Anal Bioanal Chem (2010) 396:1273–1280
DOI 10.1007/s00216-009-3292-9

ORIGINAL PAPER

Quantitative analysis of urinary phospholipids found in patients with breast cancer by nanoflow liquid chromatography–tandem mass spectrometry: II. Negative ion mode analysis of four phospholipid classes

Hye Kyeong Min · Gu Kong · Myeong Hee Moon

Received: 21 September 2009 / Revised: 2 November 2009 / Accepted: 3 November 2009 / Published online: 25 November 2009

Abstract Analysis was performed on four different categories of phospholipids (phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA)) from urine in patients with breast cancer. This quantitative analysis was conducted using nanoflow liquid chromatography–electrospray ionization–tandem mass spectrometry (nLC-ESI-MS-MS). This study shows the profiling of the phospholipids (PLs) that can be identified by the negative ion mode of MS. A previous study (Kim et al. Anal. Bioanal. Chem. 393:1649, 21) focused on only two PL classes: phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) and were identified by positive ion mode. PLs were extracted by lyophilization of 1 mL of urine from both healthy normal females and breast cancer patients before and after surgery. Separation of PLs was performed by nLC followed by structural identification of PLs using data-dependent collision-induced dissociation. A total of 34 urinary PL molecules (12 PSs, 12 PIs, four PGs, and six PAs) were quantitatively examined. Among the four PL categories examined in this study, most PL classes showed an increase in the total amounts in the cancer patients, yet PIs exhibited some decreases. The present study suggests that the lipid composition found in the urine of breast cancer patients can be utilized for the possible development of disease markers, when the analysis is performed with negative ion mode of nLC-ESI-MS-MS.

H. K. Min · M. H. Moon (✉)
Department of Chemistry, Yonsei University,
Seoul 120-749, South Korea
e-mail: mhmoon@yonsei.ac.kr

G. Kong
Department of Pathology, College of Medicine,
Hanyang University,
Seoul 133-791, South Korea

Keywords Phospholipids · Quantitative analysis · nLC-ESI-MS-MS · Urine · Breast cancer

Introduction

The composition of phospholipids (PLs) is very complicated due to their diverse combinations of polar head groups and acyl chain lengths with variations in the degree of unsaturation. While PLs are major constituents of cellular membranes, they also play important roles in cell proliferation and death, as well as in signal transduction [1–3]. Because of the potential of PLs as biomarkers related to adult diseases, lipidomics research is of interest to determine the relative changes in composition and concentration of lipids in cells and biological fluids.

Analytical lipidomics can be carried out with a number of methods such as gas chromatography–mass spectrometry (GC-MS) [4, 5], thin layer chromatography (TLC) with matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) [6, 7], densitometry [8, 9], and high-performance liquid chromatography (HPLC) with spectrometric detections [10, 11] or electrospray ionization MS (ESI-MS) [12, 13]. Studies on the analysis of PLs using ESI-MS alone were also carried out extensively to demonstrate the structural characterization of PLs [14–16]. In terms of lowering the detection limit and achieving structural identification of the PL mixture, nanoflow LC with ESI–tandem mass spectrometry (nLC-ESI-MS-MS) has shown a powerful capability for the simultaneous quantitative analysis of PL mixtures when applied to tissues and urine samples [17–20].

Our group recently reported the quantitative analysis of phosphatidylcholines (PCs) and phsophatidylethanolamine

(PEs) from urine samples of patients with breast cancer before and after their surgery using nLC-ESI-MS-MS in positive ion mode [21]. In this study, we report the second part of the quantitative study for the rest of PL species that were not dealt in the previous work [21]. Compared to the previous study, the negative ion mode of MS detection was employed to analyze phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA). The quantitative analysis of the four categories of PLs was carried out with urine samples from patients with breast cancer and normal healthy controls. The samples were obtained before and after surgery. The results from the patient samples were compared with the results from the healthy controls.

Materials and methods

Materials and urine samples

Calibration standards added to urine samples were selected for at least three species for each PL category: 12:0/12:0-PS, 14:0/14:0-PS, 16:0/16:0-PS, 18:0/18:0-PS,16:0/16:0-PI, 16:0/18:2-PI, 18:0/18:0-PI, 12:0/12:0-PG, 16:0/16:0-PG, 18:0/18:0-PG, 12:0/12:0-PA, 14:0/14:0-PA, 16:0/16:0-PA, and 18:0/18:0-PA. 14:0/14:0-PG was used as an internal standard by adding a fixed amount (0.50 pmol) to each PL mixture extracted from the urine samples in order to compensate for possible fluctuation in the MS signals of a urine sample. The experimental setup for nLC-ESI-MS-MS was identical to that used for the previous study [21]. The capillary LC column was prepared in-house with a silica capillary (75 μm i.d., 360 μm o.d., 17.0 cm length) from Polymicro Technology LLC (Phoenix, AZ, USA) by packing with the reverse-phase resin Magic C18, 5 μm-100 Å from Michrom Bioresources Inc. (Auburn, CA, USA) with He under 1,000 psi. Before packing, one end of the column tip was pulled by flame to make a sharp self-emitter for ESI. HPLC-grade solvents (CH_3CN, CH_3OH, $CHCl_3$, isopropanol, and dH_2O) were used.

The urine samples used were the same as those for the previous study [21] and were collected at Hanyang University Hospital, Korea. The control subjects were five premenopausal women (age range 30–46 years), and the patients with breast cancer were five premenopausal patients (age range 30–44 years). Samples from the breast cancer group were obtained both before and after breast cancer surgery. Each urine sample was pooled, resulting in three groups of urine samples (control, breast cancer patients before surgery, and breast cancer patients after surgery). For extraction of lipids, a lyophilization method was used with 1 mL of each sample. Details of the extraction of urinary lipids can be found in our previous report [21]. The final extracted lipid mixture was dissolved in CH_3OH/CH_3CN (1:1) in a final volume of 0.5 mL and was stored at −20 °C. This mixture was then diluted to a concentration of 5.0 μg/μL with CH_3OH/CH_3CN (9:1) for nLC-ESI-MS-MS analysis, and 1 μL of the diluted mixture was injected for each nLC run.

Nanoflow LC-ESI-MS-MS

A model 1200 capillary pump system from Agilent Technologies (Palo Alto, CA, USA) equipped with an autosampler was interfaced with an LCQ Deca XP MAX ion-trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA). The pulled-tip capillary analytical column used for PL separation was connected with two capillary tubes (one from the HPLC pump and the other for venting the split flow) and with a Pt wire for electrical contact by using a PEEK microcross obtained from Upchurch Scientific (Oak Harbor, WA, USA). Mobile-phase solutions for binary gradient LC separation in the negative ion mode of MS detection were the same as that used for the positive ion mode except for the modifier for negative ionization: 50/50 (*v*/*v*) CH_3CN/dH_2O for A and 90/10 isopropanol/CH_3CN for B, both with 0.05% NH_4OH. Lipid extract was directly loaded on the analytical column with mobile phase A at a rate of 0.3 μL/min for 10 min while the on–off valve, which was located at the end of the vent tubing, was closed. After loading, a binary gradient elution began at a flow rate of 7 μL/min from the pump with the on–off valve opened so that only 300 nL/min of flow was delivered to the analytical column for separation. The role of the vent tubing (20 μm i.d., 360 μm o.d.) was to split the flow so that only a small portion of the migration flow was delivered to the analytical column, which enabled us to manipulate a relatively high-speed flow from the pump to the microcross. This procedure helped to minimize the dwell time. For the negative ion mode, a gradient elution began at 100% mobile phase A, ramped to 55% mobile phase B over 1 min after sample loading, and then linearly increased to 90% of B over 60 min. For electrospray ionization of the nLC eluant, a potential of 3.0 kV (vs. 2.0 kV for positive ion mode in the previous work) was applied via the Pt wire at a capillary temperature of 200 °C. The mass ranges of MS detection were 500–1,000 amu for the precursor scans and 200–900 amu for the MS-MS runs. For collision-induced dissociation, data-dependent analysis mode was used for three prominent ions from each precursor scan under 45% normalized collision energy (vs. 40% in the positive ion mode).

Results and discussion

Lipid mixtures extracted from urine were examined first with the negative ion mode of nLC-ESI-MS-MS for the

structural identification of the different classes of PL species using a data-dependent CID experiment. Then, the same mixture samples were analyzed by running only precursor scans with nLC-ESI-MS for the quantitative analysis of the identified PL species. Figure 1 shows the comparison of the base peak chromatograms (BPCs) of lipid mixtures from the control and breast cancer patients before and after their surgeries in negative ion mode of nLC-ESI-MS-MS. In Fig. 1, the two time frames marked with dotted lines are indicated. In the cases of t_r=13.0 and 14.6 min, clear differences in the peak appearances were seen among the three samples. While the apparent elution patterns of the three samples appeared to be similar to each other, the quantitative analysis of each characterized PL molecule exhibits a significant variation depending on the status of the patient. For the identification of PL species, three CID runs were carried out at each precursor scan during the elution of the PL species by nLC. Figure 2 shows the precursor scan MS spectra (left) at the time frame of 12.06 min of the BPC for the control sample, which exhibits a small number of prominent ions. The two CID spectra obtained for the two precursor ions were marked with acyl chain numbers. Among the few prominent precursor ions, the two ions marked with *m/z* 788.5 and 833.7 were identified as $[M\text{-}H]^-$ ions of 18:0/18:1-PS and 16:0/18:2-PI by their corresponding CID spectra shown at the right side of Fig. 2. The CID spectra of the precursor ion *m/z* 788.5 can be interpreted with the characteristic fragment ions as follows. The base peak fragment ion *m/z* 701.8 represents $[M\text{-}H\text{-}87]^-$ indicating the loss of serine ($C_3H_4NO_2$, 87 Da), the fragment ions of *m/z* 436.8 and 435.9 reflect the loss of a fatty acid in the form of ketene as $[M\text{-}H\text{-}87\text{-}R_2'CH{=}C{=}O]^-$ and $[M\text{-}H\text{-}87\text{-}R_1'CH{=}C{=}O]^-$, respectively. The ion *m/z* 418.9 represents the loss of a fatty acid from the base peak as $[M\text{-}H\text{-}87\text{-}R_2COOH]^-$. Moreover, ions of *m/z* 283.7 and *m/z* 281.5 were found to be the two cleaved carboxylate anions of $[R_1COO]^-$ and $[R_2COO]^-$, respectively. For CID spectra of the precursor ion *m/z* 833.7 shown in the lower right of Fig. 2, the fragment ions *m/z* 571.2 and 553.3 reflect the loss of a fatty acyl chain in two different forms as $[M\text{-}H\text{-}R_2'CH{=}C{=}O]^-$ and $[M\text{-}H\text{-}R_2COOH]^-$, respectively. Likewise for the fragment patterns of PS, ions of *m/z* 415.3 were produced from the loss of carboxylic acid as $[M\text{-}H\text{-}162\text{-}R_1COOH]^-$ and *m/z* 391.1 as $[M\text{-}H\text{-}162\text{-}R_2COOH]^-$, along with the free fatty acid as a form of carboxylate anions such as $[R_2COO]^-$ for *m/z* 279.7 and $[R_1COO]^-$ for *m/z* 255.4. The loss of *m/z* 162 represented the loss of an inositol head group (162 Da). For both PS and PI molecules, shown in Fig. 2, the MS intensity of $[R_1COO]^-$ at negative ion mode was higher than that of $[R_2COO]^-$ in this study.

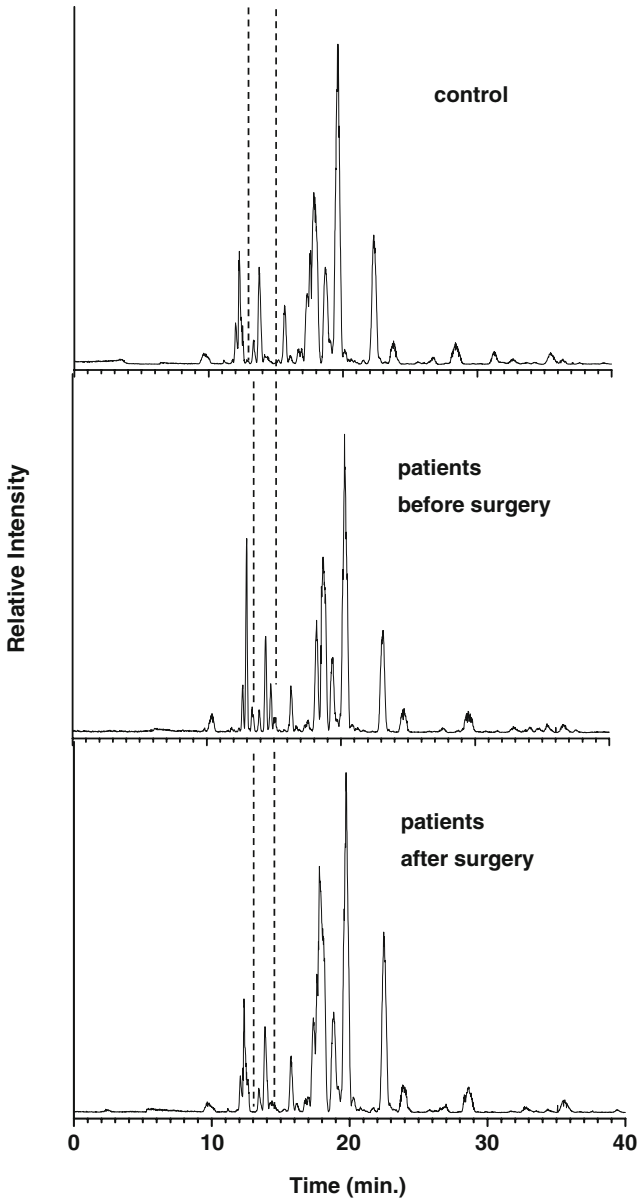

Fig. 1 Comparison of base peak chromatograms (BPCs) of PL extracts from the controls and breast cancer patients before and after surgery by nLC-ESI-MS-MS in negative ion mode

Characterizations of PG and PA molecules, shown in Fig. 3, were performed in a similar way as described for PS and PI molecules. The two prominent ions shown in the precursor scan MS spectra observed at t_r=17.97 min of the control sample in the left of Fig. 3 were identified as 18:0/16:3-PG for *m/z* 715.4 and 20:2/20:4-PA for *m/z* 747.4 from their corresponding CID spectra. For the case of PG, the loss of *m/z* 74 represents the loss of a glycerol group attached to a phosphate. In the case of PA, a relatively simple fragment ion spectra appeared, since there were no attached head groups other than phosphate. The CID spectra of 20:2/20:4-PA at the lower right of Fig. 3 shows the characteristic loss of carboxylic acid as a form of ketene and carboxylic acid. Also seen were the preferential

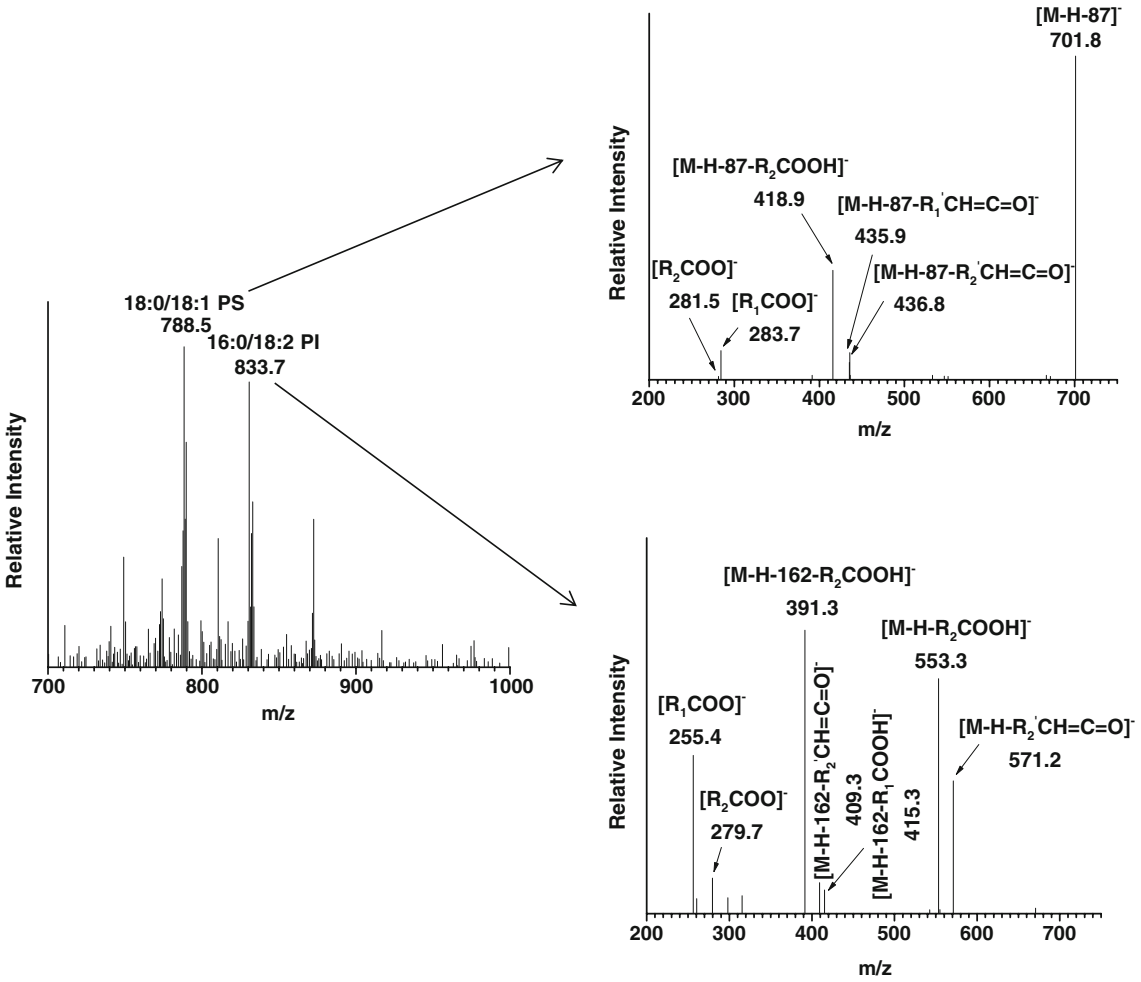

Fig. 2 MS spectra of a precursor scan (*left*) at 12.06 min and the CID spectra of the two precursor ions, *m/z* 788.5 and 833.7, identified as 18:0/18:1-PS and 16:0/18:2-PI, respectively

formation of the ions of $[M\text{-}H\text{-}R_2'CH{=}C{=}O]^-$ > $[M\text{-}H\text{-}R_1'CH{=}C{=}O]^-$ due to fact that loss of ketene is sterically more favorable at sn-2 [22]. For both PG and PA molecules, free carboxylate anions appeared with a higher abundance of $[R_1COO]^-$ compared to $[R_2COO]^-$. Structural identification of PL molecules from the control urine sample resulted in the determination of a total of 34 molecules (12 PSs, 12 PIs, four PGs, and six PAs) by nLC-ESI-MS-MS. Details will be discussed later.

For the quantitative analysis of identified PL molecules, the three PL extracts from each urine sample (control and breast cancer patients before and after surgery) were examined by nLC-ESI-MS in precursor scan mode in order to measure the peak areas of identified PL ions. For the compensation of intensity fluctuations of MS signals, an internal standard (IS, 14:0/14:0-PG) was added to all samples at a fixed amount (0.5 pmol). Then, the relative peak area (vs. IS) was calculated for all identified species for comparison. For the determination of concentration of each species, three or four calibration standards for each type of PL category (refer to the "Materials and methods" section) were added to each PL extract sample by varying the concentration for standard addition calibration. When each sample was analyzed by nLC-ESI-MS in precursor scan only, the ratio of the peak area of calibration standard to that of IS was calculated first and then the two-dimensional calibration curves were established, as shown in Fig. 4. At first, linear regressions (dotted line in Fig. 4) were achieved for each acyl chain length of the PS standards (expressed as total carbon number of two acyl chains in the horizontal axis of Fig. 4) by varying the concentrations that were added to the urine sample. From the relationships between the relative peak areas and carbon numbers, concentration calibration curves were deduced for each carbon length and were plotted in Fig. 4. The regression parameters for the calibration curves of PS molecules for the acyl chain length dependency are listed in Table 1 along with other

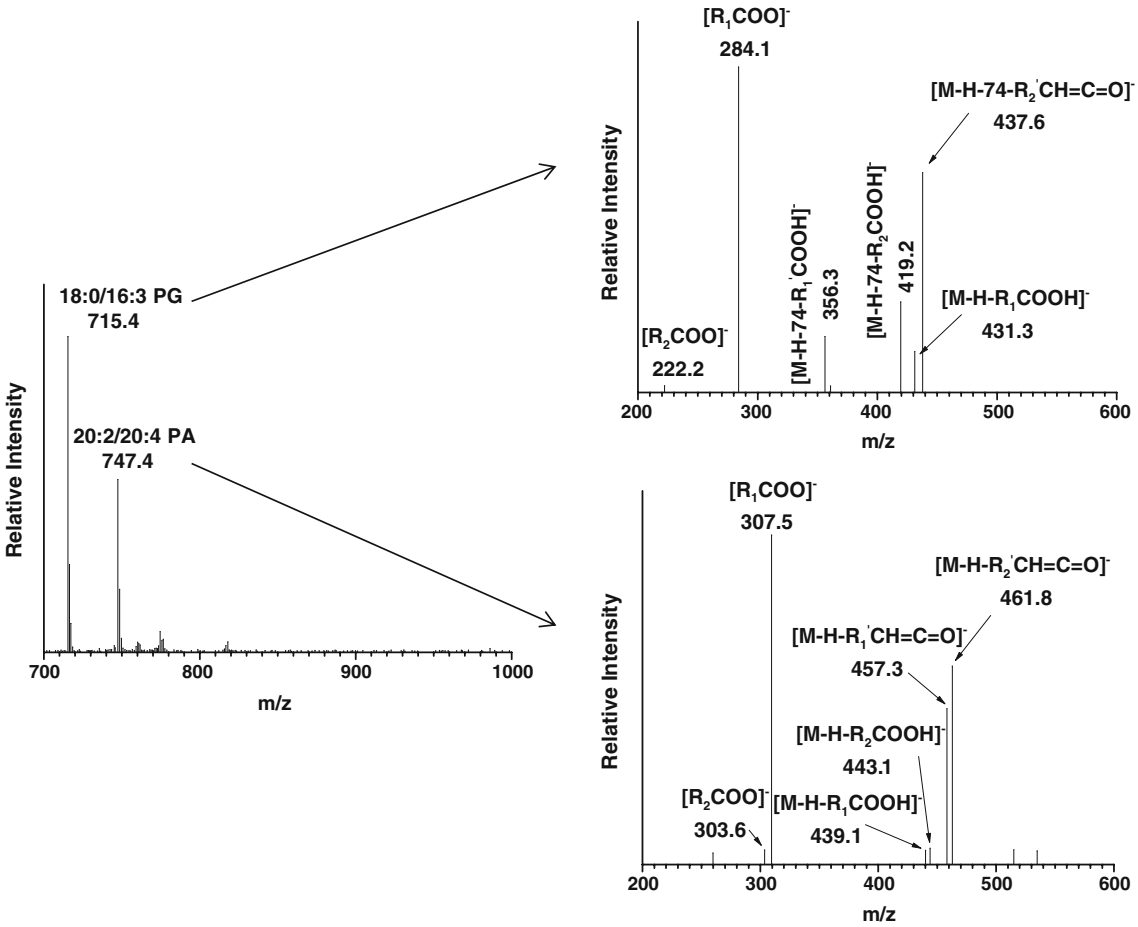

Fig. 3 MS spectra of a precursor scan (*left*) at 17.97 min and the CID spectra of the two precursor ions, *m/z* 715.4 and 747.4, identified as 18:0/16:3-PG and 20:2/20:4-PA, respectively

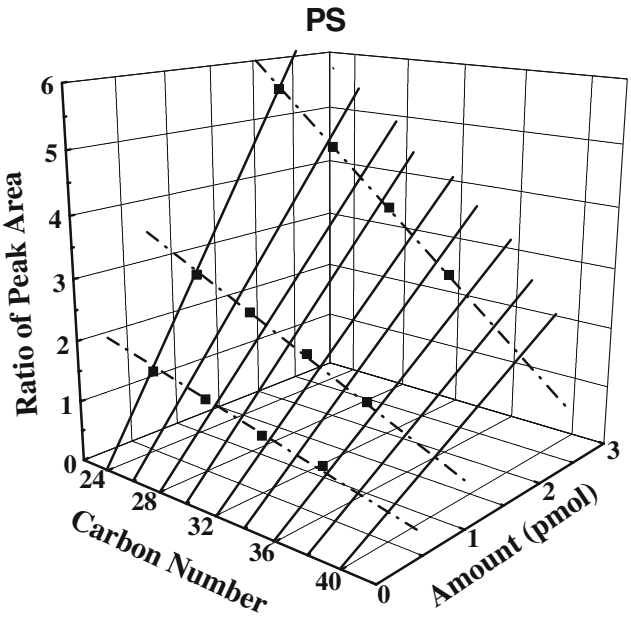

Fig. 4 Calibration curves of the concentration dependency (*solid line*) of each acyl chain length of PS standard deduced from the relationship (*dotted lines*) between relative peak area (vs. IS) and acyl chain length

three PI, PG, and PA series. Calibration parameters for the concentration dependency curves of each species are listed in Table 2. Except for a few species, most results showed good linearities (>0.999). Similar experiments that were carried out with PC and PE series in our earlier report [21] showed that they exhibited good linearities for injection amounts up to 40-fold of the concentration range. Based on this calibration procedure, the concentrations of the identified species (PSs, PIs, PGs, and PAs) from the urine sample were determined.

Identified PL molecules and their concentrations for the three samples in negative ion mode of nLC-ESI-MS-MS are listed in Table 3. For the breast cancer patient samples, the relative ratios of concentration of each molecule to that of control sample are listed together. All molecules except two PI species (16:0/18:1 and 18:6/22:0) were found for the three samples. Among them, few species exhibited a significant change in their concentrations for breast cancer patients. The two PSs (18:1/18:1 and 18:2/18:0) and one PA (18:1/20:1) of the breast cancer patient sample showed concentration increases of more than five times that of the

Table 1 Slopes and intercepts of the linear regression (broken lines in Fig. 3) of the relative peak area vs. carbon length of the acyl chain

Amount injected (pmol)	Regression equation (x = carbon number, y = normalized value; n=5)	R^2
PS		
0.5	$y = -(0.050 \pm 0.004)x + (2.563 \pm 0.133)$	0.9923
1.0	$y = -(0.107 \pm 0.003)x + (5.334 \pm 0.098)$	0.9991
2.0	$y = -(0.215 \pm 0.002)x + (10.754 \pm 0.074)$	0.9998
PI		
0.5	$y = -(0.110 \pm 0.002)x + (4.379 \pm 0.083)$	0.9998
1.0	$y = -(0.147 \pm 0.009)x + (6.344 \pm 0.031)$	0.9980
2.0	$y = -(0.305 \pm 0.071)x + (12.547 \pm 0.020)$	0.9977
PG		
0.5	$y = -(0.041 \pm 0.010)x + (1.789 \pm 0.021)$	0.9988
1.0	$y = -(0.074 \pm 0.009)x + (3.399 \pm 0.041)$	0.9975
2.0	$y = -(0.173 \pm 0.005)x + (7.424 \pm 0.027)$	0.9954
PA		
0.5	$y = -(0.040 \pm 0.002)x + (1.701 \pm 0.006)$	0.9971
1.0	$y = -(0.075 \pm 0.002)x + (3.438 \pm 0.006)$	0.9992
2.0	$y = -(0.121 \pm 0.004)x + (6.678 \pm 0.012)$	0.9988

Relative peak area was obtained in comparison to that of the internal standard. Calibration of each class of phospholipids (PS, PI, PG, and PA) was conducted at three different concentrations

control sample, but the levels decreased to the normal level after surgery except 18:1/20:1-PI which exhibited a further increase. On the other hand, the concentration of 18:0/20:4-PI was found to be decreased more than five times for the breast cancer patient sample.

In Table 4, the overall concentrations of the four PL classes examined for the three sample categories are listed. While the initial calculated total concentrations of PSs, PGs, and PAs from urine samples were increased for breast cancer patients (31.1~67.7%), they appeared to decrease after surgery. However, the total concentration of PIs was reduced by 16.5% in the breast cancer patient pool and decreased further after surgery. It was also noted that the total concentration of PIs was about three to seven times larger than those of the other three categories due to the presence of relatively high-abundance PI species: 18:0/20:4, 16:1/20:2, 20:2/20:2, 20:5/22:1, and 20:2/20:1, for which concentrations were much larger (ten to 50 times) than those of the other species (see Table 3).

Conclusions

This study demonstrates the capability of qualitative and quantitative analysis of 12 PSs, 12 PIs, four PGs, and six PAs in urine samples of patients who have breast cancer using negative ion mode of nLC-ESI-MS-MS. In this study, two PS molecules (18:1/18:1 and 18:2/18:0)

Table 2 Slope values of the calibration plots (solid lines in Fig. 3) of peak area ratio vs. concentration of standard added to each urine sample

Carbon number	PS		PI		PG		PA	
	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
24	2.787±0.010	1.0000	2.690±0.013	0.9997	1.632±0.005	1.0000	1.657±0.048	0.9989
26	2.574±0.008	1.0000	2.381±0.017	0.9997	1.464±0.004	0.9999	1.529±0.039	0.9992
28	2.359±0.006	0.9999	2.067±0.019	0.9996	1.297±0.010	0.9997	1.401±0.030	0.9994
30	2.145±0.005	0.9999	1.762±0.005	0.9999	1.129±0.017	0.9992	1.273±0.021	0.9996
32	1.931±0.005	0.9997	1.452±0.007	0.9999	0.961±0.024	0.9987	1.145±0.012	0.9998
34	1.717±0.006	0.9995	1.143±0.014	0.9997	0.793±0.030	0.9975	1.016±0.005	0.9999
36	1.503±0.008	0.9991	0.833±0.012	0.9998	0.625 ±0.038	0.9964	0.888±0.008	1.0000
38	1.289±0.010	0.9985	0.524±0.022	0.9984	0.458±0.052	0.9957	0.760±0.017	0.9999
40	1.075±0.012	0.9972	0.214±0.037	0.9879	0.290±0.047	0.9961	0.632±0.026	0.9996

Table 3 Calculated concentrations of PS, PI, PG, and PA contained in urine samples of patients with breast cancer using the standard addition method

Class	Molecular species	*m/z*	t_r (min)	Control	Patients before surgery		Patients after surgery	
				Concentration (μg/g)	Concentration (μg/g)	Ratio to control	Concentration (μg/g)	Ratio to control
PS	18:0/20:5	808.6	9.56	3.51±0.31	3.55±0.43	1.01±0.47	3.12±0.33	0.89±0.12
	18:0/20:4	810.5	9.62	1.33±0.36	1.10±0.32	0.83±0.63	1.03±0.07	0.77±0.22
	16:0/20:4	782.6	11.81	0.45±0.07	0.54±0.06	1.20±0.62	3.32±0.56	7.29±1.70
	18:1/18:1	786.6	12.04	0.39±0.04	2.20±1.21	5.61±4.51	0.44±0.10	1.13±0.27
	18:0/18:1	788.5	12.06	1.79±0.38	1.36±0.21	0.76±0.46	1.28±0.05	0.72±0.16
	16:0/18:2	758.7	12.28	6.10±1.32	8.17±2.15	1.34±0.93	6.47±1.47	1.06±0.33
	16:0/18:1	760.5	12.44	2.07±0.50	4.16±1.48	2.01±0.55	1.61±0.19	0.78±0.21
	18:2/18:0	786.6	12.90	0.38±0.04	2.09±0.14	5.57±2.32	0.54±0.11	1.43±0.33
	18:1/18:2	784.6	13.34	1.43±0.23	1.14±0.08	0.80±0.38	1.39±0.34	0.97±0.28
	18:0/20:3	812.5	13.75	0.28±0.07	0.69±0.03	2.44±1.29	0.23±0.04	0.82±0.25
	18:1/20:0	816.7	13.81	1.32±0.28	0.49±0.13	0.37±0.26	1.25±0.02	0.95±0.20
	22:1/18:1	842.6	14.26	0.58±0.25	0.28±0.06	0.48±0.38	0.28±0.02	0.49±0.21
PI	16:0/18:2	833.7	12.07	0.35±0.10	0.46±0.15	1.32±1.02	0.21±0.02	0.59±0.17
	16:0/18:1	835.6	13.50	0.64±0.05	0.63±0.47	0.99±0.90	N.D	N.D
	18:0/20:3	887.6	13.90	0.92±0.13	1.16±0.04	1.25±0.52	1.10±0.21	1.19±0.28
	18:0/20:4	885.6	13.92	10.60±1.07	1.94±0.40	0.18±0.05	1.03±0.13	0.10±0.16
	16:1/20:2	859.6	15.65	18.83±2.41	17.71±3.70	0.94±0.55	13.61±2.03	0.72±0.14
	18:0/18:2	861.6	15.66	2.89±0.11	2.62±0.44	0.91±0.41	2.16±0.43	0.75±0.15
	16:0/20:4	857.6	15.68	1.22±0.30	1.65±0.28	1.35±0.87	0.86±0.06	0.71±0.18
	18:0/18:1	863.6	16.89	2.04±1.22	1.40±0.68	0.69±0.27	1.58±0.34	0.78±0.49
	18:6/22:0	909.8	17.58	2.76±0.22	1.72±0.02	0.62±0.19	N.D	N.D
	20:2/20:2	913.6	19.34	19.79±7.85	23.40±2.22	1.18±0.05	14.32±1.69	0.72±0.02
	20:5/22:1	937.6	19.44	28.45±3.02	19.04±1.72	0.67±0.30	23.20±2.68	0.82±0.13
	20:2/20:1	915.7	19.62	39.39±9.77	35.05±12.59	0.89±0.39	34.57±6.78	0.88±0.28
PG	18:0/14:3	767.2	15.56	1.74±0.37	1.69±0.29	0.97±0.60	1.62±0.13	0.93±0.21
	18:0/16:2	745.5	17.78	4.20±1.44	5.71±0.51	1.12±0.75	4.62±0.57	1.34±0.48
	16:3/18:1	715.4	17.83	11.59±1.15	16.65±1.17	1.44±0.17	17.01±2.80	1.47±0.28
	16:2/20:4	741.8	17.89	0.36±0.01	0.87±0.16	2.40±1.08	1.28±0.40	3.51±1.10
PA	20:1/20:4	749.5	17.85	4.35±1.85	8.06±1.78	1.85±1.49	6.19±1.11	1.42±0.66
	20:2/20:4	747.5	17.89	8.28±3.94	14.96±3.67	1.81±0.97	10.94±0.19	1.32±0.63
	18:1/20:1	727.4	26.05	0.04±0.01	0.99±0.35	24.07±17.63	1.35±0.59	32.75±15.54
	20:1/18:2	725.6	26.65	0.32±0.06	0.78±0.09	2.44±1.32	0.68±0.12	2.13±0.53
	18:0/18:1	701.7	27.71	0.17±0.09	0.55±0.28	3.27±3.36	2.71±0.15	15.06±7.57
	18:0/20:4	723.6	28.39	3.95±0.37	3.36±0.74	0.85±0.48	3.85±0.35	0.97±0.13

N.D. not detected

Table 4 Total amounts of four phospholipid classes contained in urine samples of patients with breast cancer obtained before and after surgery

Species	Total amount (μg/g) ± SD (n=5)		
	Control	Patients before surgery	Patients after surgery
PS	19.63±1.6	25.77±2.94	19.96±1.66
PI	127.86±13.22	106.72±13.46	92.64±7.77
PG	17.89±1.88	24.92±1.32	24.53±2.89
PA	17.11±4.37	28.70±4.17	25.72±1.33

showed significant increases in initial concentration for the breast cancer patient sample; however, their concentrations were found to be reduced to the normal levels for the same patient sample after surgery. In addition, one PI molecule (18:0/20:4) showed a significant decrease in the breast cancer patient sample. Compared to the positive ion mode analysis (for PCs and PEs) for the same samples in the previous report [21], negative ion mode analysis provided a faster separation of urinary PLs (40 min vs. 80 min). While the current study employed only pooled samples, the method can be utilized for high-speed diagnostic purposes for those molecules showing significant changes in abundance once a systematic study with multiple numbers of individual patient samples is carried out.

Acknowledgments This study was supported by a grant (2008-03136) from the Korea Ministry of Science & Technology (MOST) and partly by a grant from the Korea Science and Engineering Foundation through the Center for Bioactive Molecular Hybrids (CBMH).

References

1. Brouwers JFHM, Vernooji EAAM, Tielens AGM, van Golde LMG (1999) J Lipid Res 40:164–169
2. Wright MM, Howe AG, Zaremberg V (2004) Biochem Cell Biol 82:18–26
3. Wolf C, Quinn PJ (2008) Prog Lipid Res 47:15–36
4. Kuksis A, Myher JJ, Marai L (1984) J Am Oil Chem Soc 61:1582–1589
5. Heller DN, Murphy CM, Cotter RJ, Fenselau C, Uy OM (1988) Anal Chem 60:2787–2791
6. Fuchs B, Schiller J, Süβ R, Schürenburg M, Suckau D (2007) Anal Bioanal Chem 389:827–834
7. Rohlfing A, Muthing J, Pojlentz G, Distler I, Peter-Katalinc J, Berkenkamp S, Dreisewerd K (2007) Anal Chem 79:5793–5808
8. Helmy F, Rothenbacher F, Nosavanh L, Lowery J, Juracka A (2007) J Planar Chromatogr Modern TLC 20:209–215
9. Murray KE, Fried B, Sherma J (2007) Acta Chromatographica 18:190–198
10. Kaduce TL, Norton KC, Spector AA (1983) J Lipid Res 24:1398–1404
11. He FQ, Wang CT, Li XK, Liu XQ (2007) J Anal Chem 62:369–372
12. Kim HY, Wang TCL, Ma YC (1994) Anal Chem 66:3977–3982
13. Koivusalo M, Haimi P, Heikinheimo L, Kostiainen R, Somerharaju P (2001) J Lipid Res 42:663–672
14. Hsu FF, Turk J (2001) J Am Soc Mass Spectrom 12:1036–1043
15. Hsu FF, Turk J (2001) J Am Soc Mass Spectrom 11:986–999
16. Hsu FF, Turk J (2005) J Am Soc Mass Spectrom 16:1510–1522
17. Taguchi R, Houjou T, Nakanishi H, Yamazaki T, Ishida M, Imagawa M, Shimizu T (2005) J Chromatogr B 823:26–36
18. Bang DY, Kang D, Moon MH (2006) J Chromatogr A 1104:222–229
19. Bang DY, Ahn E, Moon MH (2007) J Chromatogr B 852:268–277
20. Kim H, Ahn E, Moon MH (2008) Analyst 133:1656–1663
21. Kim H, Min HK, Kong G, Moon MH (2009) Anal Bioanal Chem 393:1649–1656
22. Hsu FF, Turk J (2000) J Am Mass Spectrom 11:797–803