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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.

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