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# Simultaneous analysis of phospholipid in rabbit bronchoalveolar lavage fluid by liquid chromatography/mass spectrometry

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**Abstract**

A method was developed to separate and simultaneously quantitate phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM) and lysophosphatidylcholine (LPC) in rabbit bronchoalveolar lavage fluid (BALF) by liquid chromatography/mass spectrometry (LC/MS). This method consisted of a simple liquid-liquid extraction procedure, separation of phospholipid classes on silica gel column by gradient mode, and detection of mass spectrometry with electrospray ionization (ESI). The precision, accuracy and recovery ranged from 1.6 to 7.6%, −0.8 to +14.7% and 69.3 to 90.0%, respectively. This method was applied to compare the content and the composition of phospholipid classes in BALF collected from inflammation-model and control rabbit. The ratio of LPC concentration to PC significantly increased in inflammation-model BALF compared with control BALF.


*Keywords:* Phospholipid; LC/MS; Simultaneous analysis; Rabbit bronchoalveolar lavage fluid (BALF); Inflammation-model

## 1. Introduction

Phospholipids, the main constituents of biological membranes, are classified into several phospholipid classes by differences in the structure of the polar head groups (Fig. 1). Phospholipid classes with the same polar head group can be classified into many molecular species by differences in the length of the alkyl chain and number of double bonds at the *sn*-1 and *sn*-2 position. Thus, phospholipids exhibit great structural diversity and complexity. The phospholipids, lysophosphatidylcholine (LPC) and sphingomyelin have been reported to be closely related to various diseases, such as inflammation [1,2], atherosclerosis [3,4] and diabetes [5]. Recently, lipidomics, a field in which changes in lipid metabolism and lipid-mediated signaling processes are simultaneously identified, is increasing in importance for elucidating the function of proteins, for example, enzyme protein and receptor protein, in proteome studies. In particular, since phospholipids serve as the ligand and the substrate of these proteins, they are the most important and notable molecules in lipids. Among the phospholipid, the receptor of LPC has been identified and reported [2,6,7]. Moreover, under a disease related to phospholipid-modifying enzyme such as phospholipase

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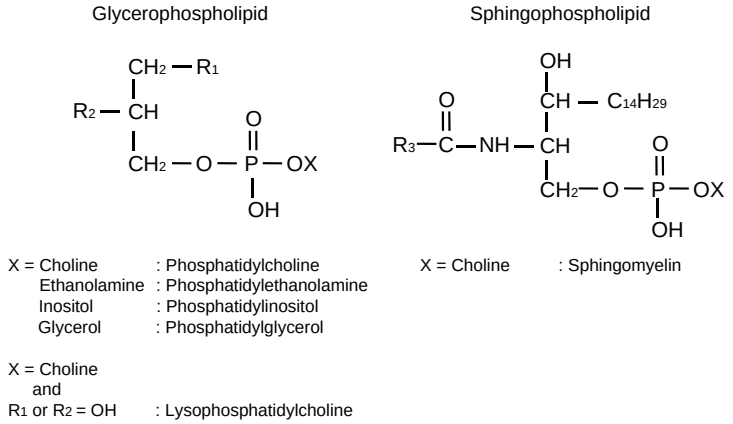


Fig. 1. Structure of phospholipids. X is polar head group, and R is side chain.

$A_1$, phospholipase $A_2$ and phospholipase C, the composition changes of all phospholipid classes must be simultaneously known. From these reasons, there is an increasing need to quantitate phospholipids and simultaneously know how change occur in phospholipid content in various biological samples due to the progression of the proteome studies.

Analytical techniques have been developed for the separation and quantification of phospholipids, such as thin layer chromatography (TLC)-phosphorus analysis [8–10], HPLC-UV analysis [11,12] and GC/MS analysis [13]. However, these methods are not suitable for the simultaneous analysis of phospholipids due to their cumbersome procedures and insufficient sensitivity and specificity. At present, mass spectrometry (MS)-based methodology has been generally the best technique for phospholipid analyses and many researchers have reported studies using MS-methods. One approach is to analyze only a specific phospholipid by the LC/MS method utilized in quantification of the drug compound in a biological sample [14,15]. This method can be useful if the target is limited. However, in most cases, it is necessary to simultaneously know the composition changes of all phospholipid classes. For these reasons, as another approach, a high through-put method was reported for simultaneous detection of some phospholipid molecular species using electrospray ionization (ESI)-MS/MS without HPLC separation [16–18], but it was difficult to quantitatively analyze phospholipids because of ion suppression by major components, such as phosphatidylcholine, and the influence of the matrix effect from biological samples. For quantifying phospholipids in biological samples, HPLC separation is very important. There is a report on qualitatively analysis of individual phospholipids by LC/MS mode [19]. However, the method had many problems in quantitative routine analysis, for example, insufficient pretreatment, very large sample volume, very long analytical time and no calibration curve. Especially, concerning the phospholipid analysis, it is difficult to obtain the well-fitting calibration curve due to formation of phospholipid dimer. To solve these problems, we tried to establish a routine analytical method to determine phospholipids. The improvement of the pretreatment procedure and HPLC conditions (column size, composition of solvent and gradient condition) with suitable internal standard made it possible to analyze biological samples continuously in 50 min cycles with reliability.

The method was applied to analysis of six phospholipid classes in bronchoalveolar lavage fluid (BALF) to compare the composition of phospholipids in inflammation-model BALF with control BALF.

To our knowledge, this is the first report based on quantitative discussion of the simultaneous determination of phospholipids in biological samples.

## 2. Experimental

### 2.1. Materials

Phosphatidylinositol (PI, from bovine liver), sphingomyelin (SM, from bovine brain), 1,2-dipalmitoyl-

phosphatidylglycerol (16:0–16:0 PG), 1,2-dipalmitoyl-phosphatidylethanolamine (16:0–16:0 PE), 1,2-dipalmitoyl-phosphatidylcholine (16:0–16:0 PC), 1-palmitoyl-lysophosphatidylcholine (16:0 LPC), 1,2-dilauroyl-PE (12:0–12:0 PE), 1,2-dilauroyl-PC (12:0–12:0 PC), 1,2-dimyristoyl-PC (14:0–14:0 PC), 1,2-distearoyl-PC (18:0–18:0 PC), 1,2-diarachidoyl-PC (20:0–20:0 PC), 1,2-dibehenoyl-PC (22:0–22:0 PC), 1,2-linoleoyl-PC (18:2–18:2 PC), 1-stearoyl-2-arachidonoyl-PC (18:0–20:4 PC) and 1-stearoyl-2-docosahexaenoyl-PC (18:0–22:6 PC) were purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure water was prepared using a Milli-Q AP TOC distillation unit (Millipore, Malborough, MA). Other organic solvents and reagents were of the highest purity commercially available.

### 2.2. Chromatographic conditions

The chromatographic system consisted of a Waters 2690 Alliance system. Phospholipids were separated into their classes using a silica column, an Inertsil SIL-100A (150 mm × 2.1 mm i.d., 5 μm, GL Sciences Inc., Tokyo, Japan). Mobile phase A was acetonitrile/methanol/1 M ammonium formate (78:20:2, v/v/v), while mobile phase B was acetonitrile/methanol/1 M ammonium formate (49:49:2 v/v/v). The flow-rate was 0.2 ml/min and the separation was performed at 30 °C. In the gradient program of the mobile phase, solvent B was increased from 0 to 15% for 5 min along a non-linear gradient curve, then increased to 80% at 5.1 min, and delivered isocratically for 10 min. Next, solvent B was increased to 100% at 15.1 min and delivered isocratically for 10 min. Finally, solvent B was decreased to 0%, and solvent A was delivered isocratically for 25 min. The total run time was 50 min. A 5-μl aliquot of assay solution was injected into the system with methanol as the wash solvent.

### 2.3. Mass spectrometry

Mass spectra were measured with a Micromass Quattro-LC (Manchester, UK). The instrument was equipped with a Z-spray ionization source, and was operated in the positive ion electrospray ionization mode. The nebulizer gas and desolvation gas were nitrogen. Typical operating parameters were as follows: capillary voltage 3.5 kV, cone voltage 30 V, resolution 14.5, source temperature 120 °C, desolvation temperature 350 °C, nebulizer gas flow 100 l/h, desolvation gas flow 650 l/h and multiplier 650 V. These conditions were optimized with 16:0–16:0 PC standard. The *m/z* range for measurement was set at *m/z* 450–1000 with 1.2 s of a scan time in the centroid mode. In quantitative analysis, *m/z* ranges set for each phospholipid class are as follows: *m/z* 720–800 for PG, *m/z* 830–940 for PI, *m/z* 680–800 for PE, *m/z* 650–850 for PC, *m/z* 700–705 + 723–732 + 753–754 + 787–850 for SM, *m/z* 450–600 for LPC. These *m/z* ranges cover all molecular species. The *m/z* range of SM was selected to decrease the influence of PC with a tailing peak. In this study, 12:0–12:0 PE was used as the internal standard (IS) for analysis of all phospholipid classes, the *m/z* range was set at *m/z* 550–600. To make the calibration curve, the peak area ratios (analyte/IS) versus the concentration of phospholipid (μg/ml) in rabbit BALF were plotted and fitted to a linear regression for PI, SM, LPC with $1/x$ weighting or quadratic regression for PG, PE, PC with $1/x$ weighting. The ranges of calibration curves in BALF were 0.250–12.5 μg/ml for PG, PE, SM, LPC, 0.375–12.5 μg/ml for PI and 0.150–7.5 μg/ml for PC.

### 2.4. Preparation of standard solution

Working standard solutions for the calibration curve were prepared by dissolution in chloroform/methanol (1:1, v/v) at 50, 40, 30, 20, 10, 5, 2.5, 1.5 and 1 μg/ml of 16:0–16:0 PG, 16:0–16:0 PE, SM and 16:0 LPC, and 50, 40, 30, 20, 10, 5, 2.5 and 1.5 μg/ml of PI and 30, 24, 18, 12, 6, 3, 1.5, 0.9 and 0.6 μg/ml of 16:0–16:0 PC. The working IS solution, 25 μg/ml of 12:0–12:0 PE was prepared by dissolving it in chloroform/methanol (1:1, v/v). Saline was used as the matrix for the calibration curve instead of BALF. All standard solutions were freshly prepared on each day of use.

### 2.5. Extraction procedure

Phospholipids were extracted by the Bligh & Dyer method [20]. In the extraction procedure for the calibration curve, BALF aliquots (400 μl) were mixed with 100 μl of working IS solution, 100 μl of working standard solution including six phospholipid

classes for the calibration curve, 900 μl of methanol and 400 μl of chloroform for 10 min. To the mixture, 500 μl of chloroform and 500 μl of water were added and then mixed for 10 min. After centrifuging at 3000 $min^{-1}$ for 5 min at 4 °C, the chloroform layer was evaporated to dryness. The samples were reconstituted with 100 μl of chloroform/methanol (1:1, v/v) by mixing for 5 min. Glassware was used throughout the assay to avoid the adsorption of phospholipid onto the container, pipet, etc.

### 2.6. Precision, accuracy and recovery

The precision was obtained as the relative standard deviation (R.S.D., %). The accuracy was obtained by calculating the bias between the mean value of the individual concentration and the respective theoretical concentration, which is the sum of the added concentration of phospholipid and endogenous concentration of phospholipid. The recovery was calculated using the following equation:

$$\text{Recovery of phospholipid (\%)} = \frac{\text{PA1} - \text{PA3}}{\text{PA2} - \text{PA3}} \times 100$$

where PA1, PA2 and PA3 are peak areas from BALF sample added phospholipids, the recovery test sample and control BALF sample, respectively. The recovery test sample was prepared with extract from BALF.

### 2.7. Preparation of BALF

BALF was collected from control rabbits infused saline ($n = 4$) and inflammation-model rabbits infused oleic acid (OA) ($n = 4$), as described previously [21]. The BALF was used after centrifuging at 3000 $min^{-1}$ for 5 min at 4 °C.

## 3. Results and discussion

### 3.1. Separation of phospholipids

To quantitate the phospholipids in their class level, HPLC separation of PG, PE, PI, PC, SM and LPC was examined. They are major phospholipid classes in BALF [22]. A diol column, a Lichrosorb Diol-10 (150 mm × 2.1 mm i.d., 5 μm, Chemco Scientific Co., Ltd., Osaka, Japan), in the normal phase mode under isocratic condition was tested and good separation was achieved. However, as PC was initially eluted under the condition, analysis of other phospholipid classes was interfered with tailing of the PC peak due to its extremely high concentration in BALF. In fact, PC occupied approximately 80% of the total phospholipid in BALF [23]. Finally, the method of separation developed by Taguchi et al. [19] was modified for routine and continuous analysis with good separation. The gradient condition and composition of the mobile phase was changed and a column with a larger internal diameter was used. These modifications resulted in good reproducibility with baseline separation of all phospholipid classes. As shown in Fig. 2, separation of six phospholipid classes was achieved within 50 min by using our HPLC method. Identification of individual phospholipid classes was performed using the mass spectra data and it was confirmed that several other peaks observed in the mass range chosen for each phospholipid were other phospholipid. Each phospholipid class generally includes various molecular species, which have alkyl chains of different length and/or different numbers of double bonds in the side chain. Therefore, the chromatographic peak of a phospholipid class from the biological samples is broader than the peak of a standard phospholipid for the calibration curve. Consequently, the method was established to be applicable to analysis of almost all biological samples with complete separation of the phospholipid classes.

### 3.2. Comparison of ionization efficiencies among molecular species of PC

Biological samples contain various phospholipid molecular species. However, it is difficult to obtain a phospholipid standard with the same composition as a biological sample for the calibration curve. As the major molecular species in BALF has been reported to be the dipalmitoyl type [23], the dipalmitoyl type of PC, PG, PE, the palmitoyl type of LPC and a mixture of molecular species for SM and PI, for which the dipalmitoyl type was not available, were used as standards for the calibration curve. If the ion efficiencies among various molecular species are significantly different, they are not appropriate as standards. Therefore, the peak area ratios to the dipalmitoyl type were determined after analyzing 50 ng of each

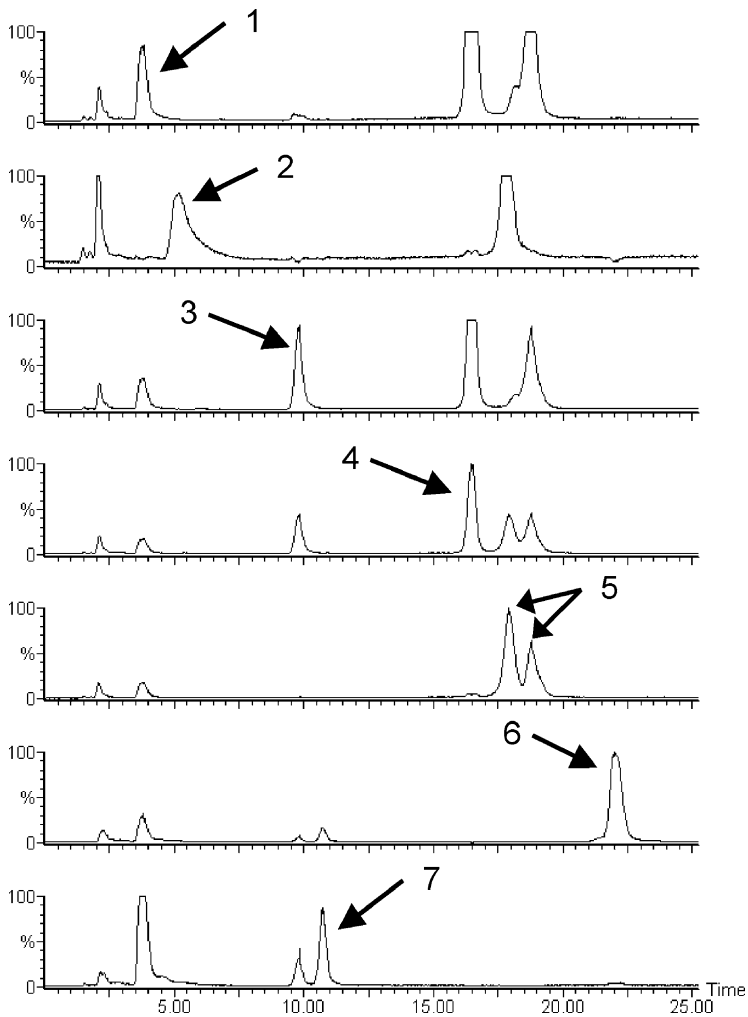


Fig. 2. Typical chromatograms of phospholipid standards in each mass range. *m/z* ranges set for each phospholipid class were as follows: *m/z* 720–800 for phosphatidylglycerol (PG), *m/z* 830–940 for phosphatidylinositol (PI), *m/z* 680–800 for phosphatidylethanolamine (PE), *m/z* 650–850 for phosphatidylcholine (PC), *m/z* 700–705 + 723–732 + 753–754 + 787–850 for sphingomyelin (SM), *m/z* 450–600 for lysophosphatidylcholine (LPC) and *m/z* 550–600 for internal standard (IS). (1) PG, (2) PI, (3) PE, (4) PC, (5) SM, (6) LPC, and (7) IS.

standard PC. The results are shown in Table 1. Significant differences were evident in the ion efficiencies of the negative ion mode, while those of the positive ion mode were almost the same. From these results, the phospholipids were detected in the positive ion mode.

### 3.3. Calibration curve

It was difficult to obtain linearity of the calibration curve of phospholipids because the response at higher concentrations extremely decreased. Therefore, the range of the calibration curve was set to the level of the concentration at which the response did not decreased to an extreme. In analyzing samples with high concentration, samples were diluted. For each phospholipid class, the peak area ratios (analytes/IS) for each corresponding calibration curve were plotted versus the concentration of phospholipid in rabbit BALF and fitted to the following equations with $1/x$ weighting: $y = 0.00542 + 0.153x - 0.00283x^2$ for PG, $y =$

Table 1
Comparison of the efficiencies of ionization among the molecular species of phosphatidylcholine

| Molecular species | Peak area ratio | |
|---|---|---|
| | Positive ion mode | Negative ion mode |
| 12:0–12:0 | 0.95 | 1.60 |
| 14:0–14:0 | 0.95 | 1.37 |
| 16:0–16:0 | 1.00 | 1.00 |
| 18:0–18:0 | 1.19 | 0.81 |
| 20:0–20:0 | 0.98 | 0.49 |
| 22:0–22:0 | 1.11 | 0.35 |
| 18:2–18:2 | 1.13 | 1.05 |
| 18:0–20:4 | 0.91 | 0.71 |
| 18:0–22:6 | 0.90 | 0.71 |

$-0.0526+0.0867x$ for PI, $y=-0.00253+0.321x-0.00379x^2$ for PE, $y=-0.0241+1.57x-0.0572x^2$ for PC, $y=-0.0870+0.519x$ for SM, $y=-0.194+0.953x$ for LPC. PE with a short chain was used as IS, which was not present in the biological samples. All calibration curves had correlation coefficients ranging from 0.9938 to 0.9998. The bias of the back-calculated concentrations of calibration curve ranged from −20.0 to +23.2.

Phospholipids are classified by differences in the structure of the polar head group, and the range of molecular weights is broad. The *m/z* of LPC is the smallest due to lack of a side chain from the diacyl type PC. PI, including inositol, is the largest *m/z*. Therefore, the scan *m/z* range for measurement was set to cover the *m/z* range for all phospholipids. The chromatogram for quantification was obtained from the total ion in the *m/z* range set for each phospholipid class (see Section 2), which is composed of a mixture of many molecular species with different side chains at *sn*-1 and *sn*-2.

### 3.4. Application to rabbit BALF

This method was applied to the analysis of rabbit BALF as one of the biological samples. BALF characteristically contains a large amount of PG, compared with other biological samples. Phospholipid levels in BALF have been analyzed by various methods, for example, TLC and phosphorus method [24–26], GC/MS method [13] and ESI/MS method [22,27], but these methods have not been evaluated quantitatively.

Initially the separation of endogenous phospholipid extracted from BALF was examined. The result is shown in Figs. 3 and 4. Identification of individual phospholipid classes was performed using the mass spectra data and the retention time. There was excellent separation of phospholipid classes on the chromatogram and no marked interference peaks around the retention time of each phospholipid class. Therefore, the analytical method was evaluated with

Table 2
Within-run precision and accuracy of the assay method for phospholipids in control rabbit bronchoalveolar lavage fluid ($n=5$)

| Compound | Added concentration (μg/ml) | Theoretical concentration (μg/ml) | Mean concentration (μg/ml) | Precision (R.S.D., %) | Accuracy (bias, %) | Recovery (%) |
|---|---|---|---|---|---|---|
| Phosphatidylglycerol | 0 | – | 1.06 | 4.7 | – | – |
| | 5.10 | 6.16 | 6.74 | 1.9 | 9.4 | 87.2 |
| Phosphatidylinositol | 0 | – | 1.31 | 6.9 | – | – |
| | 5.20 | 6.51 | 7.10 | 4.5 | 9.1 | 85.1 |
| Phosphatidylethanolamine | 0 | – | 0.820 | 5.6 | – | – |
| | 5.15 | 5.97 | 6.03 | 4.1 | 1.0 | 86.5 |
| Phosphatidylcholine | 0 | – | 25.0[a] | 7.6 | – | – |
| | 101 | 126 | 124[a] | 1.6 | −0.8 | 90.0 |
| Sphingomyelin | 0 | – | 1.73 | 5.2 | – | – |
| | 5.20 | 6.93 | 7.14 | 3.4 | 3.0 | 69.3 |
| Lysophosphatidylcholine | 0 | – | 0.507 | 3.2 | – | – |
| | 5.00 | 5.71 | 6.08 | 3.9 | 6.5 | 85.1 |

[a] 40-fold diluted.

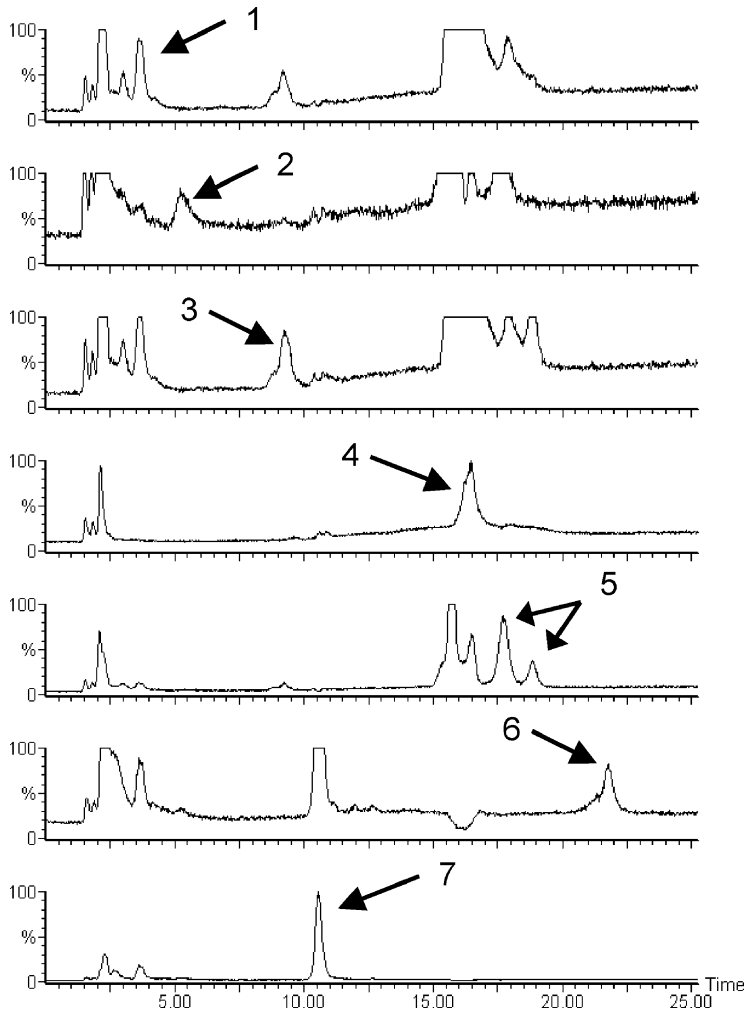


Fig. 3. Typical chromatograms of endogenous phospholipids and IS in control BALF at the respectively selected mass ranges. *m/z* ranges set for each phospholipid class were as follows: *m/z* 720–800 for phosphatidylglycerol (PG), *m/z* 830–940 for phosphatidylinositol (PI), *m/z* 680–800 for phosphatidylethanolamine (PE), *m/z* 650–850 for phosphatidylcholine (PC), *m/z* 700–705 + 723–732 + 753–754 + 787–850 for sphingomyelin (SM), *m/z* 450–600 for lysophosphatidylcholine (LPC) and *m/z* 550–600 for internal standard (IS). (1) PG, (2) PI, (3) PE, (4) PC, (5) SM, (6) LPC, and (7) IS.

the BALF collected from rabbit infused saline and rabbit infused OA as the matrix. Since biological samples contain endogenous phospholipid, the basal concentrations of endogenous phospholipids ($n = 5$) were determined. The precision was calculated using the determined basal value. Next, the concentration of phospholipids was determined in BALF to which known amounts of phospholipid standards had been added. As the concentration of PC was significantly higher than other phospholipids in the biological samples, the assay sample for PC analysis was prepared by diluting the extract 40-fold. Tables 2 and 3 show the results obtained from intra-day examination. The assay performance for phospholipids was determined by assessing the precision, accuracy and recovery. The developed method had precision ranging from 1.6 to 7.6% and from 1.7 to 6.8%, accuracy ranging from −0.8 to +9.4% and from +2.7 to +14.7%, recovery ranging from 69.3 to 90.0% and from 71.9 to 88.6%, in control BALF and inflammation-model

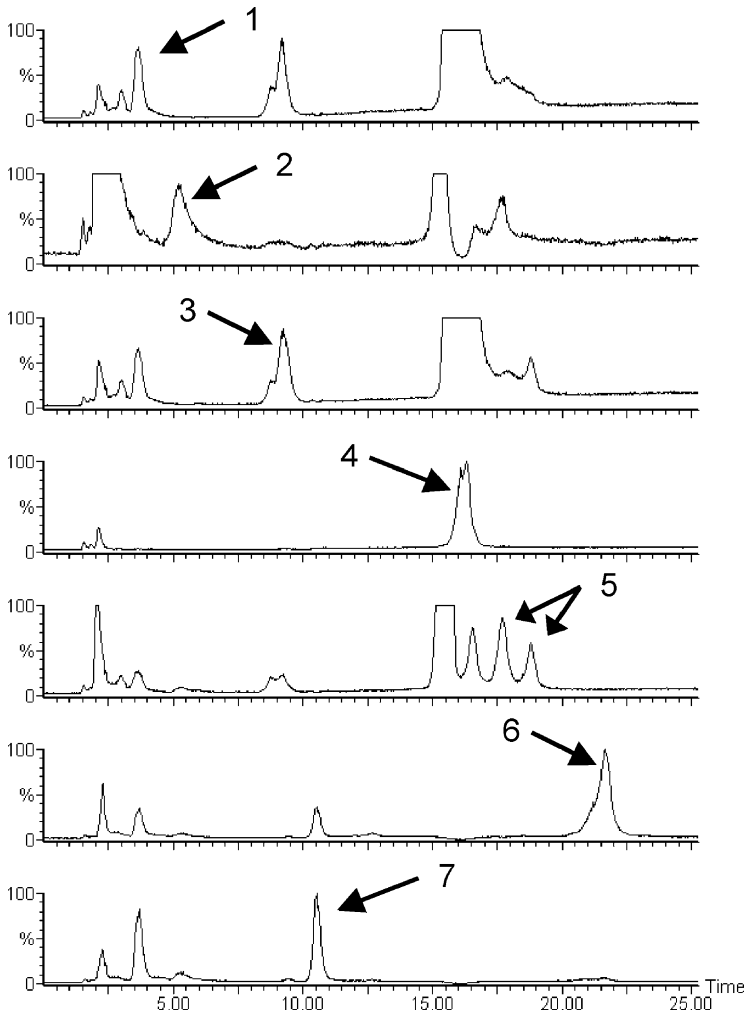


Fig. 4. Typical chromatograms of endogenous phospholipids and IS in inflammation-model BALF at the respectively selected mass ranges. *m/z* ranges set for each phospholipid class were as follows: *m/z* 720–800 for phosphatidylglycerol (PG), *m/z* 830–940 for phosphatidylinositol (PI), *m/z* 680–800 for phosphatidylethanolamine (PE), *m/z* 650–850 for phosphatidylcholine (PC), *m/z* 700–705 + 723–732 + 753–754 + 787–850 for sphingomyelin (SM), *m/z* 450–600 for lysophosphatidylcholine (LPC) and *m/z* 550–600 for internal standard (IS). (1) PG, (2) PI, (3) PE, (4) PC, (5) SM, (6) LPC, and (7) IS.

BALF, respectively. These results demonstrate that our method is quantitative. If BALF without endogenous phospholipid was available as the matrix for the calibration curve, more accurate values should be obtained because the matrix effect could be cancelled.

Using this method, the amount of phospholipids was determined in BALF collected from inflammation-model rabbit ($n = 4$) and control rabbit ($n = 4$). The results showed that the concentration of all phospholipid classes was higher in inflammation-model BALF than in the control. However, since the deviation of concentrations among individuals was higher, the inflammation-model BALF was compared with the control by using the ratio of the concentration of each phospholipid to PC (Fig. 5). This evaluation demonstrated that LPC is significantly increased in the inflammation-model BALF compared to control BALF. The mass spectral data could be used to obtain information on the molecular species though they should be confirmed by quantitative

Table 3
Within-run precision and accuracy of the assay method for phospholipids in inflammation-model rabbit bronchoalveolar lavage fluid ($n = 5$)

| Compound | Added concentration (μg/ml) | Theoretical concentration (μg/ml) | Mean concentration (μg/ml) | Precision (R.S.D., %) | Accuracy (bias, %) | Recovery (%) |
|---|---|---|---|---|---|---|
| Phosphatidylglycerol | 0 | – | 6.46 | 1.9 | – | – |
| | 5.10 | 11.6 | 12.2 | 4.1 | 5.2 | 81.8 |
| Phosphatidylinositol | 0 | – | 4.86 | 6.6 | – | – |
| | 5.20 | 10.1 | 11.1 | 2.7 | 9.9 | 88.6 |
| Phosphatidylethanolamine | 0 | – | 6.42 | 2.2 | – | – |
| | 5.15 | 11.6 | 13.3 | 6.8 | 14.7 | 87.4 |
| Phosphatidylcholine | 0 | – | 119[a] | 1.7 | – | – |
| | 101 | 220 | 247[a] | 5.7 | 12.3 | 83.8 |
| Sphingomyelin | 0 | – | 2.49 | 4.8 | – | – |
| | 5.20 | 7.69 | 7.90 | 2.7 | 2.7 | 80.0 |
| Lysophosphatidylcholine | 0 | – | 6.28 | 2.9 | – | – |
| | 5.00 | 11.5 | 12.5 | 4.8 | 8.0 | 71.9 |

[a] 40-fold diluted.

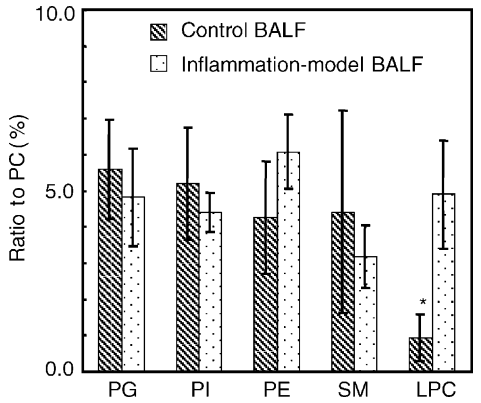


Fig. 5. Comparison of concentration of each phospholipid [phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), sphingomyelin (SM), lysophosphatidylcholine (LPC)] to phosphatidylcholine (PC) between control bronchoalveolar lavage fluid (BALF) ($n = 4$) and inflammation-model BALF ($n = 4$) ($^*P < 0.01$).

analysis using the LC/MS/MS system with the MRM mode. For example, the composition of 18:2-LPC and 18:1-LPC increased in inflammation-model BALF.

## 4. Conclusion

In this study, a new LC/MS method was developed to simultaneously quantitate six phospholipid classes and its application to rabbit BALF was described. This method makes it possible to analyze phospholipids in various biological samples, and to obtain information on changes in the content or composition of phospholipid classes. This method should be useful for simultaneously examining the change of phospholipid content in various diseases, for example, inflammation and atherosclerosis, to which phospholipids and phospholipid-modifying enzyme were related.

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