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ESTIMATION OF AMNIOTIC FLUID PHOSPHOLIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have developed a high-performance liquid chromatographic (HPLC) method for the analyses of surface-active amniotic fluid phospholipids, lecithin (L), sphingomyelin (S), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), and phosphatidyl serine (PS), which are important in the prediction of fetal lung maturity. The method incorporates an internal standard in the amniotic fluid extract, and utilizes a 10-μl aliquot of a 2:1 chloroform—methanol extract of amniotic fluid injected onto a 5-μm DIOL or CN HPLC column, and a variable-wavelength detector set at 203 nm.

Amniotic fluid phospholipid estimations were determined on 40 amniotic fluid samples by the HPLC method and by the routine thin-layer chromatographic (TLC) method. Good agreement was observed between the two methods for the L/S ratio, PG, and PI (r_{PG} 0.94, r_{PI} 0.95, $r_{L/S}$ 0.97).

The advantages of the HPLC procedure include: (i) Selective separation for PG, PI, PS, and PE, as well as L and S at the same time. (ii) The internal standard allows individual concentration of phospholipids to be estimated. (iii) The procedure is rapid: 16 min for a single assay compared with 50 min for the standard TLC procedure.

INTRODUCTION

Immaturity of the fetal lung leading to respiratory distress syndrome (RDS) is the principal cause of death in the premature neonate. RDS is primarily due to a lack of pulmonary surfactant. Recent advances indicate that surfactants appear in the amniotic fluid (AF) during gestation and their quantity and pattern are determined by their production in the lung. Surfactant enables a low, stable surface tension of the air—water interface within the alveoli to be maintained, it decreases the amount of pressure needed to distend the lung and prevent alveolar collapse. Therefore, in the clinical situation, rapid analysis of amniotic fluid phospholipids for the detection of lung maturity is important in the management of the premature infant.

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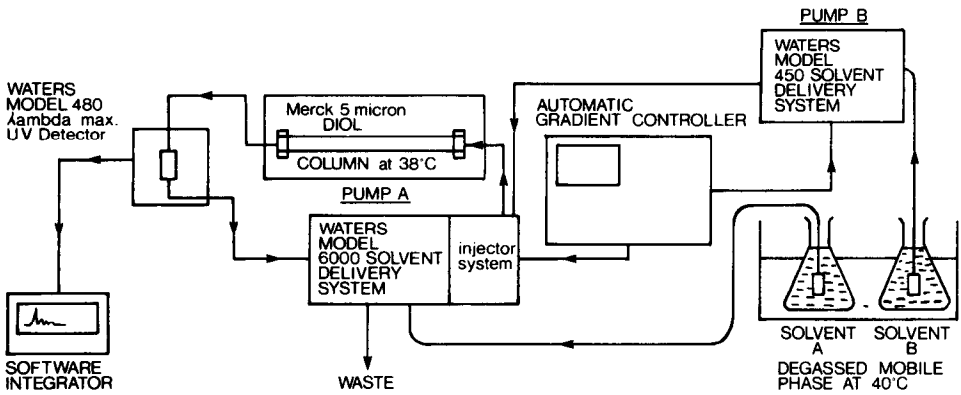

Fig. 1. A line diagram of the HPLC apparatus used for the analyses of the amniotic fluid phospholipids.

Thin-layer chromatography (TLC) was first established by Gluck et al. in 1971 [1] for the separation and measurement of the predominant amniotic fluid phospholipids lecithin (L) and sphingomyelin (S) as the L/S ratio and later by Hallman et al. in 1976 [2] for the estimation of two other important phospholipids, phosphatidyl glycerol (PG) and phosphatidyl inositol (PI) by two-dimensional TLC. However, TLC is a relatively laborious procedure and gives limited information. High-performance liquid chromatography (HPLC) is a much faster procedure than TLC but its application to lung maturity studies of amniotic fluid phospholipids has to date resulted in very limited progress. The present paper describes the development of an HPLC procedure for separation and quantitation of all the amniotic fluid phospholipids of interest in the study of fetal lung maturity, namely L, S, PG, PI, phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE).

MATERIALS AND METHODS

A line diagram of the current HPLC apparatus is shown in Fig. 1. Apart from an ultraviolet (UV) detector, it comprises the following solvent delivery system: a Waters Model 6000A pump and a Waters M45 pump with a U6K injection system and a Waters automatic gradient controller for gradient elution. The column used is a 5-μm DIOL 12.5 cm × 4.6 mm and is upplied by Merck—BDH (Australia). A Merck 3 cm × 4.6 mm guard column packed with 5-μm silica Si60 was fitted between the analytical column and the precolumn filter and injection system. The column and guard column were maintained at an oven temperature of 38°C while the solvents were kept at 40°C in a water bath. All solvents were HPLC grade and, prior to use, were filtered through a 0.2-μm Durapore filter and degassed by sonication for 0.5 h. Regeneration of the column was carried out periodically in accordance with the Merck literature on column care.

The choice of detectors is a major problem with the HPLC separation of phospholipids. Of the common detectors available, the differential refractometer is probably the most suitable, since it allows the use of solvents such as chloroform and ammonium hydroxide which are commonly used in TLC sep-

arations of phospholipids; its major drawback is that it is less sensitive than most other detectors. Fluorescent detectors require derivatisation of the phospholipids and, for this reason, were not considered. The detector used in our system was a Waters Lambda Max-480 UV detector (190—380 nm). The direct detection of phospholipids at low UV wavelength according to Geurts van Kessel et al. [3] is dependent not only on the degree of unsaturation of the fatty acid side-chains of the phospholipids, but also on the functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium of each molecule. This accounts for the difference in response to UV absorption for the individual phospholipids.

Standards

Table I shows the concentration of a typical working calibration standard used in routine analyses. The seven phospholipids, PG, PI, PS, PE, L, S, and the internal standard (IS) γ-capryloyl lysolecithin were of the highest purity available from either Calbiochem—Behring or PL-Biochemicals. Each individual standard was made up to 1 μg/μl in a 2:1 (v/v) chloroform—methanol solvent. In order to prepare a composite working standard, aliquots of each individual standard were mixed together and dried under nitrogen in a water bath at 50°C, then reconstituted in 1.0 ml of 2:1 chloroform—methanol mixture. The working standard (2 μl) was injected onto the column at the beginning of each run and then after every third sample. The working standard phospholipids were stored at −10°C when not in use.

TABLE I

TYPICAL WORKING CALIBRATION STANDARD

A typical calibration standard of phospholipids found in amniotic fluid which includes the internal standard γ-capryloyl lysolecithin: 2 μl of this standard are injected initially in the column and then after every third specimen.

Phospholipid	Concentration (μmol/l)
Phosphatidyl glycerol	1.92
Phosphatidyl inositol	2.26
Phosphatidyl ethanolamine	0.29
Phosphatidyl serine	2.56
Lecithin	2.66
Sphingomyelin	0.70
γ-Capryloyl lysolecithin	39.50

Extraction of phospholipids from amniotic fluid

Fig. 2 shows a flow diagram of the extraction procedure for the phospholipids.

Duplicates of 1.5 ml of centrifuged amniotic fluid were extracted with an equal volume of methanol, followed by vortexing for 30 sec, then addition of twice the volume of chloroform and then vortexed again for 30 sec. After centrifugation for 10 min at 1500 *g*, the supernatant aqueous methanol layer was aspirated to waste. The lower chloroform layer was withdrawn and evaporated to dryness under nitrogen on a water bath at 50°C. The lipid residue was ace-

tone-fractionated, using ice cold anhydrous acetone according to the well established procedure of Gluck et al. [1].

The acetone precipitate after thorough drying was taken up in 20 μl of 2:1 chloroform—methanol mixture containing 22.5 μmol/l internal standard γ-capryloyl lysolecithin. Of this 10 μl were injected onto the HPLC column. For the present study, the second replicate was taken up in 10 μl of chloroform and spotted on a prepared TLC plate.

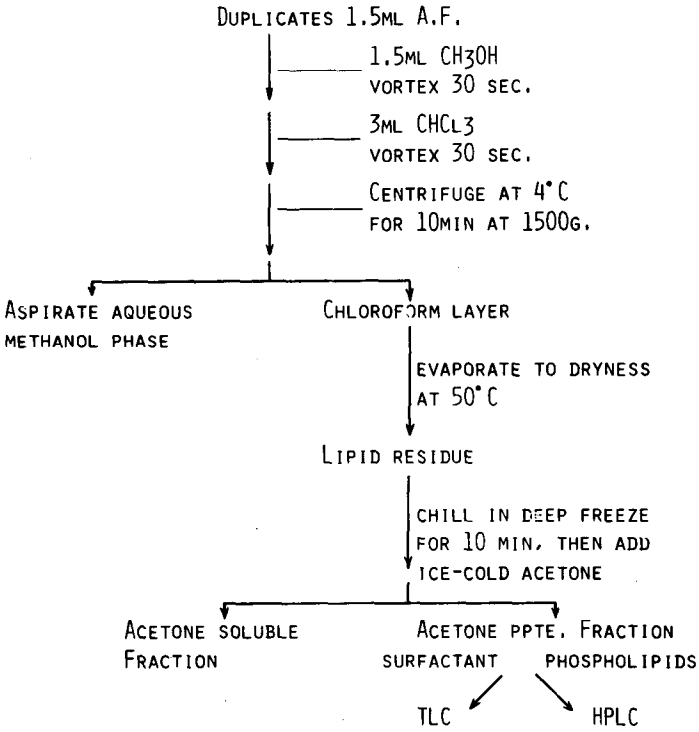

Fig. 2. Flow diagram for the extraction procedure of the phospholipids from amniotic fluid.

HPLC procedure

The HPLC system was set up as follows for gradient elution of phospholipids. Initial conditions consisted of solvent A (acetonitrile 100%) 88% and solvent B (a mixture of acetonitrile—water in the ratio 3.5:1) 12%. A linear gradient was run from 4.20 to 12.20 min when the final conditions were 25% solvent A and 75% solvent B. The flow-rate was constant at 2.0 ml/min and the maximum allowable back-pressure on the column was 14 MPa. The column effluent was monitored at 203 nm and the detector was set at 0.02 a.u.f.s. deflection. Quantitation was by integration of peak areas using the Waters Data Module and a Hewlett-Packard 85 computer.

To determine the best system for the separation of amniotic fluid phospholipids, several columns and solvent systems were explored using an isocratic system. The best separation achieved by this procedure was with a Waters μPorasil 60 A°GPC column using hexane—isopropanol—water (6:8:1.15) as the mobile phase, illustrated by Figs. 3 and 4. While this gave adequate separation

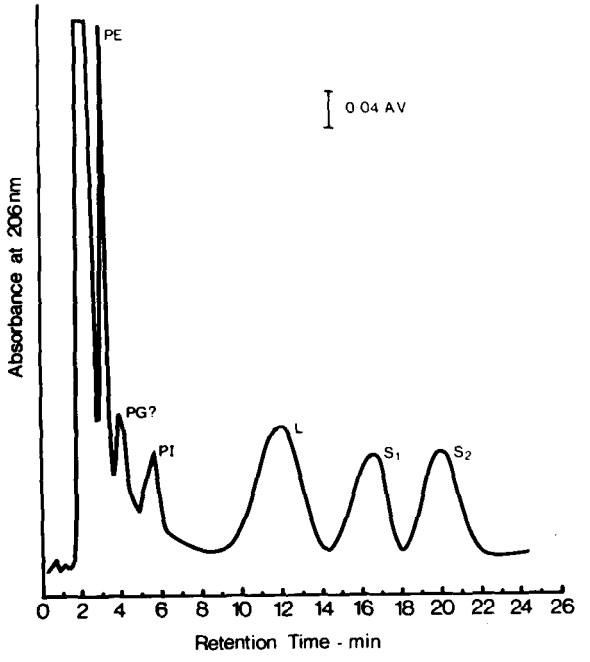

Fig. 3. Separation of amniotic fluid phospholipids before lung maturity using an isocratic system. Mobile phase: *n*-hexane—2-propanol—water (6:8:1.15, v/v/v). Note the broad peaks of both lecithin (L) and sphingomyelin which is split into two peaks (S_1 and S_2). PE = Phosphatidyl ethanolamine; PG = phosphatidyl glycerol; PI = phosphatidyl inositol; PS = phosphatidyl serine.

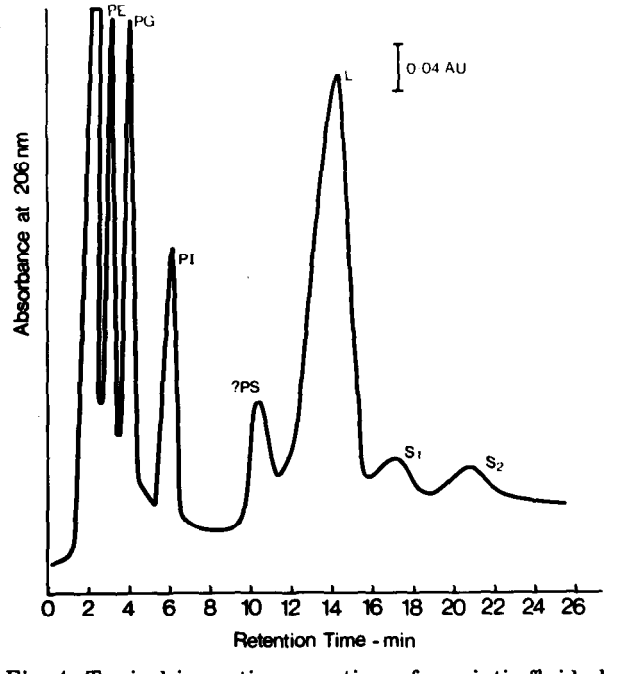

Fig. 4. Typical isocratic separation of amniotic fluid phospholipid observed after lung maturity is attained. Mobile phase: *n*-hexane—2-propanol—water (6:8:1.15, v/v/v). The large peak due to the lecithin (L) and the very flat broad peaks of sphingomyelin (S_1 and S_2) cannot be resolved by the integrator to give an accurate estimation of lecithin and sphingomyelin. For peak identification, see Fig. 3.

of the phospholipids, the lecithin and two sphingomyelin peaks (S_1 and S_2) were too broad to be adequately quantitated by the integrator.

Gradient elution using the DIOL column and the conditions already described allowed the lecithin and sphingomyelin to elute much closer to the earlier eluting phospholipid peaks, i.e. less than 16 min. In addition, PG, PI and PE all eluted much further away from the solvent front, allowing baseline resolution to occur before peak detection began. The broader peaks that were observed in the isocratic system were much sharper, and sphingomyelin eluted as a single peak as shown in Fig. 5.

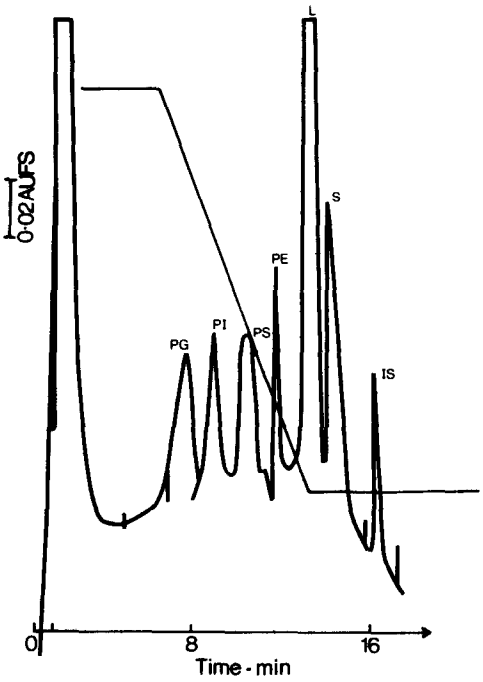

Fig. 5. Gradient elution chromatogram of a typical calibration standard containing the internal standard (I.S.), γ-capryloyl lysolecithin. All the peaks are well resolved and sphingomyelin (S) appears as a single peak. Initial conditions: 88% solvent A (100% acetonitrile) and 12% solvent B (acetonitrile—water, 3:5:1). Final conditions: 25% solvent A and 75% solvent B. Flow-rate: 2.0 ml/min. UV detector: 203 nm. For peak identification, see Fig. 3.

Fig. 6 shows a comparison of another separation procedure using a CN column instead of a DIOL column. The same aliquot of amniotic fluid was injected into the two different systems. Although initial conditions are slightly different and solvent B has much less water (5:1 acetonitrile to water) the phospholipids elute in the same order. The advantage of the CN column is that it is a more versatile column and easier to re-equilibrate than the DIOL column.

Internal standard

The reason for the use of an internal standard in the HPLC separation of amniotic fluid phospholipids is two-fold: (i) it allows the absolute concentration of individual phospholipids to be estimated, and (ii) it acts as a reference peak for all the other phospholipids. The choice of internal standard was made after

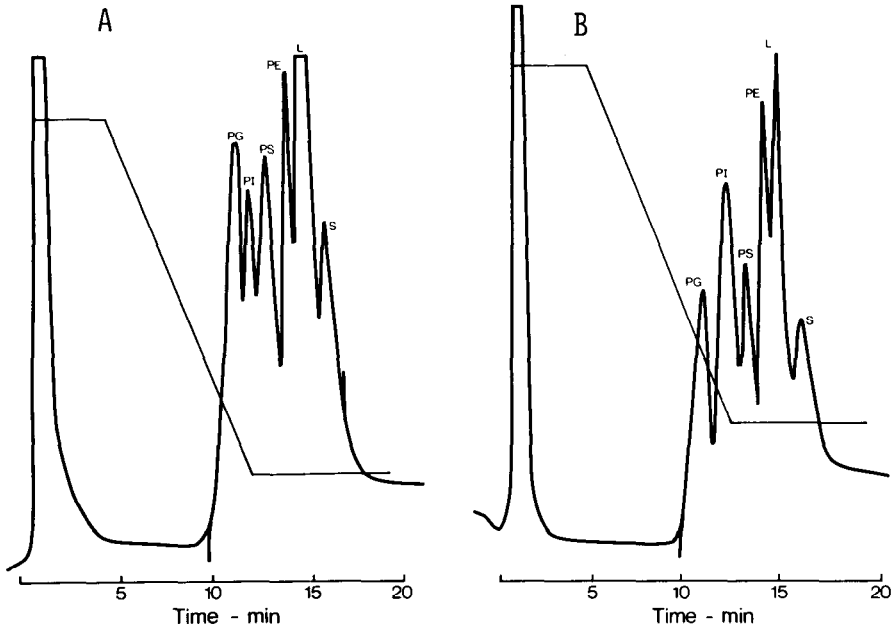

Fig. 6. Comparison of the same gradient elution procedures, with two different columns (CN and DIOL), using similar solvents but containing different concentration of water in solvent B. The phospholipids separate in the same order but are better resolved on the CN column. (A) 5-μm CN column (25 cm). Initial conditions: 90% solvent A (100% acetonitrile) and 10% solvent B (acetonitrile—water, 5:1). Final conditions: 25% solvent A and 75% solvent B. (B) 5-μm DIOL column (25 cm). Initial conditions: 88% solvent A (100% acetonitrile) and 12% solvent B (acetonitrile—water, 3.5:1). Final conditions: 25% solvent A and 75% solvent B. For peak identification, see Fig. 3.

observation that lysolecithin does not appear in physiological or pathological conditions in the acetone precipitate fraction of amniotic fluid. For this reason γ-capryloyl lysolecithin was chosen as an internal standard. Fig. 5 shows a typical calibration standard, the internal standard appears as a sharp, single peak eluting after sphingomyelin at 16 min.

For the gradient elution procedure, using individual phospholipid standards of varying concentrations, the response factor (RF) for each of the phospholipid standards was established as shown in Fig. 7. Each point represents a mean of five injections at that point. A linear relationship with widely different sensitivity was found between peak areas and concentration for each of the phospholipids. Linearity was observed over the range of working standards and these cover the physiological/pathological ranges for amniotic fluid phospholipids in the system.

Stability of standards

The stability of the prepared calibration standards is quite good, provided they are stored in a freezer when not in use. However, the quality of PS varied considerably from batch to batch. At times the sensitivity of detection was so low that very high concentrations of PS standard in excess of 40 μmol/l had to be injected onto the column before a peak could be detected. In most of the amniotic fluids analyzed, the concentration of PS was low by both HPLC and TLC.

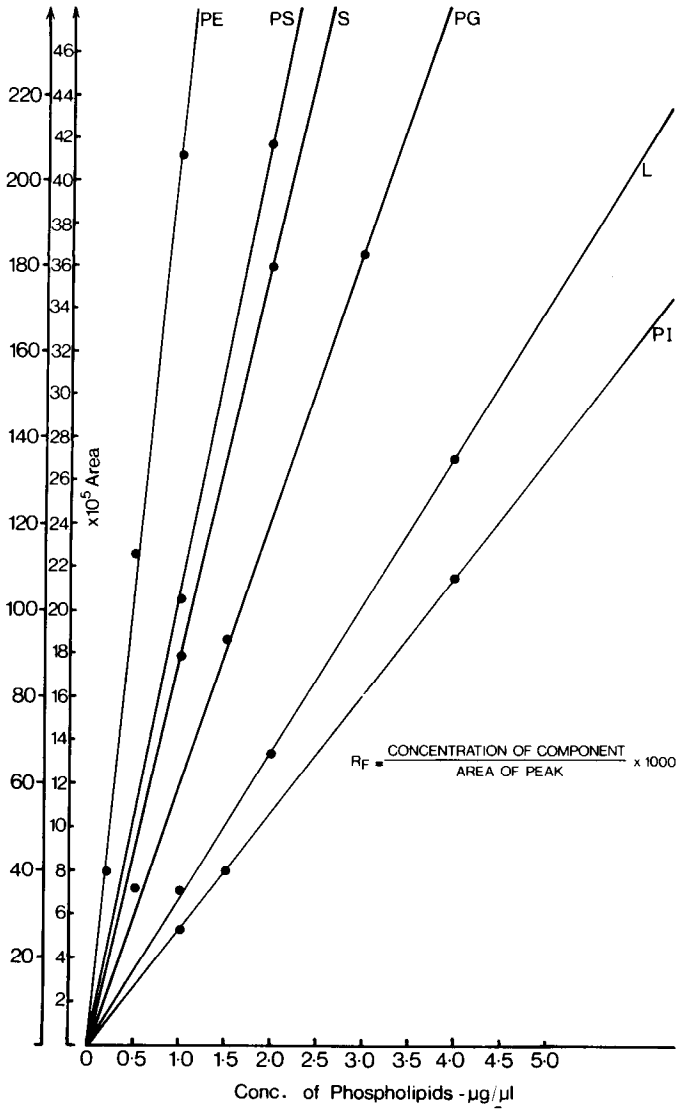

Fig. 7. Standard calibration curves of the six phospholipids showing the plot of the area $\cdot 10^5$ as measured by the integrator versus the concentration of the individual phospholipid at that point. Each point represents a mean of five injections. The formula for the calculation of the response factor (RF) of each peak is shown. For abbreviations, see Fig. 3.

TLC procedure

The TLC estimations on the amniotic fluid specimens were determined for comparison with the HPLC procedure. The TLC procedure was an adaptation of the method of Painter [4]. One-dimensional TLC was carried out using borosilicate TLC plates coated with silica gel 60 F254 and 1 g/l copper chloride utilising chloroform—ammonium hydroxide—methanol (65:3:25, v/v/v) as the mobile phase. The plates were run for 50 min, sprayed with cupric acetate—8% phosphoric acid stain, followed by charring on a hot plate and quantitation using a densitometer.

RESULTS

The sensitivity of the HPLC method was established for each phospholipid in the amniotic fluid. Sensitivity depends not only on the peak area and the concentration, but also on the baseline noise and quantity of amniotic fluid extracted. These factors must be optimized for the particular analytical column in use over the physiological/pathological concentrations of phospholipids found in amniotic fluid. Mean values for each phospholipid measured in four "immature" amniotic fluids (L/S ratio by TLC < 1.0:1) are shown in Table II.

TABLE II

SENSITIVITY OF THE HPLC PHOSPHOLIPID METHOD

The sensitivity of the HPLC method depends on the peak area, weight ratio for each phospholipid, baseline, and the quantity of amniotic fluid (AF) extracted.

Phospholipid	Towards lower limit of detection (1.5 ml of extracted AF in μmol/l)
Phosphatidyl glycerol	0.19
Phosphatidyl inositol	0.40
Phosphatidyl ethanolamine	0.01
Phosphatidyl serine	12.6
Lecithin	0.50
Sphingomyelin	0.20
γ-Capryloyl lysolecithin	19.9

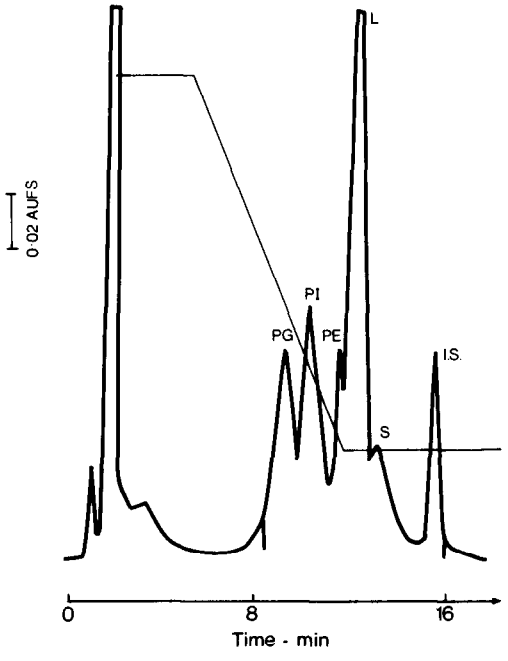

Fig. 8. Typical HPLC chromatogram of a mature amniotic fluid (L/S 3.9:1) using gradient elution. The concentration of individual phospholipids expressed as a percentage of the total phospholipid concentration are L 48.82%, S 12.48%, PG 7.11%, PI 16.26%, PE 15.31%, PS < 0.01%. Gestation 37 weeks. For abbreviations, see Fig. 3.

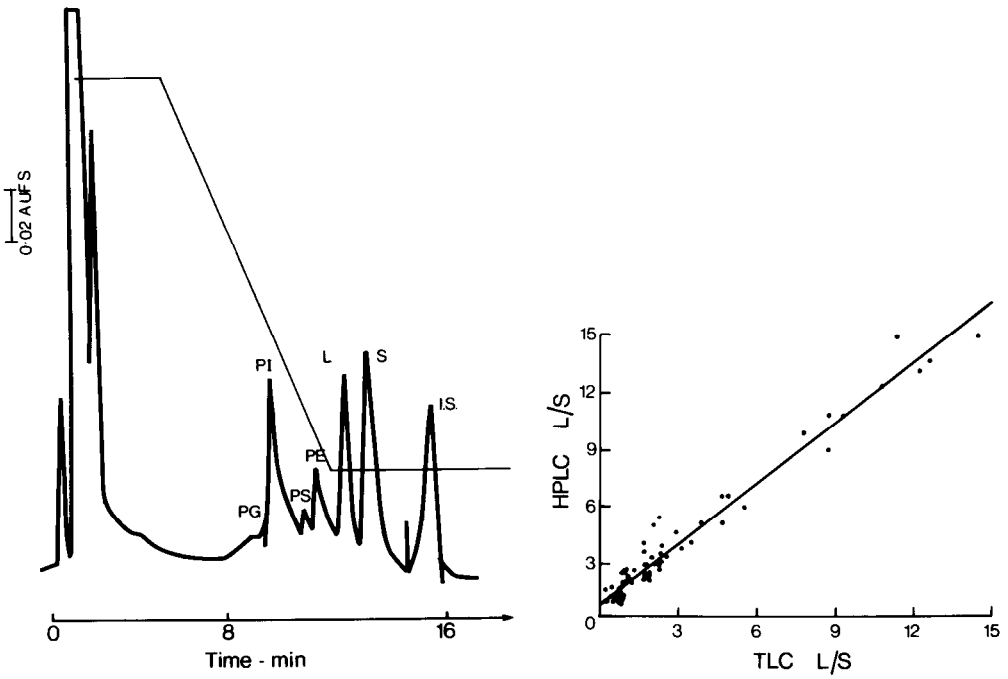

Fig. 9. HPLC trace of an immature amniotic fluid (L/S 1.2:1) using gradient elution. Phosphatidyl glycerol (PG) is not present in this trace. Initial conditions: 88% solvent A (100% acetonitrile) and 12% solvent B (acetonitrile—water, 3.5:1). Final conditions: 25% solvent A and 75% solvent B. Flow-rate: 2.0 ml/min. UV detector: 203 nm. For peak identification, see Fig. 3.

Fig. 10. Comparison of the L/S ratio by both HPLC and TLC procedures in the acetone precipitate of amniotic fluid. $Y = 1.08\ X + 0.52$; $r = 0.97$; $n = 40$.

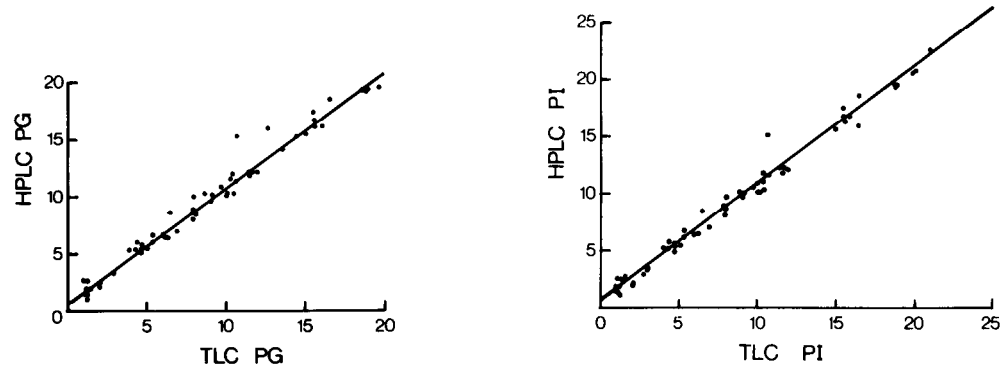

Fig. 11. Comparison of phosphatidyl glycerol (PG) by both HPLC and TLC procedures in the acetone precipitate of amniotic fluid. PG is expressed as a percentage of the total phospholipid concentration in the amniotic fluid. $Y = 1.02\ X + 0.8$; $r = 0.94$; $n = 40$.

Fig. 12. Comparison of phosphatidyl inositol (PI) by both HPLC and TLC procedures in the acetone precipitate of amniotic fluid. PI is expressed as a percentage of the total phospholipid concentration in the amniotic fluid. $Y = 1.01\ X + 0.86$; $r = 0.95$; $n = 40$.

In a preliminary patient study, 40 amniotic fluids were analysed by both the HPLC and the TLC procedures. Typical chart traces for the mature and immature amniotic fluids are shown in Figs. 8 and 9, each chromatogram being completed in 20 min running time. The retention times of the separated phospholipids are similar to the retention times observed in the standard chromatogram of phospholipids (Fig. 5). Note that interference by other peaks, which is a common problem observed in many HPLC separations of biological extracts, is minimal in this separation of the phospholipids, because of the acetone precipitation step which, as shown in Fig. 2, extracts only the surfactant phospholipids from the amniotic fluid.

Clinically the most important parameters are the L/S ratio, and proportions of PG, and PI. Figs. 10—12 illustrate the correlation coefficients and regression lines for these estimations. PG and PI are expressed as a percentage of the total phospholipid fraction estimated by both the HPLC and TLC procedures (r = 0.97 for the L/S, r = 0.94 for PG, and r = 0.95 for PI).

DISCUSSION

As can be seen from the present study from the comparison between quantitation by HPLC and quantitation by TLC, the HPLC procedure gives results which compare well with the longer established TLC technique.

Recently several authors (Paton et al. [5] and Briand et al. [6]) using similar procedures and columns but different detectors, have used HPLC to separate phospholipids in biological fluids. Paton et al. [5] stated that the HPLC method was less sensitive than their TLC method even though large volumes of amniotic fluid (5 ml) were used to overcome the lack of sensitivity of the detector. Our preliminary observations (Table II) show that the HPLC method with respect to the phospholipids PG, PI, L and S is much more sensitive. However, as mentioned previously, PS is difficult to detect, owing partly to its presence in low concentration in amniotic fluid and partly to the variability in the purity of the commercial standards. This problem was also observed by Paton et al. [5] and Briand et al. [6].

Preliminary evidence obtained from the comparison of the CN column with the DIOL column (Fig. 6) shows that it may improve the resolution, not only of PS but all the phospholipids. Further work is continuing in this area. A disadvantage of the HPLC procedure compared with TLC is the initial preparation of the solvents which require filtering and degassing for lengthy periods to remove all dissolved oxygen and other impurities which may cause variation in baseline and spurious peaks due to UV absorption at very low wavelengths.

Certain maternal conditions such as diabetes, severe hypertension, and Rhesus haemolytic disease are known to accelerate or retard lung maturity. The L/S ratio is known to be unreliable in such cases; however, several authors have stated that the presence of PG and PI, despite an immature L/S ratio, i.e. a value of less than 2.0:1, will preclude RDS from a premature neonate. In our preliminary study, all those infants who had a PG value of greater than 2.5% of the total phospholipid content by TLC did not develop RDS, irrespective of the value of the L/S ratio. It was also noted that PI was present in excess of 5% of the total phospholipid content in this study. Comparison of the two

procedures as shown in Figs. 10 and 11 of the L/S ratio and PG indicates that an L/S ratio of 2.0:1 by the TLC is associated with an HPLC L/S ratio value of 2.7:1 and a PG value of 2.5% of the total phospholipid value is associated with an HPLC PG value of 3.4%.

Although an internal standard has been used in our HPLC procedure and absolute concentrations may be estimated, to allow for the variability of total phospholipid concentration caused by differences in amniotic fluid volume, the "amniotic fluid phospholipid profile" as presented in our hospital is reported as a percentage of the total phospholipid assayed in amniotic fluid.

For all analysis runs, the concentration of each component is calculated as follows by the computing integrator:

$$\text{Concentration of phospholipid} = \frac{RF \times \text{area}}{1000} \times \frac{\text{area of I.S. in calibration standard}}{\text{area of I.S. in sample}} \times 66.7\ \mu\text{mol/l}$$

$$\text{Percentage phospholipid} = \frac{\text{concentration of individual phospholipid} \times 100}{\text{total concentration of phospholipid}}$$

An example of a typical calculation of "mature" amniotic fluid phospholipid profile is shown in Fig. 8. The total phospholipid concentration for this specimen was 149 μmol/l.

The comparison between quantitation by the TLC and HPLC procedures gave results that compare favourably with longer established TLC techniques. Although HPLC may require more expertise to set up, its advantages are that it enables a full amniotic fluid phospholipid profile to be separated in 16 min after extraction, and quantitated by an internal standard, compared to the 50 min for the same separation by TLC. As well as this, it is non-destructive and can also be used to collect the individual phospholipid fractions for further study, for example, of their fatty acid constitutents.

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