Variables Affecting Resolution of Lung Phospholipids in One-Dimensional Thin-Layer Chromatography

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Resolution of the confusion in the literature about the separation of lung phospholipids in thin-layer chromatographic systems has awaited a systematic study of the variables that potentially affect this separation. In this study I show that: (a) incorporation of ammonium sulfate into silica gel "GHL" has a dramatic effect on separation of lung phospholipids; (b) this effect is equally dramatic but different in activated and nonactivated gels; (c) when it picks up moisture, ammonium sulfate-activated gel very rapidly loses its ability to resolve lecithin from phosphatidylglycerol; (d) in gel containing ammonium sulfate, small amounts of phosphatidylethanolamine are hydrolyzed to lyso-phosphatidylethanolamine.

Additional Keyphrases: variation, source of · ammonium sulfate added to silica gel · lecithin/phosphatidylglycerol ratio

Part of the controversy regarding whether one-dimensional thin-layer chromatography (TLC) produces results identical to two-dimensional TLC in identifying and quantifying lung phospholipids (1–6) is based on the various chromatographic conditions used, many of which are poorly described (1, 7–11). Two major differences among the various systems reported are (a) the presence or absence of ammonium sulfate in the gel and (b) whether or not the gel is activated. Originally, Walker (12) incorporated ammonium sulfate into silica gel to make the phospholipids visible after charring. Upon heating, ammonium sulfate decomposes to ammonia and sulfuric acid; the ammonia vaporizes, and the sulfuric acid chars the phospholipids. Although Walker suggested that the addition of ammonium sulfate provided superior resolution of lung phospholipids, this finding was not fully explained. Others have reported that the resolution of phospholipids is dramatically affected by atmospheric humidity (13, 14). With the method in use in our laboratory (15), the migration of phospholipids has shown marked variability, which could not be controlled. In this study I have attempted to address these problems by studying (a) the effect of ammonium sulfate in the unactivated plate, (b) the effect of different concentrations of ammonium sulfate, (c) the effect of varying the duration and temperature of activation, (d) the effect of exposing the plates to different relative humidities for various periods.

Materials and Methods

I used the method of Tsai and Marshall (15) with the following modifications: (a) thin-layer silica gel "GHL" plates (Analtech Inc., Newark, DE) were dipped in ammonium sulfate solution; (b) developed plates were charred on a hot-plate stirrer; (c) the solvent was chloroform/methanol/water/acetic acid (55/16/2.5, by vol); (d) the plates were activated by heating at 100 °C for 1 h unless otherwise stated. I quantified results by transmission densitometry.

Phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (S), dipalmitoylphosphatidylcholine (lecithin, L), dioleoylphosphatidylcholine, and dipalmitoylphosphatidylglycerol (PG) were all purchased from Sigma Chemical Co., St. Louis, MO.

In the experiment examining the effect of ammonium sulfate concentration, the 20 × 20 cm plates were cut into 1.5-cm strips and dipped into solutions of various concentrations of ammonium sulfate (10–100 g/L). To determine the effect of rehydration of activated TLC plates, I exposed the plates to moisture in a closed tank with 100% or 32% relative humidity (16). I inserted the plates into this chamber at various times so that they could all be removed and used in an assay simultaneously. To determine the moisture content of the gel, I removed a portion of the gel from the top of the glass plate, weighed the gel, reactivated it at 100 °C, and weighed it again. The percentage of moisture is expressed as the difference in the weighings divided by the weight of the activated gel.

For all experiments I applied mixtures of the pure phospholipids L, S, PI, PE, PS, and PG to the TLC plates. Where the Rf of a particular phospholipid was in doubt in any experiment, I confirmed the Rf by repeating the experiment in a separate run with only the phospholipid in question.

I also repeated these experiments but used silica gel "H" (Analtech Inc.) to see if it behaved like silica gel GHL. This silica gel H as purchased did not contain any ammonium sulfate.

Results and Discussion

Adding ammonium sulfate to silica gel GHL plates dramatically affects the migration of the phospholipids in amniotic fluid (Figure 1). The activated TLC plate containing ammonium sulfate effectively separates all the major phospholipids except PE and PS. This performance is acceptable because these two phospholipids are not important for assessing fetal pulmonary maturity. However, an activated plate not containing ammonium sulfate has two serious drawbacks: it does not clearly separate PG from PE and PS; and the PI and L co-migrate, which may lead to overestimation of the L/S ratio.

Using silica gel with added ammonium sulfate but without heat activation results in migration of PG, PI, and PE such that all five major phospholipids have Rf values <0.5. This is unfavorable because the migration of PI between L and S increases the possibility that PI might erroneously be included with either L or S. Consequently, slight overloading of the plate or small errors in densitometric quantification of either L or S could cause large errors in determining the L/S ratio.

Given major differences in migration of phospholipids on the various TLC plates, I studied the effect of concentration of ammonium sulfate in the TLC plate. As Figure 2 shows, in the range of 0–50 g/L concentrations of ammonium sulfate...
Running fate (Figure 1).

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**Table 1.** One-dimensional TLC of phospholipids on plates pretreated as indicated.

<table>
<thead>
<tr>
<th>Application Point</th>
<th>Not activated</th>
<th>Activated at 100°C - 1H</th>
<th>Not activated</th>
<th>Activated at 100°C - 1H</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (NH₄)₂ SO₄</td>
<td>No (NH₄)₂ SO₄</td>
<td>10% (NH₄)₂ SO₄</td>
<td>10% (NH₄)₂ SO₄</td>
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**Fig. 1.** One-dimensional TLC of phospholipids on plates pretreated as indicated.

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**Fig. 2.** Effect on the migration of phospholipids of the concentration of ammonium sulfate incorporated into the silica gel. Each point shows results for one plate that was dipped in aqueous ammonium sulfate before activation for 1 h at 100°C.

The migration of PG and PI continues to vary, but at concentrations >50 g/L, the migration of phospholipids remains constant. All subsequent experiments were run with ammonium sulfate at 100 g/L, so that small changes in ammonium sulfate concentrations would have virtually no effect on the migration of the phospholipids.

Because the activation process is important for good separation in plates containing ammonium sulfate, I investigated the effect of temperature of activation and found (Figure 3, top) that 100–120°C is required for activation. At 100°C, the activation of plates containing ammonium sulfate is complete by 30 min (Figure 3, bottom).

The migration of the phospholipids was confirmed by running the individual phospholipids in separate experiments. A run with pure PE showed the presence of a minor component, with Rᵣ of 0.16. This minor component was apparent only on the plates containing ammonium sulfate (in either H or GHL gels). Subsequent investigations suggested that this minor component is lyso-PE, based on a comparison of chromatographic properties. This minor component has also been detected in clinical specimens; because it migrates between L and S, it may interfere with quantification of the L/S ratio.

When plates containing 100 g/L ammonium sulfate are activated at 100°C and then exposed to an atmosphere having a relative humidity of 32%, they rapidly absorb water, as much as 30% by weight (Figure 4); under these conditions L and PI co-migrate. In a separate experiment (results not shown) the plates were exposed to 100% relative humidity for as long as 24 h. Again, PI and L co-migrated when the moisture content of the plate exceeded 30% migration of the other phospholipids was not affected until the moisture content exceeded 70%. Because activated plates rapidly lose their ability to separate PI from L when exposed to high humidity, TLC plates for determining L/S ratios must be absolutely dry.

The gel on the GHL plates is sturdy and not easily damaged, whereas the silica gel H is fragile and difficult to handle without damaging. Nonetheless, I performed parallel experiments with silica gel H instead of silica gel GHL because Gluck et al. (17) reported that silica gel G produces

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more diffuse spots than silica gel H. I found the exact opposite; i.e., silica gel GHL had much more compact spots and hence produced superior resolution. Moreover, all the phospholipids migrated with identical RF values when ammonium sulfate was incorporated into both GHL and H types of gels and the gels were then activated. Gluck et al. (7) also indicated that silica gel G and H plates did not char in a similar fashion; they used 50–70% solutions of H2SO4 and temperatures of 180–280 °C. I found no differences in RF ratios for standards containing both saturated and unsaturated lecithins as well as for clinical specimens.

For samples from three pools of amniotic fluid the GHL and H plates produced L/S ratios of 1.3, 2.1, 4.0 and 1.4, 2.2, and 4.3, respectively; for samples from three selected patients the respective L/S ratios were 0.6, 2.4, 6.6 and 0.7, 2.6, and 6.4. Quality-control samples produced L/S values of 2.3 (SD 0.1) and 2.2 (SD 0.2) (n = 6) on GHL and H plates, respectively. The phospholipids in the control samples migrated to give patterns as diffuse as those from clinical specimens because both saturated and unsaturated lecithins from commercial sources were included.

These results demonstrate that the chromatographic system described adequately resolves phospholipid standards and has allowed more consistent separation of amniotic fluid phospholipids in my laboratory; however, it is essential that the gel plate be activated just before use and that the sample be applied under a hot-air fan to ensure that the plate stays dry. The results obtained by this system remain to be clinically validated.

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References

GLUCK ET AL. used silica gel G plates (AG Merck, Darmstadt, R.G.), which contain CaSO4 binder.