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Original Paper

Quantitative analysis of phosphatidylcholine in rat liver tissue by nanoflow liquid chromatography/tandem mass spectrometry

A quantitative method was developed for the determination of phosphatidylcholine (PC) species concentration using nanoflow LC-ESI-MS/MS. In this study, a calibration method is developed to determine the effect of PC carbon chain length on MS peak intensity. Using the multiple standard addition method, a relationship between the peak intensities of different PC species from nanoflow LC-MS and carbon chain length is established first using different injection amounts of PC standards. From this relationship, a calibration curve for each carbon chain length can be obtained for the concentration calculation. It was found that the MS peak area of PC species analyzed by nanoflow LC-MS linearly decreased with increased acyl carbon numbers, and that the effect of the degree of acyl chain unsaturation on MS peak intensity was minimized when the injection amount was maintained at less than 1 pmol. The method was applied for the quantitative calculation of 34 PC species from rat liver, which were identified from data-dependent MS/MS analysis during nanoflow LC separation.

Keywords: LC-MS/MS / Nanoflow LC / Phosphatidylcholine / Quantitative analysis / Rat liver / Tandem mass spectrometry

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1 Introduction

Phospholipids (PLs) are essential in living organisms as building blocks of the cellular membrane as well as playing crucial roles in signal transduction, apoptosis, membrane trafficking, and protein sorting [1 – 4]. As lipid species in higher organisms are so diverse in their compositions, the functions exerted by or involved with these lipid species also vary greatly. However, the quantitative and qualitative analyses of PL compositions and their changes have not been straightforward. This difficulty is mainly due to the complexity of PLs in cells or biological fluids, and due to the lack of a simple yet comprehensive and highly sensitive analytical method. A recent advancement in MS coupled with ESI offers a reliable basis in analyzing PLs with high sensitivity and speed [5 – 7]. While ESI-MS provides an outstanding performance in the resolution and speed of lipidome analysis with advantages of its soft ionization and structural determination using CID, ESI-MS analysis of the complicated mixture of lipids cannot assure an equal efficiency of detection for different lipid species since MS signal intensities may be influenced by acyl chain length, degree of acyl chain unsaturation, and concentration effect [7 – 9]. When HPLC is connected with an ESI-MS, it increases the selectivity of the lipid characterization by reducing spectral congestions, which decrease the ionization suppression of low abundance species [3, 10 – 13]. Despite the added power of LC-ESI-MS, it is still not widely applicable since a sophisticated and computerized analyzing method is not available. For example, an extensive database search of fragmentation ion spectra is needed to characterize complicated lipid mixtures, which vary with polar head groups, chain length, degree of unsaturation, and relative abundances. It is not yet quite clear to explain why MS signals are influenced by acyl chain length and unsaturation. However, it has been reported [7] that ionization of lipid molecules with shorter acyl chains, being more surface active, is more efficient than that of longer acyl chains. It also applies to unsaturated lipid species due to the large surface activity and in addition, double bonds could weaken the intermolecular

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Abbreviations: BPC, base peak chromatogram; **IS,** internal standard; **PC,** phosphatidylcholine; **PE,** phosphatidylethanolamine; **PL,** phospholipid

interactions in droplet surface layer which enhances the evaporation during ESI.

Quantitation of lipid species is of interest since acute or chronic changes of PL compositions are involved in pathological mechanisms and crucial cellular functions such as signal transduction, apoptosis, and protein sorting [7, 14]. Quantitative study of PLs using HPLC-ESI-MS was carried out by calculating the relative MS ion intensity of bacterial PLs in comparison to that of an internal standard (IS) [15], and by simply calculating the peak area of a neutral loss scanning mass chromatogram [16] or peak area of an extracted ion chromatogram [13]. A new method has been developed to quantitatively analyze PLs by utilizing multiple ISs along with the development of software tools [12]. It demonstrated the possibility of analyzing complex lipidomes in an automated way, but the assignment of lipid species still mostly relies on mass measurement.

In this study, a quantitative analysis method along with the simultaneous identification of lipid structures was evaluated using nanoflow LC-ESI-MS/MS. Based on our earlier studies on the separation and characterization of PLs using a shotgun method [13, 17], the current study was focused to develop a quantitative method based on the addition of multiple standards to compensate for the dependency of MS signals on acyl chain carbon number and the total lipid concentration. Our method was applied to phosphatidylcholine (PC) species extracted from rat liver, of which PC species were identified from the data-dependent CID spectrum.

2 Experimental

2.1 Materials and reagents

Seven PC standards were utilized in this study: 12:0/12:0 and 20:0/20:0 were purchased from Avanti Polar Lipids (Alabaster, AL, USA), while 14:0/14:0, 18:0/18:0, 18:0/18:1, 18:0/18:2, and 18:2/18:2 were purchased from Sigma–Aldrich (St. Louis, MO, USA). A 12:0/12:0-phosphatidylethanolamine (PE) was used as an IS in order to correct the peak recovery variation of each PC component from every LC separation. Silica capillaries (20, 50, and 75 μm id, 360 μm od for all) for homemade RPLC columns and for tubing connections from an HPLC pump were purchased from Polymicro Technology LLC (Phoenix, AZ, USA). Packing material for the LC column was RP resin (Magic C18, 5 μm–100 Å) from Michrom Bioresources (Auburn, CA, USA). All solvents (H_2O, CH_3CN, CH_3OH, $CHCl_3$, HCOOH, and isopropanol) used were of HPLC grade.

2.2 Lipid extraction from rat liver

Lipid mixtures were extracted from rat liver by the Bligh and Dyer method [1]. The tissue sample was prepared from a rat liver by mincing and mixing properly, and about 0.1 g of the tissue mixture was homogenized and vortexed with 0.75 mL of $CHCl_3/CH_3OH$ (1:2). Then, 0.25 mL of $CHCl_3$ was added and vortexed again. The solution mixture was washed with 0.25 mL of water. After the mixture was centrifuged at 13 000 rpm for 5 min at room temperature, the upper phase was removed and the lower phase was evaporated by SpeedVac. The extracted lipid powder was weighed as 4 mg in total and was redissolved using chloroform/methanol (5:5), and diluted with methanol/acetonitrile (9:1) in preparation for HPLC separation.

2.3 Nanoflow LC-ESI-MS/MS

RPLC separation was carried out with a model 1200 capillary pump system from Agilent Technologies (Palo Alto, CA, USA), which was equipped with an autosampler. The capillary RPLC column (170 mm × 75 μm id, 360 μm od) was prepared in-house by packing a methanol slurry of 5 μm 100 Å Magic C18 resin at a constant pressure (1000 psi) of He [18]. Before packing, one end of the capillary was first pulled by a flame to make a needle-like emitter having an inner diameter of about 10 μm, and then prepared to make a sol–gel frit 3 mm in length at the needle using a potassium silicate solution. Then, the capillary was packed with C18 resin as explained in earlier reports [19]. The pulled tip capillary column was connected to a PEEK microcross, which was connected with capillary tubing to an HPLC pump, an on–off valve for venting, and to a Pt wire, which applied electric voltage for ESI. The detailed configuration of the column connection and Pt wire can be found in an earlier report [17]. A sample injection was made directly to the capillary column at 300 nL/min for 10 min with the on–off valve closed. After injection, a binary gradient elution began with the following mobile phase compositions: 50:50 CH_3CN/H_2O (solution A), 90:10 isopropanol/CH_3CN (solution B), and both mobile phase solutions contained 0.1% formic acid. The binary gradient condition was varied according to the sample complexity. During gradient elution, mobile phase at a flow rate of 7 μL/min was delivered to the microcross with the on–off valve open, so that only a small portion of flow was transferred to the column at 300 nL/min, and the rest was drained through vent tubing. The flow rate to the RPLC column was adjusted by controlling the length of vent tubing, which was a narrow bore capillary with a 20 μm id and 360 μm od.

For nanoflow LC-ESI-MS/MS analysis, we utilized an LCQ Deca XP MAX IT mass spectrometer from Thermo Finnigan (San Jose, CA, USA). ESI was carried out in the positive ionization mode at a potential of 2.0 kV applied via Pt wire with a capillary temperature of 200°C. For a precursor scan, eluting ions were scanned from 600 to

900 amu first. Following each precursor scan, three tandem (MS/MS) mass spectrometric analyses were carried out in a data-dependent mode (40% normalized collision energy) with the scanned mass ranging from 200 to 900 amu. Identification of PC species was manually made from the characteristic fragment ions in the CID spectrum along with the parent mass recorded during the precursor scan.

3 Results and discussion

Since it is known that acyl chain length has a significant influence on the MS signal intensity of PLs [7, 9], this effect is expected to dominate the LC-ESI-MS analysis of PLs. In order to evaluate the relationship of acyl chain length and the MS signal intensity from an LC-ESI-MS run in detail, experiments were carried out by measuring the relative peak area of four standard phosphatidylcholine (PCs) compared to that of an IS added for the compensation of peak recovery variations in nanoflow LC separations. Figure 1 shows the base peak chromatograms (BPCs) of an equimolar mixture of four PC standards (noted by total acyl chain carbon number with the number of unsaturation; 12:0/12:0, 14:0/14:0, 18:0/18:1, and 20:0/20:0) and an IS (12:0/12:0-PE) obtained by nanoflow LC-ESI-MS. The two chromatograms were obtained from different injection amounts of the standard mixtures (4.0 and 2.0 pmol *per* species, respectively) but from a fixed amount (1.0 pmol) of the IS. Injection volume was fixed at 2 μL throughout all experiments. The injection amount of each sample component, represented with solid lines in Fig. 1, was 2.0 pmol. Separation of PC standards was carried out with a binary gradient elution, which began at 100% mobile phase A (50:50-CH_3CN/H_2O with 0.1% formic acid), was then immediately ramped to 55% mobile phase B (90:10-IPA/CH_3CN with 0.1% formic acid) over 1 min after sample loading, and was then ramped to 90% of B over 60 min. A gradient elution may complicate the evaluation of the carbon chain length effect on ionization efficiency due to the influence of solvent composition. However, gradient elution provides a higher efficiency of the PC separation by reducing peak broadening, along with a decrease in separation time compared to an isocratic elution. By introducing nanoflow separation using a capillary LC column at 300 nL/min, it is expected that this problem can be minimized by reducing the injection amount along with an improved separation resolution and an increased ionization efficiency with a direct nanoflow ESI.

In order to test the relationship between the signal intensity of each PC species and acyl chain length, experiments were further carried out by reducing the amount of PC mixtures injected (fixed injection volume) to 1.0 and 0.5 pmol *per* species. The relative peak area of each

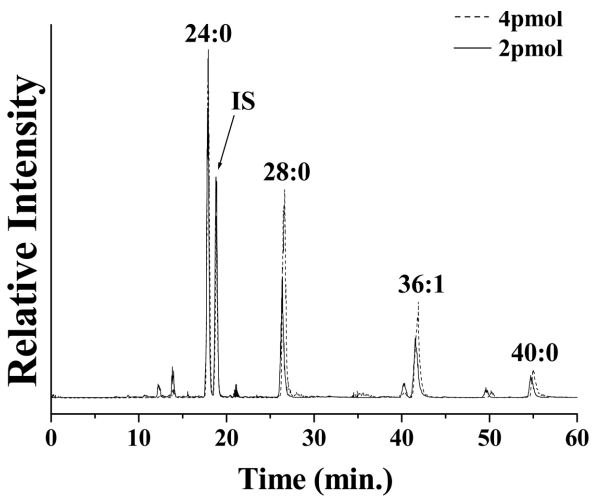

Figure 1. BPCs of an equimolar mixture of four PC standards and an IS (12:0/12:0-PE, 1.0 pmol) by varying injection amounts obtained by nanoflow LC-ESI-MS at positive ionization mode. Binary gradient elution condition can be found in the text.

individual PC species compared to the IS was calculated and the resulting plots of peak area ratio *versus* the total number of acyl chain carbons are shown in Fig. 2a. The 12:0/12:0-PE utilized as an IS was selected because retention of this species during nanoflow LC separation did not overlap with most of the other PC species from rat liver extract. Figure 2a indicates that the relative peak area ratio decreases when acyl chain length increases. The error bars indicate the SD ($n = 5$). Upon decreasing the injection amount to 0.5 pmol *per* species, a good linear relationship between the relative peak area and the carbon number was observed as shown in Fig. 2a, and the correlation coefficients listed in Table 1a appear to be greater than 0.9938. However, the relationship becomes nonlinear when the injection amount of each PC species was greater than 2 pmol. As the injection amount increases to 4 pmol *per* species, the relative peak area for the short acyl chain species (bi-24:0-PC) did not appear as large as expected. While the MS signal intensity significantly decreases with increased acyl chain length as previously reported [7], the decreasing pattern at larger injection amounts (>4.0 pmol) shown in Fig. 2a is less linear than the exponential decrease of a previous report. It was noted that the PC standard containing 36 carbons contained one unsaturation in the acyl chain, but at low injection amounts (≦2 pmol) it was not significantly different from the linear expectation. This is similar to the observations in which the effect of lipid unsaturation and concentration on the MS response was not significant at concentrations less than 0.4 pmol/mL *per* species [7]. In our experiment, we included only one unsaturated lipid standard in the standard mixtures. However, the linear relationship between the peak area and concentra-

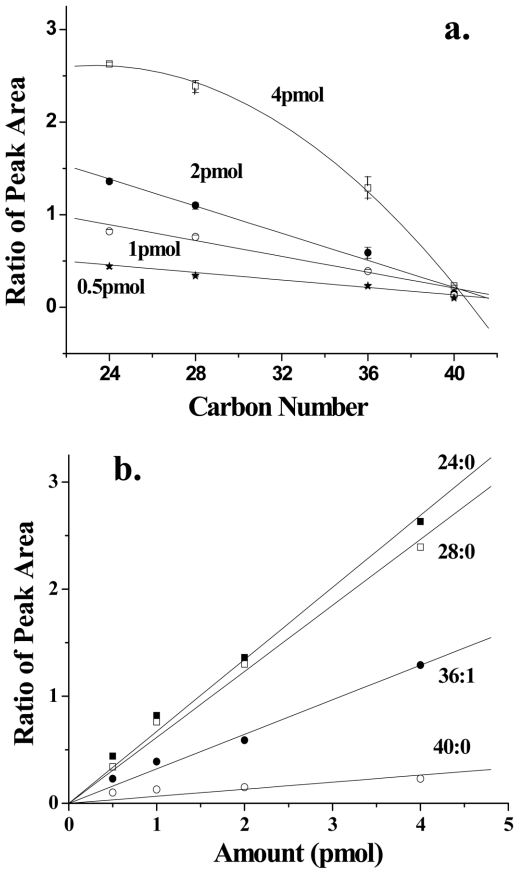

Figure 2. (a) Plots of the ratio of peak area of PC (to IS) at different injection amounts *vs.* carbon number of acyl chain and (b) the calibration plots of the ratio of peak area *vs.* injection amount of each different PC standard.

tion was maintained when the injection amount *per* species was maintained at less than 2 pmol (equivalent to 2 μL injection of 1 pmol/μL PC standard). The effect of unsaturated acyl chains on peak intensity will be discussed later in a separate experiment. The linearity of the relative peak area with respect to the injection amount is examined in Fig. 2b. For the same standard mixtures examined in Fig. 2a, peak area showed a relatively good linear relationship over the range of injection amounts (0.5 – 4.0 pmol) without a serious saturation of MS signal for the IT instrument used in this study. However, a considerable plateau was observed at greater injection amounts (8 pmol) of the shorter PC species (24:0 and 28:0), but this observation was not included in our calibration. The slope values of the calibration curves shown in Fig. 2b are listed in Table 1b. The calculated correlation coefficient values, R^2, listed in Table 1b are greater than 0.9925, and also showed a good linearity. For the calculation of the LOD, 24:0-PC species were tested at low femtomolar injections, and the LOD was calculated as 2.22 ± 0.33 fmol (n = 9) based on S/N = 3.

Table 1. Calibration curve parameters of (a) the ratio of PC peak area (to IS) *vs.* carbon number obtained at different injection amounts and (b) the ratio of peak area *vs.* injection amount shown in Fig. 2

(a) Calibration plot parameters of the carbon number dependence shown in Fig. 2a (n = 5)

Amount injected (pmol)	Type of regression	Regression equation	R^2
0.5	Linear	$y = -0.017x + 0.842$	0.9938
1.0	Linear	$y = -0.043x + 1.918$	0.9857
2.0	Linear	$y = -0.073x + 3.144$	0.9933
4.0	Polynomial-2nd	$y = -0.009x^2 + 0.399x - 2.050$	0.9989

(b) Calibration plot slope values of the injection amount dependence shown in Fig. 2b

Carbon number	Slope	R^2
24	0.672	0.9993
28	0.616	0.9978
36	0.322	0.9946
40	0.066	0.9925

The IS used was 12:0/12:0-PE at a fixed amount of 1 pmol (y is the ratio of peak area and x is the acyl chain carbon number).

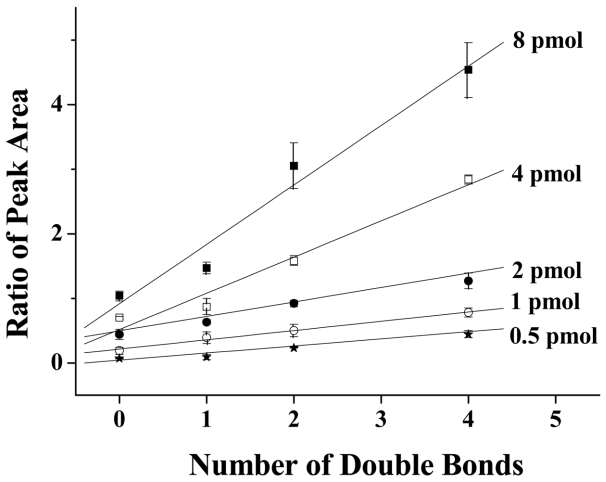

Figure 3. Effect of acyl chain unsaturation on peak intensity (relative to IS) at different injection amounts.

Effect of acyl chain unsaturation on peak intensity in nanoflow LC-ESI-MS analysis was further evaluated using PC standards having the same acyl chain length, but different numbers of double bonds under the same gradient elution condition utilized in Fig. 1. The PC standards used for this examination were 18:0/18:0, 18:0/18:1, 18:0/18:2, and 18:2/18:2. Figure 3 represents the relationship between the relative peak area of each PC species (1 pmol) and the number of double bonds at different injection amounts. When the injection amount of each

Table 2. Linear calibration plot constants of the peak area ratio of 36:*x*-PCs (to IS) *vs.* the number of acyl chain degree of unsaturation at various injection amounts

Amount injected (pmol)	Type of regression	Regression equation	R^2
0.5	Linear	$y = 0.042x + 0.110$	0.9904
1.0	Linear	$y = 0.143x + 0.214$	0.9953
2.0	Linear	$y = 0.224x + 0.496$	0.9958
4.0	Linear	$y = 0.561x + 0.516$	0.9847
8.0	Linear	$y = 0.921x + 0.914$	0.9843

The IS used was 12:0/12:0-PE at a fixed amount of 1 pmol (y is the ratio of peak area and x is the number of unsaturation).

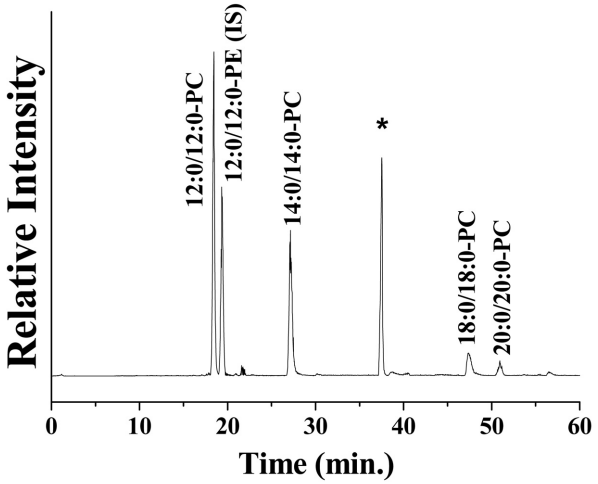

Figure 4. BPC of a 3 pmol of 18:1/16:0-PC test material with four calibration PC standards (marked with chain length) and the same IS (1 pmol fixed). The injection amount of the four calibration PC standards was 1 pmol each. Binary gradient elution condition was the same as used in Fig. 1.

species was 8.0 pmol, the peak area significantly increases as the number of double bonds increases. However, as the injection amount decreases, the slope of the linear calibration curve decreases to 0.143 when 1.0 pmol of each species is injected, and further decreases to 0.42 at a 0.5 pmol injection. The slope values are listed in Table 2. It was found that the effect of acyl chain unsaturation can be less significant when the injection amount of lipid molecules is minimized. Based on this experiment, quantitative studies of rat liver PC extracts were carried out with the least amount of material required.

In order to test the validity of this quantitative method, a known concentration of 18:1/16:0-PC sample was evaluated on a nanoflow LC-ESI-MS. The test sample was mixed with the same calibration standards used in Fig. 2 along with the IS (1 pmol throughout). The 18:1/16:0-PC standard solution to be tested was prepared in triplicate at a fixed concentration of 1.75 pmol/μL, while the calibration standards (12:0/12:0, 14:0/14:0, and 20:0/20:0-PC) were added to each of the three solutions at varying concentrations of 0.25, 0.5, and 1.0 pmol/μL *per* species. Each sample mixture was run (2 μL of each injection) in triplicate using the same gradient elution condition used in Fig. 1. The MS chromatogram is shown in Fig. 4. From the carbon chain length influence on the relative peak area (from the IS peak area) similar to Fig. 1a, three calibration curves were established (plots not shown) and parameters of the calibration curves are listed in Table 3. From the carbon chain dependency, a secondary calibration curve of the relative peak area dependent upon the injection amount was established for the case of PC having 34 carbon chains: $y = 0.351x$ ($R^2 = 0.9821$), where y is the peak area and x is the injection amount. The resulting calculation for the 34:1-PC species was found to be 3.54 ± 0.82 pmol ($n = 5$) and the difference from the original injection amount (3.50 pmol) was less than 2%. In this case, the effect of unsaturation on ionization efficiency was not shown to be critical since the PC species used to test the developed calibration method had one double bond in the *sn*-1 acyl chain (18:1/16:0-PC).

Table 3. Calibration curve parameters (peak area *vs.* carbon number) obtained with multiple internal PC standards

Amount injected (pmol)	Type of regression	Regression equation	R^2
0.5	Linear	$y = -0.029x + 1.198$	0.9984
1.0	Linear	$y = -0.044x + 1.936$	0.9818
2.0	Linear	$y = -0.069x + 2.964$	0.9992

The same IS was used as in Table 1 (y is the ratio of peak area and x is the acyl chain carbon number).

The developed calibration method was utilized to quantify PC species contained in the PL extracts from rat liver tissue. A sample of PL mixtures (100 ng) from rat liver, extracted as previously reported by our laboratory [17], was injected for nanoflow LC-ESI-MS/MS analysis in a positive ionization mode. For calibration, the three calibration standards (12:0/12:0, 14:0/14:0, and 20:0/20:0-PC) were added to the three equal aliquots of the diluted PL extract with various concentrations of the calibration standards, however, the IS concentration was fixed. Figure 5 shows the BPC of the rat liver PL extract along with the three PC standards and an IS obtained using an extended gradient elution condition, beginning with 100% A and immediately ramped to 55% B, then increased to 90% B during 90 min. In this experiment, PE species were detected together, but only PC species were examined for quantitative analysis. Injection amounts for the calibration standards shown in Fig. 5 were 2.0 pmol of each and the same BPCs were obtained by decreasing the injection amount of each standard to 1.0 and 0.5 pmol, respectively (results are not shown). In Fig. 5, a PC standard (36:1-PC) was not included as a calibration standard in

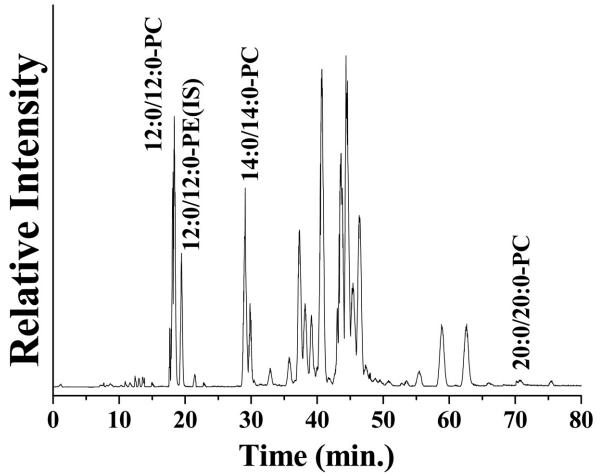

Figure 5. BPC of the rat liver PL extract along with the three PC standards (2 pmol each) and an IS (1 pmol) obtained using the same gradient elution conditions as employed in Fig. 4.

Table 4. Calibration slopes and intercepts obtained for (a) the calibration of PC standards with the IS (same as used in Table 1) dependent upon acyl chain length and (b) for the plot of peak area ratio *vs.* concentration of the added standard PC species in rat liver PL extracts

(a) Calibration constants of the plots shown in Fig. 6a. (*y* is the peak area ratio and *x* is the acyl chain carbon number)

Amount injected (pmol)	Type of regression	Regression equation	R^2
0.5	Linear	$y = -0.029x + 1.184$	0.9999
1.0	Linear	$y = -0.055x + 2.277$	0.9898
2.0	Linear	$y = -0.118x + 4.856$	0.9957

(b) Slope values of the linear plots shown in Fig. 6b

Regression number	Carbon number	Slope	R^2
1	24	0.951	0.9990
2	26	0.881	0.9991
3	28	0.812	1.0000
4	30	0.648	0.9990
5	32	0.531	0.9991
6	34	0.415	0.9990
7	36	0.298	0.9989
8	38	0.181	0.9986
9	40	0.050	0.9773

order to avoid possible peak overlap with other PC species from the PL mixture sample. Initial experiments were processed to identify PC species in which the mass spectrometric analysis was carried out with a precursor scan first, followed by three data-dependent MS/MS scans. From the measured *m/z* values of the PC species from each precursor scan and its characteristic fragment ions observed in tandem MS analysis, 34 PC species were identified. The detailed procedure to identify PC species was explained in earlier publications [13, 17]. The linear relationship between the peak area ratio (from precursor run) of each calibration standard and carbon number was established as shown in Fig. 6a using the three different injection amounts. The resulting calibration curves for calculating the amount of unknown species were plotted for each carbon number in Fig. 6b. The calibration parameters for Fig. 6 are listed in Table 4. The linearity between the peak area and injection amounts as shown in Fig. 6b (correlation coefficients listed in Table 4b) was fairly good except for the PC species having 40 carbons (line 9). The identified 34 PC species are listed in Table 5 along with the calculated values expressed as the number of moles *per* milligram of extracted lipid mixtures. The calculation of each species in Table 5 was originally made using less than 4 pmol *per* 100 ng of lipid mixture injected by dilution. For instance, the first eluted PC species, which is 18:2/22:6-PC in Table 5, was calculated as 0.361 pmol out of 100 ng of PL mixture from the calibration curves. In this case, the influence of acyl chain unsaturation on MS

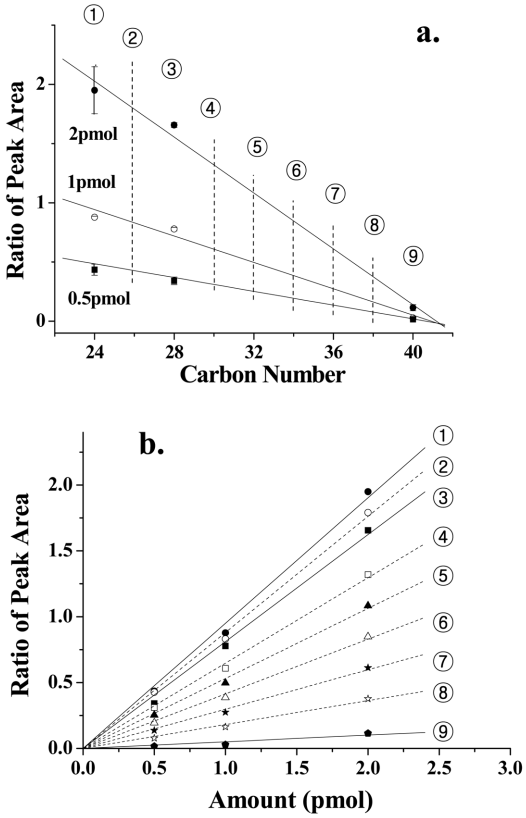

Figure 6. (a) Plots of the ratio of peak area (to IS) of the three calibration standards in Fig. 5 at different injection amounts *vs.* carbon number of acyl chain and (b) calibration plots of the ratio of peak area *vs.* injection amount of each different PC standard marked with numbers (corresponding to the numbers used for PCs of different carbon numbers as listed in Table 4b).

Table 5. Calculated concentration (nmol/mg of the extracted PL mixtures) of 34 PC species extracted from rat liver using a standard addition calibration according to the calibration parameters in Table 4

Class	Molecular Species	*m/z*	Carbon-number	t_r (min)	Amount (nmol/mg)	RSD (n = 9)
PC	18:2/22:6	831	40	32.65	3.61	2.31
	20:4/20:4	831	40	32.71	3.59	1.90
	16:1/20:4	781	36	33.12	0.31	2.84
	18:2/20:4	807	38	37.02	1.66	2.73
	18:1/20:5	807	38	37.25	4.70	2.00
	16:0/22:6	807	38	37.52	18.88	3.90
	18:1/22:6	833	40	37.63	47.34	0.76
	18:1/20:4	809	38	37.72	12.37	1.44
	18:0/22:6	835	40	37.98	33.88	5.14
	16:1/18:2	757	34	38.17	1.95	4.25
	16:0/16:1	733	32	38.51	0.71	2.92
	16:0/20:4	783	36	38.79	11.80	0.96
	16:0/22:5	809	38	38.89	3.51	1.57
	16:0/20:3	785	36	38.95	24.82	0.79
	18:2/18:2	783	36	39.16	4.00	0.69
	16:0/16:0	735	32	39.68	0.61	3.95
	16:0/18:2	759	34	40.02	12.12	3.32
	16:0/18:1	761	34	40.07	12.08	3.81
	18:1/18:2	785	36	40.41	8.16	3.39
	18:0/20:5	809	38	42.67	4.85	3.29
	18:1/20:3	811	38	42.88	9.20	4.06
	18:0/20:4	811	38	43.53	47.69	3.87
	16:0/18:0	763	34	45.79	4.43	1.40
	20:3/20:4	833	40	46.15	0.95	5.36
	18:0/20:3	813	38	46.53	4.20	4.37
	20:2/20:4	835	40	46.59	30.80	3.02
	18:1/18:1	787	36	47.32	6.83	5.19
	22:3/18:4	833	40	47.54	1.18	4.19
	18:0/18:2	787	36	47.92	26.62	1.20
	18:0/18:1	789	36	48.04	25.68	3.05
	16:0/22:4	811	38	48.27	5.87	2.83
	18:0/22:5	837	40	50.16	20.12	3.55
	18:0/20:2	815	38	50.39	8.21	2.35
	18:0/18:0	791	36	53.87	1.54	3.89

intensity can be decreased when the amount of PC species is minimized as explained earlier. However, PC species calculated to be larger than 40 nmol/mg are likely to be overestimated since the original measured value was greater than 4 pmol out of a 100 ng injection as indicated from the calibration curves in which MS intensity varies with the number of double bonds as shown in Fig. 3. They are 18:1/22:6, 18:0/22:6, and 18:0/20:4-PC as shown in Table 5. The overestimated concentration of highly abundant and unsaturated species can be determined if the injection amount is further reduced to minimize the influence of unsaturation. While the calibration methods examined in this study can be applied only for PC species that are sufficiently diluted, this study provides a possibility to estimate the relative PL abundances among different cellular or different physiological states.

4 Concluding remarks

This study demonstrates the use of nanoflow LC-ESI-MS/MS for the quantitative analysis of phospatidylcholines in biological tissue, which can be extended to other PL species in the future. The calibration method used in this study was found to be useful to compensate for the difference in MS intensities of PC species having different acyl chain lengths. While the degree of acyl chain unsaturation influences the ionization efficiency to some degree, it was found that this effect can be minimized if the injection amount is maintained at a low pmol level. This is a limitation of the current method in which experiments should be done with a minimized injection amount, however, this can be overcome by the use of nanoflow LC. In this study, it was demonstrated that both quantitative and qualitative analysis of PC species from complicated lipid mixtures can be achieved by nanoflow LC/MS/MS.

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