Evaluation of Detection Systems Used to Determine Lecithin/ Sphingomyelin Ratios in Amniotic Fluid

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Introduction

Hyaline membrane disease has been a major cause of infant mortality and morbidity, especially in infants of low birth weight that have been delivered prematurely. In 1959 it was discovered that certain components were notably deficient in pulmonary surfactant fluid taken at autopsy from infants with hyaline membrane disease. Analysis of pulmonary surfactant revealed that the lecithin levels in these fluid samples were severely reduced (4, 5). Since that time it has been discovered that during fetal development the lecithin is produced almost exclusively by the Type II alveolar cells of the developing fetus (4). Furthermore, this fluid is released into the amniotic fluid contents by ultrafiltration during fetal development, and it was discovered in 1971 that meaningful thin layer chromatographic determination of phospholipids in amniotic fluid could be used as an index for fetal lung development *in utero* (4, 5). Various studies have shown that another phospholipid, sphingomyelin, is produced at relatively constant rates in various compartments and is present at a relatively constant level in the amniotic fluid throughout gestation. In contrast, the production of lecithin increases asymptotically beyond the 34th week of gestation (5).

Statistically, it has been shown that lecithin/sphingomyelin (L/S) ratios in amniotic fluid greater than 2 are indicative of mature fetal lung development before delivery. In 1973, it was determined that treatment with cortisol stimulated the synthesis of lecithin in the developing fetal lung and cortisol treatment along with fetal retentive drugs have been used in permitting the additional normal lung development of fetuses before delivery (4). Obviously, the organic solvent extracts of amniotic fluid could be concentrated and quantitatively determined by a liquid chromatographic method. However, most hospitals have neither the monetary or personnel investment for these kinds of research procedures to become widely used at the present time.

Most of the clinical laboratory methods for determining L/S ratios involve thin layer chromatographic (TLC) separations of various phospholipid components in the organic solvent extracts from amniotic fluid. Finally, a particular detection method is used and the relative cross sectional areas for these two lipid spots compared with various standards migrations are determined. These thin layer chromatographic methods are simple, inexpensive, and rapid to use. Many reports are found in the literature indicating that lecithin and sphingomyelin standards are easily separated (3, 7, 8, 10, 12). Unfortunately, lecithin and sphingomyelin are not as easily resolved from various other phospholipids such as phosphatidyl inositol and phosphatidyl glycerol. There have been suggestions recently that some of these other minor phospholipids may have clinical significance also in assessing fetal lung maturity (6).

Thus, it has been worthwhile to improve the thin layer chromatographic resolution of lecithin and sphingomyelin from other amniotic fluid phospholipids.

This has been approached by the evaluation of various absorbent systems as well as utilization of various resolving solvent mixtures. Finally, a number of detection systems reported in the literature have been evaluated with respect to sensitivity and also linearity of response over a comparable concentration range for each phospholipid. Two fluorescent spray reagents are also evaluated for this purpose.

Materials and Methods

Various phospholipid standards have been purchased from Sigma Chemical Company, St. Louis, Missouri, and have been used throughout these experiments. The compounds include lecithin (LEC) (P-6138), sphingomyelin (SPH) (S-7004), lysophosphatidyl choline (LPC) (L-4129), phosphatidyl ethanolamine (PE) (P-5263), phosphatidyl inositol (PI) (P-0639), phosphatidyl glycerol (PG) (P-2892), and phosphatidyl serine (PS) (P-6641). Various absorbents have also been evaluated for L/S separation, including plates prepared from Brinkmann silica gel G (40 grams per 70 ml of distilled water). After plates were coated with a 250 micron layer, they were permitted to air dry overnight. Other Brinkmann silica gel G plates were activated at 70°C for 30 minutes and stored in a plate desiccator upon returning to room temperature and, finally, some silica gel plates were thoroughly activated by heating at 100°C for 60 minutes and then stored in a TLC plate desiccator. Brinkmann cellulose MN300 was also used to prepare plates with a 250 micron layer which were air dried overnight. The concentration of the slurrry was 15 grams per 90 mls. of distilled water. Brinkmann MN300 cellulose coated sheets with 100 micron layers were also used. Whatman precoated silica gel plates (LK5DF and LK6DF), as well as Whatman high performance silica gel plates (HP-KF) and Whatman KC18F reverse phase plates were evaluated in the present experiments. Organic solvent systems, which were reported in the literature, or developed in our labs include those listed in Table I. The various detection systems reported in the literature or developed in this lab are listed in Table II. The various reagents used in these systems were purchased from Sigma Chemical Company, American Scientific Products, or Fisher Scientific.

Results

Many of the solvent systems listed in Table I have value in separating lecithin standards from sphingomyelin standards on thin layer chromatography plates. Unfortunately, many of these solvent systems incompletely resolve the other phospholipid components from these two principal phospholipids. Many of the phospholipids also show considerable tailing with these various solvent systems. The systems which appear to have the greatest merit include the chloroform/methanol/water mixture (65:25:4) reported by Gluck *et. al.* (1971) (4, 5), and the chloroform/methanol/30% ammonium hydroxide mixture (68:28:4). In all of our experiments, we have used this latter solvent system.

Among the various absorbents investigated, only silica gel G plates coated in our labs were useful for the present purpose. There was a tremendous amount of tailing of most phospholipids on the cellulose plates, and the various Whatman plates were of no value whatsoever for resolution of phospholipids either directly from amniotic fluid samples or from solvent extracts of amniotic fluid samples. Utilizing the silica gel plates, the lowest degree of plate activation was found to be most satisfactory. Thus, for the remaining experiments which evaluated the use of the various detection systems, only silica gel plates which were coated with 250 micron layers and which were air dried overnight were used. The use of silica gel G supplied by other vendors such as Mallinckrodt and Sigma Chemical Company showed no significant differences in resolution or detection sensitivity.

TABLE I: *Solvent Systems Evaluated For L/S Separation*:

CH_2Cl_2:EtOH:HOH (70:16:2) (3)
$CHCl_3$:MeOH:NH_4OH (85:10:1.5) (3)
CH_2Cl_2:EtOH:NH_4OH (70:16:2) (3)
CH_2Cl_2:EtOH:CH_3COOH (70:16:2) (3)
CH_2Cl_2:EtOH:HOH (51.5:12:1.5) (11)
$CHCl_3$:MeOH:HOH (80:30:5) (12)
$CHCl_3$:MeOH:CH_3COOH:HOH (50:30:12.5:5)
$CHCl_3$:MeOH:HOH (95:35:4)
$CHCl_3$:MeOH:30% NH_4OH:HOH (34:14:2:0.75)
$CHCl_3$:MeOH:30% NH_4OH (68:28:4)
$CHCl_3$:MeOH:HOH (65:25:4)
C_6H_6:MeOH (95:5)

(The single numbers in parentheses indicate literature cited.)

Using various amounts of each phospholipid over a 100-fold concentration range, the following limits of detection have been determined for each detection system and for each phospholipid, as is shown in Table III. Finally, the linearity of response of the various phospholipid spot sizes, as concentration is varied, and using each of the detection systems, is shown in Table IV.

Discussion

The use of Rhodamine B spray reagent was reported by Blass *et. al.* (1973) (1) and by Blass *et. al.* (1974) (2). In these two papers, it was indicated that linearity of response for lecithin was observed in the concentration range of 3 to 20

TABLE II: *Spray Reagents Evaluated for L/S Separation*

1) *Rhodamine B* (50 mg) is dissolved in 10 ml methanol. Dark pink spots on a pink background are revealed under a short wavelength UV source.
2) *Phosphomolybic acid* (5 grams) is dissolved in 100 ml of isopropanol/methanol mixture (70:30). After spraying, the plates are heated at 190° for 20 minutes; black spots on a green background are revealed.
3) *Potassium Permanganate* is dissolved as 0.1 *M* solution in distilled water. Spraying yields yellow spots on a violet background.
4) *1-Aniline-8-naphthalene sulfonate* (50 mg) is dissolved in 100 ml distilled water. Under a long wavelength UV source, spots exhibit blue florescence.
5) *2, 7-Dichlorofluorescein* (250 mg) is dissolved in 100 ml distilled water. After spraying, plates are exposed to ammonia vapor for 20 minutes to develop orange-green spots on a yellow background.
6) *Bromothymol Blue* (50 mg) is dissolved in 50 ml distilled water/8 ml of 1 *N* NaOH and diluted to 120 ml final volume with water. pH is adjusted with addition of 1.25 grams of boric acid. Yellow spots are observed on a blue background.
7) *Iodine vapor* saturating a TLC chamber develops brown-yellow spots on plates within 25 minutes.
8) *Cyclodextrin* (#3-4667, Supelco, Inc., Bellefonte, PA 16823) is sprayed directly onto TLC plates and dried for 10 minutes. Exposure to iodine vapor for 20 minutes produced large brown yellow spots.
9) A 50% dilution of concentrated *sulfuric acid* reveals black spots after heating to 120°C for 20 minutes.
10) *1, 6-Diphenylhexatriene* (10 mg) is dissolved in 100 ml of dichloromethane. This reagent yields purple spots under a long wave length UV source.

TABLE III: *Limits of Detection for Various L/S Sprays (In Micrograms)*

	LEC	SPH	LPC	PE	PI	PC	PS
Rhodamine	2	1.5	3	4	—	2	3
Phosphomolyb	1	1.5	1.5	7	0.7	—	1.5
$KMnO_4$	10	2	2	7	1.5	2	0.4
ANS	0.2	1	0.3	0.7	0.7	2	0.4
DC-Fluor	0.4	10	7	—	15	7	3
BR-Thy	2	1.5	0.3	2	1.5	—	1.5
Iodine	4	10	1.5	4	—	—	2
Cyclodex-I_2	0.2	2	0.4	2	—	2	0.3
H_2SO_4	2	1.5	20	—	—	—	10
DPH	0.4	1.5	7	1	0.7	0.3	1

Key to symbols used:
Phosphomolyb = Phosphomolybdic acid
ANS = 1,8-Anilino naphthalene sulfonate
DC-Fluor = Dichlorofluorescein
BR-Thy = Bromothymol blue
Cyclodex-I_2 = Cyclodextrin iodine

TABLE IV: *Linearity of Response for Various L/S Sprays*

	LEC	SPH	LPC	PE	PI	PC	PS
Rhodamine	NI/LS	NI/HS	NI/LS	NI/LS	—	NI/LS	NI/LS
Phosphomolyb	NI/HS	NI/HS	NI/LS	NI/LS	Z/LS	—	NI/HS
$KMnO_4$	NI/LS	NI/LS	NI/LS	NI/LS	Z/HS	Z/LS	NI/HS
ANS	Z/HS	Z/HS	Z/HS	Z/HS	Z/HS	Z/HS	Z/HS
DC-Fluor	Z/HS	NI/LS	NI/LS	—	NI/LS	NI/LS	Z/LS
BR-Thy	Z/LS	NI/LS	NI/LS	Z/LS	NI/LS	—	NI/HS
Iodine	NI/HS	NI/LS	Z/LS	NI/LS	—	—	Z/LS
Cyclodex-I_2	NI/LS	NI/LS	NI/HS	NI/HS	—	NI/HS	NI/HS
H_2SO_4	NI/HS	NI/HS	—	—	—	—	NI/LS
DPH	Z/HS	NI/HS	Z/LS	Z/LS	NI/LS	NI/HS	Z/HS

Key to symbols used:
NI = non zero intercept
Z = zero intercept
LS = low slope
HS = high slope

micrograms, and linearity of response was observed for sphingomyelin in the concentration range of 3 to 12 micrograms. We found this reagent to give somewhat linear response with both phospholipids in the range of 1.5 to 20 micrograms. However, we noticed that there was sufficient background color with this reagent and the linearity of response curves determined for both lecithin and sphingomyelin had non-zero intercepts. The slope of the curve for sphingomyelin was also much steeper than that for lecithin. Finally, we determined that the lowest limits of detection with this spray reagent were 2 micrograms in the case of lecithin and 1.5 micrograms in the case of sphingomyelin. This agrees approximately with Blass *et. al.* (1973) (1) who report a low limit of detection of 1 microgram for both phospholipids. In our present results non-zero intercepts were also yielded by the other phospholipids when Rhodamine B was used as a detection reagent. Also, it is shown that the low limit of detection was not as significant for these other minor phospholipids. Although no results are shown, Blass *et. al.* (1974) indicate that Rhodamine B is more sensitive than 50% sulfuric acid which, in turn, was reported as a more sensitive reagent than bromothymol blue (2). Sulfuric acid charring was also reported by Lemmons and Jaffe (1973) (9), by Morrison *et. al.* (1974) (10), and by Sass *et. al.* (1976) (11). None of these reports give any discussion of sensitivity, selectivity or linearity of response with these particular spray reagent methods.

Our present results indicate that only lecithin and sphingomyelin are significantly detected by bromothymol blue reagent. It can also be seen that lysophosphatidyl choline and phosphatidyl serine are detected by this method but only at ten-fold greater concentrations. The other phospholipids are not even revealed in high concentration by this particular reagent. Furthermore, it is shown in Table IV that the slopes of the linearity of response curves once again have non-zero intercepts. The use of bromothymol blue as a detection reagent is reported by Stuber *et. al.* (1976) (12), once again without any discussion of sensitivity, selectivity or linearity of response for this particular spray reagent.

In our present studies, we found bromothymol blue to be a moderately sensitive reagent yielding linearity of response curves with non-zero intercepts for most of the phospholipids. Iodine vapor was reportedly used by Hallman *et. al.* (1976) as a detection method for phosphatidyl inositol and phosphatidyl glycerol in amniotic fluid (6). We have found iodine to be rather insensitive to low amounts of lecithin or sphingomyelin and iodine also yields linearity of response curves with non-zero intercepts. With the exposure time for iodine that we are presently using, we did not even detect phosphatidyl inositol and phosphatidyl glycerol under these present conditions. Sass *et. al.* (1976) report the usage also of dichlorofluorescein reagent as a detection system (11). They also compare it verbally with other detection methods. They ruled out use of the iodine method because of the iodine toxicity and the fact that the spots fade after a relatively short time period. They also indicated that phosphomolybdate spray gave results that were difficult to reproduce from one series of experiments to another. They concluded in their comparison that dichlorofluorescein was the most useful reagent. We found phosphomolybdate spray to be a relatively sensitive reagent under our conditions; however, the majority of the phospholipids gave linerarity of response curves with non-zero intercepts and variable slopes. Also, phosphatidyl glycerol was not even detected at our highest concentration (20 micrograms) by phosphomolybdate. We disagree about the value of dichlorofluorescein. It seems to be sensitive only in the detection of small amounts of lecithin and phosphatidyl ethanolamine is not detected at all by this particular reagent. Furthermore, the majority of the phospholipids give line-

arity of response curves with non-zero intercepts when this spray reagent is used. Our results also show that a potassium permanganate spray is much more sensitive to small amounts of sphingomyelin than it is to lecithin. The majority of the phospholipids gave linearity of response curves with non-zero intercepts also for potassium permanganate sprays. Also the cyclodextran-iodine detection system designed in this lab was somewhat sensitive for certain phospholipids, but the linearity of response curves were not as encouraging.

The fluorescent compound, 1, 6-diphenylhexatriene (DPH), was reported by Hyslop and York (1980) as a useful reagent for the detection of lipids on thin layer chromatography plates (8). Without showing data, they claim as little as ten nanograms of total phospholipid can be detected with this reagent. We found the reagent to be most sensitive for phosphatidyl glycerol, but the smallest quantity we detected was 300 nanograms. We did however use a different solvent system in which to dissolve the reagent. A report by Heyneman *et. al.* (1972), discusses the use of 1, anilino-8-naphthalene sulfonate (ANS) as a detection reagent (7). The plates were sprayed with this reagent and then scanned with a fluorescence densitometer. Cusick also reports the use of this ANS reagent (3). Of the various reagents investigated in the present report, we have also found that ANS is the most sensitive in terms of lowest limits of detection for the seven phospholipids currently under investigation. We also found that all of the linearity of response curves for the various phospholipids when ANS detection was used gave zero intercepts and all of these curves gave steep slopes over the concentration range reported. We have concluded that the separation of lecithin and sphingomyelin can be easily accomplished on the organic solvent extracts of amniotic fluid and can be easily and semi-quantatively detected on silica gel G plats if ANS is used as a detection reagent. We are currently investigating the actual separation of these phospholipids from amniotic fluid samples and plan to report our results in the future.

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