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Characterization of phosphatidylcholines in rabbit lung lavage fluid by positive and negative ion fast-atom bombardment mass spectrometry

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ABSTRACT

The relative distribution of intact diacylphosphatidylcholine species isolated from the lung lavage fluid of rabbits has been investigated by positive ion fast-atom bombardment (FAB) mass spectrometry. Two different isolation/purification methods were applied and evaluated prior to mass spectrometric analysis. The first method consisted of a Bligh and Dyer extraction of the lung lavage fluid followed by isocratic high-performance liquid chromatographic (HPLC) separation. In the second method a thin-layer chromatographic purification step was introduced between the extraction procedure and the HPLC separation. Further, the FAB matrices glycerol and 3-nitrobenzyl alcohol were used, and their influence on the diacylphosphatidylcholine molecular ion species was studied. The Bligh and Dyer extraction followed by the simple HPLC separation was the method of choice to obtain stable, long-lasting protonated molecular ions and diagnostic fragment ions, which permitted the identification of the polar head-group. In combination with 3-nitrobenzyl alcohol as liquid matrix we established a procedure that yielded a fast sample preparation method, a good signal-to-noise ratio for detecting minor species, and reduced formation of $[M+H-2H]^+$ ion species. The relative fatty acid composition of the diacylphosphatidylcholine fractions isolated from rabbit lung lavage fluid was determined by negative ion FAB mass spectrometry using the carboxylate anions. The mass spectrometric results were compared with those acquired by gas chromatographic determination of the fatty acid methyl esters. Close agreement was found between the data obtained by the two independent methods.

INTRODUCTION

A number of pathological processes, such as the respiratory distress syndrome (RDS) of infants [1,2] or the adult RDS [3], are closely related to the pulmonary surfactant complex. The main components of this phospholipid (PL)–protein complex are diacylphosphatidylcholines (PCs), and there is strong evidence that the PC distribution in lung lavage fluid is characteristic for the damage to the surfactant system by RDS or the improvements after parenteral nutrition support

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[4]. Therefore, it is of importance to have qualitative and quantitative information on the relative distribution of PCs as well as of fatty acids. The method of obtaining these data must be fast and as accurate as possible.

The structural analysis of polar, thermolabile compounds, such as PLs, by mass spectrometry (MS) has been difficult for a long time. Conventionally, the analytical methods for PL characterization by MS relied on extensive sample preparation involving organic solvent extraction [5], followed by thin-layer chromatography (TLC) [6,7] or high-performance liquid chromatograpy (HPLC) [8–10] and afterwards degradative and/or derivatization techniques [11,12]. The resulting products were analysed by gas chromatography–electron impact-mass spectrometry (GC–EI-MS) [13,14] or GC–chemical ionization (CI)-MS [15,16]. All these methods were time-consuming and required a large amount of sample, and represented a challenge until the introduction of soft ionization techniques in MS.

Field desorption (FD) MS has been successfully applied by Wood and Lau [17] to characterize intact PL species without chemical or enzymic degradation or derivatization. This soft ionization technique has not been widely used owing to extensive prior sample purification that is necessary, and because of the detection of several supramolecular ions such as $[M+Na]^+$, $[M+K]^+$, $[M+CH_3]^+$ and [M + choline–water]$^+$ [18,19]. Several years later Fenwick *et al.* [20] demonstrated the power of fast-atom bombardment (FAB) MS for the determination of abundant quasimolecular PC ions. Further, characteristic fragment ions were formed under FAB conditions. Analysis of different PL species isolated from biological extracts by FAB-MS has been described by several groups [21–24]. Negative ion FAB-MS is also a suitable technique for the profiling of intact PLs [25] and fatty acids [26,27] from different lipid pools. Fatty acid profiling in the negative ion mode is based on the formation of carboxylate anions $[RCOO]^-$, which are formed directly from the C-1 and C-2 positions of the PCs [25].

In spite of the numerous papers, the usefulness of FAB-MS for PC and fatty acid profiling has to be evaluated in great detail in terms of necessary sample isolation/purification prior to FAB-MS analysis and application of liquid FAB matrices. We report here the use of FAB-MS in the analysis of mixtures of diacylphosphatidylcholines isolated from lung lavage fluids of rabbits. Our special interest was focused on the necessary extent of purification and separation prior to FAB-MS. Results are presented for two sample preparation methods (extraction–HPLC and extraction–TLC–HPLC), and two FAB matrices (glycerol and 3-nitrobenzyl alcohol) were evaluated for positive ion FAB-MS of the intact PC molecular species. Further, the relative fatty acid distribution of rabbit lung lavage fluid was assessed by applying two independent analytical methods: negative ion FAB-MS with triethanolamine as the matrix for intact PCs, and GC with flame ionization detection (FID) of fatty acid methyl esters (FAMEs).

EXPERIMENTAL

Reagents

Glycerol and 3-nitrobenzyl alcohol were obtained from Merck (Darmstadt, F.R.G.). The FAB matrix glycerol was used after vacuum distillation for further purification. Triethanolamine was supplied Loba Chemie (Vienna, Austria). The diacylphosphatidylcholine standards were purchased from Sigma (St. Louis, MO, U.S.A.) and the FAMEs from Supelco (Bellefonte, PA, U.S.A.). Other reagents and solvents used were commercial products of analytical-reagent grade or better. The nomenclature of the fatty acids as follows: 14:0, myristic; 16:1, palmitoleic; 16:0, palmitic; 18:3, linolenic; 18:2, linoleic; 18:1, oleic; 18:0, stearic; 20:4, arachidonic acid.

Bronchoalveolar lavage preparation

After anaesthetization, the lung tissue of rabbits was lavaged with physiological saline solution (five times with 20 ml). The pooled lavage fluid was spun down to remove all cells and debris. The supernatant was centrifuged at 100 000 *g* for 2 h. The pellet was resuspended in water (2 ml), and extracted according to the method of Bligh and Dyer [5].

Sample preparation procedure by TLC and HPLC

The crude Bligh and Dyer lipid extract was fractionated into purified PL classes by silica gel 60 TLC (Merck) using chloroform–methanol–water (68:40:3, v/v/v). TLC purification of PCs was carried out by removing the appropriate band after visualization of a reference band by staining with acidic molybdate solution [28]. Afterwards the PCs were eluted from the unstained silica gel with chloroform–methanol (1:1, v/v).

The next purification step for the diacylphosphatidylcholine species was HPLC separation based on a modified method described by Andrikopoulos *et al.* [29]. Briefly, the apparatus consisted of a 510 pump, a U6K injector and a Lambda Max 481 variable-wavelength detector, all from Waters (Milford, MA, U.S.A.). The system was equipped with Whatman (Maidstone, U.K.) Partisil SCX analytical column (250 mm × 4 mm I.D., 10 μm particle size). The HPLC column was equilibrated with acetonitrile–water (86.2:13.8, v/v) at room temperature. The flow-rate was 2.0 ml/min, and the effluent was monitored at 206 nm.

Half of the resulting HPLC fraction was taken directly for FAB-MS analysis, and the other part was used for saponification and conversion into FAMEs prior to GC analysis.

Another approach was to omit the TLC purification before HPLC separation of the crude Bligh and Dyer lipid extract. The resulting fraction was used as described for FAB-MS and GC–FID analysis.

Gas chromatography

GC-FID measurements of the FAMEs were made with a Dani 3800 gas chromatograph (Monza, Italy). It was fitted with a 12 m × 0.22 mm I.D. fused-silica capillary column chemically bonded with Carbowax 20M (Macherey-Nagel, Düren, F.R.G.) stationary phase (0.25 μm film thickness). The carrier gas was hydrogen. The chromatograph was used in the split injection mode with a split ratio of 1:20 and an injection port temperature of 250°C. The column temperature was set for 3 min at 150°C and increased to 230°C at 5°C/min. The flame ionization detector was held at 250°C. The GC data presented are the average of at least two analysis.

The FAMEs were formed by treating the PC fraction with methanolic sodium hydroxide at 60°C for 30 min. The solution was extracted twice with *n*-hexane, and the resulting FAMEs were analysed by GC–FID.

FAB-MS

Positive and negative ion FAB-MS analyses were performed on a Finnigan MAT 8230 mass spectrometer (Bremen, F.R.G.) equipped with a saddle field FAG gun B11NF (Ion Tech, Teddington, U.K.). Xenon was used as the primary particle source at an energy of 7.0 kV. The scan rate was 30 s/decade with a resolution of 1500 (10% valley definition) and an interscan time of 1 s. The spectrometer was operated at room temperature with an accelaration voltage of 3 kV. PC fractions were dissolved in 40 μl of chloroform–methanol (85:15, v/v). An aliquot of 10 μl was mixed with 10 μl of the FAB matrix and 4 μl of this mixture were deposited on a gold target attached to the direct insertion probe, which was kept at constant temperature (22°C) by a circulating cooling fluid. The matrix was triethanolamine for the negative ion mode and glycerol as well as 3-nitrobenzyl alcohol for the positive ion mode. Data were collected and processed on an SS-300 data system (Finnigan MAT). The ion intensities, which were the basis for the calculation of the relative PC and fatty acid distribution, were the average of twenty successive scans, and all samples were measured at least twice.

RESULTS AND DISCUSSION

Fig. 1A shows the cation-exchange chromatogram of the crude Bligh and Dyer extract isolated from the rabbit lung lavage fluid, containing the PL mixture. Additional peaks were found, which correspond to sphingomyelins and lysophosphatidylcholines. A representative liquid chromatogram of the lipid extract after Bligh and Dyer extraction, TLC separation, and chloroform–methanol elution is shown in Fig. 1B. The hatched parts in both chromatograms (Fig. 1A and B) were used for further FAB-MS and GC–FID analysis, respectively. As can be seen clearly, HPLC purification without the TLC step gives sufficient separation of the different PL classes. The positive ion FAB mass spectra exhibited abundant $[M+H]^+$ ions and diagnostically important fragment ions in the region *m/z* 100–

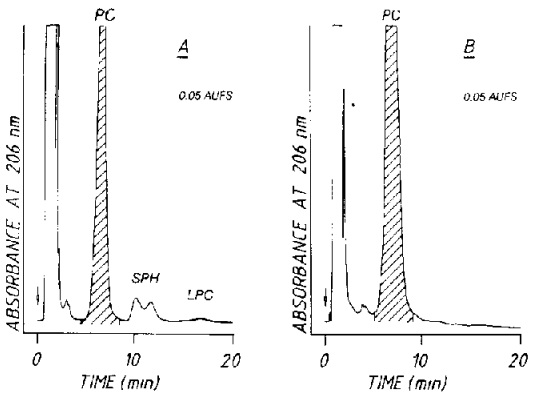

Fig. 1. Magnified part of the cation-exchange HPLC separation of the PC fraction isolated from rabbit lung lavage fluid after Bligh and Dyer extraction (A) and after Bligh and Dyer extraction followed by TLC purification (B). The hatched parts were used for FAB-MS and GC–FID analysis. Chromatographic conditions as described in Experimental. Peaks: PC = phosphatidylcholine; SPH = sphingomyelin; LPC = lysophosphatidylcholine.

300. The base peak in all mass spectra was at m/z 184, corresponding to the protonated choline phosphoester. This diagnostic peak was used to reveal whether the isolated samples contained only phosphocholine-containing species. Detection of significant abundant polar head-group ions, *e.g.* m/z 142 or m/z 226, would indicate the presence of phosphatidylethanolamines or phosphatidylserines. All isolated lipid samples showed only the polar head-group ion characteristic for PCs.

Fig. 2 displays the PC molecular ion region of lavage fluid isolated from rabbits with 3-nitrobenzyl alcohol as FAB matrix. The isolation procedure in this

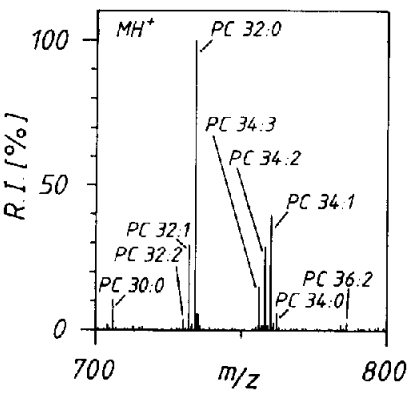

Fig. 2. Positive ion FAB mass spectrum of the PC molecular ion region obtained with 3-nitrobenzyl alcohol as liquid matrix. The PC fraction was isolated from rabbit lung lavage fluid after Bligh and Dyer extraction and subsequent HPLC purification.

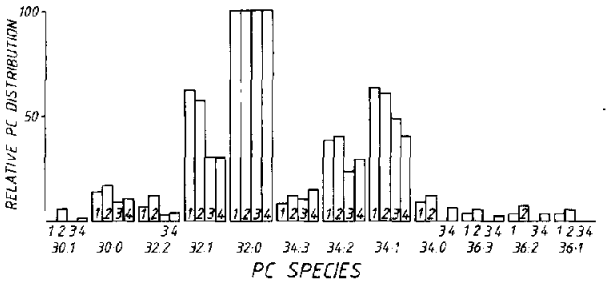

Fig. 3. Relative PC molecular ion species distribution of different purified PC fractions (TLC–HPLC and HPLC) isolated from rabbit lung lavage fluid determined by positive ion FAB-MS with two different matrices (glycerol = G and 3-nitrobenzyl alcohol = NBA). The most abundant PC molecular ion species *m/z* 734 was chosen as base peak, corresponding to PC 32:0. (1 = TLC–HPLC and G, 2 = HPLC and G; 3 = TLC–HPLC; NBA; 4 = HPLC and NBA).

case consisted of a Bligh and Dyer extraction followed by HPLC separation (See Fig. 1A). The molecular ions were interpreted as a PC series with C-1 and C-2 fatty acids of increasing chain length (from PC 30:0 to PC 36:2). Positive ion mass spectra of all PC fractions exhibited as most abundant ion *m/z* 734, in the pseudo-molecular ion region (*m/z* 600–1000). This ion corresponds to the protonated molecular PC species 32:0. The PC species distribution shown (see Fig. 3) is relative to *m/z* 734. The lipid samples that were purified only by extraction and HPLC provided, in all cases, a higher number of PC species than those subjected to an additional TLC purification step. This finding can be attributed to adsorption on the TLC material of PC species present in low concentration, which could not be removed by elution. Suppression effects, based on the different behaviour of PCs in the liquid FAB matrix, are another possible explanation of this observation [30,31]. No minor components were lost due to the TLC area, which was selected for scraping and elution. The dominant PC species were identified as PC 31:1 (*m/z* 732), PC 32:0 (*m/z* 734), PC 34:2 (*m/z* 758) and PC 34:1 (*m/z* 760). The diacylphosphatidylcholine with the highest molecular weight identifiable in the lung lavage fluid of rabbits was PC 36:1 (*m/z* 788). Based on these data we suggest that the Bligh and Dyer extraction followed by HPLC purification is the method of choice, because of higher yield of low-concentration PC species and the dramatically reduced sample preparation time.

Two different FAB matrices, glycerol and 3-nitrobenzyl alcohol, have also been investigated to evaluate their performance for the positive ion mode. FAB mass spectra of PC fractions using glycerol as matrix exhibited a total ion current percentage of 25–31% (mass range *m/z* 600–1000) for the $[M+H]^+$ ion (*m/z* 734). The detection of low-intensity PC molecular ion species in this mass range was extremely difficult because signals at each mass from the glycerol matrix were observed. The intensity and number of ions derived from radiation-induced damage to the matrix, as well as clusters, was significantly reduced by the use of

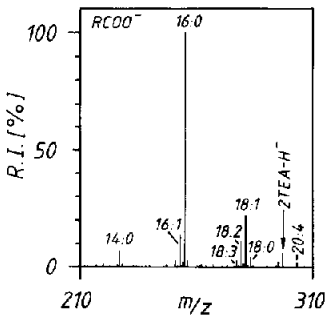

Fig. 4. Negative ion FAB mass spectrum of the fragment ion $[RCOO]^-$ (R = fatty acid moiety) region obtained with TEA as liquid matrix. The PC fraction was isolated from rabbit lung lavage fluid after Bligh and Dyer extraction and subsequent HPLC purification.

3-nitrobenzyl alcohol. The percentage of the total ion current due to the ion *m/z* 734 increased to 39–50% (*m/z* 600–1000). The PC signal-to-background ratio was improved by the second matrix, but matrix-related ions could not be eliminated completely (see Fig. 2). Further, when 3-nitrobenzyl alcohol was used as matrix, reduced formation of $[M+H-2H]^+$ ions [20,25] in comparison with the use of glycerol [32] was observed. Therefore, the PC distribution obtained with 3-nitrobenzyl aclohol (see Fig. 3) reflects to a higher extent the true relative PC distribution in the rabbit lung lavage fluid.

Fig. 4 shows the negative ion FAB mass spectrum from *m/z* 210 to *m/z* 310 of intact molecular PC species. This region exhibits abundant fragment anions, which correspond to the C-1 and C-2 attached fatty acids ($[RCOO]^-$). The presence of myristic (14:0), palmitoleic (16:1), palmitic (16:0), linoleic (18:2), oleic

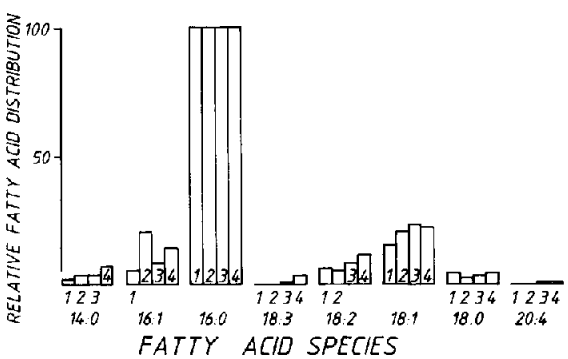

Fig. 5. Relative fatty acid distribution of different purified PC fractions (TLC–HPLC and HPLC) isolated from rabbit lung lavage fluid determined by negative ion FAB-MS using carboxylate anions $[RCOO]^-$ (R = fatty acid moiety) and GC–FID using FAMEs. All area counts obtained by GC–FID were relative to the most abundant FAME, corresponding to palmitic acid (16:0). The most abundant $[RCOO]^-$ ion species *m/z* 255 was chosen as base peak, corresponding to C 16:0 (palmitic acid). (1 = TLC–HPLC–GC; 2 = TLC–HPLC–FAB-MS; 3 = HPLC–GC; 4 = HPLC FAB-MS).

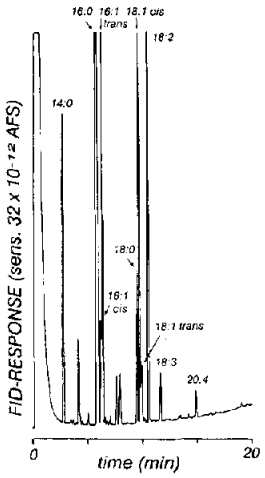

Fig. 6. Chromatogram (GC–FID) obtained from rabbit lung lavage fluid after Bligh and Dyer extraction, followed by HPLC purification and subsequent treatment with methanolic sodium hydroxide to form FAMEs. Chromatographic conditions as described in Experimental.

(18:1) and stearic (18:0) acids (*m/z* 227, 253, 255, 279, 281 and 283, respectively) was evident in all PC samples. The major fatty acid in all fractions was palmitic acid. The ions at *m/z* 277 and *m/z* 303 were detected only in samples not purified by TLC, and were identified as linoleic (18:3) and arachidonic (20:4) acids. Triethanolamine (TEA) was chosen as the FAB matrix for the negative ion mode because of the resulting low background in the relevant mass range. The cluster ion $[2TEA–H]^-$ at *m/z* 297 (see Fig. 4) did not interfere with any carboxylate anions, which are commonly known to be present in lung lavage fluids. These carboxylate anions have been used to quantify fatty acids. The relative fatty acid distribution obtained from PC samples, which were purified by Bligh and Dyer extraction followed by HPLC separation, was almost identical with that from samples treated by Bligh and Dyer extraction followed by TLC and HPLC purification (see Fig. 5). The calculated correlation coefficients averaged 0.996, which indicates no significant change in the fatty acid distribution of the PC samples during the different sample preparation methods. Further, the relative fatty acid distribution was obtained by GC–FID of FAMEs (see Fig. 6). The data agreed with the distribution found in negative ion FAB-MS. The correlation coefficients indicate the excellent agreement between the two methods (1–2: 0.993 and 3–4: 0.998, see also Fig. 5). The combination of Bligh and Dyer extraction followed by a HPLC separation of rabbit lung lavage fluid with negative ion FAB-MS is very suitable for determining the relative fatty acid distribution without any chemical or enzymic manipulation prior to analysis.

CONCLUSION

Positive ion FAB-MS with 3-nitrobenzyl alcohol as liquid matrix permits the reliable determination of the relative PC distribution in the lung lavage fluid of rabbits. Bligh and Dyer extraction combined with isocratic HPLC separation prior to FAB analysis turned out to be the method of choice, because the sample preparation was simple and fast and also the minor PC molecular species were detected. Continuous flow FAB [33], thermospray [34] or plasma spray MS [35] could be used for on-line combination of HPLC and MS to quantify intact PL species from lung lavage fluids with minimal sample purification and possible automatization. The relative fatty acid distribution of a PC fraction can be determined directly from the carboxylate anions found in the negative ion FAB mass spectrum, using triethanolamine as FAB matrix. A major advantage is that no chemical or enzymic degradation or derivatization steps were necessary prior to FAB analysis, in contrast to the GC–FID method. The agreement between the quantitative data obtained by the two techniques based on different analytical principles was found to be excellent. A short purification (extraction and isocratic HPLC separation) is still a prerequisite for the MS analysis, because otherwise the correct relative fatty acid distribution of the appropriate PL class cannot be obtained.

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