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Quantitation of surfactant protein B by HPLC in bronchoalveolar lavage fluid

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Abstract

A sensitive reversed phase HPLC method with evaporative light scattering detection (ELSD) was developed for the determination of the hydrophobic surfactant protein B (SP-B) in human bronchoalveolar lavage fluid. Samples were extracted two times with $CHCl_3$:MeOH:HCl (2:3:0.005N) solution in a ratio of 1:2 by volume. The extract of the lower phase was separated on a C4 butyl silica gel column with an isocratic elution using a mobile phase, consisting of 97% methanol, 2.75% chloroform and 0.25% 0.1 M trifluoroacetic acid (by volume), at a flow rate of 1 ml/min. SP-B was detected by ELSD and quantified by comparison to an external standard. The duration of a run was 7 min, the quantification limit 30 ng and the limit of detection was at about 15 ng of SP-B. This method is suitable for the rapid routine quantification of SP-B in human bronchoalveolar lavage fluid samples.
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Keywords: Surfactant protein B; Lipid extraction

1. Introduction

Human surfactant protein B (SP-B) is a 8 kDa hydrophobic protein, produced in the lungs by alveolar type II epithelial cells [1]. Under normal conditions the majority of SP-B in the alveolar space is present as a homodimer [2]. The main function of SP-B is to accelerate the formation of a surface active film composed of phospholipids at the air-water interface by means of an increase in the adsorption rate by a factor of >150 [3]. Together with SP-A, SP-B is essential for the formation of tubular myelin figures [4]. Mutations of the SP-B gene *SFTPB* may lead to a deficiency of surfactant protein B [5], the complete absence of SP-B being lethal in humans [6] and the partial deficiency may be associated with impaired respiratory function [7].

Currently available methods for the quantification of SP-B in bronchoalveolar fluid are based on an ELISA technique [8] and on HPLC or FPLC [9–12]. Unfortunately, the HPLC methods are not sufficiently sensitive for the quantification of SP-B in all human BAL fluid samples. The lower limits of detection are given as 600 ng of SP-B [11] and 1 μg of SP-B [12]. The ELISA method is more sensitive (lower limit of detection about 10–20 ng/ml), however, includes several washing procedures, to differentially remove the lipids without loss of SP-B, which are cumbersome and the method is also dependent on the availability of the specific antibody [8].

Here we describe a novel HPLC method, which is sufficiently sensitive to directly quantify SP-B at its concentrations present in all human bronchoalveolar lavages. The method allows a rapid and accurate determination of SP-B levels in BAL fluid from patients of all ages.

2. Materials and methods

2.1. Reagents

Chloroform, Methanol (LiChrosolv© gradient grade) and analytical grade HCl were obtained from Merck (Darm-

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stadt, Germany). HPLC grade trifluoroacetic acid was purchased from Fluka (Buchs, Switzerland). Distilled water, used in the mobile phase, was supplied by Braun (Melsungen, Germany).

2.2. Sample preparation

The lavage was performed through a bronchoscope or an endhole catheter wedged in the middle lobe or lingula and 0.9% NaCl warmed to body temperature was instilled. In children 4× 1 ml/kg body weight, and in adults 160 ml (8 times 20 ml) were instilled and recovered with a 20 ml syringe under manual control [3].

For extraction of the lavages, an aliquot, usually 1–2 ml was mixed with MeOH:$CHCl_3$:HCl (2:3:0.005N) solution in a ratio by volume of 1:2, vortexed vigorously for 10 min and centrifuged for 10 min at $1.000 \times g$ to separate the phases. The organic (lower) layer, containing phospholipids, SP-B and SP-C was removed. The aqueous (upper) layer was re-extracted an additional time with the same solvent. Both lower phases were combined and evaporated to dryness under N2. The recovery of SP-B in the lower phase of the lipid extraction was $99.2 \pm 0.28\%$ ($n = 6$). For injection into the HPLC system the dried residue was resuspended in the mobile phase.

2.3. Chromatographic system

HPLC was performed using the LaChrom HPLC system by Merck (Darmstadt, Germany), consisting of a Rheodyne manual injector (7725 I), a pump (L-7100), vacuum degasser (L-7614) and UV-Detektor (L-7400). A pre-packed C4 butyl silica gel column and its corresponding guard column were used (Grace Vydac, Hesperia, CA, USA). The column dimensions were 250 mm × 10 mm with a 5 μm particle size stationary phase. An external column thermostat, Jetstream plus was supplied by Thermotechnic Products GmbH, (Langenzersdorf, Austria) and used to maintain the column temperature at 5 °C during analysis. Data were acquired and processed using the HSM D-7000 Chromatography Data Station Software by Hitachi Inst., San José, CA, USA. The mobile phase was pumped through the column at a flow rate of 1 ml/min, the injection volume was 20 μl and each run required 7 min. The separation was performed by isocratic elution using a mobile phase, consisting of 97% methanol, 2.75% chloroform and 0.25% 0.1 M trifluoroacetic acid.

2.4. Detector

For detection a Sedex© Model 75 (Sedere, France) evaporative light scattering detector (ELSD) was used. The evaporator tube was set to a temperature of 50 °C and nitrogen was used as the carrier gas at a pressure of 3.5 bar. The photomultiplier gain was set at the highest sensitivity of 12.

2.5. SDS-PAGE

Protein electrophoresis was performed by one-dimensional PAGE using NuPAGE Novex 10% Bis–Tris gels (Invitrogen, Carlsbad, CA, USA) under non-reducing conditions. MultiMark 12TM Wide-Range served as molecular weight standards. The gels were either silver stained [13] or electrophoretically transferred onto nitrocellulose and incubated with an antibody against surfactant protein B (polyclonal, rabbit anti SP-B antibody, C329, a gift of Byk Gulden, Konstanz, Germany), as the first antibody and a peroxidase conjugated antibody (AffiniPure F(ab′)2 Fragment Goat Anti-Rabbit IgG (H + L) (Dianova, Hamburg, Germany)) was used as second antibody. Detection was performed by ECL chemiluminescence fluid (Amersham Pharmacia Biotech, Uppsala, Schweden).

2.6. Calibration

For quantification, known amounts of human and porcine SP-B were used (gift of Dr. R. Schmidt, Giessen). The protein concentration was determined by the method of Bradford [14] using BSA as protein standard. Results were verified by quantitative amino acid analysis.

2.7. Statistical analysis

Statistical analysis was performed with Prism 4.0 (Graph Pad Software, San Diego, USA). The tests used are indicated in the legends to the figures or tables. A p-value of <0.05 was considered significant.

3. Results

3.1. Sample preparation

For this separation method it was essential to separate the hydrophobic SP-B from non-specific serum proteins by organic extraction, in order to remove other hydrophilic proteins which might elute within the peak of SP-B (Fig. 1). The standard procedure according to Bligh and Dyer [15] at neutral pH did not lead to satisfying results (A1, A2). With acidification and change of the ratio of the extraction solution, SP-B completely partitioned preferentially into the lower phase (A3, A4), interfering proteins remaining in the upper phase (B1, B2).

3.2. Separation method

Separation of SP-B from other hydrophobic surfactant constituents was performed within 7 min (Fig. 2). Verification of the peaks showed selective separation of SP-B in peak B and silver staining ascertained SP-B to be the sole protein component (Fig. 2). SP-C was only eluted in fraction C. As Fig. 3 shows, BSA and the phospholipids also eluted in this

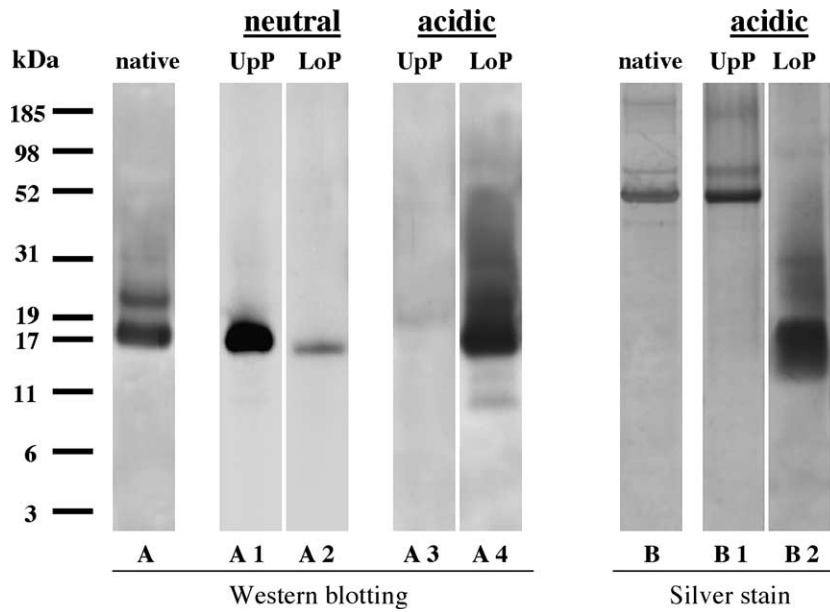


Fig. 1. Effect of different extraction methods for bronchoalveolar lavage samples on the recovery of SP-B. BAL fluid containing about 400 μg of protein was extracted according to the method of Bligh and Dyer [15] without (neutral A1, A2) and with (acidic A3, A4, B1, B2) acidification and different ratio. (A) Western blot analysis of the native lavage (5 μg of total protein added to the lane) and of the upper phases (UpP) and lower phases (LoP) of the extraction with a polyclonal SP-B antibody. (B) Silver stained SDS PAGE gel of (A) A3 and A4, corresponding to (B) B1 and B2.

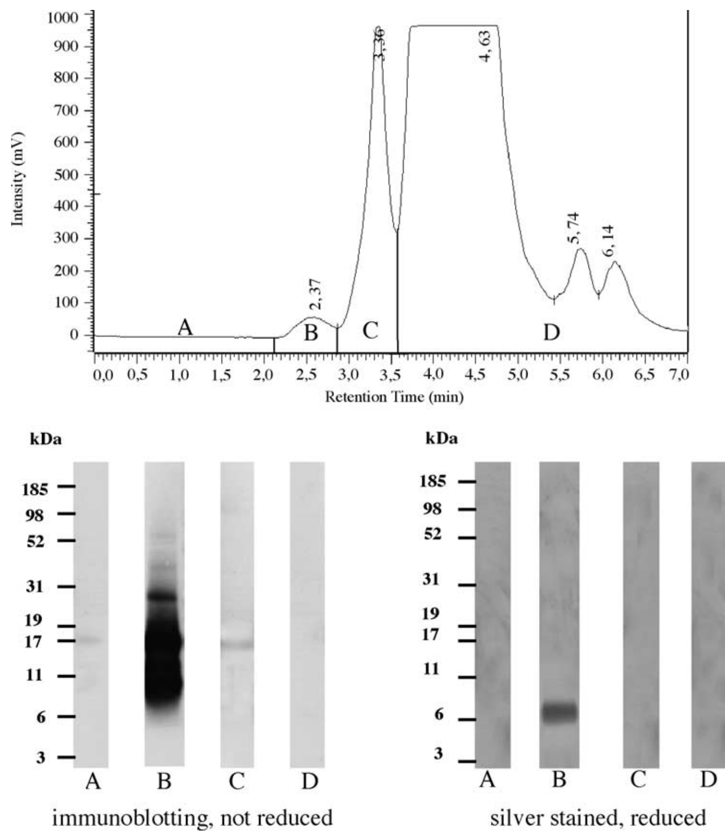


Fig. 2. HPLC separation of the acidified lipid (lower) phase of 2 ml of crude BAL fluid, containing about 160 ng SP-B. HPLC-fractions labeled A–D were analyzed by immunoblotting with a polyclonal SP-B antibody (left side) and silverstained under reducing conditions (right side). All SP-B was recovered in fraction B. HPLC conditions, see materials and methods.

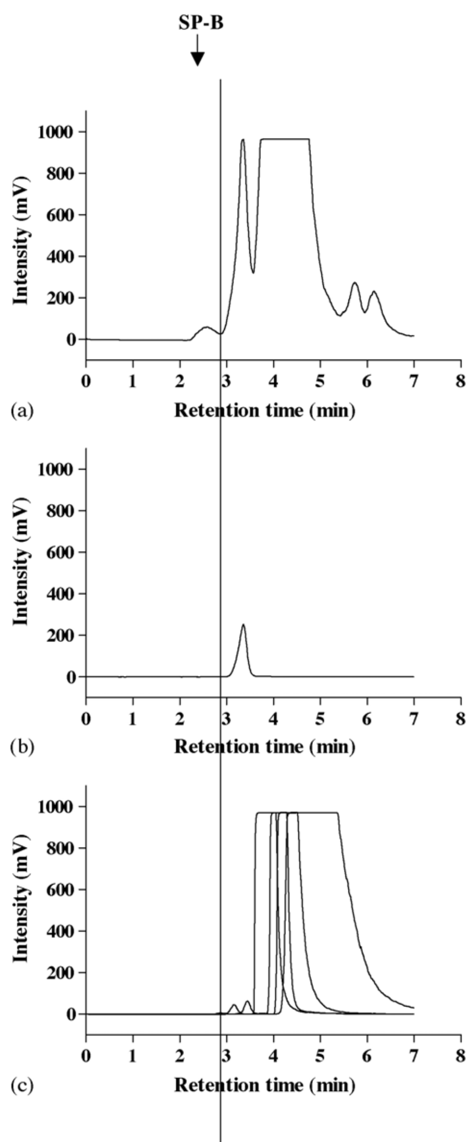


Fig. 3. HPLC separation of the acidified lipid (lower) phase of 2 ml of crude BAL fluid, containing about 160 ng SP-B (a), HPLC separation of BSA (b) and separation of cholesterol, DPPC, palmitin acid and phosphatidyl-oleyl-palmitoyl-glycerol (c). HPLC conditions, see Section 2.

fraction. This demonstrated, that the method is not suited for SP-C quantification. Reduction with dithiothreitol (DTT) showed no change in peak intensity and retention time, confirming the existence of the reduced and the non-reduced form of SP-B in a single peak (peak B, Fig. 2). The SP-B peak was clear of phospholipids and neutral lipids, which is demonstrated in Fig. 3c by the elution profiles of the major lipids. Retention times showed, no interference with the elution of SP-B.

3.3. Calibration

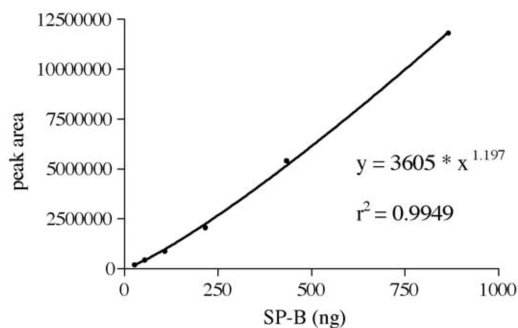
According to the principles of ELSD, the detector response is given by the equation $I = k \times m^b$ with the detector response I, mass of the scattering particles m and the constants k and b. Therefore, a plot of peak area against sample concentration is not linear, however a double logaritmic plot of the peak area against sample concentration is linear (Fig. 4). The calibration curve was constructed with an external standard at 6 different concentrations (865.79–27.06 ng). Each data point represents the average area obtained from 3 replicate injections. Non-linear curve fitting gave an acceptable correlation ($R^2 = 0.9949$) and 3605 was obtained as the value for the constant k and 1.197 for the exponent b.

3.4. Precision

Intra-day precision was measured by making replicate injections ($n = 9$) of 3 standard preparations on 1 day. The percent relative standard deviation (R.S.D.) for the peak area, the amount of SP-B and retention time were very low (Table 1). The inter-day studies ($n = 24$ analyses), showed a R.S.D. of <3% for all variables (Table 1). The results demonstrate a good reproducibility for this analytical method.

3.5. Detection limit and lower limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were evaluated according to the International Conference of Harmonization guideline [16], which defines the LOD as a signal to noise ratio of not less than three and the LOQ as a signal to noise ratio of at least 3. For this application



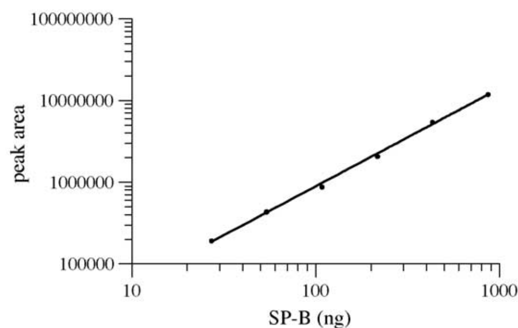


Fig. 4. Standard curve for SP-B. Increasing amounts of purified human SP-B were applied to the HPLC-column and eluted as described above. The ELSD response was recorded. Peak areas were calculated and expressed as a function of the amount of SP-B. Calibration curve follows the parabolic function $y = 3605 \times x^{1.197}$ (left panel), which is linear when expressed in double logarithmic presentation (right panel).

Table 1
Precision of the SP-B determination by HPLC and ELSD

	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)
Peak area	2.4 ± 0.96 (1.3–3.7)	1.9 ± 0.71 (0.9–3.3)
Amount of SP-B	1.9 ± 0.58 (1.0–3.0)	1.6 ± 0.58 (0.8–2.7)
Retention time	1.4 ± 0.05 (1.3–1.5)	1.8 ± 0.59 (1.0–2.9)

Relative standard deviations (R.S.D.) of peak area, amount and retention time for intra- and inter-day measurements. Human SP-B standard was injected on 4 days with 3 different concentrations (200, 300 and 600 ng) in duplicates for inter-day tests and for intra-day R.S.D. Three different concentrations (200, 300 and 600 ng) were measured 9 times. Data are given as mean ± std error (range).

the quantification limit was 30 ng and the detection limit was at about 15 ng of SP-B.

3.6. Validation

SDS-PAGE and immunoblotting were used as an independent second method to validate the results of the HPLC quantification. The amount of SP-B in the bronchoalveolar lavages from five patients with chronic obstructive bronchitis was measured by this HPLC method and directly compared to the signal obtained by immunoblotting (Fig. 5).

4. Discussion

Precise quantitative information on the concentration of SP-B in BAL fluid is of diagnostic importance in states of primary deficiency, i.e. hereditary SP-B deficiency [5] or different secondary changes, i.e. with different disease status [17] or lung injury [18]. There have been estimates that at least 25% of the normal SP-B level has to be present, in the alveolar space, in order to avoid clinical symtoms [19]. Some infants with partial SP-B deficiency and chronic respiratory tract symptoms have been shown to have SP-B level of about 10–15% of normal [20]. This newly established HPLC method has several advantages over the other methods currently available. Compared to previously published HPLC methods [9–12], the method described here has a much higher sensitivity and 30 ng of SP-B can clearly be quantitated. This sensitivity enables to quantify the amount of SP-B in normal BAL fluid, which contains about 740 ± 85 ng/ml [8]. Unfortunately this method is not usable for quantification of SP-C, because various proteins and some phospholipids are eluted within the same retention time as SP-C, which makes it impossible to determine the amount of SP-C (Fig. 3). The dynamic range of the assay is spanned over two log-units (Fig. 4). In addition, the sample purification procedure proposed here, consists of two extraction steps, which lead to a complete quantitative extraction of SP-B without any loss of SP-B, as demonstrated by most sensitive Western blotting with enhanced chemiluminescence. This and the sensitivity is achieved by evaporative light scattering detection, which provides a sensitive method that depends on the concentration and not on the chromophoric properties of the eluent, as UV detection does. This is of particular advantage, as SP-B is

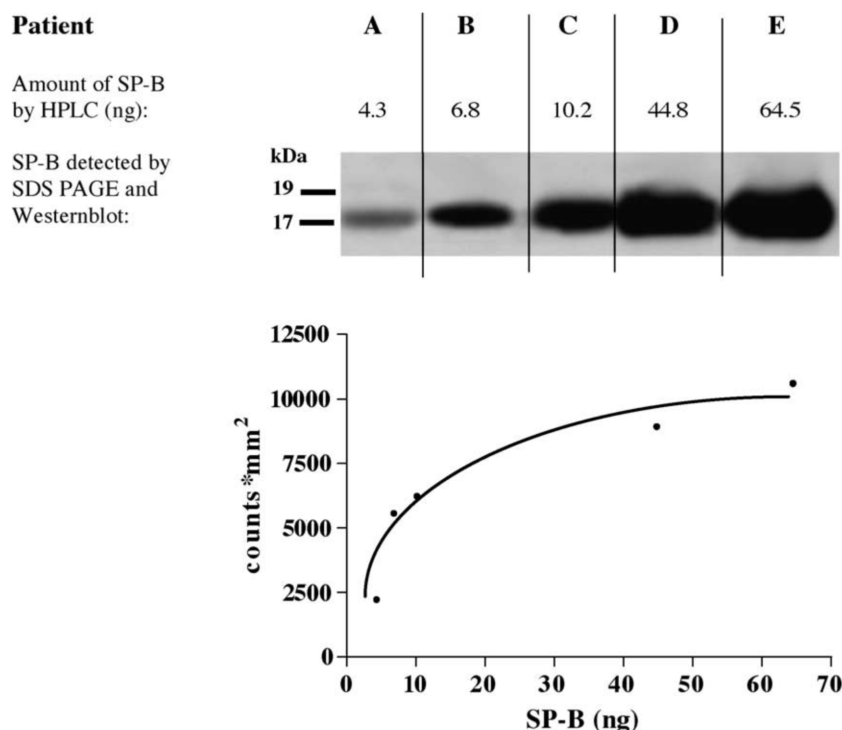


Fig. 5. Correlation between the amount of SP-B quantified by HPLC and by SDS-PAGE and immunoblotting. Bronchoalveolar fluid containing 5 μg of total protein from five different patients with chronic bronchitis (subjects A–E), which differed greatly with respect of their SP-B concentrations, were extracted by the method of Bligh and Dyer with acidification and aliquots of the lower phase were used for quantification by HPLC and Western blotting. HPLC peaks were quantified using the product of their density and area. Due to non-linearity of the quantification of the gel spots a hyperbolic function resulted.

a small hydrophobic protein lacking abundant chromophoric properties. The method may easily be adopted to other biological fluids, like cell culture supernatant, serum, tissue extract or pleural fluid. Lastly, the extraction procedure can easily be used to concentrate large samples and to enrich for SP-B, as it was shown in publications of similar extraction procedures [15].

In summary, a simple and highly sensitive isocratic HPLC method with ELSD detection was developed and validated for the routine quantification of SP-B in human bronchoalveolar lavage samples.

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