Determination of phosphatidylcholine and disaturated phosphatidylcholine content in lung surfactant by high performance liquid chromatography

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Summary A rapid isocratic method for determining the total phosphatidylcholine and disaturated phosphatidylcholine levels in lung surfactant preparations by high performance liquid chromatography (HPLC) is described. The analysis was performed on a 3.9 × 300 mm μ-Porasil® column with detection by refractive index. The lipids were eluted with a solvent system of chloroform–acetonitrile–methanol–water–85% phosphoric acid 650:650:500:130:2 (v/v/v/v/v). A 4.6 × 30 mm silica guard column was used in place of an injector loop which served as a sample concentrator and purifier. Phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and phosphatidylglycerol, all known components of lung surfactants, were eluted from the loop column and were prevented from reaching the analytical column. Sphingomyelin and lysophosphatidylcholine elute later than the phosphatidylcholines on the analytical column. The method was developed so that phosphatidylcholines elute as a single peak regardless of the fatty acid chain length (C12–C20). When the sample was first oxidized with a potassium permanganate–potassium metaperiodate solution, and potentially interfering oxidation products were removed by extraction into a basic aqueous phase, then only the disaturated phosphatidylcholines were analyzed.— **Scarim, J., H. Ghanbari, V. Taylor, and G. Menon.** Determination of phosphatidylcholine and disaturated phosphatidylcholine content in lung surfactant by high performance liquid chromatography. *J. Lipid Res.* 1989. **30:** 607–611.

Supplementary key words lysophosphatidylcholine • sphingomyelin • dipalmitoyl phosphatidylcholine • phospholipids

Treatment of Respiratory Distress Syndrome (RDS) in premature infants by surfactant replacement therapy is currently a topic of wide investigation (1–4). The therapeutic action of these surfactants is to lower surface tension in the alveoli. The major phospholipid component present and the one most responsible for surface tension lowering is phosphatidylcholine. In particular, the disaturated phosphatidylcholines have been demonstrated to be of major importance.

Previous work in this laboratory to determine the disaturated phosphatidylcholine content of a lung surfactant replacement was performed by two-dimensional TLC of the oxidized sample, isolation and digestion of the DSPC area, and then a colorimetric determination for phosphorus (5). The procedure was both labor- and time-intensive. A simpler and more accurate analytical technique was desirable.

A number of methods have been proposed for quantifying the phospholipids contained in biological samples by high performance liquid chromatography (6–15). Generally, these methods use gradient elution and detection by low wavelength ultraviolet absorbance. The absorbance arises primarily from double bonds in the fatty acid moieties, which accounts for the extremely poor response to fully saturated species. The use of refractive index (RI) detector offers an improved response and removes the restriction to ultraviolet-transparent solvents. Due to the design of the refractive index detectors currently on the market, analyses are restricted to the isocratic mode.

The procedure described herein presents an analytical technique that separates phosphatidylcholines from all the other components of lung surfactant. In addition, the disaturated species can be isolated by sample manipulation prior to the HPLC analysis.

MATERIALS AND METHODS

The surfactant preparation under investigation was obtained from bovine lung extracts that were further fortified with lipids and prepared as an aqueous suspension. More than 80% of the phospholipids present were phosphatidylcholines and of those approximately 60% were disaturated. The remaining phospholipids were comprised of phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), lysophosphatidylcholine (LPC), and sphingomyelin (SPH). In addition, the sample also contained free fatty acids, triglycerides, cholesterol, protein, and sodium chloride.

HPLC grade chloroform, acetonitrile, and methanol and certified ACS grade potassium permanganate were obtained from Fisher Scientific Co. (Fairlawn, NJ). Reagent grade potassium metaperiodate and phosphoric acid were purchased from G. Frederick Smith Chemical Co. (Columbus, OH) and J. T. Baker (Phillipsburg, NJ), respectively. Short range indicator paper (pH 5–10) strips were obtained from MCB Reagents (Gibbstown, NJ). DPPC (synthetic), PE (egg), PG (egg, ammonium salt), PI (soybean, sodium salt), PS (bovine brain), LPC (egg), SPH (egg), and PC of various fatty acid substitution were all purchased from Sigma (St. Louis, MO).

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; loop column, guard column used in place of sample loop in manual injector; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine; SPH, sphingomyelin; PG, phosphatidylglycerol; RI, refractive index; FFA, free fatty acid; TG, triglyceride; DSPC, disaturated phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine.

Chromatographic conditions

The HPLC system was comprised of a Waters (Milford, MA) model 6000A pump operating at 2 ml/min, a Hewlett-Packard (Palo Alto, CA) model 1037A refractive index detector operated at 35°C and 2 × 10^{-5} RIU sensitivity, and a Spectra-Physics (Santa Clara, CA) model SP4100 computing integrator.

The injector system was a Rheodyne (Cotati, CA) model 7125 manual injector with a Brownlee (Santa Clara, CA) guard column (10 μ spherical silica, 4.6 × 30 mm) and holder plumbed in place of the injector loop (which will be referred to as a loop column). The analytical column was a Waters, μ-Porasil, 10 μ, silica, 3.9 × 300 mm. The solvent system consisted of chloroform–acetonitrile–methanol–water–85% phosphoric acid 650:650:500:130:2 (v/v/v/v/v).

Sample preparation

The sample under investigation in this laboratory contained approximately 25 mg/ml phospholipids in normal saline. A 5-ml aliquot of sample was diluted to 100 ml with chloroform–methanol 2:1.

Total phosphatidylcholines

A 10-ml portion of the sample preparation was extracted twice with 10-ml volumes of methanol–water 65:50 to remove water from the sample preparation. The resultant organic layer was diluted to 25 ml with chloroform–methanol 2:1.

Disaturated phosphatidylcholines

A 10-ml portion of the sample preparation was evaporated, suspended in 2 ml of 90% acetic acid, oxidized with 3 ml of 0.5% $KMNO_4$–0.5% KIO_4 1:1 for 45 min, decolorized with 0.6 ml of 20% sodium bisulfite, and slowly neutralized over ice with 3 ml of 50% ammonium hydroxide. The phospholipids were extracted into chloroform. Chromatographically interfering oxidation products were removed from the chloroform layer by extraction with a 10-ml portion of methanol–water–ammonium hydroxide 65:50:2 followed by repeated washings with methanol–water 65:50 until the pH of the aqueous layer was 6.5–7 (as determined by submerging pH-indicating strips into the upper layer). The chloroform layer was diluted to 25 ml with chloroform–methanol 2:1.

These preparations were injected as described below and quantified; a preparation of 0.25 mg/ml dipalmitoyl phosphatidylcholine (DPPC) in chloroform–methanol 2:1 was used as a standard.

Injection technique

The Rheodyne injector was fitted such that port #2 was connected to the pump, port #3 to the analytical column, ports #5 and #6 to waste, and ports #1 and #4 to the loop column. The injector was placed in the load position and then 1 ml of acetonitrile was injected to displace the eluent. Next 0.5–1.0 ml of sample in chloroform–methanol 2:1 was injected followed by a 1.0-ml aliquot of acetonitrile. Finally 0.75 ml of HPLC eluent was injected prior to switching to the injection position. Configured in this fashion the phosphatidylcholines were loaded and then eluted from the same end of the loop column. The more polar lipids PI, PS, PE, PG, FFA, and TG were washed through the loop column and discarded. The addition of HPLC eluent as the final step also reduced the intensity of the solvent front peak.

RESULTS

The detector response for DPPC concentrations of 0.048–0.605 mg/ml in chloroform–methanol 2:1 was shown to be linear and passed essentially through the origin. The method was designed so that standard and sample concentrations are approximately equal and a single standard was used for quantification. Peak areas of replicate injections were reproducible within 2%.

Fig. 1 shows the separation obtained between DPPC, SPH, and LPC. **Fig. 2** shows typical sample and standard chromatograms. The method was designed so that phosphatidylcholines with fatty acid moieties of C12–C20 coelute. Results are calculated as total phosphatidylcholines or disaturated phosphatidylcholines versus dipalmitoyl phosphatidylcholine. If additional information is required, the eluted peak can be collected and its fatty acid profile can be determined by gas–liquid chromatography.

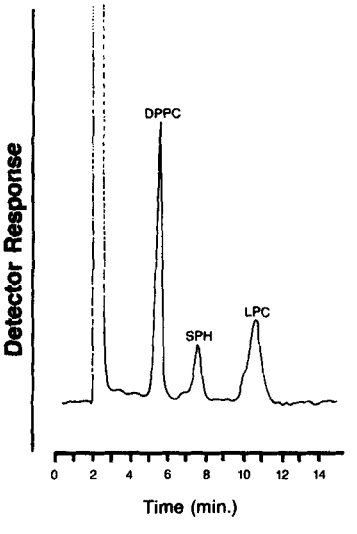

Fig. 1. Separation of a phospholipid standard mixture. The mobile phase consisted of chloroform–acetonitrile–methanol–water–85% phosphoric acid 650:650:500:130:2 flowing at 2 ml/min. Injection volume was 0.5 ml (DPPC, 0.25 mg/ml; SPH, 0.07 mg/ml; LPC, 0.17 mg/ml). Detection was by refractive index (35°C, 1 × 10^{-5} RIU). Analytical (3.9 × 300 mm, silica, 10 μ) and loop columns (4.6 × 30 mm, silica, 10 μ) at room temperature.

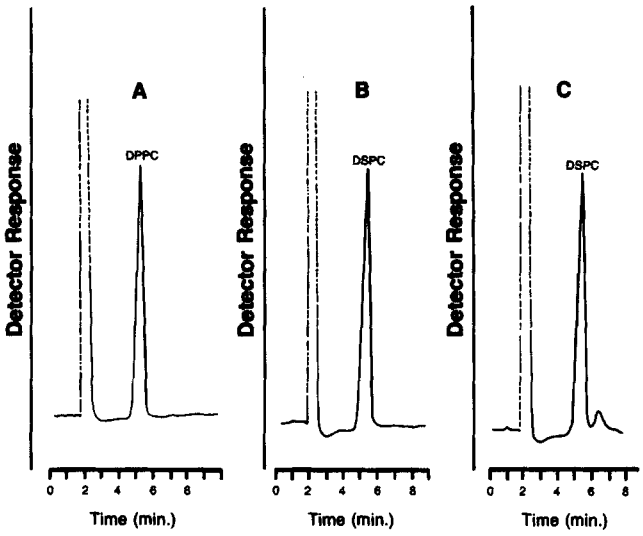

Fig. 2. Typical chromatogram of dipalmitoyl phosphatidylcholine (A), lung surfactant sample prepared for DSPC analysis (B), and lung surfactant prepared for DSPC determination depicting the incomplete clean up of oxidation products (C). The mobile phase consisted of chloroform-acetonitrile-methanol-water-85% phosphoric acid 650:650:500:130:2 flowing at 2 ml/min. One ml of preparations was injected into the loop column (approximately 0.25 mg/ml). Detection by refractive index (35°C, 2 × 10^{-5} RIU). Analytical (3.9 × 300 mm, silica, 10 μ) and loop columns (4.6 × 30 mm, silica, 10 μ) at room temperature.

The fatty acid profile of the disaturated phosphatidylcholines in the lung surfactant, as analyzed by gas–liquid chromatography, revealed that typically more than 85% of the fatty acids were palmitic (C16) and none of the other fatty acids represented more than 5%. A mixture of disaturated C12–C20 phosphatidylcholines was prepared to contain 58% dipalmitoyl, 8.4% dilauroyl, 8.7% dimyristoyl, 9.2% distearoyl, 7.5% diarachidoyl, and 8.4% dibehenoyl. Analysis of this mixture by the present method resulted in a single peak, which was broader than DPPC alone but was easily integrated. This system was suitable for the analysis of most of the naturally occurring phosphatidylcholines. It is important to note, however, that another mixture consisting of 80% dipalmitoyl and 20% dilauroyl phosphatidylcholines resulted in a split peak with the dilauroyl PC eluting on the tail end of the DPPC peak. There is, therefore, some discrimination by fatty acid moiety, and for this reason it is recommended that the DPPC elution time be restricted to 4.5–6 min. If necessary, the HPLC eluent can be modified to accomplish this (more water in the eluent decreases the retention time of the DSPC).

Additional studies were performed to demonstrate that the sample preparation was capable of discriminating saturated phosphatidylcholines (dipalmitoyl) from unsaturated (dioleoyl) or mixed (palmitoyl/linoleoyl) phosphatidylcholines. Dioleoyl phosphatidylcholine and 1-palmitoyl, 2-linoleoyl-*sn*-glycero-3-phosphocholine co-elute with dipalmitoyl phosphatidylcholine on the chromatographic system. The oxidation and extraction steps remove the phosphatidylcholines with unsaturated fatty acid moieties prior to the HPLC finish.

One study was performed on approximately 6 mg of dioleoyl phosphatidylcholine (18:1/18:1). This level represents the worst case scenario of all phosphatidylcholines in the sample being fully unsaturated. The sample preparation should completely remove these oxidized phosphatidylcholines and no peak should be observed in the chromatogram corresponding to DSPC. This analysis resulted in a recovery of 0.7% (quantitated as DPPC). The same preparation was also analyzed by GLC and found to contain only trace amounts of saturated fatty acids (indicating a disaturated impurity). The above procedure was repeated with 1-palmitoyl, 2-linoleoyl-*sn*-glycero-3-phosphocholine (18:2/16) with similar results. A suspected disaturated impurity representing less than 2% was observed by GLC (predominantly C16 with a trace of C18).

When the same manipulations were performed on oleoyl/palmitoyl phosphatidylcholines (18:1/16), 4–7% recoveries (as DPPC) were observed. The fatty acid profile of the original material demonstrated an approximate 1:1 ratio of C16 to C18:1, while the same material after the oxidation/extraction steps showed only saturated fatty acids. Both results could not be true unless the starting materials contained some disaturated (16/16) and diunsaturated (18:1/18:1) phosphatidylcholines. Finally, the 18:1/16 was oxidized but not carried through the extraction procedure. The fatty acid profile of this solution showed C16 and the expected residues that would result from the double bond oxidation of oleic acid (nonanoic and azelaic acids). The HPLC analysis of this preparation resulted in a chromatogram with a double peak: a small peak corresponding to the disaturated impurity and a peak that eluted slightly later suspected of being the palmitoyl azeloyl phosphatidylcholine (this later peak is removed by extraction with base).

Single sample preparations are sufficient for good precision data. In a preliminary experiment, triplicate analyses from a single lung surfactant preparation by two analysts resulted in relative standard deviations of 0.7 and 1.3% with the average value from both analysts differing by only 0.7%. The precision of the method was determined in a more rigorous fashion by the analysis of individually vialed preparations stored under nondegrading conditions and analyzed over a period of time. The average relative standard deviation from four lots studied was 3.3% (lot A: n = 6, RSD = 3.83%; lot B: n = 6, RSD = 1.46%; lot C: n-6, RSD = 6.40%; lot D: n = 3, RSD = 1.58%).

The accuracy of the method was determined in a number of ways. First, a DPPC reference material was analyzed. Recoveries of 96–100% were obtained indicating

that neither losses in the extraction steps nor degradation were occurring. Secondly, standard addition/recovery experiments were performed. An aliquot of the sample (DSPC content known from the precision study) was mixed with a spiking solution that contained the components of lung surfactant; tripalmitin, palmitic acid, cholesterol, sphingomyelin, DPPC, and sodium chloride. Analysis of various combinations of sample and spike solution showed DSPC recoveries of 97.3–99.8% (**Table 1**). Finally, samples were analyzed by both the HPLC method and the TLC/colorimetric procedure with good correlation (**Table 2**).

DISCUSSION

The method previously used by this laboratory to quantify the DSPC levels in lung surfactant samples involved an oxidation/cleavage with $KMnO_4/KIO_4$, extraction of the phospholipids into chloroform, and a two-dimensional TLC separation to isolate the disaturated phosphatidylcholines. The disaturated phosphatidylcholines were then scraped and eluted from the plate material and analyzed for phosphorus content by the formation of a molybdenum blue complex after an overnight digestion in perchloric acid. This method was both labor- and time-intensive. Replicates of the TLC and color development steps were necessary to achieve an acceptable precision for the method. The current method significantly reduces the number of sample manipulations (and eliminates the need for the meticulous cleaning of glassware required for colorimetric phosphorus methods).

It was of major interest to this laboratory to develop a reliable HPLC procedure for the routine analysis of DSPC. Since the disaturated species exhibit such poor ultraviolet absorbance, a refractive index detector was employed. Typically, RI detectors are not very sensitive and require relatively high analyte levels. The more current RI detectors are temperature-controlled to reduce noise and thereby increase sensitivity, but this can be offset by the tendency of some solvents to vaporize or outgas at higher temperatures which results in noise. The HPLC eluent was chosen as a compromise to these opposing problems. The chloroform–acetonitrile combination achieves good phospholipid solubility and minimizes the generation of bubbles that occurs with warmed chloroform alone. The methanol and water add the necessary polarity and the phosphoric acid greatly improves the peak shape.

To determine the total phosphatidylcholines, the sample preparation simply involves the removal of water by extraction. This is necessary because the 5% water present in the diluted sample preparation would retard the concentration of PC on the loop column and result in a broad distorted peak. The sample preparation for the DSPC determination involves the oxidation of the unsaturated species, extraction into chloroform, and the removal of chromatographically interfering oxidation products. Fig. 2 also shows a chromatogram of an oxidized surfactant sample that was incompletely cleaned.

The present method has been shown to separate disaturated phosphatidylcholines from all the other components in a lung surfactant preparation. The oxidation/extraction steps separate the disaturated phosphatidylcholines from the mixed (one saturated and one unsaturated fatty acids) or fully unsaturated phosphatidylcholines. The injection technique removes phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, free fatty acids, and triglycerides. The chromatography separates phosphatidylcholine from lysophosphatidylcholine and sphingomyelin.

Quantification of SPH and LPC in surfactant preparations could be achieved by concentrating larger sample volumes onto the loop column. The PI, PS, PE, PG fraction eluting from the loop column could be collected for analysis with a less polar mobile phase. Currently, we are

TABLE 1. Standard addition/recovery of DPPC to lung surfactant

DSPC From Sample	DPPC From Spike Solution[a]	Total DSPC Expected	Total DSPC Found	% Recovery
	mg			
3.432	1.947	5.380	5.234	97.3
3.432	3.245	6.678	6.549	98.1
3.432	4.543	7.976	7.958	99.8
3.432	1.947	5.380	5.362	99.7
3.432	3.245	6.678	6.658	99.7
3.432	4.543	7.976	7.781	97.6

[a]This solution also contained 0.07 mg/ml tripalmitin, 0.10 mg/ml palmitic acid, 0.01 mg/ml cholesterol, 0.64 mg/ml sphingomyelin (to represent all other phospholipids), 0.45 mg/ml sodium chloride, and 5% water. These levels represent those found in a typical sample diluted 1/20 with chloroform–methanol 2:1.

TABLE 2. Comparison of TLC/colorimetric and HPLC methods for the determination of DSPC in lung surfactant

Sample No.	HPLC	TLC/Colorimetric	HPLC/TLC × 100
	mg/ml		
1	13.41	13.30	100.8
2	12.97	13.99	92.7
3	10.06	10.64	94.5
4	15.10	13.88	108.8
5	13.95	13.58	102.7
6	9.74	9.16	106.3
7	13.99	13.99	100.0
8	14.58	13.94	104.6
9	13.37	14.20	94.2
10	13.73[a]	14.16	97.0
11	12.96	12.74	101.7
		Mean	100.3

[a]Average of six determinations from precision study.

testing silica columns from various manufacturers in an attempt to find a column with the proper selectivity for baseline resolution of DSPC and the oxidation products formed during the sample treatment. This would further reduce the sample manipulations.

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