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Quantitative analysis of pulmonary surfactant phospholipids by high-performance liquid chromatography and light-scattering detection

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Abstract

An improved high-performance liquid chromatographic method for the separation and quantitation of nine phospholipid classes is described. It is based on normal-phase chromatography with silica gel as stationary phase and a binary gradient with mixtures of chloroform, methanol and water as mobile phase. The response of the evaporative light-scattering detector was non-linear. Peak areas were proportional to the power 1.7 of the masses. Phospholipids in lung lavage samples were enriched by liquid extraction prior to HPLC analysis. The described method is a rapid and accurate procedure for the quantitative analysis of phospholipid classes in biological samples.

1. Introduction

Phospholipids are extremely complex mixtures of closely related compounds, which are constituents of biological membranes. Phospholipids are also major constituents of pulmonary surfactant, which lines the alveolar surfaces of all mammalian lungs, and maintains a low surface tension at changing lung volumes. This biophysical activity makes breathing possible and depends particularly on a specific phospholipid composition, which is surprisingly similar in many different species, varying only during lung development or acute lung injury [1]. In order to estimate this function, it is important to determine phospholipid classes in samples that contain pulmonary surfactant, e.g., lung lavage or lung tissue samples.

Previous quantitations of phospholipids have been obtained with thin-layer chromatography [2–5]. In recent years the application of high-performance liquid chromatography (HPLC) became more important because of better reproducibility and reduced analysis time. The determination of phospholipid classes is achieved exclusively with normal-phase chromatography. Stationary phases are mostly silica gels [6–17,25–37] or chemically modified silica gels, particularly diol [18–22], cyanopropyl [23] and aminopropyl phases [24].

Most of the published HPLC methods used detection by ultraviolet absorbance at low wavelength [6–24] and gradient elution with different solvents (acetonitrile, methanol, ethanol, hexane, isopropanol and water). UV detection, however,

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has two essential disadvantages. Firstly, the absorbance of phospholipids arises primarily from double bonds in the fatty acid moieties, which accounts for the extremely poor response of fully saturated species. Consequently the accurate calibration for the analysis of phospholipid classes requires the availability of standards of the same origin [10]. Secondly, the choice of mobile phase is limited to solvents with low absorbance in the range 195 to 210 nm, thus the use of chloroform as solvent is not feasible.

Refractive index detection permits chloroform as solvent, but only in the isocratic elution mode [25–27]. None of these methods enables a complete separation of natural phospholipids. Quantitative analysis is therefore extremely difficult.

Some of these problems could be overcome with the evaporative light-scattering detector. It enables gradient elution even with solvents of low spectral transparency to improve resolution [28–37]. In addition, the degree of saturation in a given phospholipid class is negligible, since the light-scattering response is primarily caused by the mass of the analyte [38,39]. We tested several HPLC methods for phospholipid class analysis using ultraviolet as well as light-scattering detection. A method described by Becart et al. [33] yielded the best resolution and was improved for our biomedical applications.

2. Experimental

2.1. Reagents

Chloroform (HPLC reagent stabilized with 0.75% ethanol) was obtained from Baker (Phillipsburg, NJ, USA), methanol (LiChrosolv gradient grade) and ammonia solution (25% Suprapur) from Merck (Darmstadt, Germany). Water was purified by means of a Milli-Q Plus Water System (Millipore, Eschborn, Germany). Phospholipid standards were purchased from Sigma (St. Louis, MO, USA) and include phosphatidylglycerol ammonium salt from egg yolk, cardiolipin from bovine heart, phosphatidylethanolamine from egg yolk, phosphatidylinositol sodium salt from soybean, phosphatidylserine from bovine brain, phosphatidylcholine type XVI-E from fresh egg yolk, phosphatidic acid sodium salt from egg yolk, sphingomyelin from bovine brain, lysophosphatidylcholine from egg yolk, and the synthetic compounds dilinoleyl-, diarachidoyl- and dipalmitoylphosphatidylcholine.

2.2. Sample preparation

Lung lavage samples were obtained from adult humans. The lavage fluid was centrifuged for 10 min at 450 *g*, 4°C, and the cell free supernatant was stored in aliquots of 10 ml at −70°C. Lipids were extracted by the method of Folch et al. [40] with some modifications. A thawed aliquot was added to 40 ml chloroform–methanol (2:1, v/v) and shaken in a separatory funnel for 3 min at 4°C. The lower lipid containing phase was separated and washed with 2 ml of pure solvent upper phase [40], centrifuged again, and dried under a nitrogen stream at 45°C. Dry samples were stored at 4°C or dissolved immediately in 500 μl mobile phase A (4°C, closed tube, 1 min vortex-mixed) and analysed by HPLC.

2.3. Chromatographic equipment and method

HPLC was performed with an HP-1090 liquid chromatograph fitted with a solvent delivery system PV5 (proportioning valve for gradient elution), helium degassing, autosampler and HP Vectra 486/33VL personal computer with HPLC ChemStation software (Hewlett-Packard, Waldbronn, Germany). An interface module HP-35900 converts the analogue signal from the evaporative light-scattering detector Sedex-45 (SEDERE, Vitry sur Seine, France) to digital data, which are transmitted to the computer.

For the stationary phase we used Encapharm 100, a spherical silica gel with high stability up to pH 10 (5 μm particle size, 10 nm pore size and 320 m^2/g surface area). The guard (20 × 2.1 mm I.D.) and the analytical column (120 × 4.6 mm I.D.) were packed with the same material (Molnar, Berlin, Germany).

The mobile phase reservoirs contained an A solvent of 80% chloroform, 19.5% methanol,

0.5% ammonium hydroxide and a B solvent of 60% chloroform, 34% methanol, 5.5% water, 0.5% ammonium hydroxide. The following linear gradient was used: 0 to 100% B from 0 to 14 min and returning to 0% B from 23 to 30 min. The time required to reequilibrate the column in a sequence of runs was 10 min. With a flow-rate of 1 ml/min and a column temperature of 30°C the pressure increased from 5.5 to 7 MPa. The evaporation temperature of the light-scattering detector was set to 50°C and the gain step to 12. The nebulization gas was nitrogen with a pressure of 0.20 MPa giving a flow of about 6 l/min.

3. Results

3.1. Separation

Separation of up to nine phospholipid classes was accomplished by the described HPLC method with evaporative light-scattering detection. The separation profile of a standard containing six natural phospholipids is shown in Fig. 1. All phospholipid classes were eluted as single peaks with the exception of sphingomyelin, which

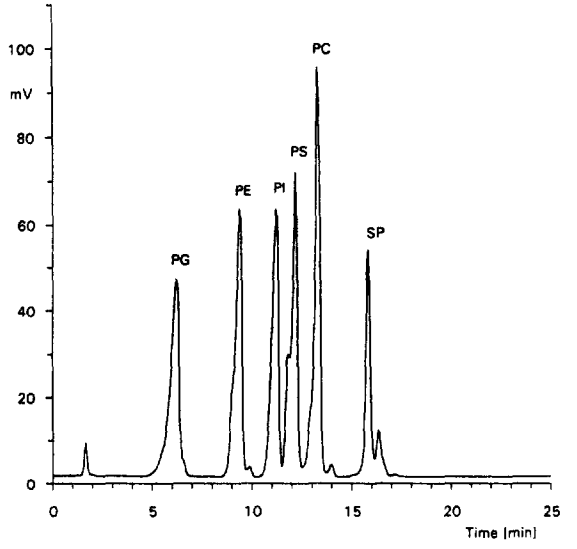

Fig. 1. Separation of six natural phospholipids by HPLC and light-scattering detection. Conditions: 8 μg of each phospholipid standard in 20 μl mobile phase A; further details are described in Section 2; for abbreviations see Table 1.

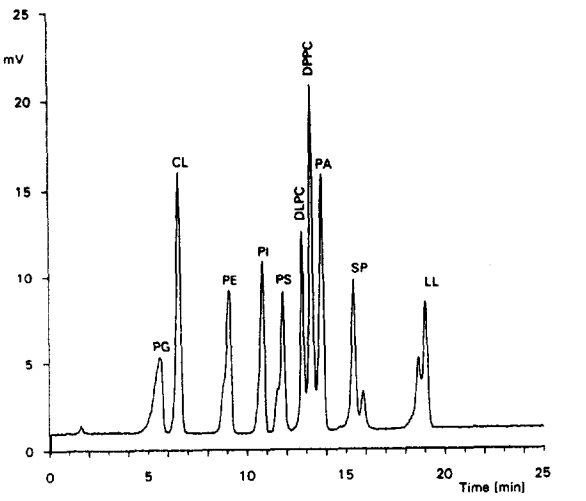

Fig. 2. Chromatogram of ten phospholipids. Conditions: 2 μg of each phospholipid standard in 20 μl mobile phase A; further details are described in Section 2; for abbreviations see Table 1.

eluted as a double peak. In Fig. 2 three additional natural phospholipids were included and natural phosphatidylcholine was replaced by two synthetic compounds (DLPC and DPPC). Table 1 summarizes our results concerning retention times of nine phospholipid classes and three synthetic phosphatidylcholines.

The retention times of most phospholipids were sufficiently reproducible with relative standard deviations of 1 to 2%. Only PG and CL showed retention times with relative standard deviations of about 8%. Therefore the time windows for these components often required correction during a sequence. We have made well over 500 injections onto one silica column without loss in resolution.

3.2. Calibration

At first we used LL as internal standard. Although only present in very small concentrations in our samples, LL plays an important role as hydrolysis indicator. Too high a content often means bad extraction or storage conditions. Therefore in this case we preferred the external standard method. Calibration curves of the light-scattering detector were non-linear (Fig. 3).

Table 1
Retention times of nine natural phospholipids and three synthetic phosphatidylcholines (mean of $n = 8$ with standard deviation and relative standard deviation)

Phospholipid	Abbreviation	Retention time (min)	S.D. (min)	R.S.D. (%)
Phosphatidylglycerol	PG	5.37	0.43	8.0
Diphosphatidylglycerol (cardiolipin)	CL	6.30	0.52	8.2
Phosphatidylethanolamine (cephalin)	PE	9.00	0.19	2.1
Phosphatidylinositol	PI	10.75	0.20	1.9
Phosphatidylserine	PS	11.81	0.11	0.9
Phosphatidylcholine (lecithin)	PC	13.11	0.18	1.3
Phosphatidic acid	PA	13.78	0.13	0.9
Sphingomyelin	SP	15.24	0.18	1.2
		15.72	0.18	1.1
Lysophosphatidylcholine (lysolecithin)	LL	18.46	0.23	1.2
		18.81	0.24	1.3
Synthetic pure compounds				
Dilinoleyl-phosphatidylcholine (C18:2)	DLPC	12.69	0.14	1.1
Diarachidoyl-phosphatidylcholine (C20:0)	DAPC	12.75	0.15	1.2
Dipalmitoyl-phosphatidylcholine (C16:0)	DPPC	13.14	0.15	1.2

Calibration functions were obtained by curve fitting using the equation:

$$a = Km^E \quad (1)$$

with peak area units a, mass of a component injected m, a constant K, and an exponent E. All measured points were weighted equally. The exponents E for the nine investigated phospholipid classes in the mass range 1–25 μg were very similar (Table 2), with a mean value of 1.73 and a standard deviation of 0.07. Fig. 3 shows the measured peak area values and the fitted calibration curve for PS as an example. When the calibration functions are plotted on a logarithmic scale they are linear (Fig. 4), following the equation:

$$\ln a = E \ln m + \ln K \quad (2)$$

with E being the slope and ln K the intercept. The logarithmic plot shows nearly the same slopes for the calibration curves of different phospholipids. The detection limits (twice the noise level) of natural phospholipids ranged from 0.03 μg for PA to 0.1 μg for PG (Table 2).

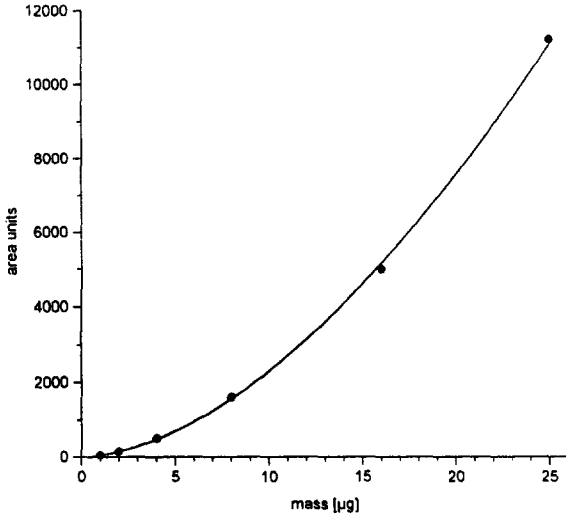

Fig. 3. Calibration curve of phosphatidylserine; measured peak areas with power function curve fitting.

3.3. Quantitation

Since light-scattering detection is not very sensitive, we enriched phospholipids in lung lavage fluids by liquid extraction (20:1 up to 100:1). Concentration c in the sample was calculated according to the relationship:

Table 2
Detection limits, calibration curve fitting results according to Eq. (1), and recoveries of nine natural phospholipid standards (for abbreviations see Table 1)

Phospholipid	Detection limit (μg)	Curve fitting			Recovery (%)
		Constant (K)	Exponent (E)	Correlation (r)	
PG	0.10	45.6	1.82	0.9996	113.4
CL	0.04	55.3	1.81	0.9993	100.7
PE	0.05	44.6	1.74	0.9998	99.5
PI	0.04	51.8	1.83	0.9988	81.5
PS	0.05	44.6	1.71	0.9998	80.6
PC	0.04	67.4	1.71	0.9997	99.8
PA	0.03	56.8	1.67	0.9944	81.4
SP	0.04	41.6	1.68	0.9989	99.1
LL	0.05	46.0	1.63	0.9986	94.6

$$c = \frac{m v_l}{v_s v_i} \quad (3)$$

where v_s is the sample volume, v_l the liquid volume for dissolution of the evaporation residue, v_i the injection volume and m the mass of a component calculated by the calibration function (Eq. 1).

The chromatogram of a lung lavage extract is shown in Fig. 5, and Table 3 gives the phospholipid class concentrations in the sample. The results of the quantitative analysis for a complex sample are adequately reproducible except for PG with a relatively high standard deviation of 14.8%, which is probably caused by varying overlap with traces of unknown components. Recoveries of sample preparation were determined by adding nine phospholipid standards of 5 μg each to a lavage sample (Table 2).

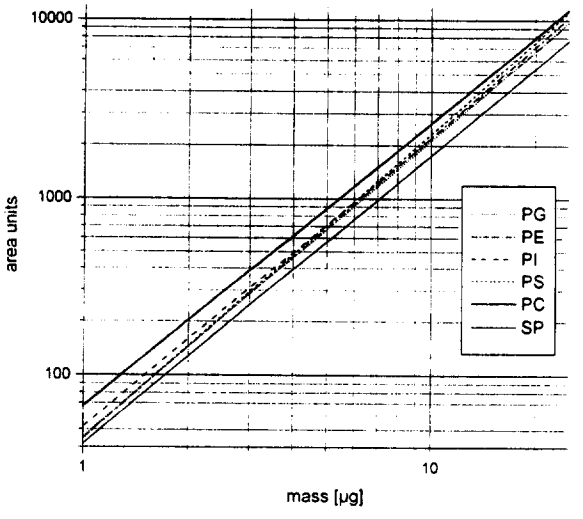

Fig. 4. Logarithmic plot of the calibration functions of six natural phospholipids in the range 1–25 μg.

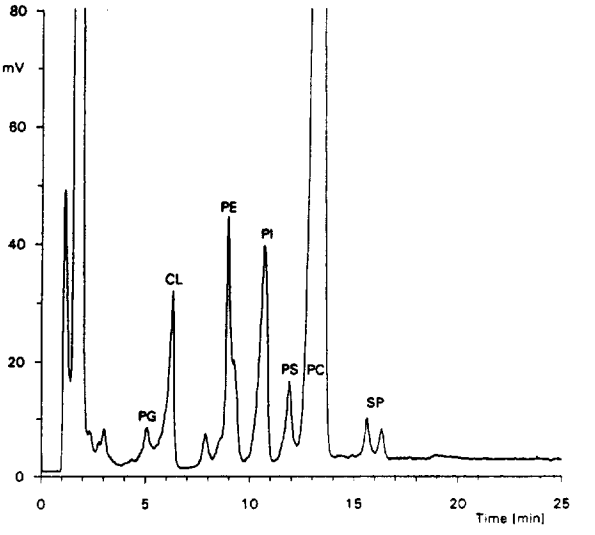

Fig. 5. Chromatogram of a lung lavage extract. Conditions: sample volume $v_s = 10$ ml, liquid volume for dissolution of the residue $v_l = 500$ μl, injection volume $v_i = 100$ μl; further details are described in Section 2; for abbreviations see Table 1. The first group of unmarked peaks represents non-polar lipids (mainly triglycerides and cholesterol).

Table 3
Quantitative phospholipid class analysis of a human lung lavage with standard deviation

Phospholipid	Concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)
PG	1.28 ± 0.19	14.8
CL	2.32 ± 0.11	4.7
PE	2.89 ± 0.18	6.2
PI	3.09 ± 0.18	5.8
PS	1.60 ± 0.07	4.3
PC	11.36 ± 0.39	3.4
SP	1.40 ± 0.03	2.4

Sample volume $v_s = 10$ ml, liquid volume for dissolution of the residue $v_l = 500$ μl, injection volume $v_i = 100$ μl, number of injections $n = 4$. For abbreviations see Table 1.

4. Discussion

Numerous techniques have been published for phospholipid analysis by HPLC. Most of them use ultraviolet detection, but the wavelength range of phospholipid absorbance limits the choice of eluting solvents. Moreover, UV quantification is not reliable, since absorbance recordings arise primarily from double bonds in the fatty acid moieties, and because natural phospholipids usually contain a variety of fatty acids, direct quantification is difficult.

The evaporative light-scattering detector overcomes some of these deficiencies. We found it necessary to use gradient elution, starting with a solvent of low polarity and ending with a solvent mixture containing water. The light-scattering detection makes gradient elution possible even with chloroform, a solvent of low UV transparency, in order to improve the separation of phospholipid classes.

In our laboratory we used a normal-phase chromatographic method with binary gradient and light-scattering detection of Becart et al. [33] and extended it for biomedical applications. We separated up to nine phospholipid classes without baseline drift in 40 min. Each peak is formed by a single phospholipid class, composed of individual molecular species with different saturated and unsaturated fatty acid moieties in their molecules. Thus the peaks do not have a Gaussian form. We found peaks with shoulders and double peaks (SP and LL) possibly caused by major subclasses.

Natural phospholipid classes can be sufficiently separated from each other, but individual molecular species of one phospholipid class lie close together. This method is capable of separating some species like DLPC and DPPC (see Fig. 2). On the other hand DLPC and DAPC form only one peak, due to their nearly identical retention times (see Table 1). The separation of natural phospholipids into individual molecular species by this method is usually not possible. Instead peak clusters are obtained, resulting from the sum of many individuals of one class with fatty acid moieties of different saturation and chain length. The retention time of such a peak cluster is therefore slightly dependent on its composition. A natural phospholipid class sometimes contains more than 20 individual molecular species, which might be separated by reversed-phase chromatography [41,42].

The response of the light-scattering detector proved to be non-linear but proportional to the power 1.7 of the mass. We used the external standard calibration method and found differences in calibration curves for various phospholipid classes. That was expected from the different shape of the peak clusters and the non-linear response. (Only in case of a linear response of an ideal mass detector, e.g., a double peak has the same total area as a single one of the same amount.) Thus, for accurate quantitation each phospholipid class needs separate calibration.

The method described in this report is a rapid and accurate procedure for the separation and quantitation of phospholipid classes in complex biological mixtures. This HPLC method is not limited to lavage fluids. It is also applicable to other biological samples; it was even used for lung tissue analysis.

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