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LC–MS analysis of phospholipids and lysophospholipids in human bronchoalveolar lavage fluid☆

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Abstract

A reversed phase HPLC method was developed for the simultaneous analysis of different phospholipids and lysophospholipids in human bronchoalveolar lavage fluid (BALF). Separation was achieved using a pellicular C8 column at elevated temperatures with an increasing gradient of acetonitrile containing 0.1% formic acid. Detection was carried out by electrospray ionization ion-trap mass spectrometry. Calibration graphs for selected phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and lysophosphatidylcholine) showed linearity up to 50 ng allowing quantitative determinations. Identification of the individual species within each class was possible with tandem mass spectrometry. Analysis of BALF phospholipids was performed after liquid/liquid extraction with a mixture of chloroform/methanol/acetic acid. Recoveries ranged from 69 to 97% with standard deviations of less than 6%. The limit of detection varied slightly between different classes but was in the range 0.05–0.25 ng total injected amount.

Keywords: Phospholipids; Lysophospholipids; BALF; HPLC/MS

1. Introduction

Pulmonary surfactant is an essential fluid produced by alveolar type II cells, covering the entire surface of the lungs [1]. It consists of a complex mixture of lipids (about 90%) and specific surfactant-associated proteins (10%). Of the lipids 80–90% are phospholipids (PLs) and 10–20% consist of cholesterol, triglycerides and free fatty acids. The principal phospholipids are phosphatidylcholine (PC) species (aproximately 85%) containing high amounts of saturated palmitic acid (C16:0) and phosphatidylglycerol (PG). Other phospholipids usually found in small amounts are phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) [2].

The most important role of surfactant is to decrease the surface tension at the air–liquid interface and, therefore, to reduce the tendency of alveoli to collapse during expiration. It also contributes to the regulation of airway fluid balance, improves bronchial clearance and sets up a barrier to inhaled agents. Apart from this biophysical function, surfactant is involved in immunomodulation [3]. Biochemical and biophysical surfactant abnormalities have been found in various lung diseases such as acute respiratory distress syndrome (ARDS), pneumonia and lung edema.

In our research we are interested in disease processes involving chronic lung inflammation, such as chronic obstructive pulmonary disease (COPD) and Bronchiolitis Obliterans (BO). A decrease in the total amount of phospholipids, which has been observed in patients with chronic lung inflammation [4], is known to enhance injury by elastase secreted from neutrophils and to induce collapse of bronchioles [5].

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Abbreviations: CID, collision induced dissociation; TIC, total ion chromatogram; EIC, extracted ion chromatogram; LC–MS, liquid chromatography–mass spectrometry; COPD, chronic obstructive pulmonary disease; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; PL, phospholipid; LPL, lysophospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; LPC, lysophosphatidylcholine

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Moreover, lysophospholipids (LPs), derived from the action of phospholipases, are involved in signalling mechanisms and inflammatory pathways [6]. Therefore, the surfactant system may be implicated in the underlying pathophysiology of lung inflammation. Hence, determination of these biomolecules in BALF may clarify the role of surfactant in chronic inflammation and provide access to novel disease biomarkers.

The aim of this work was to develop an analytical method for the quantitative determination of different phospholipids in human bronchoalveolar lavage fluid (BALF) with special emphasis on the ratio of phospholipids/lysophospholipids.

Several methods have been utilized to analyse phospholipids, many of them requiring the use of derivatization [7]. Initially thin layer chromatography was routinely employed for the separation of different phospholipid classes, however, real identification required further hydrolysis and HPLC or GC analysis making the method laborious and time consuming [8]. GC–MS has been a robust technique used in many laboratories for lipid analysis. Phospholipids are not amenable to direct GC due to their lack of volatility. Therefore, hydrolysis is required followed by trans-methylation of the released fatty acids and measurement of the corresponding methyl esters [9]. Unfortunately, this leads to the loss of information about the attachment site of the fatty acids.

Liquid chromatographic methods became a better alternative offering higher selectivity. Normal phase HPLC has been more extensively used than reversed phase for the separation of phospholipids into different classes [10]. UV absorbance [11], fluorescence [12], refractive index [13] and evaporative light scattering [14,15] detection have been common ways of detection, with the corresponding limitations regarding sensitivity and/or lack of identification.

In the last years, the use of mass spectrometry in phospholipid analysis has become more widespread providing structural information. Matrix assisted laser desorption ionization (MALDI) [16] as well as electrospray ionization (ESI) [17] are currently used approaches. Also NMR has found its application in the analysis of phospholipids [18] but its sensitivity is considerably lower than MS.

Here we report the use of reversed phase liquid chromatography coupled to ESI mass spectrometry for the direct analysis of underivatized phospholipids and lysophospholipids in human BALF using common mobile phases without the necessity for special additives or derivatizations. This should prove useful in studying the role of PLs and LPLs in chronic lung inflammation.

2. Experimental

2.1. Chemicals

Formic acid, chloroform, methanol, acetonitrile, dimethyl sulfoxide (DMSO) and acetic acid (glacial) were obtained from Merck (Darmstadt, Germany).

1,2-Diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE-C17:0/C17:0), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC-C17:0/C17:0), 1,2-diheptadecanoyl-sn-glycero-3-phosphate (Na^+ salt) (PG-C17:0/C17:0) and 1,2-diheptadecanoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (Na^+ salt) (PA-C17:0/C17:0) were obtained from Avanti Polar Lipids (Instruchemie, Delfzijl, The Netherlands).

L-α-Lysophosphatidylcholine, heptadecanoyl (LPC-C17:0) and L-α-lysophosphatidylcholine, palmitoyl (LPC-C16:0) were purchased from Sigma (Zwijndrecht, The Netherlands).

Bronchoalveolar lavage fluid (BALF) supernatant samples were obtained from the Groningen Academic Hospital from patients who underwent bronchoscopy due to different types of lung disease. BAL was performed by instillation of a saline solution. The lavage fluid was centrifuged and the supernatant stored at −80 °C until use.

2.2. High performance liquid chromatography/mass spectrometry

The HPLC part of the analytical system consisted of an Agilent Series 1100 capillary LC system (Waldbronn, Germany) comprising a degasser, a binary pump, a thermostatted autosampler and a thermostatted column compartment. Chromatographic separation took place on a reversed-phase column (Poroshell 300SB-C8, 5 μm, 1 mm i.d. × 75 mm length) (Agilent, Waldbronn, Germany).

Mobile phase A consisted of 0.1% formic acid in ultrapure water. Mobile phase B was 0.1% formic acid in acetonitrile. Separation was performed with an increasing gradient of B (25–95% in 50 min). Flow rate employed was 60 μl/min and the column temperature was 60 °C. The analytes were detected by an Agilent SL ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source operated in the positive mode. MS data were acquired over a scan range of 100–1200 amu and 5500 *m/z* per second scan rate.

2.3. BALF sample preparation

Extraction of lipids from biological material is commonly done following two different methods described by Folch et al. [19] and Bligh and Dyer [20]. Both methods are based on liquid/liquid extractions using mixtures of chloroform/methanol/water in different ratios. In our case, Folch's method provided higher recoveries and therefore it was chosen.

The final extraction protocol was as follows: 1.5 ml of a mixture of chloroform/methanol/50 mM acetic acid 8/4/0.5 (v/v/v) was mixed with 0.5 ml of BALF supernatant and manually shaken for 2 min. The mixture was centrifuged for 5 min at 1500 g, and the organic layer separated. The aqueous phase was extracted again and both organic extracts were pooled together and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 μl

of DMSO by ultrasonication and vortex mixing. 5 μl were injected in the HPLC/MS system.

Glassware was used throughout the whole sample preparation procedure.

3. Results and discussion

Initial experiments focused on optimizing the separation of the different phospholipid classes using a Zorbax C18 column (3.5 μm, 0.5 mm i.d. × 150 mm length; 25 °C) with a gradient of acetonitrile in 0.1% aqueous formic acid. While this method showed good separation of lysophospholipids (data not shown), very broad peaks were obtained for diacylphosphatidylcholines (Fig. 1) with extensive tailing. In addition no separation between the different phospholipid species due to interaction of the phospholipid headgroups with the stationary phase was obtained. Evaluation of different mobile phase compositions (acetonitrile, methanol, acetic acid, etc.) did not give satisfactory results.

Significantly better chromatographic performance was obtained through the use of a "pellicular" C8 column (Poroshell SB-C8). Poroshell particles are 5 μm in diameter with a rigid core and a 300 Å porous surface coating of microspherical particles. Due to the thin surface coating, molecules diffuse through a short porous layer and can not penetrate the core, providing fast mass transfer and allowing the use of high flow rates [21]. Increased flow rates increase the gradient volume resulting in increased resolution and in short analysis times [22].

The increased stability of the Poroshell material allows also to work at elevated temperatures up to 90 °C. Performing phospholipid separations at different temperatures (30, 40 and 60 °C) showed that peak shape and width improved with higher temperatures, probably due to a better solubility of the lipids. Finally 60 °C was chosen after checking during 18 h that no degradation of the analytes occurred.

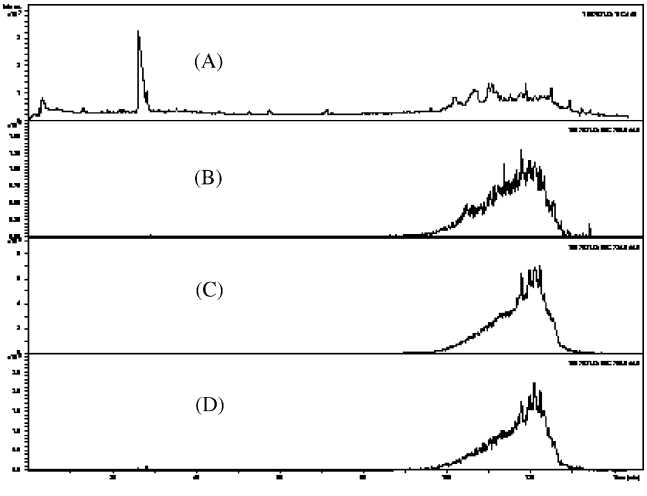

Fig. 1. (A) Chromatogram obtained from the injection of 5 μl of BALF (after liquid/liquid extraction and preconcentration of 1 ml) in a reversed-phase C18 column eluted with a 1%/min increasing gradient of acetonitrile containing 0.1% formic acid. Temperature: 25 °C. Extracted are the ions corresponding to different PC species: (B) C16:0/C14:0 PC (*m/z* 706.6), (C) C16:0/C16:0 PC (*m/z* 734.6) and (D) C16:0/C18:1 PC (*m/z* 760.6).

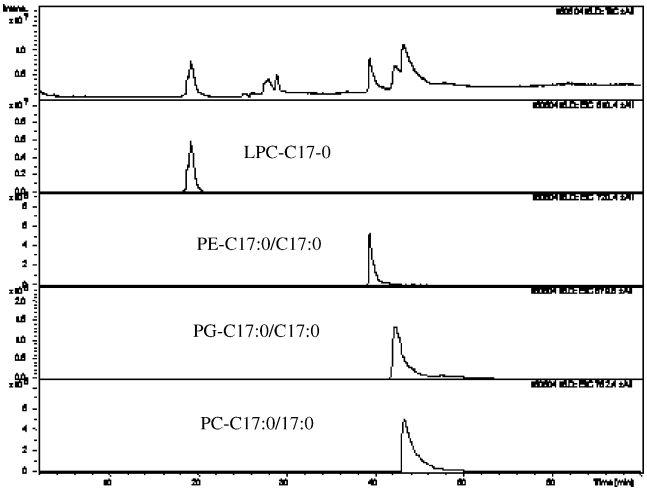

Fig. 2. Total ion chromatogarm (TIC) and extracted ion chromatograms (EIC) corresponding to the different synthetic phospholipids and lysophospholipid used as internal standards. Injected amounts: 15 ng (PC, PE and PG) and 5 ng (LPC).

Four phospholipids PC-C17:0/C17:0, PE-C17:0/C17:0 PG-C17:0/C17:0 and PA-C17:0/C17:0 and one lysophospholipid LPC-C17:0 were selected as representative of the most common phospholipids detected in BALF [23,24]. Since C17:0 is not a fatty acid present in BALF these compounds can be used as internal standards.

Injection of a mixture of the five standards showed that separation of the different PL classes was possible (Fig. 2), but as expected, PA was not detected with positive ESI. It is interesting to note that in the case of LPC, there are two separated chromatographic peaks with exactly the same mass (isomers). This is most likely due to the different position of the fatty acid chain in sn-1 or sn-2 of the glycerol group. Using isomerically pure standards it was established that the peak with the shorter retention time corresponds to the isomer containing the fatty acid in position sn-2 while the other isomer (sn-1) elutes later. Since the LPC-C17:0 standard was not pure, both isomers were considered together for quantitative purposes.

Increase of the pH of the mobile phase to 5.0 (using ammonium acetate 50 mM instead of formic acid) and use of the same gradient but with negative ESI allowed good separation and detection of the 4 PLs including PA (Fig. 3), however sensitivity was much lower. Considering our interest in measuring the ratio of phospholipids/lysophospholipids and the fact that PC is the most abundant PL in BALF, we decided to use low pH and positive ESI compromising the detection of PA.

3.1. Calibration graphs

Although some authors have reported quantitative analysis of phospholipids by flow injection directly coupled to

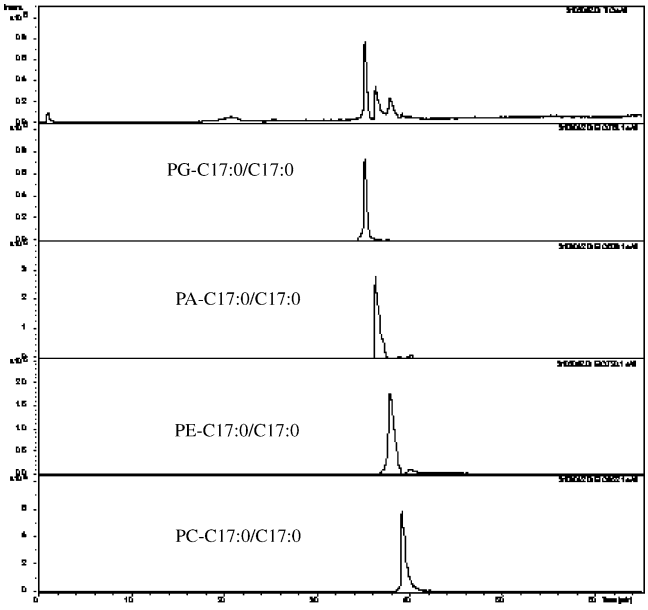

Fig. 3. Separation of a mixture of PC, PG, PE and PA C17:0 standards (25 ng of each compound injected) with detection by negative ESI (mobile phase pH 5.0, ammonium acetate 50 mM).

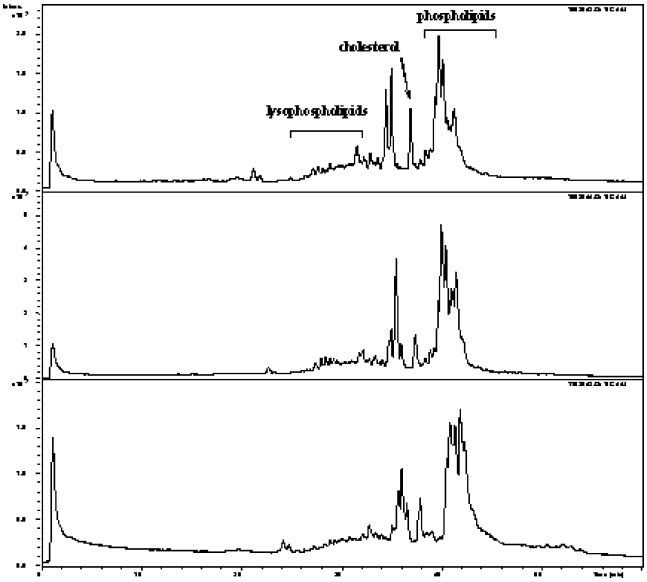

Fig. 4. TIC chromatograms corresponding to the injection of 5 μl of extract of BALF samples from three different Bronchiolitis Obliterans patients.

MS [25], chromatographic separation is required to avoid ion suppression effects from highly abundant species and matrix components. To assess the linear range of the LC–MS method, calibration graphs were first obtained for standard PC, PE, PG and LPC containing C17:0, in phosphate buffered saline solution (0.01 M, pH 7.4). Working standard solutions of the diacylphospholipids were prepared by dissolution of the dry powder in chloroform to a final concentration of 5 mg/ml. Further dilutions were made with DMSO. Lysophospholipid standards were dissolved directly in ultrapure water.

Calibration graphs (peak area versus total amount of PL injected in the column) were linear up to 25 ng (PC and PE), 50 ng (PG) and 150 ng (LPC). The following equations were obtained: $y = 5 \times 10^6 x + 3 \times 10^6$ for PC ($r^2 = 0.9984$), $y = 3 \times 10^6 x + 9.6 \times 10^5$ for PE ($r^2 = 0.9982$), $y = 3 \times 10^6 x + 1.5 \times 10^5$ for PG ($r^2 = 0.9989$) and $y = 2 \times 10^6 x + 2 \times 10^6$ for LPC ($r^2 = 0.9959$).

In order to check the applicability to real samples, BALF was spiked with the standard solutions and the calibration checked again. The same linear ranges were confirmed with regression coefficients $r^2 > 0.993$. Limits of detection were calculated as the minimum injected amount which gave a response in the detector higher than 3 times the signal/noise ratio, using the corresponding extracted ion chromatograms. The limits of detection found were: 0.05 ng (PC), 0.10 ng (PE), 0.15 ng (PG) and 0.25 ng (LPC).

3.2. Recoveries

Recoveries of the liquid/liquid extraction procedure were calculated by spiking known amounts of C17:0 PL and LPC standards (points chosen on the lower (1 ng), medium (20 ng) and higher (40 ng) level of the calibration graphs) in BALF. Since these fatty acids (C17:0) do not occur in BALF, the amount of endogenous phospholipids does not have to be considered. Highest recoveries were obtained for PC and lowest for LPC. In Table 1 the different values are collected together with the percentage of variation.

The same liquid/liquid extraction procedure but without addition of acetic acid increased the recovery of LPC to 96%, however the recovery of the diacylphospholipids decreased dramatically. In order to use the same sample preparation procedure, a slightly lower extraction yield for LPC was accepted, since reproducibility of the recovery was satisfactory (see below).

Precision and reproducibility of the whole procedure were evaluated in a similar way by spiking two different amounts of a mixture of all the internal standards (5 and 25 ng) and

Table 1
Recovery of different synthetic phospholipids and lysophospholipids in BALF

Phospholipid	%Recovery 1 ng spiked ($n = 3$)	%Recovery 20 ng spiked ($n = 3$)	%Recovery 40 ng spiked ($n = 3$)
PC-C17:0/C17:0	95.6 ± 3.5	97.1 ± 2.7	93.3 ± 4.0
PE-C17:0/C17:0	69.3 ± 4.5	74.3 ± 4.1	73.0 ± 3.1
PG-C17:0/C17:0	75.0 ± 4.3	80.6 ± 3.5	77.0 ± 5.2
LPC-C17:0	64.6 ± 4.1	67.3 ± 5.6	65.0 ± 4.6

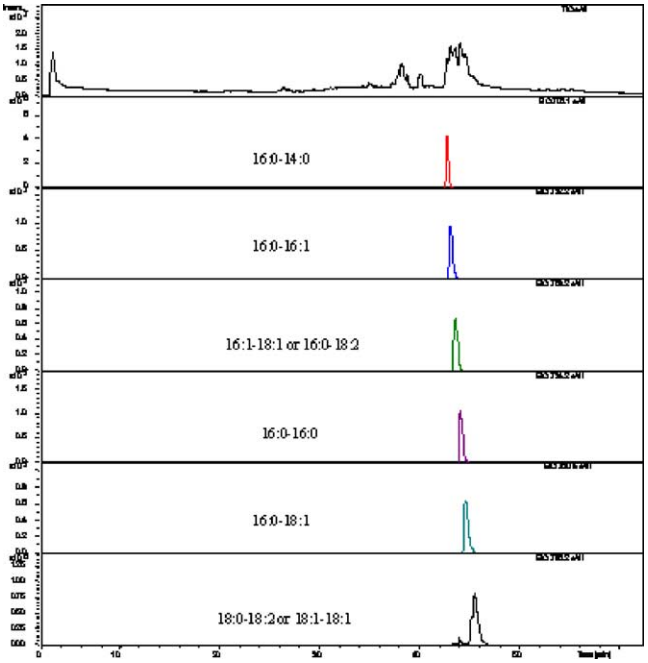

Fig. 5. EIC chromatograms corresponding to the most abundant PC species detected in BALF from a Bronchiolitis Obliterans patient (see Table 2 for relative amounts).

repeating the analysis 5 times. Precision (in terms of % R.S.D.) was better than 6% in every case.

3.3. BALF samples

Application of the method to BALF samples obtained from 3 different Bronchiolitis Obliterans patients showed similar overall chromatographic profiles (Fig. 4). The most abundant phospholipids and lysophospholipids detected belong to the phosphatidylcholine class. Detection of the different species within each class is possible by extracting the corresponding ion chromatograms (EIC). In Fig. 5 the EICs of the main PCs found in these samples are displayed: PC-C16:0/C14:0 (*m/z* 706.1), PC-C16:0/C16:1 (*m/z* 732.2), PC-C16:0/C16:0 (*m/z* 734.2), PC-C16:1/C18:1 or C16:0/C18:2 (*m/z* 758.2), PC C16:0/C18:1 (*m/z* 760.4) and PC-C18:0/C18:2 or C18:1/C18:1 (*m/z* 786.2) with PC-C16:0/C16:0 being the most abundant. Some other species (such as PC-C18:1/C18:2) were also detected in lower concentration, although to simplify the picture they are not displayed. Table 2 shows the relative abundance of the major phosphatidylcholine species for 3 different Bronchiolitis Obliterans patients.

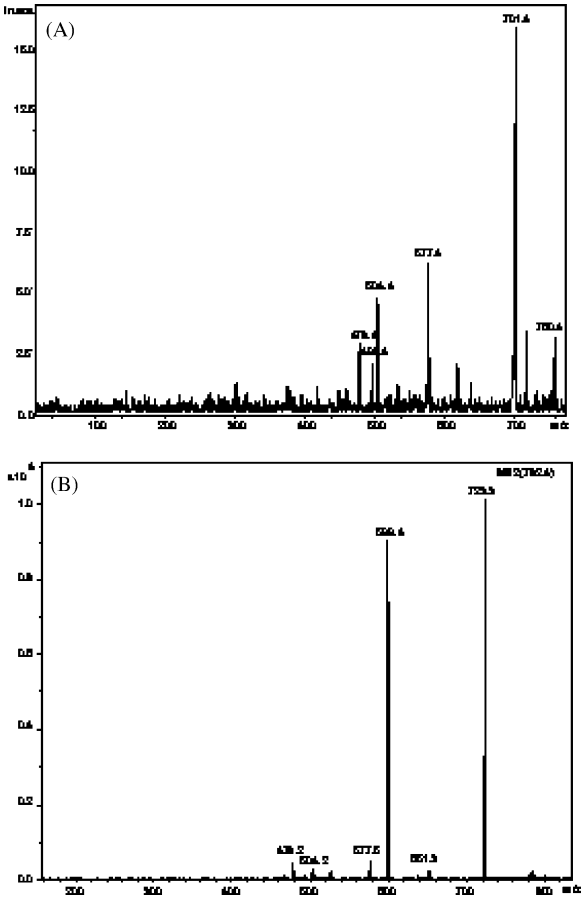

Fig. 6. MS/MS spectrum of the $[M + H]^+$ ion (A) and $[M + Na]^+$ ion (B) of PC-C18:1/C16:0 (*m/z* 760.4 and 782.4, respectively). In both cases the loss of triethylamine (M^+-59) and phosphocholine (M^+-183) is detectable.

Their mass spectra show a single intense ion corresponding to the protonated form $[M + H]^+$ with a very small contribution of the sodiated adduct $[M + Na]^+$. CID of the protonated diacylglycerophosphatidylcholines (Fig. 6A) produced very low intensity fragment ions, while their corresponding sodiated ions (Fig. 6B) fragmented easier producing mainly the $[M + Na\text{-}59]^+$ and $[M + Na\text{-}183]^+$ fragment ions corresponding to the loss of trimethylamine and phosphocholine, respectively. This different behavior may be due to stabilization of the phosphate group by the influence of the sodium ion [26].

Table 2
Distribution of the different major PC species found in BALF relative to the total amount of PC for three different Bronchiolitis Obliterans patients

	Patient 1 (%)	Patient 2 (%)	Patient 3 (%)
m/z 706.6 PC C16:0/C14:0	16.2	13.9	9.5
m/z 732.6 PC C16:0/C16:1	22.9	20.6	18.3
m/z 734.6 PC C16:0/C16:0	29.9	36.9	31.2
m/z 758.6 PC C16:1/C18:1 or PC C16:0/C18:2	14.8	11.8	17.6
m/z 760.6 PC C16:0/C18:1	16.2	16.8	23.4

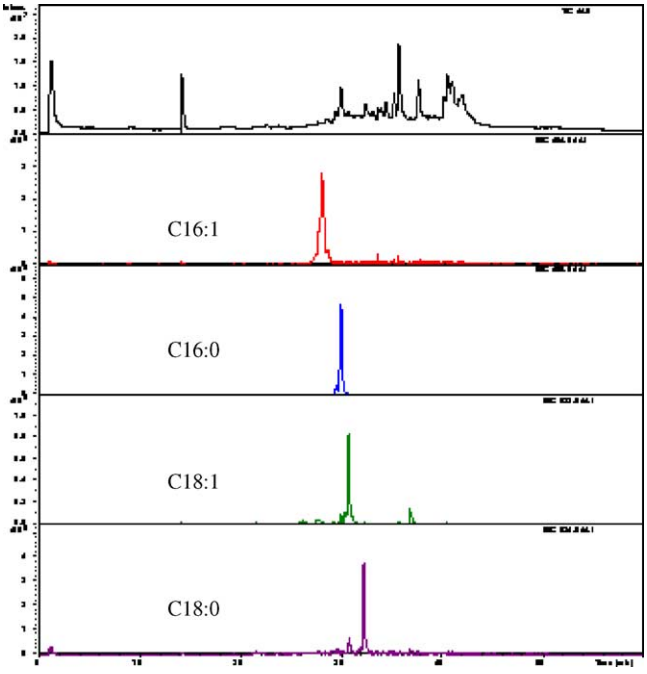

Fig. 7. EIC chromatograms corresponding to different LysoPC species detected in BALF from a Bronchiolitis Obliterans patient.

Regarding LPC, in Fig. 7 the extracted ion chromatograms corresponding to several species found in BALF are presented. The most abundant LPC is LPC-C16:0 (*m/z* 496.4 for the protonated form) but LPC-C14:0 (*m/z* 468.4), LPC-C18:1 (*m/z* 522.4) and LPC-C18:0 (*m/z* 524.4) can be observed as well. Their mass spectra show a single peak (the protonated molecular ion) in all the cases, uncomplicated by further fragmentation due to the low energy of electrospray ionization. These compounds were further characterized by subjecting each ion to CID. As examples, Fig. 8 shows the MS/MS spectra corresponding to C16:0-LPC (*m/z* 496.4) and

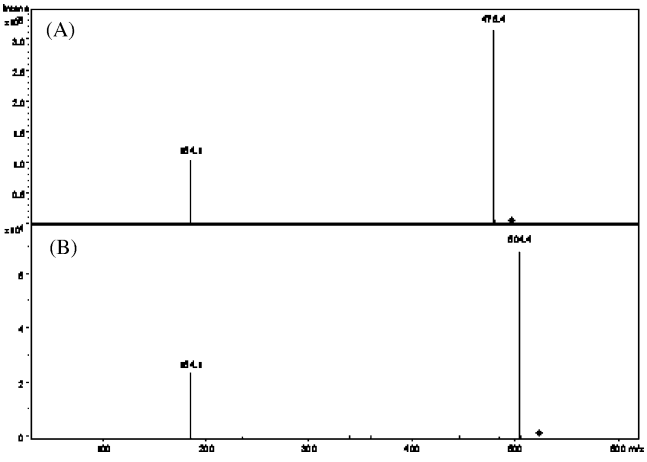

Fig. 8. MS/MS spectrum corresponding to (A) LPC-C16:0 (*m/z* 496.4) and (B) LPC-C18:1 (*m/z* 522.4) showing the release of water and the phosphocholine moiety (*m/z* 184.1).

Table 3
Ratio PC/LPC in three different Bronchiolits Obliterans patients calculated from the total PC containing the fatty acids C14:0, C16:0 and C18:1

PC/LPC ratios	Patient 1	Patient 2	Patient 3
C14:0	195.7	1114.8	447
C16:0	81.7	268.4	146.5
C18:0	234.5	2854.5	642.1

C18:1-LPC (*m/z* 522.4). As observed, the fragmentation produced is always the same: loss of water (generating the ion at *m/z* 478.4 in the case of C16:0-LPC and *m/z* 504.4 in the case of C18:1-LPC) and loss of the polar head group (phosphocholine) which retains the charge and gives the typical *m/z* 184 ion. LPC standards were also checked by MS/MS to confirm the assignments.

Phosphatidylglycerol species: PG-C16:0/18:1, PG-C16:0/18:2, and PG-C18:1/18:1 were detected in much lower amounts. The MS spectra of these phospholipids do not show an intense peak corresponding to $[M + H]^+$. The main peak is a product ion due to the loss of the phosphoglycerol group (*m/z* = 171), (e.g. *m/z* = 580 for PG C17:0/C17:0) due to in-source fragmentation. Phosphatidylethanolamines were not detected in any of the BALF samples. If present, they must be at concentrations below 2 ng/ml in original BALF. MS^2 of the standard PE-C17:0/C17:0 exhibited the same behavior as PC with loss of the phosphoethanolamine group (*m/z* = 141). Cholesterol was detected eluting at a retention time between lysophospholipids and phospholipids (see Fig. 4). In this case, the $[M + H\text{-}H_2O]^+$ (*m/z* 369.4) ion was observed.

Application of the method to the analysis of BALF samples allowed calculation of the relative concentrations of PCs and LPCs. In Table 3 these ratios are calculated for 3 different Bronchiolitis Obliterans patients. Due to the low and varying amounts of LPC found in these samples there are big variations in the PC/LPC ratios.

At the same time a major difficulty in analyzing BALF samples became apparent: variation in the concentration of BALF between different patients. Although this problem can be partially overcome by calculating relative phospholipid ratios, it is still problematic from a practical point of view. In some cases processing of 0.5 ml BALF produced final extracts with too high concentrations of PCs, thus overloading the analytical column. In that case some carry-over of the most abundant species was detected and a blank injection of DMSO was run between consecutive samples. The same amount of BALF in other cases gave very small peaks, making the detection of low abundant classes such as PG difficult. Therefore, a normalization procedure is needed to estimate the concentration of BALF prior to analysis and to compensate for the different amounts of saline solution recovered during the washing procedure. Some authors have used the amount of urea in BALF as an endogenous marker of epithelial lining fluid (ELF) dilution [27]. The original concentration of urea is the same in serum and in ELF. Therefore, the ratio between BALF-urea and serum-urea

gives an estimation of the dilution of the original volume of ELF by BALF saline. We will investigate whether this approach can be used for normalization prior to performing the analyses.

4. Conclusions

We have developed a new method to separate and quantify the main phospholipid species present in BALF in a single run. Qualitative analysis of BALF lipids has been previously performed by MALDI-TOF-MS and TLC [23] but these methodologies can only give a semiquantitative indication of BALF composition.

Quantitation of BALF phospholipids has been initially done by measuring the total lipid phosphorus [28], which does not differentiate between the different classes. In this method, the lipids are extracted using chloroform/methanol, and the organic material in the extract is degraded to inorganic phosphate with perchloric acid. Phosphate concentration is then determined by formation of a molybdenum blue complex, and quantified from the absorbance at 830 nm compared with dimyristoyl phosphatidylcholine (PC14:0/14:0) and dipotassium hydrogen orthophosphate standards.

This simple methodology has been sometimes complemented with direct infusion-ESI MS for the further analysis of the different molecular species [29,30]. Concentrations of individual phospholipid species within a phospholipid class (PC, PG, PI) are calculated from their ion currents relative to those of the relevant internal standards, after correction for the contribution from the ^{13}C isotope effects. Phospholipid class concentrations are calculated from the sum of their respective individual species and expressed relative to each other. However, there are several uncertainties concerning the reliability of quantitation using this procedure. Matrix effects as well as the influence of the different PL classes on the ionization of each other (e.g. suppression) can lead to distorsion of the obtained results.

More advanced methods have used chromatographic separation before quantitation with different types of detection principles, however only the use of MS can identify the separated species. Only one paper has so far reported the quantitative analysis of different phospholipids in rabbit BALF by LC–MS [31]. Using normal phase HPLC/MS the authors quantify the total contribution of all the species inside one class. Our method goes further since we can measure the different species within each class. The use of reversed instead of normal phase provides also superior resolution. General problems encountered in reversed phase separations of phospholipids such as excessive tailing are minimized through the use of a “pellicular” packing material at elevated temperatures without addition of special ion-pairing reagents to the mobile phase.

Recoveries obtained after liquid/liquid extraction are reproducible, which is important for the quantitative analysis, although some PLs and especially LPCs are recovered at only about 70%. Limits of detection varied between 0.05 and 0.25 ng, but improvements are possible using small diameter columns. This appears not to be necessary for quantitation of the major PL and LPC species in BALF.

The main phospholipid and lysophospholipid species we detected in human BALF were phosphatidylcholine derivatives containing C16 and C18 (saturated and unsaturated) fatty acids as previously reported by other authors. Our method has been shown to be applicable to their quantitative analysis using as internal standards the corresponding synthetic phospholipids with C17:0 fatty acids without any derivatization.

LPC was not detected in all BALF samples analysed and in the case of PC, variations in the relative concentrations of the different species between different patients were observed. Whether this can be correlated to disease progression is still under study.

In the future, the method will be applied to longitudinal studies of BALF samples obtained from patients suffering from diseases involving chronic lung inflammation such as chronic obstructive pulmonary disease (COPD) and Bronchiolitis Obliterans (BO). The focus will be to find variations in the distribution of different species as well as to quantify the ratio of phospholipids to lysophospholipids and to correlate these values to disease progression measured by other clinical parameters. This may provide access to early diagnosis of disease with improved possibilities for therapy.

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