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REVIEW

PHOSPHOLIPIDS IN AMNIOTIC FLUID WITH SPECIAL REFERENCE TO THE LECITHIN/SPHINGOMYELIN RATIO

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CONTENTS

LIST OF ABBREVIATIONS

GC	Gas chromatography
HMD	Hyaline membrane disease
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
L	Lecithin
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine

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RDS	Respiratory distress syndrome
S	Sphingomyelin
TLC	Thin-layer chromatography
TTN	Transient tachypnea of newborn

1. INTRODUCTION

1.1. Surfactant biochemistry and fetal lung maturity

The basic etiologic defect for the respiratory distress syndrome (RDS) is fairly well documented. With measurements of phospholipids in the amniotic fluid, even before parturition it is possible to predict those infants who are likely to develop RDS after birth. RDS is a disorder of the lungs which is responsible for more deaths in the pediatric groups than any other disease [1]. Gluck [2] points out that in RDS, in spite of the fact that many aspects of the disorder are unclear, one thing is common: "all are born too soon". RDS is not so much a disease as a consequence of developmental immaturity.

The early work of Avery and Mead [3] showed that surfactant deficiency is consistently found in the lungs of neonates dying of RDS. It has been suggested that fetal respiratory movements result in extrusion of endotrachial fluid containing pulmonary surfactant into amniotic fluid. These observations led directly to the study of amniotic fluid composition. The early studies of Gluck et al. in 1967 [4] provided the stimulus for use of the phospholipid concentrations in detection of RDS.

Since that time, however, advances in the knowledge of RDS have led to a proliferation of amniotic fluid tests. Measurement of phospholipids is the most direct method of evaluating pulmonary maturity. These assays are prone to effects of variability in amniotic fluid volume, sample collection modes, use of centrifugation and contamination with blood or meconium. When performed properly, results correlate with clinical evaluation.

Unfortunately, no method has acquired universal acceptance. Prediction of mature lungs and immature lungs can be accurate. Among these reports there are probably certain ones which, on proper evaluation, may eventually be the universal one.

With this background, an attempt is made in this review to point out the present status of the determination of phospholipids in amniotic fluid for determination of the lecithin/sphingomyelin (L/S) ratio for evaluation of fetal lung maturity. Planar and high-performance liquid chromatography (HPLC) are discussed. Creer and Gross [5] described the use of gas chromatography (GC) for the determination of the long-chain fatty acids after hydrolysis of the phospholipid side-chains. This method needs further investigation. The discussions in this review are limited to those methods which apppear practical and cost-effective for the determination of phospholipids in amniotic fluid for assessment of fetal lung maturity. GC thus far has not seen any acceptance in this area.

2. SAMPLE PREPARATION

Sample handling and preparation for chromatography is perhaps the most controversial area of phospholipid determination. Here is the step where many of the differences in the results that are obtained are most apparent especially in cases of the use for clinical evaluations. There are many areas where reproducibility of results from different laboratories may be at fault because of the different methods used. The chromatographic procedure, the detection methods and the quantitative aspects also differ. These areas will be reviewed in that order.

2.1. Effect of acetone precipitation on phospholipid recovery

The original method for acetone precipitation of amniotic fluid proposed by Gluck et al. [6] has been widely accepted as being indispensible. The rationale for this step has been that total amniotic fluid lecithin is not representative of "surface-active" lecithin. Acetone precipitation supposedly separates the "active" lecithin from the other lecithin components. However, it has been shown that the precipitation does not provide a pure sample of disaturated lecithin which is the desired effect [7]. Unfortunately, the method described by Gluck et al. [6] used a detection method which measured only the unsaturated lecithin. For description of the relative effects of copper acetate versus copper sulfate as charring reagent in assay of phospholipids, see the discussion in Section 3.1.

Oulton [8] reported that centrifugation of amniotic fluid alters its L/S ratio. The various methods for preparing amniotic fluids for phospholipid determination involve a wide range of centrifugation conditions, which alone may account for differences in results by different laboratories. These results were confirmed by Touchstone et al. [9]. During early investigation of the effects of precipitation with acetone losses of as much as 30% of the phospholipid were indicated. This led to the development of the direct application of amniotic fluid to the thin layer as described later.

More recently Hobson et al. [10] made a retrospective study of the effect of acetone precipitation on the clinical prediction of RDS. It was concluded that although there was a significant difference in the numeric value between results obtained with and without precipitation as in the Gluck procedure, the clinical prediction of the RSD was not enhanced by acetone precipitation. The opinion was that the Gluck acetone precipitation does not improve the clinical evaluation. Other investigations have come to the same conclusion. Mallikarjuneswara [11] and Painter [12] after some early evaluation of the Gluck procedure excluded the acetone precipitation step in their methods. Wagstaff et al. [13] showed that centrifugation even at 750 *g* results in a loss of as much as 71% of lecithin from the supernatant and that with increasing *g* forces the L/S ratio decreases dramatically. These observations were confirmed by Freer and Statland [14].

2.2. Removal of phospholipids from amniotic fluid by solid-phase extraction [15]

The following procedure has been developed for removal of phospholipids from amniotic fluid and blood plasma for subsequent assay using chromatographic

methods. It has proven viable for use with thin-layer chromatography (TLC). The results indicate that better separation of the phospholipids occurs since the bulk of extraneous material is removed. It has also been used to separate neutral lipids from the more polar analogues, i.e., cholesteryl esters from phospholipids.

The solid-phase extraction cartridges were Spice C_{18} from Analtech (Newark, DE, U.S.A.). These are placed in a vacuum manifold also from Analtech. The procedure in sequence is as follows:

1. The sorbent in the cartridge was conditioned by washing successively under vacuum with 2-ml volumes each of hexane, chloroform and methanol. The effluents are discarded.
2. The amniotic fluid (in this case, 1 ml) is added directly to the cartridge, and the aqueous part allowed to flow through. This is discarded.
3. The sample is then washed under vacuum with successive 1-ml volumes of hexane and 2-ml volumes of chloroform which are discarded.
4. The cartridge is dried by aspirating air through for 5 min.
5. The analyte is then eluted with 2 ml of chloroform–methanol (1:1).
6. The eluent is evaporated and reconstituted for analysis in 100 μl of chloroform. Aliquots of 5–10 μl are sufficient to visualize phospholipids in term amniotic fluid.

This procedure has been used for preparation of amniotic fluid samples for both TCL and HPLC. Perhaps when more experience has been obtained with this method for phospholipids, it may overcome some of the shortcomings of sample preparation and result in a more practical method.

3. THIN-LAYER CHROMATOGRAPHY

3.1. Mobile phase

There are numerous mobile phases reported that will achieve separation of phospholipids. Table 1 lists some of those reported to give complete separation of the six major phospholipids using silica gel layers.

The mobilities of the various phospholipids using any particular mobile phase will vary according to the conditions under which the chromatography is carried out. Krahn [18] reported the results of a survey of factors effecting resolution of phospholipids on thin layers. Since many investigators used ammonium sulfate incorporated into the layers these were evaluated with the following conclusions. The ammonium sulfate in the layer effects the migration. All phospholipids except phosphatidylethanolamine (PE) and phosphatidylserine (PS) are well separated. Heat activation is necessary with the layer containing ammonium sulfate. A temperature of 100–120 °C for 30 min was necessary for activation. The effect of atmospheric humidity was pronounced. As much as 30% moisture was found in exposure of the plates. Using these layers activation may be required. These experiments point out reasons why variability of results even in the same laboratory have been noticed among the numerous methods that have been reported.

For reproducibility within the laboratory consideration should be given to the

TABLE 1

MOBILE PHASES FOR TLC OF PHOSPHOLIPIDS USING SILICA GEL LAYERS

Source	Mobile phase	R_F for phospholipid*					
		S	L	PI	PS	PE	PG
Pappas et al. [16]	Chloroform–light petroleum–methanol–acetic acid (50:3:16:1)	0.08	0.90	0.34	0.51	0.81	0.90
Touchstone et al. [9]	Chloroform–ethanol–triethylamine–water (30:34:30:8)	0.23	0.30	0.52	0.47	0.59	0.78
Hobson et al. [10]	Chloroform–isopropanol–triethylamine–methanol–water (30:25:25:9:7)	Not given					
Painter [17]	Chloroform–methanol–ammonia (60:34:15)	Not given					

* S = sphingomeylin; L = lecithin; PI = phosphatidylinositol; PS = phosphatidylserine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol.

factors discussed above. It should be remembered that the sorbents of different manufactures used in the layers are not the same and separation will not be the same when layers from different manufacturers are compared. These differences may be overcome by slight modifications in the mobile phases. The literature abounds with compositions for mobile phase for phospholipid separations.

3.2. Detection reagents for phospholipids

The results of quantitation of phospholipids by TLC can be tainted by improper use of detection reagents. The results depend greatly on the method used. A list of some reagents that have been used for detection of phospholipids on TLC is shown in Table 2. There does not appear to be more recent improvements in this technology.

Problems in use of these various reagents are due to the differences in the reactivity of the various phospholipids. The most evident difference is probably due to the differences in the degree of saturation. Touchstone et al. [9] compared the relative reactivity of saturated and unsaturated phospholipids towards cupric acetate and cupric sulfate. It was shown that cupric acetate reacted only with unsaturated species while cupric sulfate reacted with both saturated and unsaturated lipids. This difference was used to quantitate these two species in amniotic fluid. Gluck et al. [6] used cupric acetate for charring. This may be the reason that subsequent investigators obtained different L/S ratios, as well as varying concentration levels. In spite of this, the majority of reports in the literature generally indicate charring in some form. Often a hot plate was used as the heat source. Plates with ammonium or copper salts required high temperatures. Herein lies one of the problems of reproducibility. The method in the report of Touchstone et al. [9] uses a specially prepared convection oven for heating and detect-

TABLE 2

REAGENTS USED FOR PHOSPHOLIPID DETECTION

Reagent	Source
Phosphomolybdic acid	Wortmann and Wortmann [19] Dittmer and Lester [20]
Ammonium sulfate	Ziminski and Borowski [21]
Cupric acetate-phosphoric acid	Fewster et al. [22]
1-Anilino-8-napthalene sulfonate	Larsen and Trostmann [24]
Bismuth subnitrate	Coch et al. [25]
Rhodamine B	Blass et al. [26]
Cupric sulfate-phosphoric acid	Touchstone et al. [9]
Molybdenum blue	Dittmer and Lester [20]
2,5-Bis[5-*tert.*-butylbenzoxazolyl (2′)] thiophene	Kraus et al. [27]
Nile red	Fowler et al. [28]

ing reagents requiring heating below 190°C. Only using these conditions reproducibility was attained. The use of a hot plate for a heat source is not recommended as it is difficult to reproduce.

The use of reagents with specificity for phosphorus (such as that of Dittmer and Lester [20]) has received some attention. Since no heating is required, reproducibility may be improved. This reagent solves some of the differential reactivity between saturated and maturated phospholipids but not the differential reactivity between the different phospholipids.

3.3. Quantitative aspects

Quantitation of phospholipids of amniotic fluids separated by TLC represent an area where much controversy can result. The earlier workers used "eyeballing" or planimetry. Even at this late date, there are investigators or clinical laboratories that still use these techniques. These methods are frought with considerable error. The variation is due to the differences in how a spot or line is visualized by the individual evaluating a chromatogram. Considering the availability of reliable densitomers it is difficult to justify the use of these techniques. There are several inexpensive scanners on the market. Visual inspection at best is only semiquantitative [29].

From the practical standpoint and the need for ready results, visualization by various methods followed by elution of the prerequisite areas and quantitation by phosphorus determination [31] is neither applicable nor accurate.

Densitometry perhaps should be a prerequisite when one is considering employing phospholipid determinations for evaluating fetal lung status using TLC. Several recent methods report the use of densitometry for this purpose [9, 10, 12, 19]. It should be pointed out that TLC is the only method in which the sorbent is constant, thus the densitometry along with this gives the potential for development of methodology for assay of many other moieties.

Recently, the Camag group has shown the results of high-performance thin-

layer chromatographic (HPTLC) methods for separating lecithin and sphingomyelin in amniotic fluid. Development time was 10 min. The detection reagent, manganese–sulfuric acid spray, permitted sensitive detection. Perhaps using the solid-phase extraction described previously, the procedures for HPLC and HPTLC can be improved since much cleaner extracts can be obtained. There is still need for further work in improving these methods. At this point, the TLC method with densitometry appears to be the one of choice, this conlcusion being from the standpoint of speed and sensitivity. With a 10-min development time and depending on the detection reagent the Camag system claims sixteen samples can be assayed in 30 min with 1–2% reproducibility.

3.4. Practical method

These various investigations tend to confirm that the acetone precipitation was unnecessary and were the basis (before and after the fact) of the method of direct application of the amniotic fluid to the sorbent of the thin layer [9]. This procedure requires the use of layers containing a "preadsorbent" or "preconcentrating zone". These modified layers are generally commercially available. The layers provided by Whatman (Clifton, NJ, U.S.A.) can be obtained with a "preadsorbent" that is double the thickness of the adsorbent layer. Using this layer, a method was developed in which the extraction of the amniotic fluid was carried out in situ on the layer. The procedure is described as follows.

Aliquots of amniotic fluid were applied directly to the preadsorbent zone of LK5 plates with a 25-μl Drummond microcapillary under a current of warm air from a hair dryer. The samples were applied across the lanes within the middle third of the application area, with drying between each application, until 50-μl (for specimens collected late in gestation) or 100-μl (earlier gestation) volumes of amniotic fluid had been applied. Standards for reference were applied on other lanes, and only alternate lanes were utilized. The samples were used in duplicate, one each on two different 20 cm $\times$ 20 cm plates.

After drying, the layers were predeveloped twice with chloroform–methanol (1:1) to the interface with the adsorbent zone, the effect being to extract the phospholipid from the sample and deposit it as a line on the starting point of the chromatogram.

The chromatogram was developed in glass tanks until the front of the mobile phase was 1 cm from the top of the plate. The mobile phase of chloroform–ethanol–triethylamine–water (30:34:30:8) was allowed to equilibrate for 10 min before the chromatogram was developed.

After development, the chromatograms were allowed to dry at ambient conditions; they were then heated for 2 min in an oven at 170°C to remove any residual solvent. Plates were sprayed with cupric sulfate reagent (10% copper sulfate in 8% phosphoric acid). The plates were allowed to air-dry for 5 min, then heated at 120°C for 5 min, followed by heating at 170°C for 10 min. The chromatograms were then scanned by densitometry. (They should be scanned within 1 h or stored in the dark until scanned.)

The chromatograms were scanned in a Kontes fiber optic scanner (Model 800),

using the white phosphor disc (440 nm). A Hewlett-Packward Model 3385A integrator was in line. The transmission mode of scanning involved double-beam operation. Scanning in the transmission mode gave higher results for the individual peaks than did scanning in the reflectance mode, an observation that accords with earlier reports [15].

Samples were either analyzed as soon as received or kept frozen. Specimens were placed in crushed ice for transport to the laboratorry, then promptly frozen; otherwise, there was loss of phospholipid.

From the standard curve obtained from scanning serial solutions (2–8 μg) of the phospholipid standards, the amounts of the individual phospholipids in the sample can be interpolated. Lower level of detection is 200 ng. Samples from a specimen of amniotic fluid were applied to ten different plates, to determine inter-plate reproducibility. The coefficient of variation for quantification of lecithin was 8.7%. For 45 samples, the within-day variation was 6.0% and for nine samples within-plate variation was 2.6%. If the plates were not washed as described above before sample application and development, the background after charring was inconsistent and results erratic. Recovery of radiolabelled dipalmitoyllecithin added to amniotic fluid, applied to the preadsorbent area and developed as described, consistently was 93%. Recoveries of radiolabelled phospholipid from the preadsorbent layer during the development were quantitative.

Fig. 1 summarizes some results obtained using this method. The standard curve for saturated lecithin using the copper sulfate reagent gave an equation with the relation $y=29.214\,x-0.375$ ($r=0.984$, $n=28$). With use of the standard curves, the amounts of phospholipid in samples of amniotic fluid can be determined. Also shown in Fig. 1 are densitometric scans of amniotic fluid treated with copper sulfate or copper acetate charring reagent. Evidently, the various "peaks" are not single components, but consist of both saturated and unsaturated homologous phospholipids since the copper acetate reacts only with unsaturated analogues. There is more "saturated" phospholipid as determined by the copper sulfate reagent minus the copper acetate result.

The amounts of total lecithin as related to gestational age are also shown in Fig. 1.

After subtracting the cupric acetate value from the cupric sulfate value the amount of "disaturated" lecithin is determined. A scattergram depicting the amounts of "saturated" lecithin versus gestational age is given in Fig. 2. At 34–35 weeks of gestation there is a sharp increase in this phospholipid. Results indicate that a value of <25 mg of saturated lecithin per liter of amniotic fluid in the latter stages of pregnancy may signal a problem. Amniotic fluid was assayed on the day the specimen was collected, whereas the clinical results were observed at birth.

The method described here is widely applicable, because saturated and unsaturated phospholipids can easily be differentiated for an entire phospholipid profile. The presence or absence of phosphatidyglycerol (PG) can be noted. Relatively little sample is required, and the minimal handling of the sample helps lead to quick and reliable results. Furthermore, no lengthy solvent partitions and evaporations are involved.

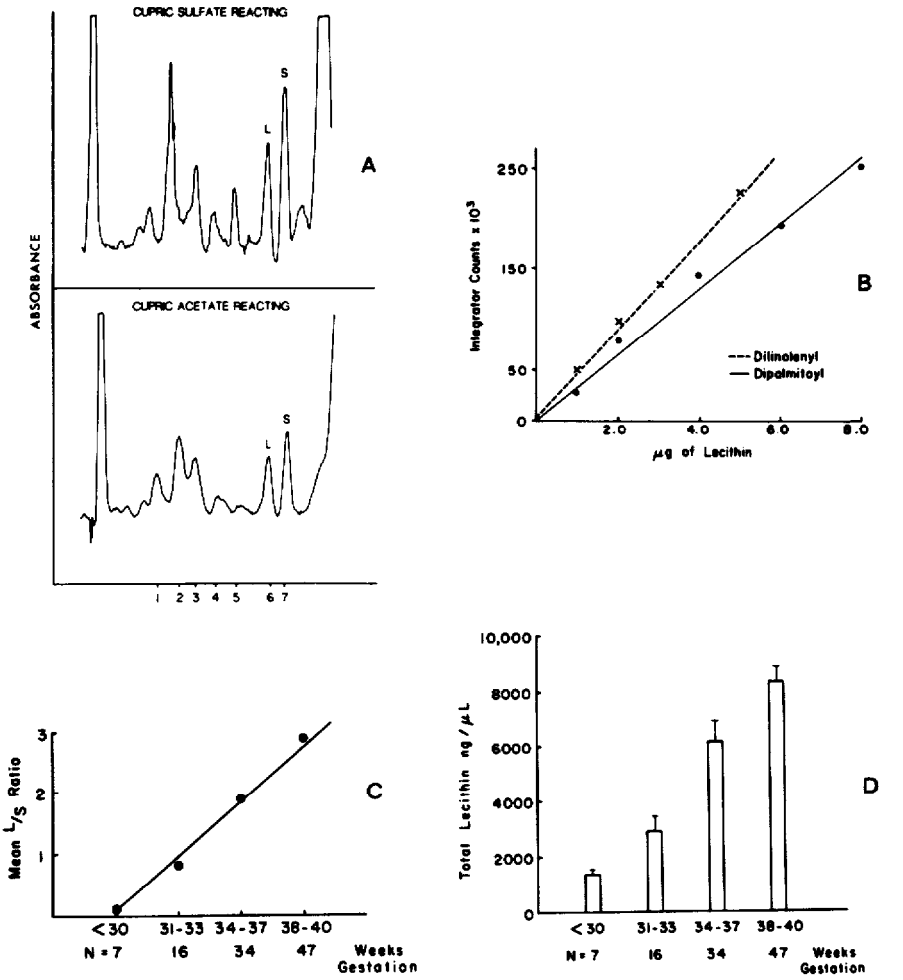
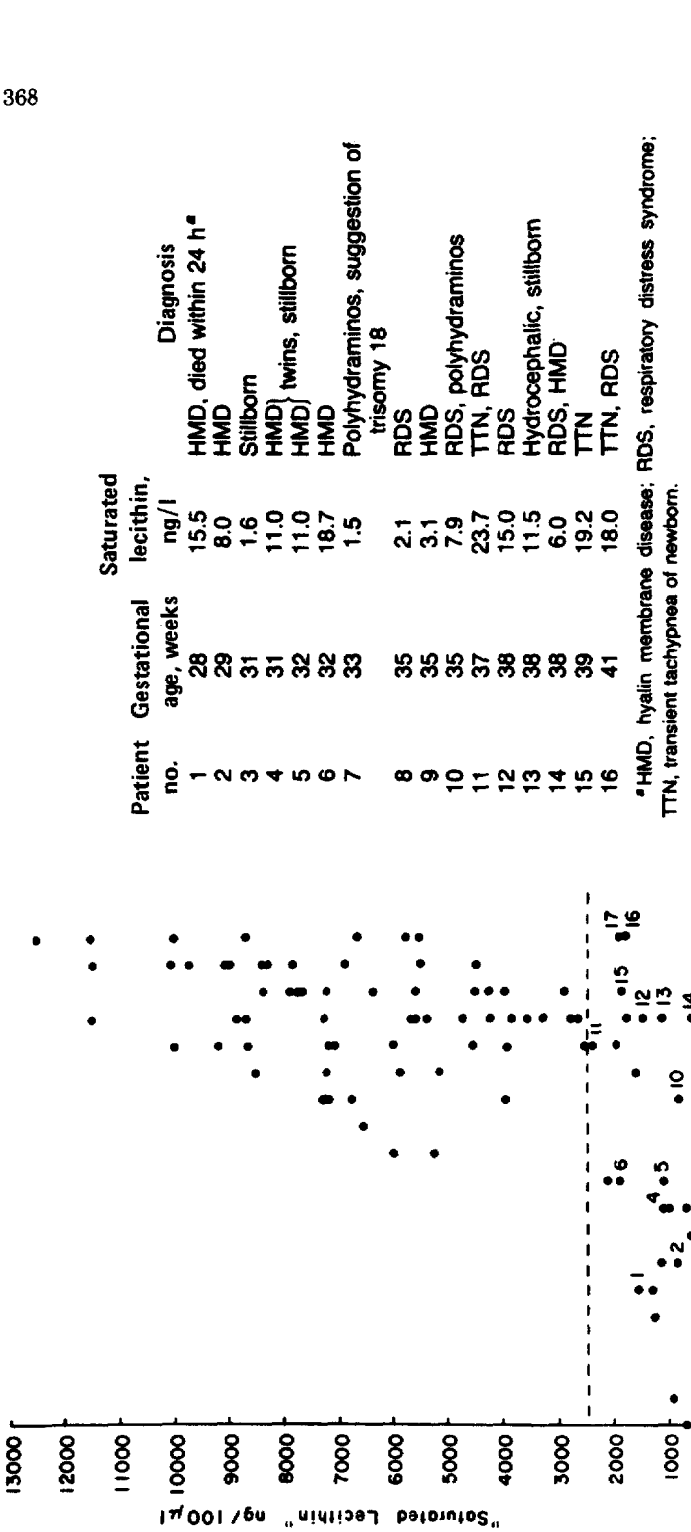


Fig. 1. Results obtained by use of direct application of amniotic fluid. (A) Comparison of cupric acetate and cupric sulfate sprays. (B) Standard curves. (C) L/S ratio using the cupric sulfate spray method. (D) Gestational age related to "total" lecithin.

4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC for the separation and quantitation of phospholipids has not replaced TLC for this determination. The application of HPLC to evaluation of lung maturity has shown little progress. Some investigators reporting HPLC methods claim that TLC methods are "not ideal" [31]. Many recent reports in TLC determinations refute this statement. Although HPLC methods have been reported, quantitation is currently a problem under investigation. Spectrophotometric methods are not sensitive and considering that the primary surfactant is saturated phosphatidylcholine this approach is not satisfactory. Detection using flame ionization [32] shows some potential but this involves specialized equipment. In HPLC detection of substances of strong aliphatic character



Patient no.	Gestational age, weeks	Saturated lecithin, ng/l	Diagnosis
1	28	15.5	HMD, died within 24 h[a]
2	29	8.0	HMD
3	31	1.6	Stillborn
4	31	11.0	HMD} twins, stillborn
5	32	11.0	HMD}
6	32	18.7	HMD
7	33	1.5	Polyhydraminos, suggestion of trisomy 18
8	35	2.1	RDS
9	35	3.1	HMD
10	35	7.9	RDS, polyhydraminos
11	37	23.7	TTN, RDS
12	38	15.0	RDS
13	38	11.5	Hydrocephalic, stillborn
14	38	6.0	RDS, HMD
15	39	19.2	TTN
16	41	18.0	TTN, RDS

[a]HMD, hyalin membrane disease; RDS, respiratory distress syndrome; TTN, transient tachypnea of newborn.

Fig. 2. Gestational age relationships with the "saturated" lecithin (cupric sulfate minus cupric acetate).

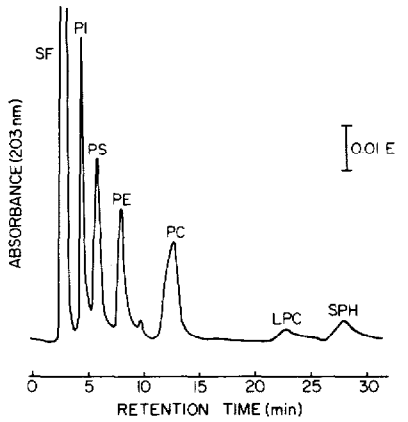


Fig. 3. Representative liquid chromatogram as noted in ref. 33. PC = phosphatidylcholine, LPC = lysophosphatidylcholine; SPH = sphingomyelin.

such as phospholipids is complicated when compared to TLC. Continuous peak monitoring is difficult for these lipids, since they absorb very weakly. The unsaturated phospholipids can show absorption in the ultraviolet near 210 nm. This will negate the use of chloroform–methanol mixtures as mobile phases since these have strong absorption below 245 nm. The use of other mobile phases [33] which would be suitable for use of ultraviolet detection has not resulted in satisfactory separation of phospholipids.

Thus, the area under a given absorbance peak will reflect mostly the unsaturation rather than the amount of phospholipid. There is a great difference between preterm and term phospholipids which indicates that this approach is not suitable. For the molecules containing the primary amino group (PE or PS) fluorescent derivatives (dansyl chloride) [34] or biphenylcarbonyl derivatives [35] can be used.

For the present, it does not appear that methods involving HPLC are suitable for routine use in the determination of L/S ratios in the clinical laboratory. Here again, it is evident that there is no universally accepted method for separation and quantitation of the phosholipids. D'Costa et al. [31] described the use of ultraviolet detection of the phospholipids but only conclude that the method "has potential" and notes that the L/S ratios obtained by HPLC with ultraviolet detection are purely empirical in nature. Paton et al. [36] described a procedure for HPLC separation of phospholipids using refractive index for detection purposes and came to conclusions that the method was insufficiently reliable to be used in place of TLC. Chen and Kou [33] used a Waters silica gel column (Micro-Pak SI-10) with a variable-wavelength detector at 203 nm. Unfortunately, in spite of some of the best separations so far reported for phospholipids in HPLC no results were reported for amniotic fluid phospholipids. Fig. 3 shows a representative chromatogram from some of the results reported in this paper.

Gebhardt et al. [37] derivatized the phospholipids with 1,6-diphenyl-1,3,5,-hexatriene for fluorimetric determination of these compounds. Using a continuous flow system, the method was adapted to the determination of phospholipids

in amniotic fluid. These results indicate that, with some improvements in the method, it might be a viable technique for determination of the L/S ratio in amniotic fluids. The use of derivative formation for enhancement of sensitivity of these compounds has not yet been perfected. Furthermore, the additional steps to the procedures only make for a less practical method.

5. SUMMARY

At the present time, it appears that the method of choice for determination of phospholipids in amniotic fluid is the thin-layer chromatographic method. Noteworthy is that presented by Touchstone et al. [9] in which a minimum of effort is required. With the direct application of the amniotic fluid the problems of recovery and time-consuming efforts are minimized, considering that extraction and chromatography are carried out on the same plate.

Another approach to the problem is shown in the reports of Krauss et al. [27] in which they showed that 24 samples can be accommodated on one plate. Sensitivity was obtained using 2,5-bis[5-*tert.*-butylbenzoxazolyl (2′)] thiophene to form fluorescent derivatives. With development times of as little as 10 min this method has not yet been perfected for use with biological samples, but indicates that some future developments using high-performance thin-layer chromatography are perhaps coming.

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