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# Quantitative analysis of hydrophobic pulmonary surfactant proteins by high-performance liquid chromatography with light-scattering detection☆

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**Abstract**

A new method for the separation and quantification of two hydrophobic lung surfactant proteins (SPs) is described. It is based on size-exclusion chromatography using the apolar stationary phase butyl silicagel with a pore size of 30 nm and isocratic elution with chloroform, methanol and trifluoroacetic acid. The samples were prepared from sheep lung lavage fluid by centrifugation and fractional extraction with butanol and chloroform–methanol. The chromatograms show three peaks in the elution order SP-B, SP-C and lipids. A small peak ahead of SP-B, which disappeared after reduction with 2-mercaptoethanol, was oligomeric SP-B. The response of the evaporative light-scattering detector was non-linear. For preparative high-performance liquid chromatography ultraviolet detection at 279 nm is recommended. 

*Keywords:* Detection, LC; Proteins; Surfactant proteins; Lipids

## 1. Introduction

Pulmonary surfactant causes alveolar stability by lowering surface tension at the air–liquid interface. Alteration of the surfactant system may cause the respiratory distress syndrome in infants and adults. Pulmonary surfactant consists of a mixture of specific lipids, mainly phospholipids, and small quantities of unique proteins. The surfactant protein (SP) A is hydrophilic, but SP-B and SP-C are hydrophobic proteins, which are extracted together with the lipids in organic solvents [1,2]. SP-B is a disulphide-linked dimer of 79 amino acid residue monomers [3] and SP-C is an extremely hydrophobic 35-residue protein [4,5]. SP-B can be determined by an antibody enzyme-linked immunosorbent assay (ELISA), while a specific SP-C antiserum cannot yet be produced due to the extreme hydrophobicity of SP-C. A fast quantification of hydrophobic surfactant proteins is required for research and therapy purposes [6], but there are no methods available in the literature.

Separation of SP-B and SP-C from lipids is achieved by low-pressure size-exclusion chromatography using hydroxypropyl dextran with a pore size of 20 or 60 nm (Sephadex LH-20 or LH-60) as stationary phase [7,8]. Van Eijk et al. [9] used Sephadex LH-60 for high-performance liquid chromatography (HPLC) of lung surfactant proteins on a preparative scale with UV detection. The peaks are broad, because Sephadex is not high-pressure resistant. The analysis time is 75 min and the detection

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limits are 1 and 4 μg for SP-B and SP-C, respectively. The hydrophobic surfactant proteins cannot be separated by reversed-phase liquid chromatography with octadecyl silica [10]. Takahashi et al. have separated SP-B and SP-C by HPLC using octyl silica [11], but the separation was not sufficient for quantification. Arjomaa and Hallmann [12] tried to separate the hydrophobic surfactant proteins using butylsilica, a water–2-propanol gradient and UV detection, but they did not succeed in separating SP-C and phospholipids by HPLC. Lee and coworkers [13] used a butylsilica column and a water–acetonitrile gradient to separate hydrophobic membrane proteins. However, the strongly hydrophobic surfactant proteins B and C are not soluble in water and acetonitrile.

We have developed a new HPLC method using butylsilica with a pore size of 30 nm and isocratic elution with chloroform–methanol and succeeded in separating SP-B, SP-C and lipids. We used evaporative light-scattering detection (ELSD) to quantify the hydrophobic surfactant proteins for the first time.

## 2. Experimental

### 2.1. Reagents

Chloroform (HPLC reagent stabilised with 0.75% ethanol) was obtained from Baker (Griesheim, Germany), and methanol (LiChrosolv gradient grade), hydrochloric acid (0.1 *M* Titrisol) and trifluoroacetic acid (Uvasol) from Merck (Darmstadt, Germany). Water was purified by means of a Milli-Q Plus Water System (Millipore, Eschborn, Germany). Dipalmitoylphosphatidylcholine was purchased from Sigma (Deisenhofen, Germany) and the stationary phase for LC Sephadex LH-60 from Pharmacia (Uppsala, Sweden).

### 2.2. Sample preparation

Lipids and surfactant proteins were separated from cell-free sheep lung lavage fluid after 2 h centrifugation at 53 000 *g*. The pellet was homogenised in 1.64 *M* NaBr buffer for density gradient centrifugation at 100 000 *g* overnight [14,15]. The pellicle was removed, washed, and homogenised in 4 ml water and extracted with 300 ml 1-butanol (30 ml butanol per mg protein). After centrifugation SP-A was in the pellet. The supernatant was aspirated carefully and the butanol was removed in a vacuum rotary evaporator at 40°C. The residue was dissolved in 50 ml chloroform–methanol (2:1) and extracted according to the procedure of Folch et al. [16]. The solvent of the lower phase was evaporated in vacuum at 40°C. The residue containing the lipids and hydrophobic proteins was weighed and stored at −20°C.

### 2.3. Preparative low-pressure liquid chromatography (LPLC)

A 100×2.6 cm glass column with adjustable adapters (Pharmacia) was filled with Sephadex LH-60 (pore size 60 nm) using the slurry method. The size of the dry spherical particles is in the range 25 to 100 μm. Fifty grammes of dry Sephadex LH-60 swells in the solvent increasing the volume to 400 ml. The mobile phase, chloroform–methanol (1:1) with 5% 0.1 *M* hydrochloric acid, was pumped by a HPLC pump Model Σ871 (Irica, Kyoto, Japan) with degasser ERC-3612 (ERMA, Tokyo, Japan) through the column. The flow was adjusted to 0.2 ml/min with a pressure of 0.1 to 0.2 MPa. The prepared sample obtained from one sheep lung lavage was dissolved in 5 ml mobile phase and injected onto the column by a six-port injection valve with 10 ml sample loop (Upchurch, Oak Harbour, WA, USA). After sample loading, fractions of 3 ml were collected for intervals of 15 min and stored at −20°C. An aliquot of each fraction was analysed by gel electrophoresis under unreduced conditions using a 15% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). The bands were silver stained [17]. SDS-PAGE is a rather poor method for quantitating proteins. We subsequently replaced it by our newly developed HPLC method (see Fig. 3).

### 2.4. HPLC equipment and method

HPLC was performed with a HP-1090 liquid chromatograph fitted with helium degassing, an autosampler, a diode array UV detector, and a HP Vectra 486/33 personal computer with ChemStation software (Hewlett-Packard, Waldbronn, Germany). An evaporative light-scattering detector Sedex-55

(Sedere, Vitry sur Seine, France) was connected to the outlet of the UV detector. An interface module HP-35900 converted the ELSD analogue signal to digital data for transmission to the computer.

As stationary phase we used Vydac $C_4$, a butylsilica gel with 30 nm pores and a particle size of 5 μm. Both the analytical (250×4.6 mm) and the guard column (10×4.6 mm) were packed with the same material (Separations Group, Hesperia, CA, USA). The mobile phase consisted of 47.5% chloroform, 47.5% methanol and 5% 0.1 *M* trifluoroacetic acid. A flow-rate of 1 ml/min at room temperature was achieved by a pressure of about 10 MPa. The wavelength of the UV detector was optimised to 279 nm (bandwidth 8 nm) with a reference of 450 nm (bandwidth 80 nm). The evaporation temperature of the light-scattering detector was set to 50°C and the gain to position 8. The nebulization gas was nitrogen at a pressure of 0.16 MPa and a flow of 4.8 l/min.

Experiments with fluorescence detection (excitation 280 nm and emission wavelength 365 nm) were based on the intrinsic fluorescence of proteins. This, however, brought no advantages in comparison with UV detection, because the peaks were smaller and the noise stronger. For monitoring of preparative HPLC separations of hydrophobic surfactant proteins we used only the UV detector. The fraction collector Model 2110 (Bio-Rad, Hercules, CA, USA) at the outlet was controlled by a time program of the HPLC system.

## 3. Results

### 3.1. Separation

The separation of the hydrophobic surfactant proteins from lipids, mainly phospholipids, in the lung lavage extract was accomplished by the described HPLC method within 5 min (Fig. 1). The time lag of the evaporative light-scattering detector behind the UV detector was 12 s, i.e. the retention times of ELSD were 0.2 min longer (Table 1). The resolution was calculated by the halfwidth method. With resolutions of about 1.5 this new method is suitable for analytical, as well as for preparative purposes. The resolution of SP-C and lipids measured by ELSD is better than that of UV detection in the same run. The huge differences of the response ratio between SP-C and lipids explain the resolution differences observed between the two detections.

The relative molecular mass of the first peak was about twice the mass of SP-B. After reduction with 2-mercaptoethanol the first peak disappeared, suggesting that it is oligomeric SP-B. We have made well over 500 injections onto one Vydac $C_4$ column without loss of resolution.

### 3.2. Calibration

Dipalmitoylphosphatidylcholine (DPPC) was used as external standard. We injected 20 μl containing 0.02 to 12 μg DPPC and measured peak areas of the different dilutions. As reported earlier, the calibration curve is non-linear [18]. The calibration function was obtained by curve fitting using the power equation

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

with peak area units $a$, mass $m$ of the component injected, a constant $K$ and an exponent $E$. All measured points were weighted equally. For the described method (see Section 2.4) we found the constant $K = 289$ and the exponent $E = 1.43$ with a correlation coefficient of 0.9999. If the calibration function is plotted as a logarithmic graph it is linear (Fig. 2), following Eq. (2)

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

After calibration of the ELSD system we analysed the hydrophobic proteins and the sum of lipids in a lung lavage extract (Fig. 1B). With the masses from the ELSD system the UV detector was calibrated using the UV chromatogram of the same run (Fig. 1A). The linearity of UV detection in this range is well known. As expected in this case, the UV detection calibration factors differed greatly for different components (Table 1). The first component has roughly the same factor as the second, further proof of identical molecules (SP-B). Detection limits (twice the noise level) of ELSD are lower than those of UV detection. The precision of ELSD (RSD 0.6 to 2.6%) was better than that of UV detection (RSD 1.4 to 6.8%; $n = 5$). Therefore, ELSD is more suitable for quantitative analyses of hydrophobic lung surfactant proteins and lipids.

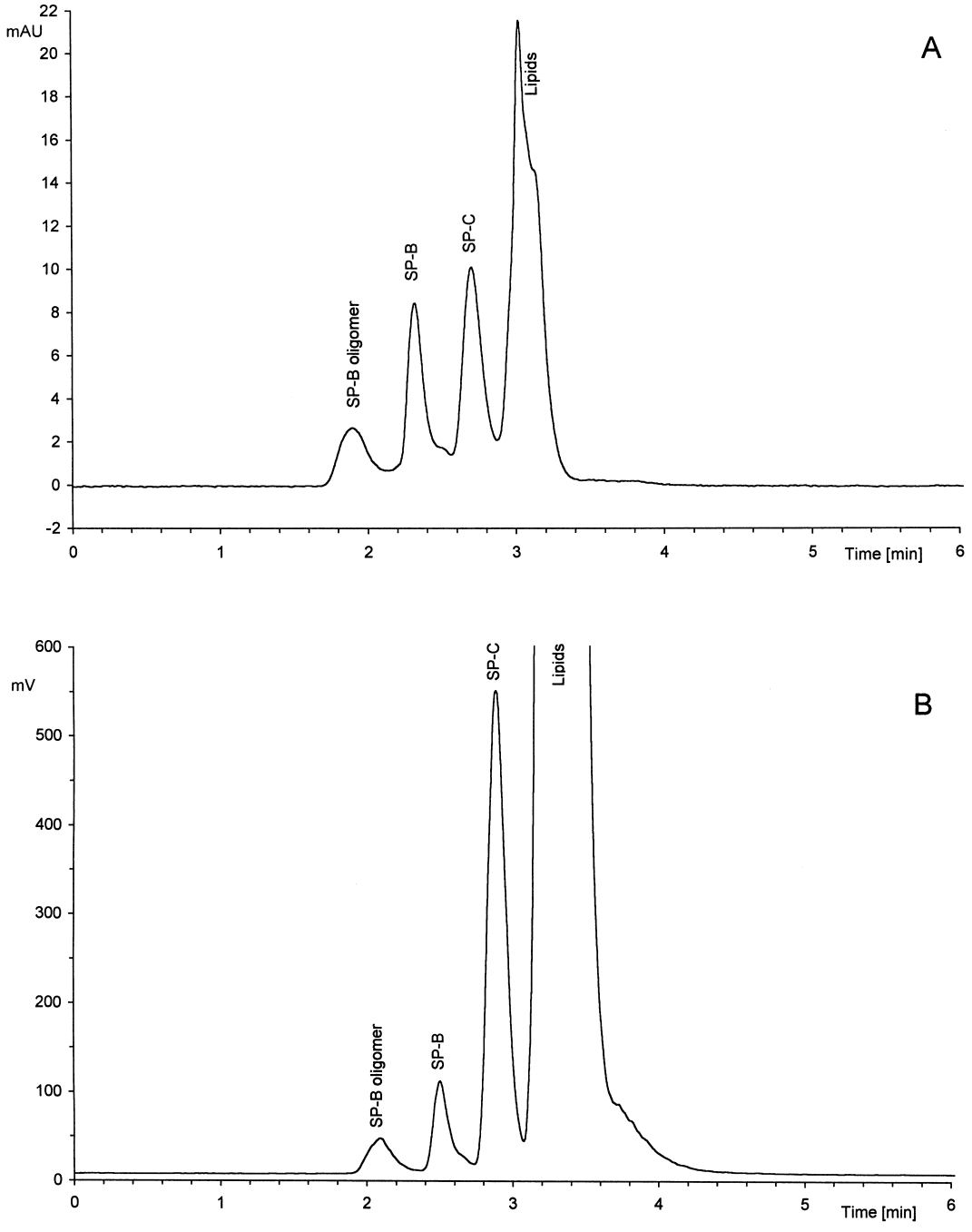


Fig. 1. Separation of hydrophobic proteins and lipids by HPLC with UV detection (A) and ELSD (B) with a time difference of 0.2 min. Sample: 20 μl of a sheep lung lavage extract containing 1.5 μg SP-B oligomer, 2.2 μg SP-B, 7.2 μg SP-C and 160 μg lipids. Further details are described in Section 2.4.

Table 1
Separation and calibration parameters of hydrophobic lung surfactant proteins and lipids (mainly phospholipids) by HPLC with UV and ELS detection. Further details are described in Section 2.4

| Component | UV 279 nm | | | | ELSD | | |
|---|---|---|---|---|---|---|---|
| | Retention time (min) | Resolution | Detection limit (μg) | Calibration factor (μg/mAUs) | Retention time (min) | Resolution | Detection limit (μg) |
| SP-B oligomer | 1.9 | – | 0.05 | 0.028 | 2.1 | – | 0.04 |
| SP-B | 2.3 | 1.5 | 0.05 | 0.027 | 2.5 | 1.6 | 0.03 |
| SP-C | 2.7 | 1.7 | 0.14 | 0.069 | 2.9 | 1.8 | 0.03 |
| Lipids | 3.1 | 1.2 | 1.15 | 0.601 | 3.3 | 1.6 | 0.09 |

### *3.3. Preparative isolation of hydrophobic surfactant proteins*

The same HPLC method was used to visualise the separation of the preparative low-pressure LC off line (see Section 2.3). Fig. 3 shows the elution profiles of SP-B, SP-C and lipids obtained by quantitative HPLC analyses of 160 LPLC fractions. This is marked progress compared to silver-stained SDS-PAGE for monitoring the separation of the proteins.

We endeavoured to replace the time-consuming LPLC by preparative HPLC. The ELSD system evaporates the analytes and therefore it is not usable as a detector when fractions have to be collected. For preparative HPLC of hydrophobic surfactant proteins UV detection at 279 nm has proved to be a useful online detection method (compare Fig. 1A).

## 4. Conclusions

In 1995 we reported a new HPLC method for the separation and quantification of the phospholipid compound classes in pulmonary surfactant [18]. However, the lack of a method suitable for protein

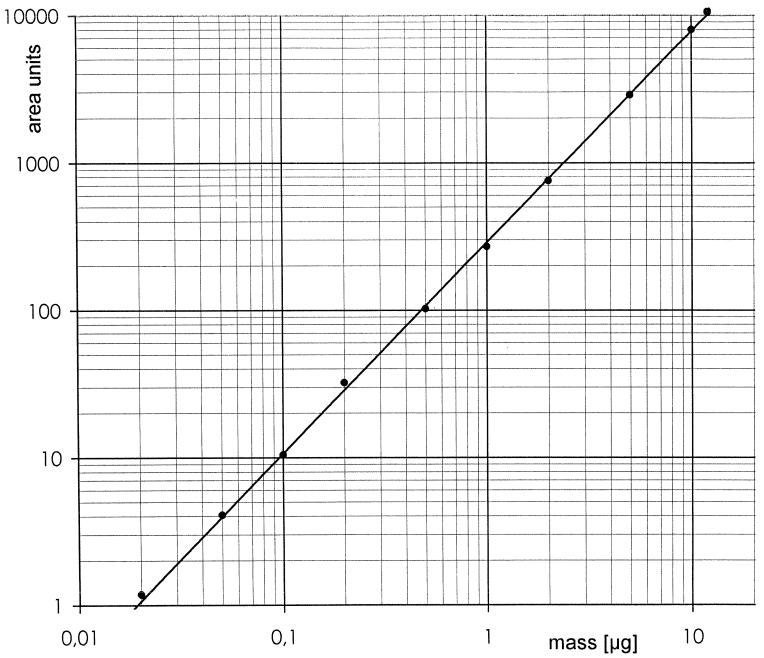


Fig. 2. Logarithmic plot of the ELSD calibration curve. Measured DPPC peak areas (●) with power function curve fitting according to Eq. (1) in the range 0.02 to 12 μg.

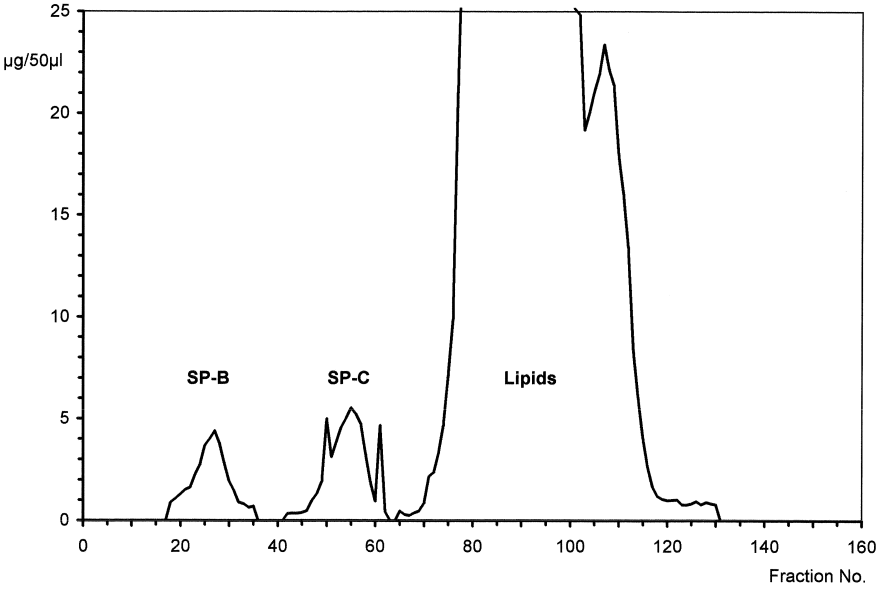


Fig. 3. Quantitative HPLC–ELSD analyses of preparative LPLC fractions showing the separation of a sheep lung lavage extract into SP-B, SP-C and lipids. LPLC column, 80×2.6 cm Sephadex LH-60; flow, 0.2 ml/min; pressure, 0.2 MPa; 160 fractions of 3 ml each collected within 40 h. Further details are described in Sections 2.3 and 2.4.

analysis of lung lavage samples has limited studies concerning the surfactant protein components. The new method presented in this paper should lead to the acceptance of HPLC in this field. The strongly hydrophobic surfactant proteins B and C are separated within 5 min from the lipids (Fig. 1).

ELSD was distinguished from UV detection by better precision and lower detection limits. SP-B and SP-C are not commercially available, and the small amounts of hydrophobic surfactant proteins found even in a whole lung lavage cannot be exactly weighed. In general, ELSD calibration curves of different compounds are similar and depend on their refractive indices. Oppenheimer and Mourey [19,20] investigated the influence of the refractive index on the response of this detector. However, the refractive indices of the proteins of interest are unknown. Therefore, we made the rough assumption of similar refractive indices of lipids and hydrophobic surfactant proteins and used the commercial phospholipid DPPC as external standard. Calibration points were measured with a dilution series of 10 concentrations, using 20 µl each (Fig. 2). With injection volumes of more than 20 µl, aberrations from the calibration function appeared, which were caused by peak broadening. Therefore, all analyses were performed with injection volumes of 20 µl or less.

The disadvantages of ELSD are the non-linear calibration curve, which requires many calibration points, as well as evaporation of the analytes, which excludes fraction collection. UV detection is a good method for preparative HPLC separations. The spectra of SP-B and SP-C have their maxima near 230 nm caused by the amide bond, but in this range the transmittance of chloroform is too low. We used a smaller local maximum at 279 nm for UV detection caused by the π-electron systems of some amino acid residues. While the response of the lipids is extremely poor, the calibration factors (Table 1) indicate most π electrons in SP-B and its oligomer. This can be explained by the higher aromatic amino acid residue content (phenylalanine, tyrosine and histidine) of SP-B [8].

The method described in this report is a rapid and precise procedure for the separation and quantification of the strictly hydrophobic pulmonary surfactant proteins. This HPLC method can be used for analysing lung lavage fluid and tissue in biomedical

research as well as for diagnostic purposes. It is also useful for the quality control of commercial preparations for surfactant substitution therapy.

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