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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE LECITHIN/SPHINGOMYELIN RATIO IN AMNIOTIC FLUID

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SUMMARY

A high-performance liquid chromatographic (HPLC) procedure has been developed for the separation of phospholipids commonly found in amniotic fluid. The chromatographic separation was achieved on a 25-cm column packed with LiChrosorb DIOL (10 μm). A 3-cm column packed with silica was fitted between the injector and the DIOL column to provide complete separation of lecithin (L) and sphingomyelin (S) from the remaining amniotic fluid phospholipids. The eluted phospholipids were quantitated employing an ultraviolet absorption detector set at 203 nm. The new HPLC separation described herein has improved the resolution and peak sharpness of L and S. Furthermore, phosphatidyl glycerol and phosphatidyl inositol were completely separated and quantitated. Amniotic fluid L/S ratios determined by this technique have been compared to those of an established thin-layer chromatographic procedure.

INTRODUCTION

Since Gluck et al. [1] first introduced the thin-layer chromatographic (TLC) separation of lecithin (L) and sphingomyelin (S) in amniotic fluid, the L/S ratio procedure has been widely used and modified for the evaluation of fetal lung maturity [2—4].

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In addition to L and S there is a group of minor phospholipids in amniotic fluid which include phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), and lysolecithin (LL) [5]. Recently, several groups of researchers have recognized the importance of these minor phospholipids, particularly PG and PI. Analyses of these phospholipids in amniotic fluid have been accepted to be of value as additional indices of fetal lung maturity [5—9].

In an attempt to improve upon the conventional TLC procedures, separations have been carried out on TLC rods precoated with silica gel, followed by quantitation using a hydrogen flame ionization detector [10]. Phospholipids have also been separated by high-performance liquid chromatography (HPLC) [11—13]. Though HPLC separations have the potential to provide more accurate and precise quantitations of L and S as a result of advances made in column technology, pumping systems and detectors, to date procedures have not been reported for the separation of the minor amniotic fluid phospholipids.

In this paper all the amniotic fluid phospholipids have been separated by HPLC and quantitated by ultraviolet absorption. A preliminary patient study has been conducted to correlate the L/S ratio in amniotic fluid determined by this technique with that of an established TLC procedure.

MATERIALS AND METHODS

Apparatus

The HPLC separations were carried out on a Hewlett-Packard (Avondale, PA, U.S.A.) Model 1084B high-performance liquid chromatograph equipped with an automatic sampler and a variable-wavelength detector (190—600 nm). The chromatographic column was 25 cm × 4.6 mm I.D. from Hewlett-Packard. The column was packed with LiChrosorb DIOL (10 μm) supplied by Merck (Darmstadt, G.F.R.). A Brownlee (Santa Clara, CA, U.S.A.) MPLC guard column, 3 cm × 4.6 mm I.D. packed with 10 μm silica (SI 60, Merck), was fitted between the injector and the DIOL analytical column. The thin-layer chromatograms were scanned by reflectance densitometry on a Joyce Loebl (Team Valley, Great Britain) Chromoscan 200 equipped with a Scan 201 TLC scanning accessory.

Reagents and samples

Water used in the HPLC solvent was distilled in the presence of $KMnO_4$ in an all glass still, and stored in a borosilicate glass bottle. HPLC grade acetonitrile, glass-distilled chloroform and Merck silica gel 60H were purchased from BDH Chemicals (Vancouver, Canada). Certified ACS grades of ammonium sulfate, methanol, and acetone were purchased from Fisher Scientific (Winnipeg, Canada). The following phospholipids were obtained from Sigma (St. Louis, MO, U.S.A.): lysolecithin (Type I, egg yolk), sphingomyelin (bovine brain), lecithin (Type III-E, egg yolk), phosphatidyl ethanolamine (Type III, egg yolk), phosphatidyl serine (bovine brain), phosphatidyl inositol (Grade III, soybean) and phosphatidyl glycerol (Grade I, egg yolk). Commercially prepared lecithin/sphingomyelin standard solutions were also obtained from Sigma.

Amniotic fluid samples were collected by amniocentesis.

Chromatographic analysis

The chromatographic mobile phase was composed of Solvent A: acetonitrile—water (80:20, v/v) and Solvent B: acetonitrile. Both solvents were maintained at 40°C. A linear solvent gradient was run from 87.5 to 25.0% B between 4.5 and 11.0 min which produced a linear gradient of water running from 2.5 to 15.0%. The flow-rate was constant at 2.0 ml/min and the column oven temperature was 35°C. The column effluent was monitored at 203 nm [11] and the detector response was set to 0.0128 a.u./cm. Quantitation was by integration of peak areas using a software integrator.

Aliquots of a commercially prepared 1:1 L/S standard solution were diluted with chloroform—methanol (2:1, v/v) to prepare a series of standards containing L and S each at concentrations of 1.0, 0.75, 0.50, 0.25 and 0.125 mg/ml of solution.

Standard solutions of each of the following were prepared at similar concentrations: LL, PE, PS, PI and PG. Twenty μl of each standard solution were injected in triplicate and the areas of the peaks averaged to produce calibration curves for each of the phospholipids.

The TLC procedure used was that described by Gluck et al. [14]. Phospholipids were extracted from 5.0 ml of amniotic fluid. Following the cold acetone precipitation, the precipitate was dissolved in 30 μl of chloroform, of which 5—10 μl were applied to the TLC plate. An additional 40 μl of chloroform—methanol (2:1, v/v) was added to the sample and a 10—30-μl aliquot was injected into the HPLC instrument.

RESULTS AND DISCUSSION

A chromatogram illustrating the separation of a mixture of standard phospholipids is depicted in Fig. 1A. When PS was chromatographed alone, a broad tailing peak was observed. The tailing of the PS is the primary reason for the baseline deviation. The baseline was much improved when PS was not included in the standard mixture, as illustrated in Fig. 1B.

In the preliminary studies, separations were performed using only a DIOL column. Sphingomyelin appeared as a split peak in the majority of chromatograms. Similar splitting of the S peak has been shown for procedures employing a silica column [11—13]. However, the primary difficulty with the preliminary separation was the appearance of PE between L and S. PE was often difficult to distinguish from S. Inclusion of a short 3-cm silica column in tandem with the DIOL column, resulted in PE eluting prior to L and S. Furthermore, this improved the resolution between the peaks and S was no longer observed to split. The elution order of the remaining 6 phospholipids in the standard mixture was unchanged. When a 25-cm silica column was connected in series with the DIOL column in place of the shorter 3-cm column, however, the separation was unsatisfactory with most of the phospholipids appearing as broad split peaks.

Solvent A was composed of acetonitrile—water (80:20, v/v) in order that the solvent delivery system would accurately deliver a relatively shallow solvent gradient running from 2.5 to 15.0% water.

Calibration curves for L and S are illustrated in Fig. 2. Linearity was ob-

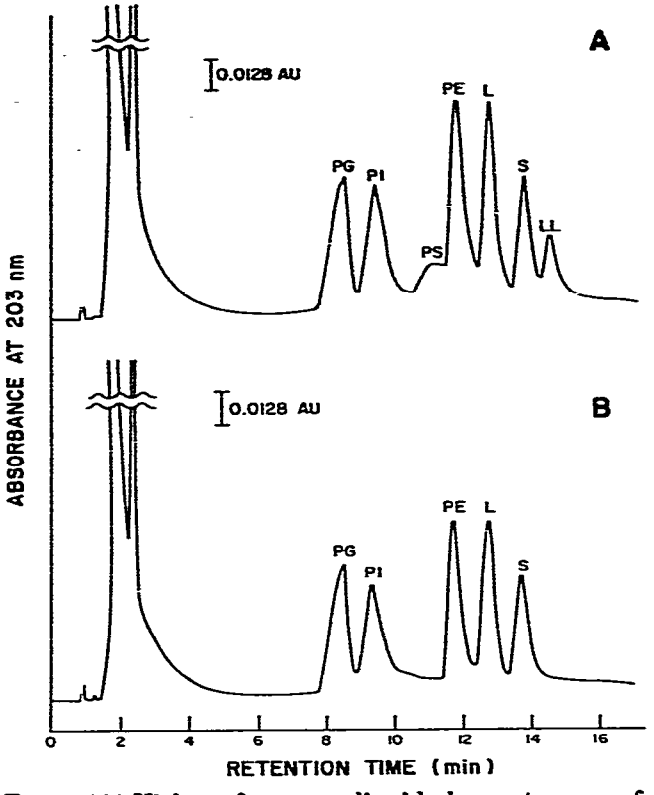

Fig. 1. (A) High-performance liquid chromatogram of a mixture of 7 standard phospholipid samples each at a concentration of 5 μg. Peaks: PG = phosphatidyl glycerol; PI = phosphatidyl inositol; PS = phosphatidyl serine; PE = phosphatidyl ethanolamine; L = lecithin; S = sphingomyelin; LL = lysolecithin. (B) High-performance liquid chromatogram of the standard phospholipids mixture excluding PS and LL.

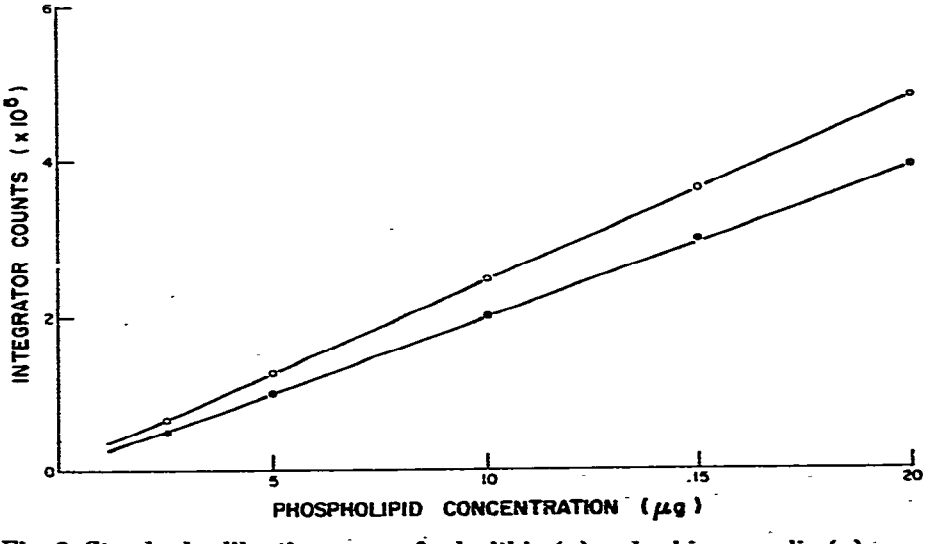

Fig. 2. Standard calibration curves for lecithin (○) and sphingomyelin (●).

served between 2.5 and 20.0 μg, which was the range of values expected for L and S in the majority of amniotic fluid samples as performed by this procedure. Of note, the linearity of the calibration curve for L was observed to extend to at least 100 μg. The calibration curves for each of the minor amniotic fluid phospholipids are depicted in Fig. 3. The linearity of these curves

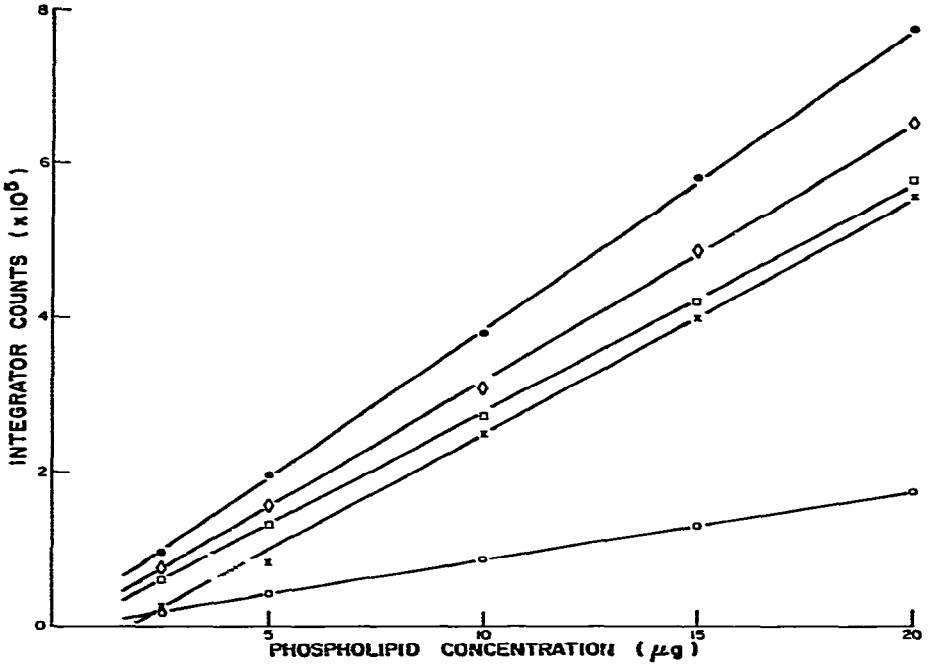

Fig. 3. Standard calibration curves for phosphatidyl glycerol (•), phosphatidyl ethanolamine (◇), phosphatidyl inositol (□), phosphatidyl serine (×) and lysolecithin (○).

was also well maintained between 2.5 and 20.0 μg. Each point on all of the phospholipid standard curves was the mean of 3 measurements, each with a coefficient of variation of less than 2.5%, with the exception of the 2.5- and 5.0-μg points on the PS standard curve which had coefficients of variation of 17.6% and 6.7%, respectively. The linear regression correlation coefficients for each of the phospholipid calibration curves was better than 0.999. The non-zero intercepts of the curves may be attributed to peak tailing. This is especially pronounced for the PS calibration curve which exhibited the greatest peak tailing.

Representative chromatographic separations of phospholipids from amniotic fluid specimens collected before and after fetal lung maturation are depicted in Figs. 4 and 5, respectively. Fifty amniotic fluid samples were analyzed by a conventional TLC procedure [14] and by the new HPLC method. The correlation between these two methods of analysis is illustrated in Fig. 6. A commercially prepared standard solution with an L/S ratio of 3.0 was tested repeatedly throughout the study to monitor the precision of the L/S ratio determination. For a total of 14 test results, the mean and coefficient of variation were 2.93 and 3.74%, respectively. The same commercial standard was also tested 14 times in succession, which produced a mean value of 2.79 and a coefficient of variation of 1.0%.

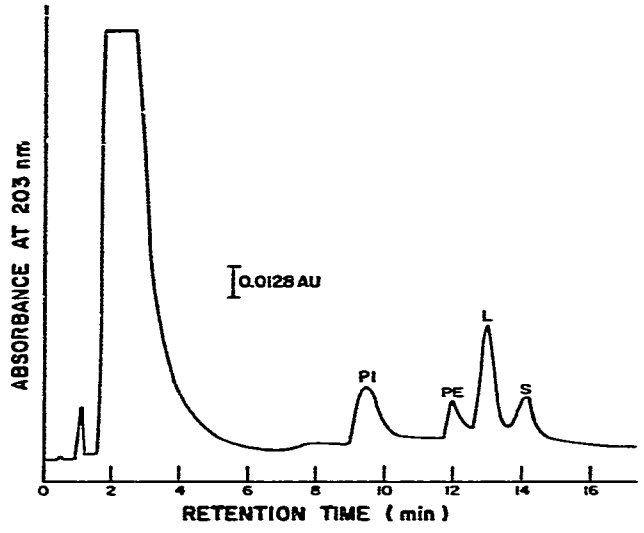

Fig. 4. High-performance liquid chromatogram of an amniotic fluid sample taken before fetal lung maturity.

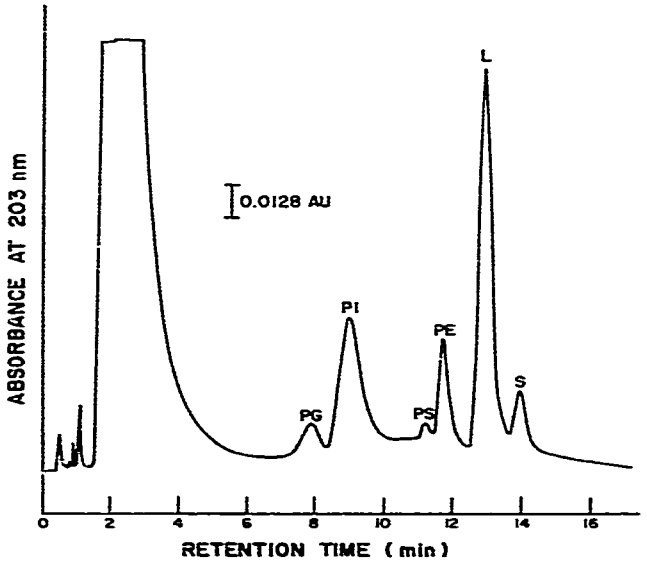

Fig. 5. High-performance liquid chromatogram of an amniotic fluid sample taken after fetal lung maturity.

Gluck et al. [15] have stated that "An L/S ratio of 4.0 by the gravimetric technique corresponds to one of 2.0 by reflectance densitometry, representing maturity of the lung". From the preliminary patient study described herein, employing only 50 patient samples, it would appear that an L/S ratio of 3.0 as determined by HPLC would correspond most closely to an L/S ratio of 2.0 by TLC and reflectance densitometry. To establish a reliable range of values for the interpretation of fetal lung maturity, a larger number of amniotic fluid

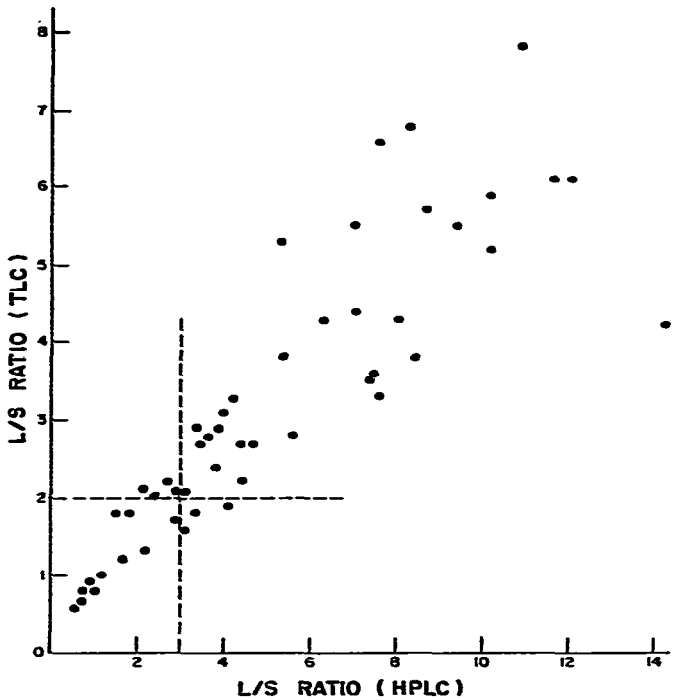

Fig. 6. Correlation of amniotic fluid L/S ratio results established by TLC and HPLC analyses.

specimens should be collected at parturition, analyzed, and the results carefully correlated with fetal lung maturity.

Jungalwala et al. [11] have shown that phospholipids containing unsaturated fatty acids gave much greater ultraviolet absorption at 203 nm than phospholipids with saturated fatty acids. These researchers suggested that if the degree of unsaturation in the phospholipids varied, quantitation by direct ultraviolet absorption would not be accurate, and that an alternative detection system would be required. However, Geurts van Kessel et al. [13] stated that the ultraviolet absorption was due not only to the presence of unsaturated centres, but also due to functional groups such as carbonyl, carboxyl, phosphate, amino, and quaternary ammonium. They went on to state, that if there were a variety of fatty acid constituents, there would be problems with quantitation by direct ultraviolet absorption. Several researchers [16—19] have determined the fatty acid composition of L. Though differences have been reported, a correlation coefficient of 0.860 was obtained by linear regression analysis between the HPLC method employing ultraviolet absorption detection and the TLC procedure using charring and reflectance densitometry (Fig. 6).

The presence and relative concentration of PG and PI in amniotic fluid have been reported to be of clinical significance for the evaluation of fetal lung maturity [5—9]. False positive L/S ratio results have been reported in pregnancies with complications, e.g. diabetes, placenta previa, and maternal fever [7,8,20]. In these circumstances analysis of the minor amniotic fluid

phospholipids is believed to be of particular importance. It has been suggested that a lung phospholipid profile would be valuable and possibly essential in institutions caring for patients with high risk pregnancies [21].

The present HPLC separation provides not only an improved separation and precise quantitation of L and S, but also the possibility of a complete lung phospholipid profile.

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