Comparison of Two Simple Methods for Determining Lecithin/Sphingomyelin (L/S) Ratios in Human Amniotic Fluid Samples

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Introduction

The ability of a neonate to survive after delivery depends largely on proper development of its respiratory system. If the Type II alveolar cells are incapable of synthesizing proper pulmonary surfactant, there is a high probability that the neonate will have respiratory distress syndrome. Proper pulmonary surfactant is rich in phospholipids, especially dipalmityl phosphatidyl choline (lecithin) and phosphatidly glycerol (PG). The latter phospholipid is present in the pulmonary surfactant in tenfold smaller molar quantities. Since the maternal amniotic fluid is in direct contact with the fetal lung compartment during gestation, the lung phospholipids are readily transferred and reflected in relative abundance in the amniotic fluid (10,2,5).

Analytical studies of phospholipids present in amniotic fluid have shown that the relative levels of sphingomyelin remain relatively constant throughout gestational development. However, the amounts of lecithin and phosphatidyl glycerol are relatively low throughout gestation until approximately week 28 when both of these compounds begin to increase in relative levels asymptotically. If an amniotic fluid sample is carefully taken by amniocentesis before delivery, it is possible to remove contaminating cells and to extract the phospholipids from the amniotic fluid. The phospholipids can be collected in a cholorform layer, concentrated, and subjected to thin layer chromatography for resolution. After separation, some detection method can be employed and the relative amounts of sphingomyelin, lecithin, and phosphatidyl glycerol can be detected either by visualization of a developed color or by a developed fluorophore (10). The detected phospholipid spots can be measured visually or with a scanning densitometer. If an L/S ratio greater than 2.0 and the presence of phosphatidyl glycerol are detected it can be concluded with some certainty that fetal lung development is normal. If a smaller L/S ratio or absence of phosphatidyl glycerol in the amniotic fluid sample is detected, it is possible to give fetal retentive drugs and to enhance the synthesis of pulmonary lecithin and phosphatidyl glycerol by administration of cortisol to the mother (5).

Various detection systems have been utilized in the past to reveal phospholipids resolved by thin layer chromatography. These detection reagents have been ionization sensitive, unsaturation sensitive, or phosphate sensitive. In recent years the Helena Laboratories (Beaumont, Texas) copper acetate reagent (8000) has enjoyed much popularity as an unsaturation sensitive detector. Using 42 human amniotic fluid samples, we have compared the results obtained for L/S ratios by the copper acetate charring detection method with the use of anilino - 1, 8 - naphthalene sulfonate (ANS) detection. We have used this latter reagent because of its reported ability to form fluorescent complexes with phospholipids and yield similar fluorescence intensities. In earlier studies in our laboratories we have also found anilino - 1, 8 - naphthaline sulfonate to be especially sensitive in detecting six phospholipid standards and we found the response for spot size to concentration load to be linear for most of the phospholipids over a 100 fold concentration range (7).

Materials and Methods

Purified phospholipid standards (lecithin, sphingomyelin, phosphatidyl glycerol) were purchased from Sigma Chemical Company, St. Louis, Missouri, and were dissolved in chloroform at concentrations of two milligrams per milliliter. Glass plates (20 centimeter X 20 centimeter) were coated with a slurry prepared by dissolving Silica Gel G (Brinkman 7731) 40 grams in 90 milliliters of demineralized water. The silica gel was coated with a Brinkman apparatus. Plates were air dried at room temperature overnight and received no additional activation at higher temperatures. Frozen amniotic fluid samples were thawed to room temperature and two ml. aliquots were placed in 60 ml. separatory funnels. Two ml. of 100% methanol was added and the mixture was shaken for 20 seconds. Two ml. of chloroform was added and the mixture was again shaken for 20 seconds. Each emulsified sample was centrifuged in a Clay Adams Clinical Centrifuge at 2000 RPM's for ten minutes. A Pasteur pipet was used to carefully remove a one ml. aliquot from the lower chloroform layer. The organic extract was evaporated to dryness under a stream of nitrogen and was reconstituted with approximately 40 microliters of chloroform.

This entire extract could be placed on a thin layer plate and chromatographed along with ten microliter samples of the respective phospholipid standards. The plates were developed in a solvent system containing 68 ml. of chloroform, 28 ml. of methanol, and 4 ml. of 30% ammonium hydroxide, in a Sigma thin layer chromatography chamber. Detection of the resolved phospholipids was accomplished by direct visualization following spraying with the Helena Laboratories copper acetate spray reagent and heating to 120°C for ten minutes. Alternatively, a similar aliquot from a given patient amniotic fluid extract was detected by spraying a solution of 50 milligrams of ANS dissolved in 100 ml. of methylene chloride. Detection of the resolved spots was obtained with a Ultra-Violet Products UVSL-25 lamp. Spots were quickly circled with pencil and a quantity proportional to spot area was calculated by multiplying horizontal diameter by vertical diameter of each spot. Finally the ratio of lecithin spot area to sphingomyelin spot area was calculated. The sub-populations of 42 patient samples detected by these two methods were statistically compared using the Students' t test.

Results

The statistical data obtained from the Students' t test is shown in Table 1. The result of comparing the two population means with each other statistically indicates in a two-tail probability that the two methods give statistically significantly different results. Of course this statistical parameter does not indicate that one method is better than the other, merely that the two sample populations are statistically different (4).

Table 1: Comparison of L/S Ratios in Amniotic Fluid Determined by Two Detection Methods on 42 Patient Samples.

	Mean of L/S Ratios	Std. Dev.
ANS Fluorescence Detection Method	2.56	1.17
Cupric Acetate Charring Method	2.07	0.94
Diff. of Means	0.49	
Std. Error Diff. of Mean	0.14	
Calc. t Value	3.39	
Two Tail. Probability	0.002 (Significantly Different)	

TABLE 2: Individual Patient L/S Ratios Determined by the Two Detection Methods

Patient No.	ANS Fluorescence	Cupric Acetate Charring
1	2.60	0.72
2	2.00	0.75
3	3.00	0.83
4	1.40	0.98
5	2.80	1.30
6	4.50	1.60
7	0.83	1.40
8	1.50	1.20
9	1.20	0.85
10	0.98	1.40
11	0.48	1.20
12	4.00	3.80
13	2.60	4.40
14	2.40	2.10
15	1.50	1.60
16	2.80	1.50
17	2.60	2.30
18	4.30	2.80
19	1.30	1.40
20	3.30	2.50
21	3.30	3.90
22	3.60	3.00
23	2.50	2.70
24	2.20	2.40
25	2.90	3.00
26	5.30	3.10
27	1.60	1.50
28	1.30	2.00
29	3.40	2.60
30	1.40	0.88
31	1.70	1.80
32	2.80	3.70
33	1.80	1.70
34	3.70	3.00
35	2.20	1.50
36	3.20	2.00
37	3.60	1.90
38	4.20	3.20
39	1.40	1.40
40	3.90	3.00
41	4.30	3.50
42	1.20	1.70

It is evident from Table 2 that in 28 of the 42 L/S ratio determinations, these ratios are larger when determined by the ANS fluorescence method.

Discussion

Published results from several laboratories suggest that the copper acetate charring method of detection is loaded with problems. Spillman, *et al.* reported that when unsaturation sensitive methods such as copper acetate are compared with unsaturation insensitive methods such as molybdate detection, that the unsaturation insensitive methods consistently give higher L/S ratios than those methods that are unsaturation sensitive (8). Touchstone, *et al.* completed a study of the reactivity of separated phospholipids toward various charring reagents. They also noted that saturated lecithins as are commonly found in mature amniotic fluids are rather insensitive to copper acetate

detection. Those lecithins containing at least one unsaturated fatty acid are responsive to the reagent and multiple unsaturated lecithins are additionally sensitive. Partially unsaturated samples of phosphatidyl ethanolamine, phosphatidyl serine and phosphatidly inositol are also responsive to copper aceteate charring reagents (9). Various researchers report that the relative abundance of saturated lecithins (as dipalmityl phosphatidyl choline) increases dramatically beyond week 30 of gestation (5). Thus, as fetal lung maturation occurs as reflected by pulmonary surfactant present in amniotic fluid one can expect the resultant lecithins to become increasingly insensitive to spray reagents such as copper acetate.

A number of authors have shown that the temperature used to complete the charring is even critical with copper acetate sprays. Mueller has shown, if sprayed plates are heated at 120°C that consistently higher L/S ratios are determined, while if plates are heated to 130°C, consistently lower L/S ratios are determined, using the phospholipid standards (6). In a classic study by Gluck, *et al.* comparing other charring methods, it was noted that differing results are obtained at different temperatures and also results are dependent on whether calcium sulfate binder is present or absent in the silica gel. Finally these results were variable with the kind of charring reagent used (3). Brown, *et al.* recently reported that as charring times are increased, the resolved sphingomyelin spot intensified in color while the resolved lecithin spot decreases in color. Thus as charring time is increased, the L/S ratio appears to decrease (1).

These published results of other researchers certainly indicate that if copper acetate charring methods are used that all the parameters, such as silica gel source, presence or absence of calcium sulfate binder, charring temperature, and charring time must be very carefully and uniformly regulated from one determination to another. In order to ultimately decide if the sensitive ANS fluorescent reagent is more effective, we will need to conduct a series of experiments comparing L/S determinations on amniotic fluid samples for the two detection methods. We will need to have an elaborate neonate follow-up after the fact to actually determine if the lung development of the newborn would parallel the estimate yielded by our respective tests.

Summary

We have extracted and chromatographed methanol-chloroform concentrates of 42 patient amniotic fluid samples on air dried silica gel thin layer chromatography plates. We have detected resolved phospholipids by either direct visualization of cupric acetate charred spots or by fluorescence of spots revealed after spraying with ANS. Calculation of spot areas in each case has permitted us to determine lecithin/sphingomyelin ratios for each sample analyzed by each detection method. We have shown that the two sample populations are statistically different and that the saturated lecithin sensitive methods (ANS detection) yields larger L/S ratios for 28 of the 42 patient samples.

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