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Short Communication

Semiautomated multisample analysis of amniotic fluid lipids by high-performance thin-layer chromatography–reflectance spectrodensitometry

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ABSTRACT

Analysis of the various lipids in amniotic fluid by multisample semiautomated quantitative high-performance thin-layer chromatography (HPTLC) is presented. Amniotic fluid (AF) lipids are extracted by liquid–liquid partition with chloroform–methanol (C–M) (final C–M–AF ratio of 4:2:1, v/v), 4-μl aliquots of the C–M extract applied by way of an HPTLC autosampler device (Camag) to Whatman HP-K silica gel plates, and the lipids separated in one dimension using chloroform–ethanol–triethylamine–water (30:34:30:8, v/v) and hexane–diethyl ether (50:5, v/v) as the initial and final mobile phases, respectively. The plates were then stained with a 10% solution of copper sulfate in 8% phosphoric acid and placed in an oven with initial and final temperatures of 24 and 120°C, respectively. This HPTLC system allowed resolution of cholesteryl palmitate, triglycerides, free fatty acid fraction, cholesterol, cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine. The stained chromatograms were scanned with a Shimadzu CS-9000U spectrodensitometer in the reflectance mode at 310 nm. The lower limit of detection was 20 ng/ml with linear detector response extended to 2 μg/ml. Use of this methodology allows the simultaneous analysis of the various lipids in AF for up to 50 samples in *ca.* 2 h. This method constitutes a sensitive, quantitative, reproducible, and high-capacity system for analysis of AF lipids.

INTRODUCTION

Thin-layer chromatographic (TLC) analysis of phospholipids in amniotic fluid (AF), including phosphatidylcholine and sphingomyelin (L/S ratio) and phosphatidylglycerol, has been the method of choice for the assessment of fetal lung maturity for over twenty years [1–4]. Phosphatidylcholine (dipalmitoylphosphatidylcholine) and phosphatidylglycerol are key components of the lung surfactant, and their presence in AF at certain levels indicates pulmonary maturity in the fetus. More recently, Ludmir *et al.* [5] have shown that cholesteryl palmitate in AF of women with uncomplicated pregnancies, as measured by high-performance thin-layer chromatography (HPTLC), constitutes a sensitive predictor of fetal lung maturity. Even in the absence of phosphatidylglycerol, cholesteryl palmitate levels

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above 41 μg/ml are indicative of fetal lung maturity [5].

Despite the significant growth that TLC has experimented in the last ten years, this chromatographic technique still remains regarded as an ancestral method of analysis in the clinical environment. HPTLC has become one of the leading chromatographic techniques due to the recent introduction of automated instrumentation that has improved considerably the precision and detection limits of this particular mode of analysis. This instrumentation includes automated multisample applicators which allow application of up to 72 different samples per plate at variable volumes and application speed, horizontal development chambers that allow sample separation in both directions with minimal solvent requirements and development time, and scanning spectrodensitometers which permit scanning of the chromatograms in the reflectance and/or transmittance modes at a scanning speed of up to 20 mm s^{-1}. The combination of scanning spectrodensitometry and copper sulfate staining of the phospholipid and cholesteryl ester fractions separated by HPTLC results in detection limits of the order of 20 ng/ml [3]. The software capabilities of the spectrodensitometer computer permit transfer of the processed data to a modem or communications network system, thus making the data directly available to a remote location bypassing a printed report. Using the aforementioned methodology, including sample preparation, a complete lipid profile report can be obtained in *ca.* 2 h for up to 50 samples.

Although some of the recently introduced tests [6–10] for the assessment of fetal lung maturity are also based on the detection of the lung surfactant-associated phospholipids, these tests do not provide quantitative data, are non-specific, detecting some general physico-chemical properties of the sample that could be ascribed to other compounds unrelated to the fetal lung surfactant (unsaturated lecithins, phosphatidylethanolamine, cardiolipin, free fatty acids, neutral glycosphingolipids, gangliosides), and the lower limit of detection is at least one order of magnitude greater (> 500 ng/ml). This lack of sensitivity often results in false negatives.

In this report, a new method of analysis of the aforementioned lipids by semiautomated HPTLC is presented. Resolution of the various AF lipids, including cholesteryl palmitate, triglyceride fraction, cholesterol, free fatty acid fraction, phosphatidylglycerol, pseudophosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine, is accomplished in one dimension allowing, therefore, multisample analysis.

EXPERIMENTAL

Chemicals and reagents

The lipid standards, including cholesteryl palmitate, trioleoin, oleic acid, cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine, were purchased from Sigma (St. Louis, MO, USA). Precoated silica gel HP-K high-performance plates (10 cm × 10 cm, 250 μm thickness) were obtained from Whatman (Clifton, NJ, USA). Solvents were EM Science chromatographic grade. Inorganic salts were from J. T. Baker (Phillipsburg, NJ, USA) and of the highest purity available.

Sample preparation

AF samples were obtained by transabdominal amniocentesis from women with gestational ages ranging between 28 and 38 weeks. Pregnancies complicated by diabetes mellitus or Rh sensitization were excluded. Non of the patients had clinical signs or symptoms of chorioamnionitis and all fluid samples had negative cultures. Aliquots of 0.1 ml of the AF samples were added to glass conical tubes having screw-cap tops lined with PTFE and extracted by liquid–liquid partition by addition of 0.6 ml of a mixture of chloroform–methanol (C–M) (2:1, v/v) to obtain a final ratio of C–M–AF of 4:2:1 (v/v). The tubes were then centrifuged at 800 *g* for 5 min, the water-saturated C–M lower phases aspirated separately and evaporated to dryness. The resulting lipid residues were redissolved in 10 μl of C–M (1:1, v/v).

High-performance thin-layer chromatography

Aliquots of 4 μl were applied to the plates as 4-mm bands, 5 mm from the lower edge of the plate and at a speed of 800 nl/s using a Camag Automatic TLC Sampler III (Camag Scientific, Wilmington, NC, USA). Following sample application, the plates were predeveloped in C–M (1:1, v/v) to *ca.* 1 cm from the lower edge of the plate, thoroughly dried, and developed in 25 cm × 25 cm size tanks using chloroform–ethanol–triethylamine–water (C–E–T–W) (30:34:30:8, v/v) for the first development (SF_1). This mobile phase allows separation of polar lipids, including sphingomyelin, phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, and phosphatidylinositol. Cardiolipin and phosphatidylglycerol comigrate as a single band following this first development. The plates were then dried at 24°C in a vacuum oven for 5 min and placed in hexane–diethyl ether (H–E) (50:5, v/v) for the second development (SF_2). This mobile phase allowed separation of the cholesteryl ester fraction and resolution of phosphatidylglycerol and cardiolipin. The plates were dipped for 5 s in a 10% solution of $CuSO_4$ in 8% H_3PO_3 [3], excess $CuSO_4$ allowed to drip off the plate, placed horizontally in an oven with initial and final temperatures of 24 and 120°C, respectively, and scanned with a Shimadzu CS-9000U spectrodensitometer at 310 nm in the reflectance mode using a beam size of 1 mm/5 mm.

In a different set of experiments, 100 ml of pooled term AF samples were extracted with six volumes of C–M (2:1, v/v), the organic phase was evaporated to dryness, and the resulting residue redissolved in 1 ml of C–M (1:1, v/v). Aliquots of 200 μl were applied with the Camag Automatic TLC Sampler III as a 18-cm streak to Whatman HP-K silica gel plates, 10 cm × 20 cm, the plates predeveloped in C–M (1:1, v/v) and developed using C–E–T–W and H–E, as indicated above. The bands corresponding to cholesteryl palmi-

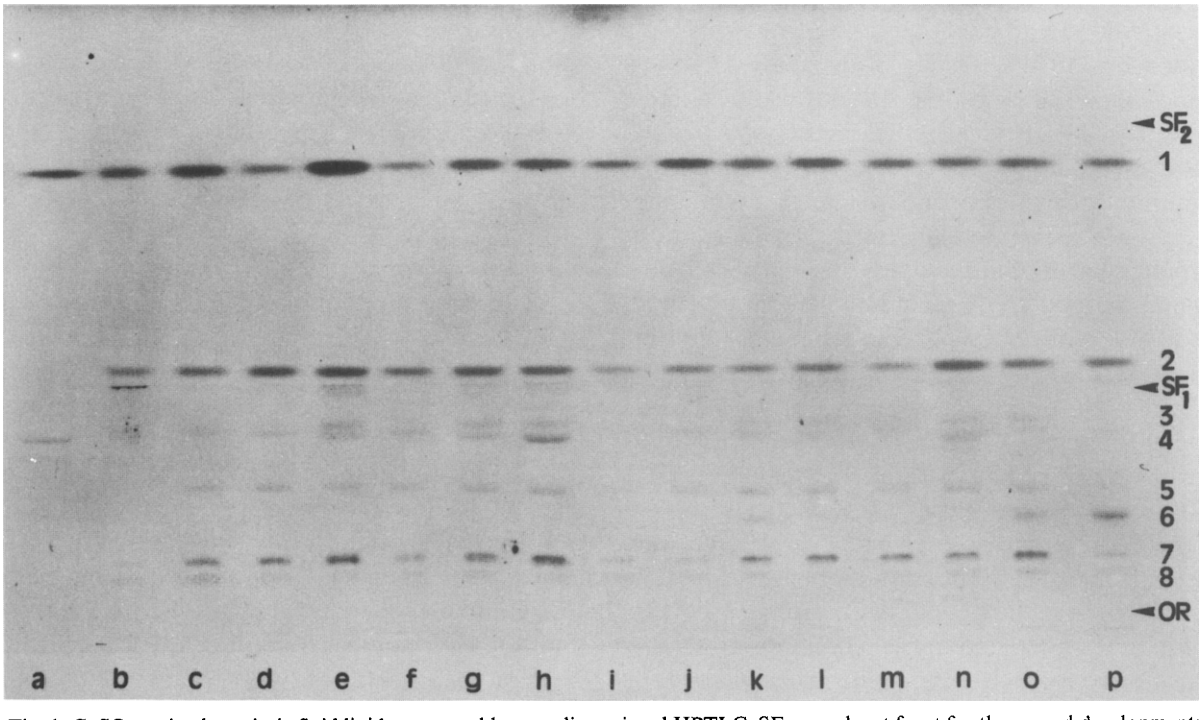

Fig. 1. $CuSO_4$-stained amniotic fluid lipids separated by one-dimensional HPTLC. SF_2 = solvent front for the second development; 1 = cholesteryl palmitate; 2 = cholesterol; SF_1 = solvent front for the first development; 3 = phosphatidylglycerol; 4 = cardiolipin; 5 = phosphatidylethanolamine; 6 = phosphatidylinositol; 7 = phosphatidylcholine; 8 = sphingomyelin; OR = origin; lane a = 0.5 μg of cholesteryl palmitate standard and 0.1 μg of cardiolipin standard; lanes b–p correspond to the different amniotic fluids analyzed.

tate, phosphatidylglycerol, phosphatidylinositol, phosphatidylcholine, and sphingomyelin were scrapped from the plate, and the lipids desorbed from the silica gel by addition of C–M–W (1:1:0.1, v/v) followed by centrifugation at 800 *g* for 5 min. The various C–M–W supernatants containing the individual lipids were evaporated to dryness and redissolved in 500 μl of C–M (1:1, v/v) to obtain a final concentration of 1 mg/ml. The purified lipids were then used to generate individual standard curve as follows: aliquots of the lipids isolated from AF dissolved in C–M were evaporated to dryness, and 0.5-ml aliquots of AF of low lipid content, as determined by HPTLC–reflectance spectrodensitometry (28–30 weeks of gestation), added to obtain concentrations that ranged from 0.04 to 4 μg/ml. The spiked samples were sonicated in a Branson 1200 ultrasonic bath (Danbury, CT, USA) for three 30-s periods at 10-s intervals and brought to 1 ml with 0.5 ml of the same AF. Aliquots of 0.1 ml were then extracted and analyzed as indicated above. Quantitation was obtained by interpolation of the integration areas of the lipids under analysis with the standard curves generated for the individual lipids purified from AF.

RESULTS AND DISCUSSION

The $CuSO_4$-stained chromatogram corresponding to the lipid profile of 15 different AF samples is shown in Fig. 1. Fig. 2 represents the HPTLC profile obtained following scanning of lane o from Fig. 1. The ultraviolet and visible spectra of the $CuSO_4$–lipid chromogen following the charring reaction is shown in Fig. 3. Maximal absorbance was observed at 310 nm. As shown in Table I, scanning of the $CuSO_4$–lipid chromogen in the reflectance mode at 400 nm resulted in a signal ratio of 1.4 as compared to scanning in the transmittance mode at the same wavelength (the differential absorption, ΔA, is maximal at 400 nm; $\Delta A = A_s - A_r$, A_s and A_r being the solute and layer absorption, respectively). Scanning in the transmittance mode at 310 nm is forbidden because of absorption of that wavelength by the glass of the plate. Scanning in the reflectance mode at 310 nm resulted in a signal ratio of 1.8 as compared to scanning in the reflectance mode at 400 nm. Therefore, scanning in the reflectance mode at 310 nm was selected for analysis of the $CuSO_4$-stained lipids.

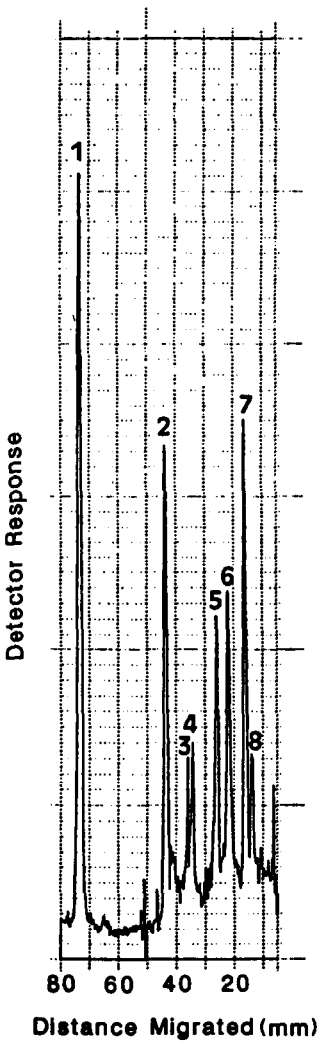

Fig. 2. Integrated HPTLC chromatogram of amniotic fluid lipids. This chromatogram corresponds to lane o of Fig. 1. Peaks: 1 = cholesteryl palmitate, 2.0 μg (50 μg/ml); 2 = cholesterol, 1 μg (25 μg/ml); 3 = phosphatidylglycerol, 0.1 μg (2.5 μg/ml); 4 = cardiolipin, 0.3 μg (7.5 μg/ml); 5 = phosphatidylethanolamine, 0.5 μg (12.5 μg/ml); 6 = phosphatidylinositol, 0.4 μg (10 μg/ml); 7 = phosphatidylcholine, 1 μg (25 μg/ml); 8 = sphingomyelin, 0.1 μg (2.5 μg/ml). The horizontal axis of the chromatogram indicates the *y*-position of each lipid on the plate in mm. The vertical axis indicates the detector response.

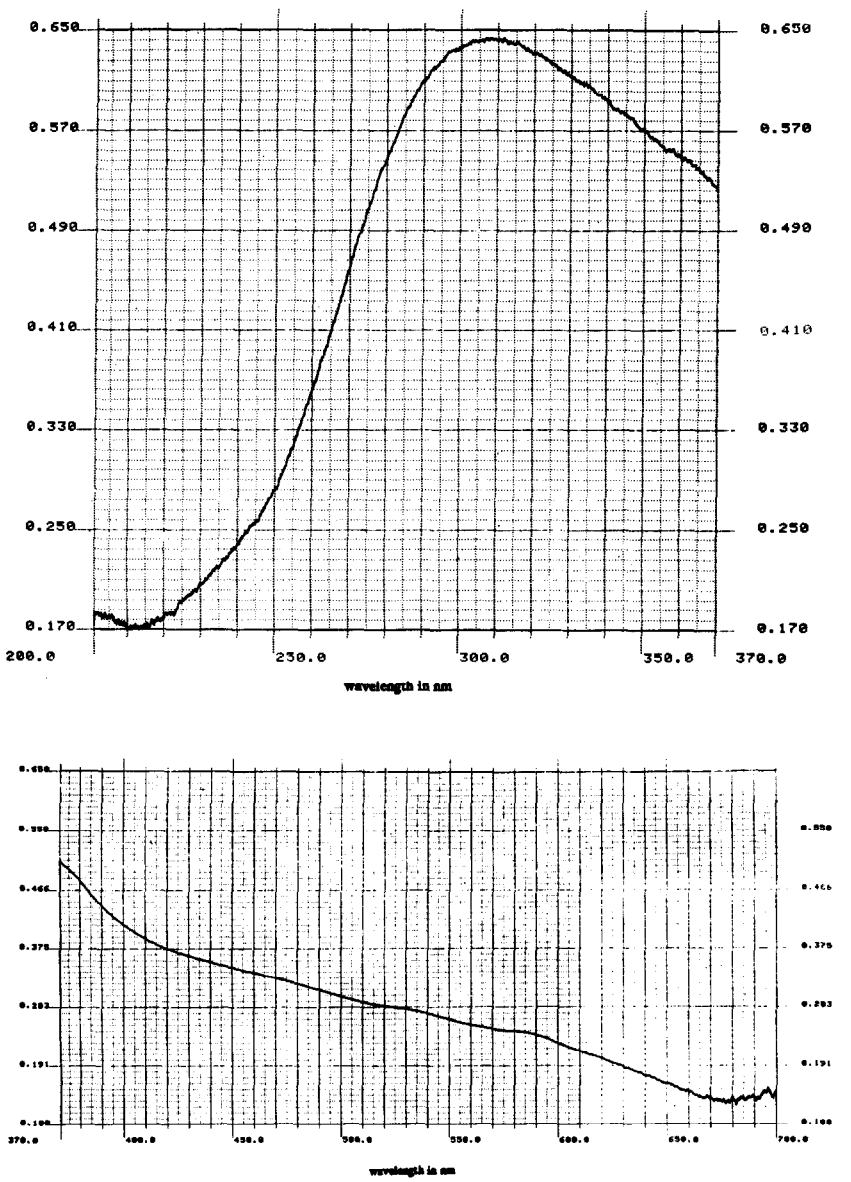

Fig. 3. Differential ultraviolet and visible spectra of the $CuSO_4$–lipid chromogen. The ultraviolet (upper) and visible (lower) spectra of the $CuSO_4$–lipid chromogen were acquired in the ranges between 200 and 370 nm and 370 and 700 nm, respectively, in the reflectance mode, using a Shimadzu CS-9000U spectrodensitometer for scanning. To obtain the differential spectrum, the spectrum of a blank area of the plate was first obtained and then subtracted by the computer of the spectrodensitometer from that obtained for the $CuSO_4$–lipid chromogen.

Sample preparation time for the processing of 50 different AF samples, including sample transfer, C–M partition, and solvent evaporation, was 45 min. Sample application time, including sample delivery and rinsing cycles for all 50 samples, was 25 min. The combined predevelopment and development time was 35 min. All 25 lanes were scanned in the automatic mode at 1 cm s^{-1} with an interlane scanning speed of 10 cm s^{-1} and data accumulation time of 20 s per chromatogram. Total estimated time for scanning and data accumulation was 13 min.

TABLE I

SCANNING OF THE $CuSO_4$–LIPID CHROMOGEN IN THE TRANSMITTANCE AND REFLECTANCE MODES

Values correspond to the detector response obtained following scanning of the $CuSO_4$–lipid chromogen at concentrations that ranged from 0.02 to 2 μg and represent the mean (± S.D.) of five experiments; N.D. = not determined.

Scanning mode	Detector response		
	310 nm	400 nm	420 nm
Reflectance	1.0 ± 0.05	0.71 ± 0.03	0.65 ± 0.03
Transmittance	N.D.	0.55 ± 0.02	0.45 ± 0.02

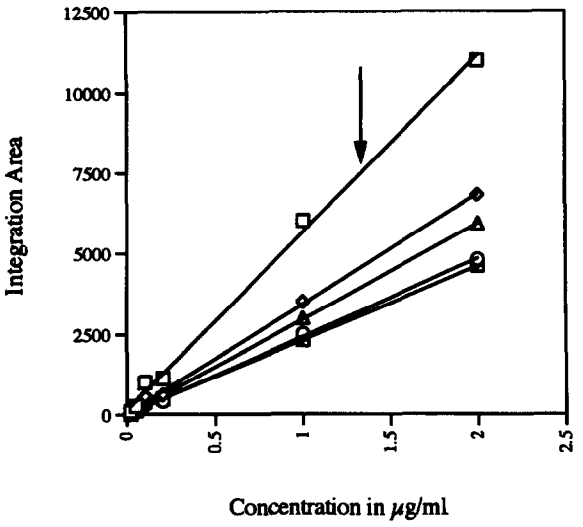

Fig. 4. Standard curves for amniotic fluid lipids. (□) Cholesteryl palmitate; (◇) phosphatidylglycerol; (△) phosphatidylinositol; (○) phosphatidylcholine; (⊞) sphingomyelin. Each point represents the mean of five experiments. Error bars are the standard deviations. The arrow indicates the value that corresponds to a cholesteryl palmitate concentration in amniotic fluid of 38 μg/ml.

The mode of analysis presented in this study differs from other TLC methods in that (1) it only requires 0.1-ml aliquots of amniotic fluid, (2) it uses 4.5 μm particle size high-performance layers that provide higher resolution only comparable to that obtained with HPLC columns, (3) sample application is automated, resulting in higher speed of analysis and precision, (4) all lipid classes, including phosphatidylglycerol and cardiolipin, often referred to as pseudophosphatidylglycerol, are separated in one dimension, therefore allowing multisample analysis, (5) the lipids are visualized with a highly sensitive lipid reagent that allows detection of picomole amounts of all lipids, (6) it provides quantitative data for lipids known to be associated with the fetal lung surfactant, and (7) a complete profile of AF lipids can be obtained in *ca.* 2 h for up to 50 samples.

The recoveries for the various lipids added to the AF following one single extraction ranged between 82 and 95%. The intra- and inter-assay variation, as measured by the relative standard deviation, were 4 and 8%, respectively. The lower limit of detection was 20 ng/ml with linear detector response extended to 2 μg/ml (Fig. 4). This method constitutes a quantitative, sensitive, selective, reproducible, and high-capacity system for the analysis of the various lipids present in AF. Work is now in progress to establish the cut-off values for the various surfactant-associated lipids that will allow accurate prediction of fetal lung maturity.

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