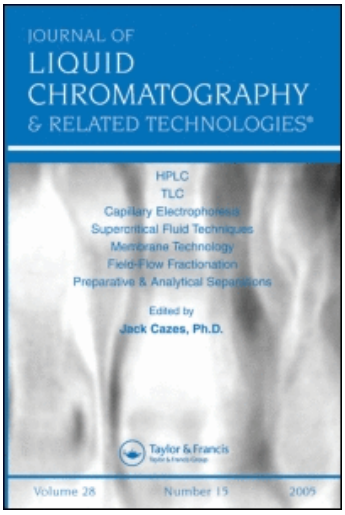

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Simultaneous Analysis of Eight Phospholipid Classes by Liquid Chromatography/Mass Spectrometry: Application to Human HDL

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Abstract: We developed a method to separate and simultaneously quantitate eight phospholipid classes, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC). A chloroform/methanol/1-propanol/ammonium hydroxide/water solvent system was used as the HPLC mobile phase. This condition was set to fulfill a fit in a calibration curve and adequate separation of phospholipids. We evaluated our analytical method for precision, accuracy, and recovery of the phospholipids for human high-density lipoprotein (HDL). The precision, accuracy, and recovery ranged from 1.4 to 22.6%, 26.9 to +30.4%, and 56.0 to 99.3%, respectively.

Keywords: Phospholipid, Simultaneous analysis, Human HDL

INTRODUCTION

Phospholipids are not only major constituents of membranes, but are also important molecules in a variety of biological actions. In recent years, lysophosphatidylcholine (LPC), which is one of the phospholipids, has been reported for a variety of diseases, for example, inflammation,[1,2] atherosclerosis,[3] and diabetes.[4] In addition to LPC, other phospholipids may play a functional role in the pathogenesis of various diseases; simultaneous analysis of phospholipid classes is important.

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In analysis of phospholipid, tandem mass spectrometry (MS/MS) would have yielded great specificity and sensitivity in the detection.[5–7] However, this detection has a disadvantage as described below. MS/MS only detects several specific phospholipid species. It is generally difficult to know which molecular species in phospholipid classes changes in an initial study. The specific method will be used for the evaluation in an higher level. Therefore, we selected an LC/MS method that provided broad profile data. Some LC/MS methods have been reported. Most of them qualitatively analyze phospholipids by comparison of peak intensity without a calibration curve.[8–12] Therefore, to resolve these issues, we have developed a simultaneous and quantitative analytical method of six phospholipid classes by LC/MS.[13] The method enabled us to obtain the absolute concentration in biological samples by a calibration curve for each phospholipid class, and was validated using rabbit bronchoalveolar lavage fluid (BALF) containing six phospholipid classes as a biological sample. However, it was difficult to analyze phosphatidylserine (PS) and phosphatidic acid (PA) because of the broad peaks in this method. PS is related to apoptosis and activation of intracellular enzyme.[14] PA is a key intermediate in lipid metabolism on the structure of all phospholipids.[15] Recently, PA has been shown to have a crucial involvement in signal transduction and cellular proliferation.[16–18] In addition, these phospholipids are the precursors of lysophosphatidylserine (LPS) and lysophosphatidic acid (LPA) that are related to a variety of biological activities.[14,19] Therefore, there is a demand to analyze these classes and analysis of the precursor of these lyso phospholipids is very significant. However, analysis of these phospholipids was difficult because of the broad peak in mobile phase, which is generally used for mass spectrometric analysis.

In order to analyze PS and PA, chloroform is considered to be one of the essential HPLC solvents.[20] LC/MS/MS analysis of six phospholipid classes using a diol column and chloroform has been reported.[21] However, since PC is initially eluted under the conditions, analysis of the other phospholipid classes interfered with tailing of the PC peak due to its extremely high concentration in biological samples. Separation of each phospholipid was also not sufficient. Therefore, we tried to establish an analytical method for eight phospholipid classes using silica columns and chloroform. Additionally, we examined the fit of the calibration curve to the data; we could not obtain a well fitting calibration curve because of an extreme decrease of the response at higher concentrations due to the formation of phospholipid clusters such as dimers. To overcome this problem, various solvents were tried for the HPLC mobile phase. The final developed method was applied to the analysis of eight phospholipid classes in human high-density lipoprotein (HDL) and was validated.

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

EXPERIMENTAL

Materials

Phosphatidylinositol (PI, from bovine liver), sphingomyelin (SM, from bovine brain), 1,2-dipalmitoyl-phosphatydilglycerol (16:0–16:0 PG), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (16:0–16:0 PE), 1,2-dipalmitoyl-phosphatidylserine (16:0–16:0 PS), 1,2-dipalmitoyl-phosphatidic acid (16:0–16:0 PA), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (16:0–16:0 PC), 1-palmitoyl-lysophosphatidycholine (16:0 LPC), and 1,2-dilauroyl-phosphatidylethanolamine (12:0–12:0 PE) were purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure water was prepared using a Milli-Q AP TOC distillation unit (Millipore, Malborough, MA). All other organic solvents and reagents were of the highest grade commercially available.

Preparation of Human HDL

High-density lipoprotein (HDL, d = 1.085–1.210 g/mL) was isolated from the plasma of healthy and fasting donors by sequential ultracentrifuging, as described previously.[22] The final concentration of the HDL solution was 32.2 mg/mL.

Chromatographic Conditions

For the LC separation, an Inertsil SIL100A column (150 × 2.1 mm I.D., 5 μm) (GL Sciences Inc., Tokyo, Japan) was used. The flow-rate was 0.2 mL/min and the separation was performed at 30°C. Mobile phase A consisted of chloroform/methanol/1-propanol/28% ammonium hydroxide (80:9:10:1, v/v/v/v), while mobile phase B consisted of methanol/1-propanol/water/28% ammonium hydroxide (83:10:6:1, v/v/v/v). The mobile phase gradient condition is specified in Table 1. A 5 μL aliquot of the assay solution was injected into the system with methanol as the wash solvent.

Mass Spectrometry

Mass spectra were measured with a Quattro-LC triple-stage quadrupole mass spectrometer (Micromass) equipped with an electrospray ion source (Manchester, UK). The instrument was equipped with a Z-spray ionization source and operated in the positive 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. The m/z range

Table 1. Gradient condition for the HPLC separation

Time (min)	A (%)	B (%)
0.0	100	0
0.1	80	20
6.0	80	20
6.1	70	30
10.0	70	30
10.1	50	50
13.0	50	50
13.1	0	100
25.0	0	100
25.1	100	0
40.0	100	0

for measurement was set at m/z 450–1000 with 1.2 s of a scan time in the centroid mode. In quantitative analysis, the m/z ranges set for each phospholipid class are as follows: m/z 545–610 for PG, m/z 690–800 for PE, m/z 550–660 for PI, m/z 730–860 for PS, m/z 660–760 for PA, m/z 730–860 for PC, m/z 675–850 for SM, and m/z 490–570 for LPC. For PG and PI, ions of diradyl glycerol were used due to the detection of some interference peaks at around m/z 700–900. 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 580–620. To make the calibration curve, the peak area ratios (analyte/IS) versus the concentration of phospholipid (μg/mL) in human HDL were plotted and fitted to a quadratic regression with 1/x weighting. The ranges of the calibration curves in human HDL are 20–600 μg/mL for PG, 6.25–125 μg/mL for PE, 20–1000 μg/mL for PI, 20–1000 μg/mL for PS, 50–800 μg/mL for PA, 10–1000 μg/mL for PC, 12.5–1250 μg/mL for SM, and 30–1000 μg/mL for LPC.

Preparation of Standard Solution

Working standard solutions for the calibration curve were prepared by dissolution in chloroform/methanol (1:1; v/v). Standard solutions were freshly prepared on each day of use. For the matrix of the calibration curve, saline was used.

Extraction Procedure

Phospholipids were extracted by the Bligh & Dyer method.[23] In the extraction procedure for the calibration curve, HDL aliquots (4 μL) were mixed with

400 μL saline, 100 μL of working IS solution, 100 μL of working standard solution, 900 μL of methanol, and 400 μL of chloroform for 10 minutes. To the mixture, 500 μL of chloroform and 500 μL of water were added and then mixed for 10 minutes. After centrifuging at 3000 rpm for 5 minutes at 4°C, the chloroform layer was evaporated to dryness. The assay samples were reconstituted with 100 μL of chloroform/methanol (1:1; v/v) by mixing for 5 minutes.

Precision, Accuracy, and Recovery of the Assay

Precision was obtained as the relative standard deviation (RSD, %) of measured concentration. The accuracy was obtained from calculation of the bias between the mean value of the measured concentration and the theoretical concentration, which is the sum of concentration of added phospholipid to HDL and endogenous concentration in HDL. The recovery was calculated using the following equation:

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

Peak areas were obtained from preparation and measurement of the following samples: PA1: phospholipid standards were added to HDL sample before extraction; PA2: phospholipid standards were added to the sample extracted from HDL sample; PA3: phospholipid standards were not added to the HDL sample.

RESULTS AND DISCUSSION

Calibration Curve and Separation of Standards

Phospholipids, the main constituents of biological membranes, are classified into several phospholipid classes by differences in the structure of the polar head groups. 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. Since phospholipids exhibit great structural diversity and complexity, it is generally difficult to know which molecular species changes in initial study. LC/MS/MS method has high specificity and sensitivity, but only detects specific molecular species. Therefore, we selected LC/MS method that provided broad profile data in this study. If the target of specific analysis is specialized in an LC/MS method or another study, it would be necessary to quantitatively and accurately analyze utilized individual calibration standards and deuterated internal standards.

The classification of phospholipid mixtures is generally accompanied by normal-phase HPLC separation using diol or silica columns. Analysis of six

phospholipid classes using a diol column has been reported.[21] However, as PC is initially eluted under the conditions, analysis of the other phospholipid classes interfered with tailing of the PC peak due to its extremely high concentration in biological samples. In addition, the separation of phospholipids was not sufficient. Therefore, we developed a method for simultaneously analyzing phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC) by LC/MS using a silica column.[13] However, in this method phosphatidylserine (PS) and phosphatidic acid (PA) could not be quantified due to their broad peaks. Therefore, we applied a chloroform solvent system, which was generally used in HPLC-UV analysis,[20,24] to our LC/MS method. The chloroform solvent system has not been generally accepted for MS analysis because of high background noise levels. However, chloroform is considered to be one of the essential HPLC solvents for analysis of PS and PA, resulting in good separations of PS and PA.

The calibration curve of PC was made in the range of 2–200 ng under the conditions described above. However, the calibration curve did not fit well because the response at extremely high concentrations decreases. The phospholipid dimer peak was detected at high intensity in the mass spectra data. It was found that the formation of phospholipid clusters, such as the dimer, extremely decreased the response of the monomer at a high concentration. In order to suppress the formation of the phospholipid cluster, primary amines (decylamine and hexylamine), which had the effect of multimer suppression,[25] were added to the mobile phase and tested. Although the linearity of the calibration curve was improved, many adduct ions with amines were detected on the mass spectra. Next, we examined the effect of various organic solvents added to the mobile phase and evaluated the linearity using the correlation coefficients (R) for the calibration curve of PC. The results are shown in Table 2. When 1-propanol was used, the calibration curve gave a better fit compared with other organic solvents. These findings suggested that an organic solvent with a high boiling point remained in the spray of the solvent into the MS

Table 2. Relationship of organic solvent and correlation coefficients in calibration curve of PC

Organic solvent	Boiling point[a] (°C)	Correlation coefficients (R)
Methanol	64.7	0.8393
Ethanol	78.5	0.9357
Acetonitrile	81.6	0.9183
2-Propanol	82.5	0.9722
1-Propanol	97–98	0.9851

[a]Quoted from Merck index.

interface and suppressed the formation of phospholipid dimer, which are insoluble in water.

The HPLC gradient conditions in the composition of the mobile phase were examined to obtain adequate separation of eight phospholipid classes and shorter run time. The improvement resulted in continuous analysis of 40 minutes cycles, as shown in Fig. 1.

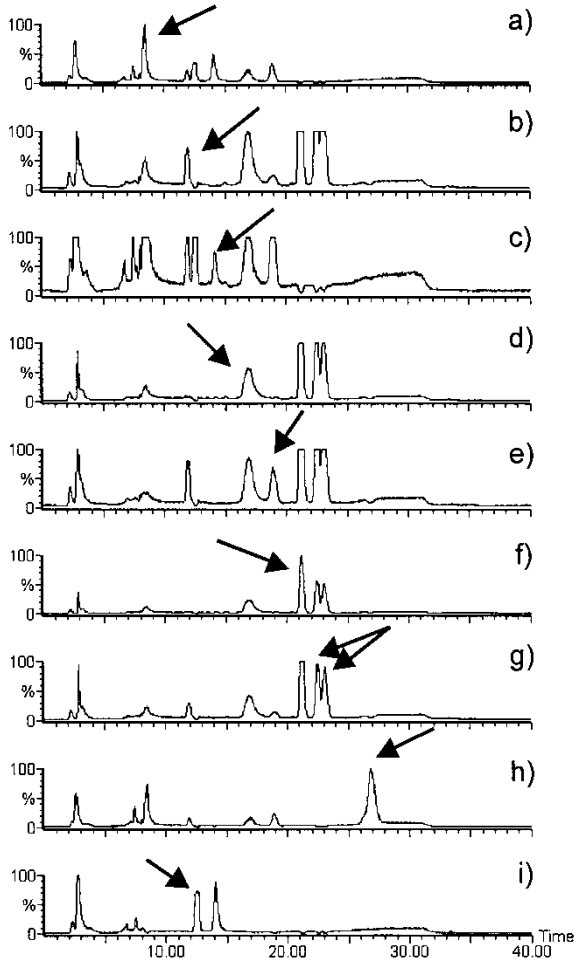

Figure 1. Chromatograms of authentic phospholipid standards at selected mass range in positive ion mode. a, phosphatidylglycerol (PG) (m/z range: 545–610); b, phosphatidylethanolamine (PE) (m/z range: 690–800); c, PI (m/z range: 550–660); d, phosphatidylserine (PS) (m/z range: 730–860); e, phosphatidic acid (PA) (m/z range: 660–760); f, phosphatidylcholine (PC); g, sphingomyelin (SM) (m/z range: 675–850); h, lysophosphatidylcholine (LPC) (m/z range: 490–570); i, 12:0–12:0 PE (m/z range: 580–620).

Quantification and Validation of Phospholipid in Human HDL

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. Therefore, we selected a standard of the dipalmitoyl type of PC, PG, PS, PA, PE,

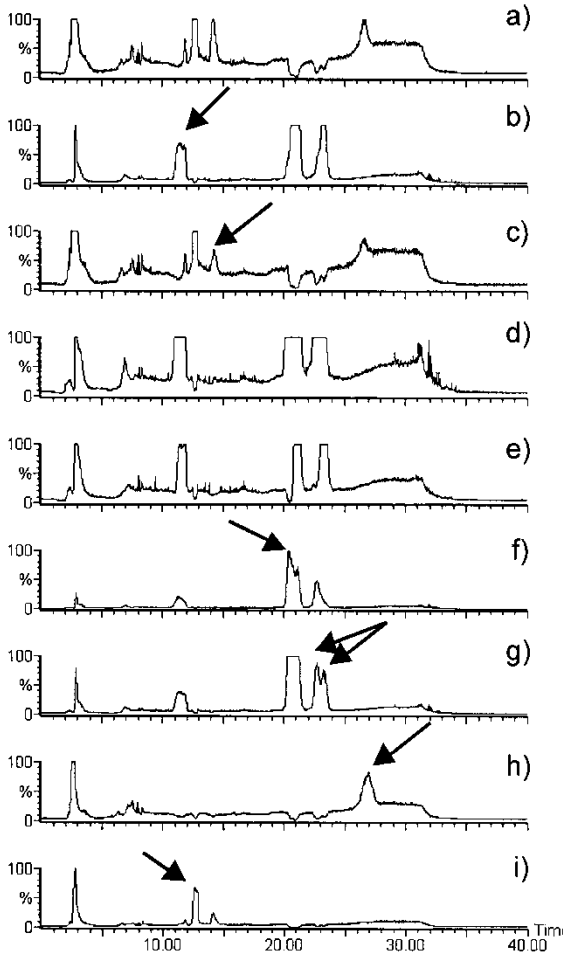

Figure 2. Chromatograms of endogenous phospholipids and internal standard at selected mass range in positive ion mode. a, phosphatidylglycerol (PG) (m/z range: 545–610); b, phosphatidylethanolamine (PE) (m/z range: 690–800); c, PI (m/z range: 550–660); d, phosphatidylserine (PS) (m/z range: 730–860); e, phosphatidic acid (PA) (m/z range: 660–760); f, phosphatidylcholine (PC); g, sphingomyelin (SM) (m/z range: 675–850); h, lysophosphatidylcholine (LPC) (m/z range: 490–570); i, 12:0–12:0 PE (m/z range: 580–620).

the palmitoyl type of LPC, and a mixture of molecular species for SM and PI, for which the dipalmitoyl type was not available. Ion efficiency of individual phospholipids was different in each molecular species. We reported the difference in ion efficiency in phosphatidylcholine molecular species.[13] The difference of ion intensity was lower than the accuracy in this method. Therefore, we concluded the difference of ion intensity was not an important factor in this method.

We applied our method to human HDL. Good separation of PE, PI, PC, SM, and LPC was obtained, but PG, PS, and PA were not detected (Fig. 2). Identification of individual phospholipid classes was confirmed using the mass spectra data and the retention time. Human HDL contains various molecular species. The PC profile is shown in Fig. 3. The chromatograms for quantification and each peak area were obtained from the total ion in the m/z range set for each phospholipid class [see the Experimental section].

For each phospholipid class, the peak area ratios (analytes/IS) for each corresponding calibration curve were plotted versus the concentration of phospholipid in human HDL and fitted to the following equations with $1/x$ weighting: $y = 0.101 + 0.00437x - 0.00000263x^2$ for PG, $y = 0.172 + 0.0115x - 0.00000170x^2$ for PE, $y = -0.0321 + 0.00514x - 0.00000193x^2$

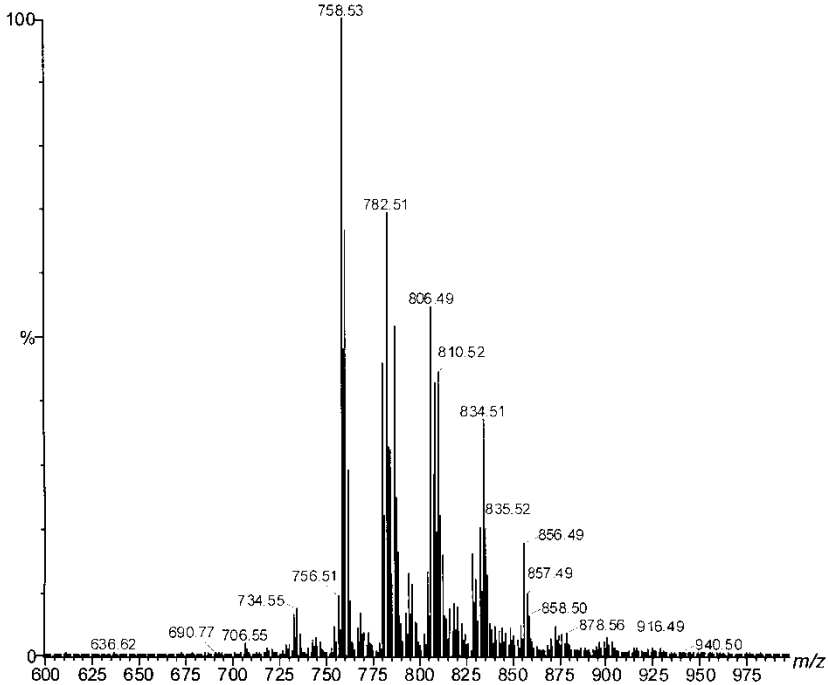

Figure 3. Mass spectrum of phosphatidylcholine (PC) in positive ion mode.

Table 3. Within-run precision and accuracy of the assay method for phospholipids in human high-density lipoprotein (n = 5)

Compound	Concentration added to HDL[a] (μg/mL)	Theoretical concentration (μg/mL)	Mean measured concentration (μg/mL)	Precision (RSD, %)	Accuracy (Bias, %)	Recovery (%)
Phosphatidylglycerol	0	—	nd[b]	—	—	—
	300	300	220	4.5	−26.7	130.2
Phosphatidylethanolamine	0	—	410	32.2	—	—
	1,000	1,410	1,580	2.5	+12.1	112.7
Phosphatidylinositol	0	—	240	6.3	—	—
	306	546	444	6.5	−18.7	115.0
Phosphatidylserine	0	—	nd[b]	—	—	—
	304	304	230	7.0	−24.3	117.7
Phosphatidic acid	0	—	nd[b]	—	—	—
	298	298	327	18.7	+9.7	107.6
Phosphatidylcholine	0	—	12,000[c]	17.2	—	—
	2,550	14,600	14,600[c]	6.2	0.0	57.0
Sphingomyelin	0	—	1,420[c]	16.2	—	—
	10,300	11,700	11,200[c]	6.3	−4.3	88.0
Lysophosphatidylcholine	0	—	211	3.8	—	—
	304	515	558	10.2	+8.3	99.1

[a]High-density lipoprotein.
[b]Not determined.
[c]Diluted 20-fold.

for PI, $y = 0.0578 + 0.00778x - 0.00000281x^2$ for PS, $y = 0.0290 + 0.00217x$ for PA, $y = -0.0109 + 0.00778x - 0.00000594x^2$ for PC, $y = -0.0381 + 0.00989x - 0.00000108x^2$ for SM, $y = -0.0109 + 0.0149x - 0.00000594x^2$ for LPC. All calibration curves had correlation coefficients ranging from 0.9815 to 0.9997. The bias of the back-calculated concentrations of calibration curve ranged from −23.9 to + 31.4%.

We evaluated our method based on the precision and accuracy of the concentration values. First, the endogenous concentration in human HDL was quantified, and the precision was calculated. Second, we quantified the concentration in human HDL to which known concentrations of phospholipid standards had been added, and the precision was calculated. The theoretical concentration for accuracy was calculated from the endogenous concentration and the added concentration. The results of the within-run precision, accuracy, and recovery of eight phospholipid classes in human HDL are summarized in Table 3. The within-run accuracy was between −26.7 and +12.1% with a precision of less than 32.2%. Recovery ranged from 57.0 to 130.2%. If human HDL without endogenous phospholipid had been available as the matrix for the calibration curve, more accurate values should have been obtained because the matrix effect would have been cancelled.

CONCLUSION

In this study, we developed a new LC/MS method to simultaneously and continuously quantitate eight phospholipid classes in biological samples within 40 min cycles with reliability, and demonstrated its applicability to human HDL. Although PS and PA were not detected in human HDL, it was demonstrated that our method makes it possible to quantitate them in the biological samples; their levels increased.

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