENSITIVE METHOD FOR ACCURATE DETERMINATION OF THE LECITHIN/SPHINGOMYELIN RATIO BY THIN-LAYER CHROMATOGRAPHY AND REFLECTANCE SPECTROFLUOROMETRY

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SUMMARY

A highly reproducible thin-layer chromatographic procedure has been developed for accurate determination of the lecithin/sphingomyelin ratio. Two interfering compounds, phosphatidyl inositol and phosphatidyl serine, have been investigated and eliminated by adsorption onto DEAE-cellulose. A uniform fluorescence staining procedure employing 2',7'-dichlorofluorescein has been developed. Accurate quantitation was performed by direct measurement of the reflected fluorescence intensity of the lecithin and sphingomyelin fluorophore spots with a spectrofluorometer equipped with a thin-layer scanning attachment. Stability and reproducibility studies are reported.

INTRODUCTION

Gluck and coworkers' [1] original lecithin/sphingomyelin (L/S) ratio procedure for the evaluation of fetal lung maturity has prompted much recent investigation of amniotic fluid phospholipids. Within the past few years, numerous thin-layer chromatographic (TLC) procedures have been developed for the determination of L/S ratios in amniotic fluid samples. Moreover, recent investigation has shown that phosphatidyl inositol (PI) and phosphatidyl serine (PS) may preclude accurate determination of the L/S ratio [2, 3]. Some of the techniques employed to achieve the separation of L and S from the aforementioned lipids include lengthy chromatographic separation time employing silica gel H chromatography plates containing 5% ammonium sulfate [4, 5], two-dimensional chromatography [3, 6], and adsorption of PI and PS onto diethylaminoethyl cellulose (DEAE-cellulose) [2].

Rapid TLC separation of L and S has been performed on silica gel impregnated glass microfiber chromatography sheets [3, 7—12]. A variety of solvent systems, visualization reagents, and quantitation methods have been employed

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TABLE I

TLC SEPARATION OF L AND S ON GLASS MICROFIBER CHROMATOGRAPHY SHEETS

Solvent system	Visualization reagent	Quantitation	R_F		Reference	Comment
			L	S		
$CHCl_3$–CH_3OH–NH_4OH (170:20:3, v/v)	I_2 vapor and/or bismuth subnitrate	Visual estimation	0.67	0.48	8, 9	
CH_2Cl_2–C_2H_5OH–H_2O (102:24:3, v/v)	Rhodamine B	Spot area (height × midpoint width)	0.27	0.12	7	
3.0 ml of CH_2Cl_2–C_2H_5OH–H_2O (34:8:1, v/v) plus H_2O optimization (2–4 drops of H_2O)	Rhodamine B	Spot area (height × midpoint width)	0.62	0.37	10	Miniature chromatography sheet (6.5 cm × 10 cm)
$CHCl_3$–CH_3OH–NH_4OH (170:20:3, v/v)	Malachite green	Densitometry			11	
$CHCl_3$–CH_3OH–NH_4OH (200:25:4, v/v)	Bismuth subnitrate	Visual comparison	0.63	0.44	12	
CH_2Cl_2–C_2H_5OH–H_2O (100:25:3, v/v), then			0.55	0.37	3	Two-dimensional chromatography
$CHCl_3$–CH_3OH–NH_4OH (170:20:3, v/v)	2',7'-Dichlorofluorescein		0.53	0.38		

(Table I). Although most detection procedures can be adapted to visualize phospholipids separated on glass microfiber sheets, further development of sensitive and accurate in situ methods of quantitation is essential.

In this paper, a rapid and highly reproducible TLC procedure is described for the determination of L/S ratios. A previously reported procedure [10] has been modified as follows: A DEAE-cellulose treatment step has been added to eliminate the interference of PI and PS; the TLC application procedure has been improved; a sensitive 2′,7′-dichlorofluorescein (DCF) detection procedure has been incorporated; and quantitation has been performed by direct scanning of the chromatogram with a spectrofluorometer equipped with a thin-layer scanning attachment.

MATERIALS AND METHODS

The following Certified ACS grade solvents were purchased from Fisher Scientific (Winnipeg, Canada): chloroform, methanol, methylene chloride, and isopropanol. Absolute ethanol was obtained from Standard Chemicals (Winnipeg, Canada). Lecithin (L-α-phosphatidyl choline, from egg yolk), sphingomyelin (from bovine brain) and commercially prepared 1:1, 1.5:1, and 2:1 L/S ratio standards were purchased from Sigma (St. Louis, Mo., U.S.A.). The following phospholipids were obtained from Supelco (Bellefonte, Pa., U.S.A.): phosphatidyl serine (bovine) and phosphatidyl inositol (plant). Synthetic lysolecithin (LL) was purchased from Calbiochem (San Diego, Calif., U.S.A.). The DEAE-cellulose (DE32) was from North American Scientific Chemical (Calgary, Canada).

ITLC-type SG chromatography sheets (20 × 20 cm) were from Gelman (Ann Arbor, Mich., U.S.A.). The sheets were cut to size 9.9 × 6.4 cm, to fit the Gelman Seprachrom micro-chromatography chamber. A Gem hand-punch (McGill Metal Products Co., Marengo, Ill., U.S.A.) was used to excise round 3-mm chromatography discs. Holes were punched 2 cm from the bottom edge and at intervals of 1 cm from the left edge of each miniature chromatography sheet. The miniature sheets were heat activated in a 110° oven for 30 min. Activated sheets were stored in a desiccator above silica gel.

The chromatography solvent system, methylene chloride—ethanol—water (100:25:3, v/v), was prepared fresh weekly. The solvent system was stored at room temperature in a tightly stoppered brown bottle. Prior to use, the solvent system was vigorously mixed for 1 min.

A standard solution of lecithin was prepared to contain 1 mg of L per ml of chloroform–methanol (9:1, v/v). Standard solutions of S and LL were similarly prepared. PI and PS standards were prepared in chloroform. Three successive 1-μl volumes of the L standard solution were applied to a blank chromatography disc with a 10-μl syringe equipped with a Channey adaptor (Hamilton, Reno, Nev., U.S.A.). Standard discs were prepared in batches of ten or more, as required. Four dry standard L discs were inserted into a miniature ITLC sheet. Three milliliters of well-mixed solvent system were transferred to a Seprachrom chromatography trough. The chamber was assembled and the solvent system was allowed to migrate to within 1.5 cm of the top of the ITLC sheet. This required approximately 3.5 min. The developed chromatogram was

immediately removed and air dried for 5 min. The chromatogram was dipped for 10 sec into a solution containing 1.5 mg of DCF per 100 ml of isopropanol. Excess stain was allowed to drain onto the edge of the staining trough. The stained chromatogram was air dried for 15 min. Lecithin fluorophore spots were visualized under 375-nm ultraviolet light.

The reflected fluorescence intensity of the chromatogram spots was measured with a Farrand Mark I spectrofluorometer and recorded on a Farrand Model 100 strip-chart recorder (Model SR-204, Heath Co., Benton Harbor, Mich., U.S.A.). To improve instrument stability, the spectrofluorometer was modified as previously described [13]. Excitation and emission slit widths were 5 and 10 nm, respectively. The Color Specification numbers for the primary and secondary filters were 7-54 and 3-73, respectively. The area of the recorded L peaks was measured with a Koizumi Compensating Polar Planimeter purchased from Reliable Drafting Supplies (Regina, Canada). The above procedure was performed repeatedly. Fluorescence intensity studies were similarly performed for S, LL, PI, and PS. Relative fluorescence intensities were calculated with respect to the L fluorophore results.

Five microliters of a standard solution of PS (1 mg PS per ml of chloroform) were added to a blank chromatography disc. A second chromatography disc was prepared to contain L, S, and PS in 6-, 3-, and 5-μg quantities, respectively. For the preparation of a third chromatography disc 1.5 μl of the PS standard solution (50 mg PS per ml of chloroform) were added to a test-tube and the DEAE-cellulose adsorption procedure was performed as described by Gosselin and Foidart [2]. The residue was concentrated at the bottom of a pear-shaped flask and redissolved in 0.04 ml of chloroform—methanol (9:1, v/v). Three microliters were sequentially applied to the chromatography disc. For the preparation of the fourth chromatography disc, 40 μl of a 2:1 L/S standard and 1.5 μl of the PS (50 mg PS per ml of chloroform) standard were mixed in a test-tube, dried under nitrogen gas, and the DEAE-cellulose adsorption procedure was performed. The final residue was concentrated at the bottom of a pear-shaped flask with 0.04 ml of chloroform—methanol (9:1, v/v). Three microliters were applied to the chromatography disc. The four chromatography discs prepared above were inserted into a miniature ITLC sheet. Chromatographic separation and visualization were performed. The chromatogram was scanned from the origin to the solvent front for each test. A blank scan was obtained by scanning between two chromatogram discs. The above procedure was performed in duplicate. Similar testing of the DEAE-cellulose adsorption procedure was performed in the presence of 14 μl of a commercially prepared PI standard (10 mg PI per ml of chloroform). L/S ratio reproducibility studies were similarly performed for each of the following: a laboratory weighed in 2:1 L/S standard after DEAE-cellulose treatment; a commercial 2:1 L/S ratio standard with PS added and DEAE-cellulose treatment; and a commercial 2:1 L/S ratio standard with PI added and DEAE-cellulose treatment.

A fluorescence stability study was performed. A commercially prepared 2:1 L/S ratio standard was used to prepare a chromatogram disc which contained 6 μg of lecithin and 3 μg of sphingomyelin. Chromatographic separation and visualization were performed as described above. The fluorescence intensity of the chromatogram spots was recorded within 0.5 h after staining and after 3, 6,

9, 12, and 24 h. Results were calculated as percentages of the fluorescence intensity of the 6 μg lecithin fluorophore as measured within 0.5 h. L/S area ratios were also calculated. The above study was performed in triplicate. A fluorescence stability study using a weighed in 2:1 L/S ratio standard [2 mg of L and 1 mg of S per ml of chloroform–methanol (9:1, v/v)] was similarly performed.

Four chromatography discs were prepared to contain L and S in the following quantities: 2 μg of L and 1 μg of S; 4 μg of L and 2 μg of S; 6 μg of L and 3 μg of S; and 8 μg of L and 4 μg of S. Chromatographic separation, visualization, and measurement of the fluorescence intensity of the L and S fluorophore spots were performed as described above. Peak area measurements of L and S were plotted against μg of lipid applied. L/S area ratios were also calculated. The aforementioned study was similarly performed in duplicate.

A 1:1 L/S ratio standard was prepared by adding 2.0 mg of sphingomyelin and 20 μl of lecithin [1 g per 10 ml of chloroform–methanol (9:1, v/v)] to a 2-ml volumetric flask which was brought to volume with chloroform–methanol (9:1, v/v). Subsequent ratios of 1.5:1, 2.0:1, 2.5:1, 3.0:1, and 3.5:1 were similarly prepared by adding 30, 40, 50, 60, and 70 μl of the commercial lecithin solution, respectively. Chromatographic separation, visualization, and measurement of the reflected fluorescence intensity were performed as previously described. L/S area ratios were calculated and plotted against L/S weight ratios applied. The above study was performed in duplicate. The following commercially prepared L/S ratio standards were similarly tested: 1.0 mg L and 1.0 mg S per ml, 1.5 mg L and 1.0 mg S per ml, and 2.0 mg L and 1.0 mg S per ml of chloroform–methanol (1:1, v/v). The ratio results were calculated and plotted as described above. This study was similarly performed in duplicate.

RESULTS AND DISCUSSION

Rapid chromatographic separation of L and S has been achieved on unactivated ITLC-type SG chromatography sheets [7–10]. However, for accurate quantitation recent investigation in this laboratory has shown that ITLC sheets activated for 30 min in a 110° oven give smoother and more reproducible strip-chart recordings [13].

Uniform staining was achieved by dipping the chromatograms for 10 sec into a solution containing 1.5 mg of DCF per 100 ml of isopropanol. Excess stain was allowed to drain onto the edge of the staining trough. This procedure eliminated variation due to uneven spraying. The day-to-day chromatogram stain density also appeared to be more uniform.

Dry, powdered DCF reagent has been observed to discolor upon storage at room temperature for many months. With the present spectrofluorometer detection system, use of discolored DCF dye has resulted in increased background fluorescence and higher noise levels.

When the DCF-stained chromatograms were viewed under a long-wave ultraviolet lamp (375 nm), bright-green phospholipid spots were observed on a dark purple background. The intensity of the reflected fluorescence was measured with a spectrofluorometer equipped with a thin-layer scanning attachment and recorded on a strip-chart recorder. Relative fluorescence intensity results were

TABLE II

FLUORESCENCE INTENSITY OF 2′,7′-DICHLOROFLUORESCEIN-STAINED PHOSPHOLIPIDS

Lipid	Quantity (μg)	Relative fluorescence* (%)	C.V.** (%)
L	3	100.0	5.2
S	3	77.8	4.8
LL	3	131.0	3.4
PS***	3	71.0	7.0
PI***	3	75.4	11.7

*Values are reported in percentages of the fluorescence of 3 μg L.
**Each of the reported C.V. were calculated from 10 test results.
***Approximate fluorescence intensity results were calculated from diffusely distributed fluorophore spots (see Fig. 1c). Refer to text for complete details.

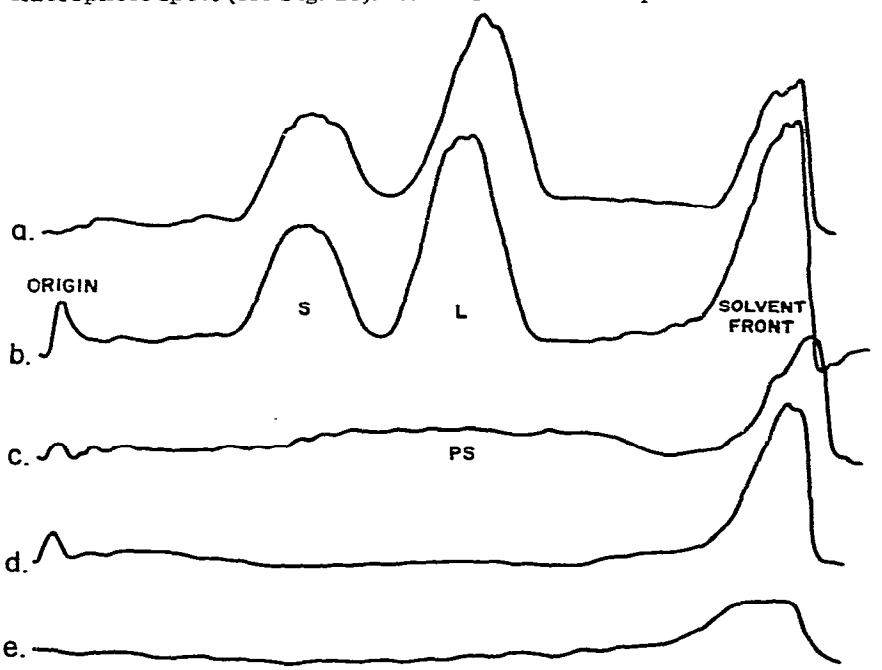

Fig. 1. Reflected fluorescence scans depicting: (a) L, S and PS; (b) L, S and PS after DEAE-cellulose treatment; (c) PS; (d) PS and DEAE-cellulose treatment; (e) blank chromatogram. Refer to text for complete details.

calculated for L, S, and LL (Table II). Very reproducible results were observed for the L, S, and LL standards. Although the fluorescence intensity of the LL fluorophore was more intense than that of the L or S, no interference was observed because of the excellent separation distances achieved; R_F values for L, S, and LL were 0.55, 0.37 and 0.23 respectively. In contrast, a similar investigation undertaken for PI and PS resulted in very elongated spot formation. A typical scan of a PS spot is depicted in Fig. 1c. In general, when L and S spots were scanned in the presence of PS or PI, the base-line intensity

increased, the noise-to-signal ratio increased, and an apparent incomplete separation was observed between the L and S spots (Fig. 1a). However, comparison of the scans depicted in Fig. 1 clearly shows that DEAE-cellulose removes the interference caused by the presence of PS. Similar results were observed for PI.

To maintain optimal reproducibility at least three rinse steps should be employed to concentrate the phospholipids at the bottom of the flask after rotary evaporation to dryness. Successive application of phospholipid material to blank chromatography discs was also observed to improve reproducibility of the L/S ratio results. In the present procedure, three successive 1-μl applications were made to the front side of blank discs.

The reproducibility of the presently developed L/S ratio procedure was repeatedly evaluated and a summary of the results is given in Table III. Very

TABLE III

L/S RATIO REPRODUCIBILITY STUDIES

n	L/S area ratio ($\bar{X}$)	S.D.	C.V. (%)	Comment***
40	1.92*	0.087	4.5	L and S, one batch per day for four days
10	2.11*	0.080	3.8	L, S, PS and DEAE-cellulose adsorption
13	2.03*	0.078	3.8	L, S, PI and DEAE-cellulose adsorption
10	2.27**	0.079	3.5	L and S
12	2.40**	0.108	4.5	L and S after DEAE-cellulose adsorption

*Commercial 2:1 L/S ratio standard.
**Laboratory weighed in 2:1 L/S ratio standard.
***Refer to text for complete details.

reproducible results were observed throughout all studies. However, the mean L/S ratio calculated for the commercial standard was consistently lower than the results calculated for the laboratory weighed in standards. Furthermore, a slight increase was observed for L/S ratio results after incorporation of the DEAE-cellulose adsorption step.

Fluorescence stability tests were conducted for a commercial L/S standard and a laboratory weighed in L/S standard. A total of six fluorescence stability tests were performed because of fluctuations encountered in the xenon arc lamp intensity during the 24-h studies; two studies are reported in Tables IV and V. In general, the L and S fluorophore intensity was observed to decrease only slightly over 24 h. During this time, highly reproducible L/S ratio results were observed (Tables IV and V).

Accessory attachments and modifications to improve stability of the Farrand Mark I Spectrofluorometer have been reported previously [13]. Excellent fluorometer stability has been observed; however, recently, large fluctuations in line voltage including power failures have caused fluctuations in the intensity of the xenon arc lamp. The increase in the relative fluorescence of the 3-h L and S fluorophore results reported in Table IV is attributed to a change in the intensity of the xenon arc lamp.

L/S ratio standards were applied to blank discs in the following quantities:

TABLE IV

FLUORESCENCE STABILITY OF A COMMERCIALLY PREPARED 2:1 L/S RATIO STANDARD

Time (h)	Relative fluorescence*		L/S ratio
	L (6 μg)	S (3 μg)	
0—0.5	100.0	48.7	2.05
3**	103.0**	56.1**	1.84
6	102.2	56.3	1.82
9	95.8	51.7	1.85
12	95.2	51.7	1.84
24	94.0	50.3	1.87

*Values are reported in percentages of the fluorescence intensity of 6 μg of lecithin as measured between 0 and 0.5 h.
**The relative fluorescence increase observed is attributed to spectrofluorometer instability. Refer to text for complete details.

TABLE V

FLUORESCENCE STABILITY OF A WEIGHED IN 2:1 L/S RATIO STANDARD

Time (h)	Relative fluorescence*		L/S ratio
	L (6 μg)	S (3 μg)	
0—0.5	100	45.9	2.18
3	99.3	46.4	2.14
6	97.9	46.4	2.11
9	96.4	45.4	2.12
12	97.1	46.2	2.10
24	96.4	45.4	2.12

*Values are reported in percentages of the fluorescence intensity of 6 μg of lecithin as measured between 0 and 0.5 h. Refer to text for complete details.

2:1, 4:2, 6:3, 8:4 μg of L and S, respectively. Corresponding L/S ratio results for each of the aforementioned tests were 3.2, 2.5, 2.3, and 2.3, respectively. The increased fluorescence intensity of the L fluorophore compared to the S fluorophore accounts for L/S ratio results above 2.0 (see Table II and Fig. 2). The L/S ratio results were particularly elevated at lower concentrations; for example, 2 μg of L:1 μg of S resulted in an L/S ratio of 3.2.

A linear relationship was obtained for L/S ratios between 1 and 3.5 when the L/S area ratios were plotted against the L/S weight ratios of the laboratory prepared standards (Fig. 3). The linear regression parameters for these results are: correlation coefficient, 0.999; y intercept, 0.467; slope, 0.920. The results were above the line of identity and converged toward the line of identity. The area ratio results above the line of identity are attributed to the greater fluorescence intensity of the lecithin fluorophore (Table II), while convergence of the results toward the line of identity is attributed to increased self-quenching of the L spots. The variation in slope between the commercial and the laboratory prepared standards is presently unexplained. Repeated testing of

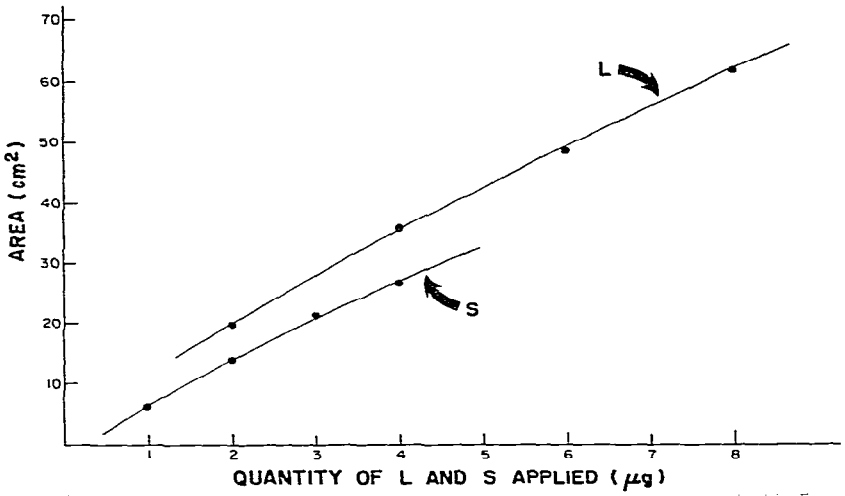

Fig. 2. L and S area measurements vs. quantity of lipid standard applied. The results depicted represent an average of two samples at each quantity studied. Refer to text for complete details.

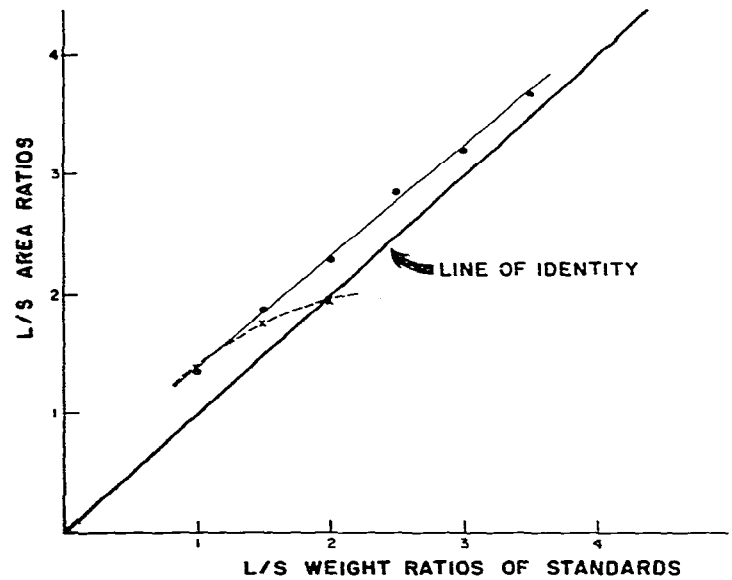

Fig. 3. Correlation of L/S area ratios vs. L/S weight ratios. The results depicted represent an average of two samples at each quantity studied. Refer to text for complete details. •——•, Laboratory prepared standards; x— — — x, commercial standards.

the 2:1 commerical standard has established a mean value of 1.92 which does not correlate with the laboratory weighed in standards (Table III). However, it should be noted that water contamination of the lecithin employed in preparation of the commerical standards is suspected.

The presently developed procedure is highly recommended for accurate and reproducible measurement of the L/S ratio.

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