Evaluation of Sample Pre-treatments as Potential Methods of Enhancing Phospholipid Extraction from Human Amniotic Fluid

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Introduction

It is well known that phospholipids present in human amniotic fluid have been transferred from the fetal lung compartment to amniotic fluid. Furthermore, these phospholipids are components of pulmonary surfactant, a fluid necessary for normal lung physiology in neonates. These phospholipids necessary in surfactant are synthesized by the Type II alveolar cells and include dipalmityl phosphatidyl choline (lecithin), sphingomyelin, and phosphatidyl glycerol. The relative levels of sphingomyelin are known to remain rather constant throughout gestational development, but the levels of both lecithin and phosphatidyl glycerol increase dramatically beyond week 28 of intrauterine life. If the phospholipids are extracted from amniotic fluid and chromatographed, it is possible to predict that normal fetal lung development is occurring when lecithin/sphingomyelin ratios are greater than 2.0 and when phosphatidyl glycerol is also detected among the chromatographically resolved spots (6).

Amniotic fluid is a complex analytical matrix composed of water, dissolved salts, various proteins, cholesterol and other neutral lipids and several kinds of phospholipids. We decided to evaluate various pre-treatment methods to determine if we could release more phospholipid from protein binding sites to enhance the extraction of the three principal phospholipids into chloroform-methanol, and to avoid the emulsions sometimes generated when amniotic fluid samples are extracted with chloroform-methanol. Potential pre-treatments could involve the quantitative destruction or removal of undesired components from the mixture (protein, cholesterol, or neutral lipids) or enhancement of the extractibility of the three principal phospholipids into chloroform-methanol (3). We have evaluated three pre-treatment procedures for this purpose: addition of ammonium sulfate to alter the activity coefficient of water and permit quantitative removal of protein components from the analytical matrix; pre-extraction with various non-polar organic solvents to quantitatively remove cholesterol and/or neutral lipids from the analytical matrix; or adjustment of the amniotic fluid pH to either acid or alkaline extremes to alter the partition coefficient of the principal phospholipids into chloroform-methanol by modification of ionization states of the principal phospholipids.

Materials and Methods

Frozen human amniotic fluid samples were obtained from Parkview Memorial Hospital, Fort Wayne, Indiana and from University Hospital, Indianapolis, Indiana. These samples were thawed, pooled and refrozen in 4 ml. aliquots. All samples were stored at $-20^{\circ}C$. Only those samples stored for periods less than nine months were used and samples obviously contaminated with blood or meconium or heme pigments were routinely discarded. Ammonium sulfate (A-5132) was obtained from Sigma Chemical Company, St. Louis, Missouri and was added as various dry powered increments to 4 ml. amniotic fluid samples which had been thawed to room temperature. After thorough mixing with the ammonium sulfate, the samples were centrifuged at 1000 RPM for five minutes in a Clay-Adams centrifuge and extracted with chloroform

and methanol according to the Helena Fetal Tek 200 Procedure (1). The remaining steps in determination of L/S ratio and detection of phosphotidyl glycerol were according to the Helena Fetal Tek 200 method also.

For organic solvent pre-extraction tests, ACS reagent grade Matheson, Coleman and Bell reagents were used, including hexanes (HX 299), ethyl acetate (EX 240), benzene (BX 220), and tricholoroacetic acid (TX 1045). For organic solvent pre-extractions, a 4 ml. sample of thawed amniotic fluid was placed in a 15 ml. liquid scintillation counting vial. The appropriate organic solvent was added in three separate portions of 3 ml. each. The vial was shaken after each addition and the top organic reagent layer was withdrawn by a Pasteur pipet. After the third extraction was completed, a 2 ml. sample of amniotic fluid was drawn off with a measuring pipet from the bottom aqueous layer and again submitted to the Helena Fetal Tek 200 Procedure. An untreated sample was also used in the Helena method to serve as a control.

For trichloroacetic acid pre-treatment, a 4 ml. amniotic fluid sample was mixed with 8 ml. of chilled trichloroacetic acid. After precipitation had occurred, the sample was transferred to centrifuge tubes equipped with Bio Analytical Systems filters. Tubes loaded with 1.5 ml. samples were centrifuged at 2000 RPM's for ten minutes. Since filters became clogged with precipitates, it was often necessary to transfer partially clarified liquid to fresh centrifuge-filter apparatus and repeat the centrifugation step a second time. The combined filtered solutions were adjusted back to pH 7 using 2 M. sodium hydroxide and glacial acetic acid. The resultant aqueous sample was submitted to the Helena Fetal Tek 200 Procedure.

Finally, amniotic fluid samples were adjusted to extremes of pH using 1 M. sodium phosphate, analytical reagent grade, supplied by Mallinckrodt, Inc. The pH altered samples were prepared as usual by the Helena Fetal Tek 200 method.

Results

The effect of ammonium sulfate precipitation of amniotic fluid samples on the L/S determination can be seen in Table 1. Four values are reported at each treatment level on four aliquots of the pooled amniotic fluid. It can be seen when ammonium sulfate is added in amounts which would bring the saturation of water from 20% to 80% that no alteration of the L/S ratio occurs. It was also noted that huge amounts of protein were precipitated, even at the lowest level of ammonium sulfate addition. Although the ammonium sulfate pre-treatment does not negatively influence the extraction of lecithin and sphingomyelin, there is no enhancement of phosphatidyl glycerol from the altered analytical matrix. Thus ammonium sulfate pre-treatment would be of no value in the present case.

TABLE 1. Effect of Ammonium Sulfate Precipitation of Amniotic Fluid on L/S Determination

Amount $(NH_4)_2SO_4$ added to 4 ml sample	L/S Ratio
untreated	----; 1.2; 1.1; 1.2
0.56g	1.1; 0.7; 1.3; 1.2
1.13g	1.6; 0.8; 1.1; 1.2
1.69g	1.2; 0.9; 1.2; 1.1
2.26g	----; 1.1; 1.2; 1.2

TABLE 2. Effect of Organic Solvent Pre-Extraction of Amniotic Fluid on L/S Determination

Organic Solvent	L/S Ratio
None	0.9; 1.1; 1.1; 0.7
Benzene	0.8; 1.0; 0.9; 0.5
Ethyl Acetate	0.7; 0.6; 0.7; 0.5
Hexanes	0.8; 0.8; 1.0; 0.7
Trichloroacetic Acid	ppt. formed

Table 2 shows the effects of various organic solvent pre-extractions of amniotic fluid upon the L/S determination. Once again, benzene and hexane are without effect on the determined L/S ratio and no additional phophatidyl glycerol was extracted into the chloroform-methanol treatment of the Helena Fetal Tex 200 Procedure. No attempt has been made to examine the extent to which cholesterol or neutral lipids may have been removed by organic solvent extraction. Table 2 suggests that ethyl acetate pre-extraction lowers the L/S ratio which is calculated. Examination of the densitometer scans show clearly that ethyl acetate differentially removes lecithin in the pre-extraction phase. Trichloroacetic acid treatment yields a copious precipitate which apparently traps phospholipids quantitatively in the precipitating mixture. We have concluded that the organic solvent pre-extractions examined at present are of no value in the pre-treatment of human amniotic fluid for the enhancement of phospholipid removal by the Fetal Tek 200 Procedure.

Table 3 shows the effect of extreme pH adjustment of amniotic fluid before extraction on the determined L/S ratios. It was seen that there is no significant alteration of the L/S ratio by adjustment of amniotic fluid pH to either pH2 or pH12. Once again no enhancement of phosphatidyl glycerol extraction into the chloroform-methanol occurred.

Discussion

Although undesirable contaminants are often removed from an analytical matrix by pre-precipitation treatments or by pre-extraction, it is clear that the three approaches presently reported have not been of value in enhancing the removal of phospholipids from human amniotic fluid. In the past, Gluck et al. have reported that acetone pre-treatment was useful in enhancing the extraction of lecithin and sphingomyelin (4). More recently, other reports suggest that acetone pre-treatment is useless (5). A recent

TABLE 3. Effect of pH Adjustment of Amniotic Fluid (before extraction) on L/S Determination

Adjusted pH of Sample	L/S Ratio
1. 2M sodium phosphate, pH2	1.4
	1.3
	1.6
	1.7
2. 2M sodium phosphate, pH12	3.1
	1.3
	1.5
	1.4

report by Duck-Chong *et al.* compared various methods of extracting phospholipids from human amniotic fluid. These authors noted that various pre-treatment exposure, such as extraction with chilled solvents or chromatography of single phased mixtures over Sephadex G25 columns caused decreases from 15 to 40% in the total phospholipid originally present in the sample (2).

This report dramatizes the fact that whatever procedure one uses in determining L/S ratios, it is necessary to rigorously standardize all conditions and to develop local standards for fetal lung maturity by comparing statistically the results obtained on many patient samples with the general health observed in the neonate during subsequent postpartum follow-up. We are continuing to evaluate other pre-treatment methods in our laboratory with the hope of realizing this desired goal of enhancing the removal of lecithin, sphingomyelin and phosphatidyl glycerol into chloroform-methanol extraction mixture.

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