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A Practical Method to Analyze the Phospholipids in the Amniotic Fluid Using HPTLC

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An operative method using HPTLC which makes possible the determination of nine phospholipids in the amniotic fluid individually is described: lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid and cardiolipine. Since a simple and reliable method for clinical practice was sought, the working techniques, materials, chromatographic solvents and staining reagents were chosen accordingly. The standardization method was made by using standard phospholipids. This method was tested with 34 samples of amniotic fluid. A discussion of the practical application in the determination of some ratios of fetal pulmonary maturity is made.

Key words: Amniotic fluid, Fetal pulmonary maturity, High performance thin-layer chromatography, Phospholipids

Since the first experiments by GLUCK *et al.* (2), a large number of chemical methods for predicting fetal lung maturity based on qualitative and quantitative measuring of amniotic fluid phospholipids levels have appeared in medical literature (4, 6, 8).

In most of these studies conventional techniques of thin-layer chromatography have been used. However, recent studies have used the high performance thin-layer chromatography **, and the best results in the phospholipids separation have been obtained (4, 13).

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** *Abbreviations:*

HPTLC	High performance thin-layer chromatography.
Sph	Sphingomyelin.
PC	Phosphatidylcholine.
PI	Phosphatidylinositol.
PS	Phosphatidylserine.
PG	Phosphatidylglycerol.
PE	Phosphatidylethanolamine.
LL	Lysophosphatidylcholine.
P Ac.	Phosphatidic acid.
Card	Cardiolipine.

Subsequent to the development of Gluck's method, new methods based on other phospholipids such as phosphatidylglycerol (14, 16, 17), phosphatidylinositol (3) and disaturated phosphatidylcholine (11, 12) have been reported.

These methods have tended to employ very different experimental conditions, making standardization difficult. Even so many of these methods pose problems of a practical nature, for example, chromatographic spots attributed to a single phospholipid correspond to several phospholipids (1).

Furthermore, the influence of other factors on the method (15), such as atmosphere humidity, should be taken into account.

In the present study, a method using HPTLC has been described, which allows the simultaneous evaluation of some rates of prediction of fetal lung maturity based on phospholipid levels.

In this paper, two basic points related to methodology —staining reagents and chromatography solvents — have been studied. Having established these two points, they were checked using 34 amniotic fluid samples, excellent results being obtained in the separation of six phospholipids normally present in amniotic fluid: Sph, PC, PI, PS, PE and PG.

Materials and Methods

Standard patrons. The following phospholipids were purchased from Sigma: L-α-lysophosphatidylcholine, L-α-phosphatidylcholine, phosphatidylethanolamine and L-α-phosphatidyl-DL-glycerol from egg yolk, sphingomyelin and L-α-phosphatidylserine from bovine brain, L-α-phosphatidylinositol from soybean, L-α-phosphatidic acid from egg lecithin, sodium salt and cardiolipine from bovine heart.

TLC plates. Merck silica gel F_{254} high performance thin-layer chromatographic plates (10 × 10 cm) especially for nanochromatography were used.

Staining reagents. Eleven of the most usual staining reagents used for the detection of phospholipids were studied (5, 7, 10) (table I).

Equipment. Centrifuge: Hettich Rotanta K centrifuge. Chromatography injector: CAMAG Nanomat 2770-1078-E with nanoapplicator 27750-278-E. Chromatography tank: CAMAG Twin Through Chamber, for HPTLC (10 × 10 cm). Densitometer: CAMAG TLC Scanner 76500, with 1 × 1.5 mm slit and 620 nm light source, equipped with an integrator Rockwell AIM 65 interface analogic-digital.

Standard mixtures. Appropriate working standards for each phospholipid were prepared weekly from pure standard stock.

To prepare a working standard, 1 µl of each phospholipid stock has been evaporated to dryness which was then dissolved in 10 ml of chloroform/methanol (95:5 by vol.).

A combined phospholipid standard is prepared by mixing equal volumes of the above mentioned phospholipid standards. The prepared working standards were stored in screw-top vials at —20°C when not in used.

Chromatographic solvents. Three chromatography solvents were studied for the HPTLC in order to obtain the best separation of different phospholipids: Solvent A: Chloroform/methanol/water (65:24:4 by vol.) (2). Solvent B: Chloroform/methanol/water/glacial acetic acid (13:5:8:1.6 by vol.) (17). Solvent C: Chloroform/hexane/methanol/glacial acetic acid (5:3:1.6:1 by vol.) (6).

Amniotic fluid samples. Thirty-four amniotic fluid samples were obtained by transabdominal amniocentesis between 27

and 42 weeks of gestation. Amniocentesis was performed for medically indicated reasons when fetal lung status might influence management. All patients underwent ultrasonography prior to the procedure. Meconium and blood-stained fluids were discarded. If the sample could not be used on the same day it was stored frozen at —20°C until required.

General procedure. Amniotic fluid samples were centrifuged (2,500 × *g* 5 min), and the supernatant fluid decanted. Two millilitre amniotic fluid supernatant were mixed with 2 ml methanol, vortexed for 10 s, then mixed with 4 ml chloroform and vortexed for 1 min before centrifugation for 5 min at 1,500 × *g*. The lower chloroform layer was carefully collected using a Pasteur pipette and evaporated to dryness at 60°C under a stream of nitrogen. The residue was dissolved in 50 μl of chloroform and was applied in duplicate on a HPTLC plate to verify a good phospholipid separation.

Results and Discussion

STANDARD RESEARCH

Detection procedures. Table I shows the results obtained with several staining reagents.

The best global results, as far as stability, simplicity of preparation, use and contrast between phospholipid spots and plate background are concerned, are undoubtedly obtained with phosphomolibdic acid (fig. 1).

Other typical staining reagents, such as sulphuric acid or potassium dicromate-sulphuric acid mixture, also provide excellent contrast for densitometry but require greater technical complexity, both

Table I. *Results obtained with eleven staining reagents examined.*

Staining reagent	Spot colour	Background colour	Reason for elimination
1. Bromothimol blue	Dark blue	Pale blue	Slight spotlessness and rapidly fading spot
2. Potassium dicromate/sulphuric acid	Brown	Green	Drying problems with the plates
3. Fluoresceine	Fluorescent yellow	Yellow	Spot measurement very difficult due to need for UV light
4. Rodamine B	Fluorescent pink	Pink	Spot measurement very difficult due to need for UV light
5. Iodine-IK	Brown	Pale brown	Rapidly fading spot
6. Metallic iodine	Brown	Pale brown	Rapidly fading spot
7. Cobalt (II) chloride	Pink	Pale blue	Irregularity in dried plates
8. Phosphomolibdic acid	Dark blue	Greenish-yellow	—
9. Sulphuric acid (A)	Brown	White	Drying problems with the plates
10. Sulphuric acid (B)	Brown	White	Drying problems with the plates
11. Tungstophosphoric acid	—	—	No results obtained

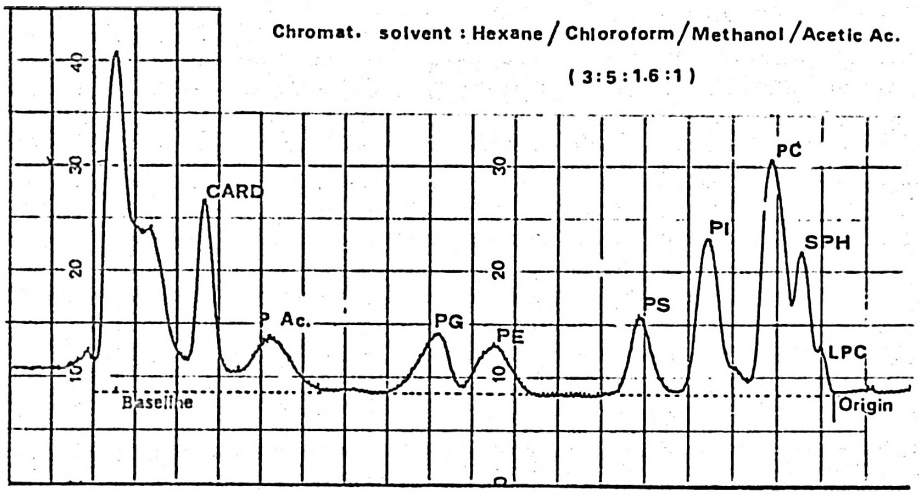

Fig. 1. *Transmission densitometric of the high performance thin-layer chromatogram of phospholipid standards.*

in the preliminary treatment of the plates and later on when they are sprayed.

In general, the other staining reagents have their drawbacks, technical spraying difficulties [Cobalt (II) chloride] and bad qualities for densitometric determinations (Fluoresceina, Rodamine B, need the use of UV light, which makes their location difficult).

Chromatography solvents: Table II shows the results obtained with the three chromatography solvents used. The Rf values for each phospholipid to determine the best separation have been used.

The results obtained with solvent A, show an agglomeration of phospholipids in the middle zone of the plate, with very small differences in the Rf values of several phospholipids. In relation to the nine phospholipids studied, a group formed by five phospholipids with Rf values of between 0.29 and 0.40 has been observed. The fact that the spots overlap makes their identification difficult.

Table II. *Rf values obtained with the chromatography solvents examined.*

Phospholipid	Solvent A standard	Solvent B standard	Solvent C standard	Solvent C samples
Lysophosphatidylcholine	0.21	0.12	—	—
Sphingomyelin	0.29	0.36	0.03	0.04
Phosphatidylcholine	0.39	0.54	0.08	0.09
Phosphatidylinositol	0.32	0.49	0.16	0.19
Phosphatidylserine	0.34	0.57	0.26	0.27
Phosphatidylethanolamine	0.63	0.78	0.47	0.53
Phosphatidylglycerol	0.46	0.74	0.56	0.66
Phosphatidic acid	0.40	0.85	0.76	—
Cardiolipine	0.51	0.92	0.85	—

On the other hand, with solvent B and solvent C, good separation was obtained with all phospholipids, greater differences between the phospholipid Rf values being obtained with solvent C.

In solvent C, petroleum ether (bp 36.3-54.4°C) has been replaced with n-hexane (bp 68.7°C). The Rf values obtained are slightly different from those of PAPPAS *et al.* (6). Table II shows the Rf values using standards and samples. Both cases are very similar.

The main adventage of this solvent is that it is easy to standardize. Moreover, as the Rf values slightly decrease interference with the other lipids, draw along by the solvent is avoided.

AGENTS THAT AFFECT PHOSPHOLIPID SEPARATION

Two of the most important agents that affect phospholipid separation are the activation of the plates and the atmosphere humidity.

A third agent, of certain importance, is the saturation time of the elution chamber.

Activation of the plates. In this study chloroform/methanol/hexane/glacial acetic acid (5:1.5:3:1 by vol.) was used as a chromatographic solvent and two different activation states were analyzed.

I. Plates without activation. II. Plates activated for about one hour at 110°C. The results are shown in table III, and slight variations can be observed in the Rf values of all phospholipids, the best separations being obtained among the phospholipids with the plate activated for about one hour at 110°C.

As a consequence of this result, a slight variation in the methanol ratio was introduced, which allows a slight increase in the separation of all phospholipids (III).

Atmosphere humidity. Atmosphere humidity is an agent that has a great effect on phospholipid separation, causing an elongation of phospholipid spot and altering their Rf values (fig. 2).
This problem was avoided by keeping the plates in a desiccator until the samples were applied and about ten minutes subsequently before elution, in order to eliminate any possible humidity acquired in the process of application.

Both the activation of the plates and atmosphere humidity are agents that have a negative effect on the success of the chromatoplate.

However, whereas the chromatoplate activation is a permanent intrinsic factor with all the plates, atmosphere humidity only appears under certain circumstances, such as, air conditioning in laboratories, and it generally depends on external conditions.

Saturation time of the elution chamber. This agent affects phospholipid separation, causing a slight variation of the phospholipid Rf values, making difficult the job of determining the areas of several pairs of phospholipids (such as Sph and PC, PE and PG).

Table III. *Effect of the activation of the plates on the Rf values of phospholipids.*

Plate	Sph	PC	PI	PS	PE	PG	P Ac.	Card
I	0.04	0.09	0.15	0.27	0.53	0.55	0.82	0.87
II	0.03	0.07	0.14	0.24	0.44	0.50	0.77	0.85
III	0.03	0.08	0.17	0.26	0.47	0.54	0.78	0.87

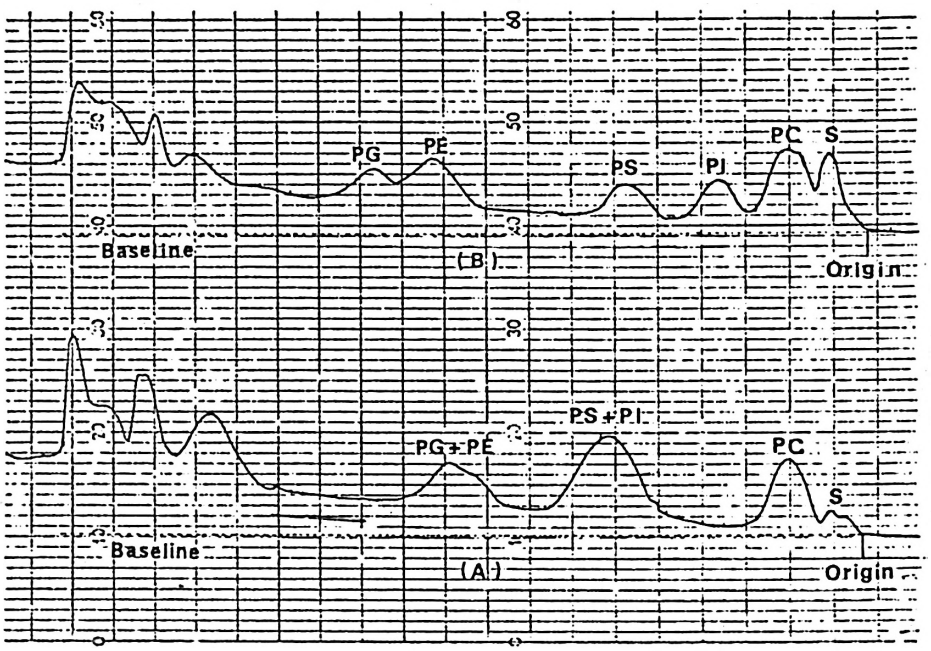

Fig. 2. *Effect of the atmosphere humidity in the phospholipid separation.* A) Transmission densitometric of the HPTL chromatograms of phospholipid standard mixtures showing the effect of atmosphere humidity. B) Plate kept ten minutes in dessicator.

In the present study, a good separation of the phospholipids studied using a saturation time of ten minutes has been produced.

Method application to amniotic fluid samples

Up to now, only standard phospholipid mixtures to establish the most suitable work conditions have been used.

These work conditions were tried out with 34 amniotic fluid samples to verify the value of chromatography.

In the 34 amniotic fluid samples studied, six phospholipids (Sph, PC, PI, PS, PE and PG) were separated satisfactorily, whereas the determination of the other three phospholipids mentioned in the bibliography (LL, P Ac., and Card) was found to be impossible.

In the case of LL, this remains in the elution origin, and its determination presents problems because of the low concentration present in the amniotic fluid, and other mechanical factors of homogeneity in the spotting point.

With regard to Card and P Ac., these phospholipids cannot be determined because they have very high Rf values and consequently interfere with other nonpolar lipids present in the amniotic fluid.

«Pseudo PG» detection. In the analysis of the 34 amniotic fluid samples, in eleven cases the presence of an unknown compound, with a Rf value slightly higher than phosphatidylglycerol (fig. 3) have been observed.

This unknown compound has been reported recently in the bibliography as «pseudo PG» (9). It can interfere with the determination of PG and appears in relation to the number of weeks of gestation in a random way.

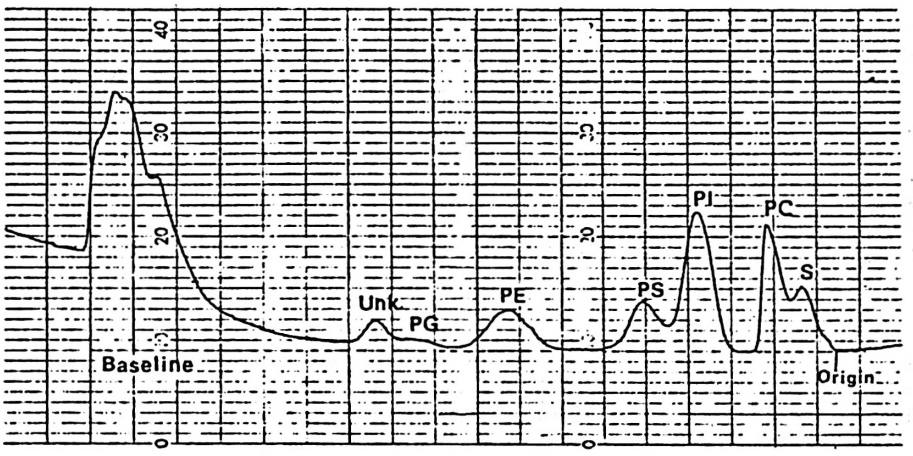

Fig. 3. *Location of the compound unknown (UNK) in the transmission densitometric of the HPTL chromatogram of amniotic fluid sample.*

The presence of this unknown compound has nothing to do with the L/S ratio found in the samples.

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Resumen

Se describe un método por cromatografía de capa fina de alta resolución, que permite determinar individualmente nueve fosfolípidos presentes en el líquido amniótico: lisofosfatidilcolina, esfingomielina, fosfatidilcolina, fosfatidilinositol, fosfatidilserina, fosfatidiletanolamina, fosfatidilglicerol, ácido fosfatídico y cardiolipina. Las técnicas de trabajo y los materiales se han seleccionado para obtener un método simple y fiable conveniente para la práctica clínica. La estandarización del método se realiza usando fosfolípidos patrones. Se discute la aplicación práctica en la determinación de algunos índices de madurez pulmonar fetal.

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