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ORIGINAL PAPER

Quantitative analysis of phosphatidylcholines and phosphatidylethanolamines in urine of patients with breast cancer by nanoflow liquid chromatography/tandem mass spectrometry

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Abstract Phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) from urine of patients with breast cancer were qualitatively and quantitatively analyzed by nanoflow liquid chromatography-electrospray ionization tandem mass spectrometry (nLC-ESI-MS-MS). Urinary phospholipids (PLs) were extracted from three different categories of patients (non-cancer controls and breast cancer patients before and after surgery) by first lyophilizing only 1 mL of urine sample to enrich PLs. Next, nLC-ESI-MS-MS analysis of intact urinary phospholipids was performed, resulting in structural identification of 21 PCs and 12 PEs, followed by quantitative analysis using a multiple standard addition method. This study demonstrated that nLC-ESI-MS-MS can be powerfully utilized for the study of relative changes in the contents and concentration of urinary PCs and PEs from breast cancer patients: total concentration of PCs and PEs of patient sample increased to (144±9)% and (171±11)%, respectively, compared to control sample but they decreased significantly following surgery.

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

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Introduction

Phospholipids (PLs) are a major group of lipids that serve as building blocks for cellular membranes (lipid bilayer) and are involved in a diverse array of functions such as cell signaling and execution of both cellular proliferation and death programs [1–3]. PLs are extremely diverse because they comprise numerous combinations of lipids that vary based on their length, degree of acyl chain saturation, and composition of the polar head group. The growing field of lipidomics studies lipid classes in relation to lipid-related metabolism and signaling processes, both qualitatively and quantitatively, by utilizing novel analytical methods [3]. Indeed, changes in the distribution and concentration of lipid molecules can serve as potential biomarkers for various diseases.

Breast cancer is by far the most common cancer among women, and the diagnosis and treatment of breast cancer continue to garner a great deal of interest within the medical community. Importantly, the phospholipid content of breast cancer tissue has been shown to be higher than that of normal breast tissue because the turnover of phospholipids is higher in carcinomas [4]. Other studies have suggested that cancerous breast tissues have a higher linoleic acid content [5] and lower stearic acid content compared with normal tissues [6]. Furthermore, StarD10, a protein over-expressed in breast cancer, has been demonstrated to selectively extract phosphatidylcholine (PC) and phsophatidylethanolamine (PE) from small unilamellar vesicles and preferentially selects palmitoyl or stearoyl chain lipid species on the sn-1 position and unsaturated fatty acyl chains (18:1 or 18:2) on the sn-2 position [7].

However, the above-mentioned studies were based on the analysis of PLs in tissues for which the sampling method is rather invasive compared to that of other biological fluids

such as blood or urine which are readily collected and reflect various clinical and physiological states. Unfortunately, attempts to study PLs in human urine samples to identify possible disease biomarkers have been ineffective due to the low concentration of PLs in urine and the lack of a suitable analytical method.

With the help of the recent advancements in mass spectrometry (MS) technologies, intact PLs can now be quantitatively and qualitatively analyzed at high speed and sensitivity using electrospray ionization (ESI) [8–10]. However, comprehensive analysis of complex PL mixtures remains beyond the capacity of ESI-MS alone, especially due to the influence of the ion suppression effect. To address this problem, separation methods such as thin-layer chromatography (TLC) and liquid chromatography (LC) have been successfully used to simplify complicated mixtures prior to MS. Furthermore, TLC has been used with phosphorous detection [11], densitometry [12, 13], and even in conjunction with MS analysis of scrapped fractions using matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [14, 15]. High-performance liquid chromatography (HPLC) interfaced with ESI-MS (LC-ESI-MS) has been used to successfully characterize PL species [16–20], while capillary LC with tandem mass spectrometry (LC-ESI-MS-MS) has been employed to analyze PL mixtures and to also provide structural identification while simultaneously lowering the detection limit [21–24]. Very recently, a simple lyophilization method was shown to be useful to extract PLs from small volume of urine for nanoflow liquid chromatography (nLC)-ESI-MS-MS analysis in comparison to conventional ultracentrifugation methods [25].

Based on our earlier studies [24, 25], it is demonstrated in this study that the developed analytical method can be applied for the simultaneous profiling and quantitative analysis of PCs and PEs from human urine samples of breast cancer patients. Human urinary PCs and PEs, extracted by lyophilizing 1 mL of urine from breast cancer patients, were identified by nLC-ESI-MS-MS during which urinary PLs were separated with a fritless pulled tip capillary column followed by data-dependent collision-induced dissociation (CID) for structural identification of PL molecules in a positive ion mode. Identified species were quantitatively analyzed using the standard addition calibration method to examine the concentration variations of patient samples in comparison to control samples.

Materials and methods

Materials and reagents

Two sets of phospholipids were used as calibration standards. The following PC and PE standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA): 12:0/12:0-PC, 18:0/18:0-PC, 20:0/20:0-PC, 12:0/12:0-PE, 14:0/14:0-PE, and 18:0/18:0-PE. To compensate for possible fluctuation in the MS signals of each PC and PE component from every LC separation, an internal standard of 14:0/14:0-PC from Avanti Polar Lipids was added to all urinary PL mixture samples. The capillary tubing used in this study was purchased from Polymicro Technology LLC (Phoenix, AZ, USA); 75 μm id for a homemade RPLC column and 20 or 50 μm id for tubing connections (all have 360 μm od) from the on–off valve to the HPLC pump. The packing material for the RPLC column was the reverse-phase resin Magic C18, 5 μm–100 Å from Michrom Bioresources Inc. (Auburn, CA, USA). All solvents used (H_2O, CH_3CN, HCOOH, NH_4OH, $CH_3CHOHCH_3$, CH_3OH, $CHCl_3$) were of HPLC grade.

Extraction of urinary lipids

Female subjects were recruited from both inpatient and outpatient pools at Hanyang University Hospital, Korea. This study was approved by the Institutional Review Board of Hanyang University Hospital, and informed written consent was obtained from all subjects. Urine samples for healthy control subjects were collected from five premenopausal women (age range 30–46 years). Urine samples for patients with breast cancer, both before and after surgery, were collected from five premenopausal women (age range 30–44 years).

A total of 1 mL of each urine sample was pooled to generate three different urine mixtures (controls, patients before surgery, and patients after surgery). For lyophilization, 1 mL of each pooled sample was placed in a vial and frozen for 15 min in a dry-ice/methanol bath. Frozen urine samples were then transferred to a lyophilizer (Bondiro Fe 5508S Freeze dryer with Shell Freezer, Ilshin Lab Co.) for 12 h to evaporate the liquid phase prior to solvent extraction. After recovering sediments, lipids were extracted with the Folch method [26]. For extraction, the sediments of the 1-mL lyophilized urine samples were re-dispersed with 0.90 mL of 2:1 (*v*/*v*) $CHCl_3$:CH_3OH by vortexing and then left at room temperature for 1 h. The solution mixture was then added to 0.18 mL of H_2O and centrifuged at 13,000 rpm for 5 min at room temperature. After removing the upper phase, the lower phase containing the lipids was recovered and dried with a SpeedVac. The final extracted lipid mixture was re-dissolved in CH_3OH/CH_3CN (1:1) in a final volume of 500 μL; these samples were stored at −20 °C. For nLC-ESI-MS-MS experiments, the final lipid solutions in storage were subsequently diluted to a concentration of 5.0 μg/μL with CH_3OH/CH_3CN (9:1).

Nanoflow LC-ESI-MS-MS

Nanoflow LC separation was carried out with binary gradient elution using a model 1200 capillary pump system

from Agilent Technologies (Palo Alto, CA, USA); the capillary pump was equipped with an autosampler. The RPLC capillary column (170 mm×75 μm id, 360 μm od) was prepared in-house. The tip at the end of the capillary tubing was pulled by flame to make the inner diameter of the tip approximately 10 μm. The capillary column with pulled tip was then packed directly with C18 resins in methanol under a constant pressure (1,000 psi) of He.

The analytical column was placed such that it made electrical contact with a Pt wire to apply electric voltage through a PEEK microcross, which was also connected to the two capillaries leading to the on–off valve through the one port and to the binary pump through the other port. The mobile phase compositions were 50:50 (*v*/*v*) H_2O/CH_3CN for solution A and 90:10 isopropanol/CH_3CN for solution B. For positive ion mode MS detection, 0.1% formic acid was added to both mobile phases. The sample was then directly loaded on the analytical column with mobile phase A at a rate of 0.3 μL/min for 10 min with the on–off valve closed. After sample loading, a binary gradient elution separation was initiated at a high-speed flow rate (7 μL/min) from the pump with the on–off valve opened. A PEEK microcross was used to connect the analytical column from the HPLC pump, which had an on–off valve for both venting (20 μm id, 360 μm od) and pressurizing, to a Pt wire for electrical contact to introduce ESI. When the on–off valve was opened, only 300 nL/min was allowed to pass through the analytical column, while the rest was drained through vent tubing (20 μm id, 360 μm od). By using this configuration, the pump flow could be delivered to the microcross at a high speed such that the dwell time was reduced during gradient elution. A detailed configuration of the column connection and Pt wire has been described previously [24].

For mass spectrometric analysis, an LCQ Deca XP MAX ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) was utilized. Positive ion ESI was carried out with a potential of 2.0 kV applied via the Pt wire at a capillary temperature of 200 °C. Eluting ions were detected first with a precursor scan from 550 to 900 amu, followed by three data-dependent CID runs (40% normalized collision energy) from 200 to 900 amu for structural identification of PC and PE molecules.

Results and discussion

Identification of urinary PC and PE by nLC-ESI-MS-MS

Quantitative analysis of PLs in urine samples from breast cancer patients started with structural identification of individual PC and PE molecules from precursor MS scans; each precursor scan was followed by data-dependent CID runs by nLC-ESI-MS-MS. Figure 1 shows the base peak chromatograms obtained from the three urine samples obtained from control sample, breast cancer patients before surgery, and breast cancer patients after surgery. The amount of lipid extract injected for each sample was 5 μg. The chromatograms in Fig. 1 show the numerous peaks that were eluted over a 70-min period, and, among these peaks, PCs and PEs were identified from CID spectra. The peaks marked by chain lengths and the number of double bonds (i.e., 24:0-PC for 12:0/12:0 PC) in Fig. 1 were from calibration standards and an internal standard (28:0-PC), respectively. These standards were selected such that they were able to identify the samples as they did not overlap with the PC or PE species found in the human urine samples. As shown in Fig. 1, the peaks that appeared at retention times of approximately 15, 40, 43, and 64 min exhibited a significant change in height among the three categories of specimens. For example, the intensities of peaks in these retention time zones were relatively higher for breast cancer patient sample before surgery (middle chromatogram in Fig. 1) than for the other two samples.

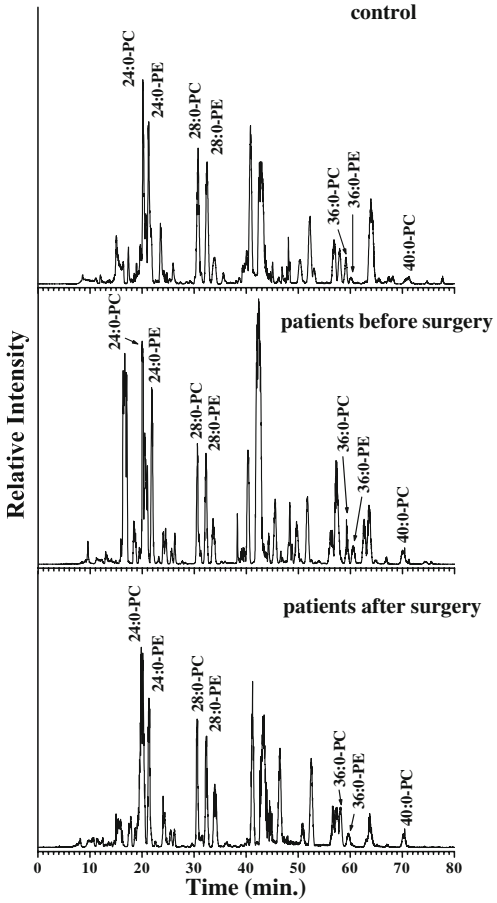

Fig. 1 Base peak chromatograms of PCs and PEs extracted from the control group and the patient group with breast cancer before and after surgery with positive ion mode nanoflow LC-ESI-MS-MS

The variation in the concentration of these species was examined next.

The identification of lipid species in this study focused on PCs and PEs that were present in urine samples because these two categories of PL molecules are favorably detected in positive ion mode. For example, a precursor MS scan of the control sample at 42.71 min is plotted in the left MS spectrum of Fig. 2 and shows three prominent ion peaks at 740.4, 758.6, and 784.5 *m/z*. Each of the precursor ions was selected in sequence for the CID experiment, but only two MS-MS spectra are shown here at the right side of Fig. 2. Since the collision energy for CID was fixed for each case, the fragmentation patterns of the two species (PE and PC) were different. The CID of *m/z* 740.4 was identified as 16:0/20:4-PE with the prominent fragment ion (*m/z* 599.8) by the loss of its polar head group, which was ethanolamine phosphate ($HPO_4(CH_2)_2NH_3$, 141 amu), while all other fragment ions were present at relatively low intensities. Fragment ions of *m/z* 502.8 and 484.7 represented the loss of a fatty acid (sn-1) in the form of ketene ($[M+H-R_1' CH=C=O]^+$) and a carboxylic acid ($[M+H-R_1COOH]^+$), respectively. The ions at *m/z* 454.8 and 436.6 exhibited a similar fragmentation pattern for the sn-2 acyl chain from the precursor. Based on these fragmentation patterns, the precursor ion *m/z* 740.4 was identified as 16:0/20:4-PE. A clearer observation of fragment ions occurred for PC molecules with the same collision energy. The precursor ion *m/z* 784.5 was identified as 18:2/18:1-PC based on the fragment ion *m/z* 601.5 for $[M+H-183]^+$ ($HPO_4(CH_2)_2N(CH_3)_3$, 183 amu) along with subsequent fragment ions of acyl chains as described for the PE case. However, the intensities of the fragment ions for the loss of the acyl chains of the PCs were relatively larger than those observed with the PEs. Furthermore, due to the difference in the optimum energies required for each of the different species, it was impossible to avoid differences in fragmentation efficiency for dual PC/PE detection. Nonetheless, using this method, lists of both PCs and PEs were prepared for more extensive quantitative analysis.

Quantitation of PC and PE of breast cancer patient urine by nLC-ESI-MS

For quantitative analysis of the PCs and PEs identified from the urine samples of the breast cancer patients, extracted lipid samples were combined with calibration standards at varying concentrations (from 0.05 to 2.0 pmol) and analyzed with nLC-ESI-MS without CID to measure the peak area ratio (with respect to an internal standard) of precursor ions that had been previously identified by nLC-ESI-MS-MS. Calibration curves were established for each acyl chain length of the PC and PE standards to account for the difference in MS intensities according to a method described previously [24]. This was achieved by first establishing the relationship between the relative peak area (the ratio of the peak area of each standard to the internal

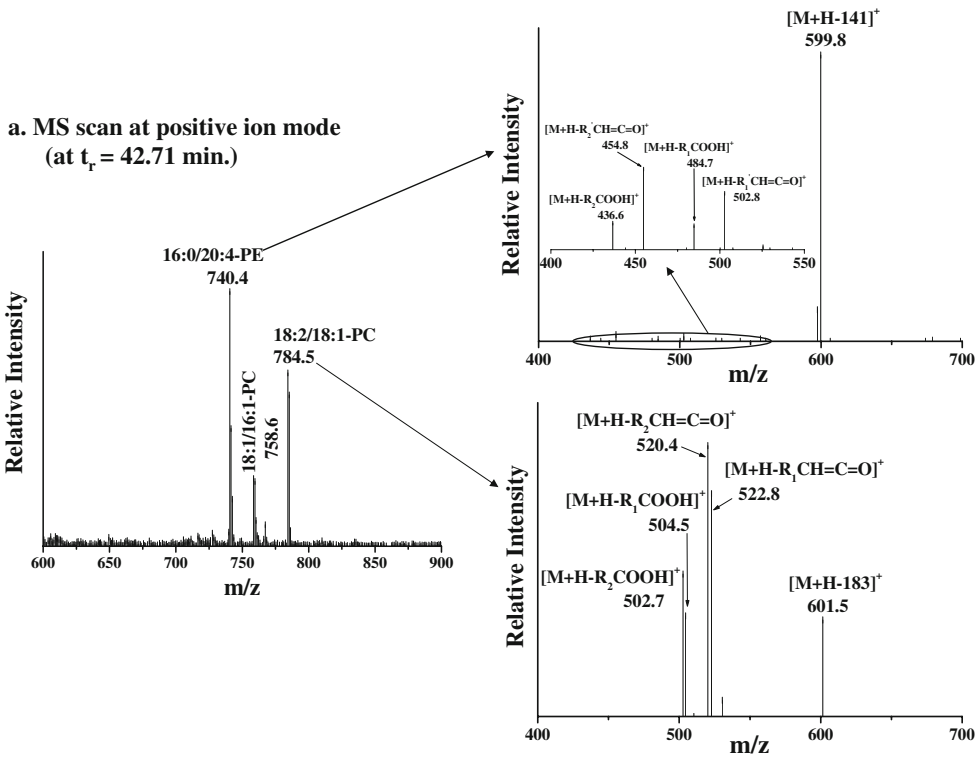

Fig. 2 Precursor MS scan of t_r= 42.71 min obtained from an nLC-ESI-MS-MS run of the patient sample before surgery and the two CID spectra of ions of *m/z* 740.4 and 784.5 by data-dependent MS-MS

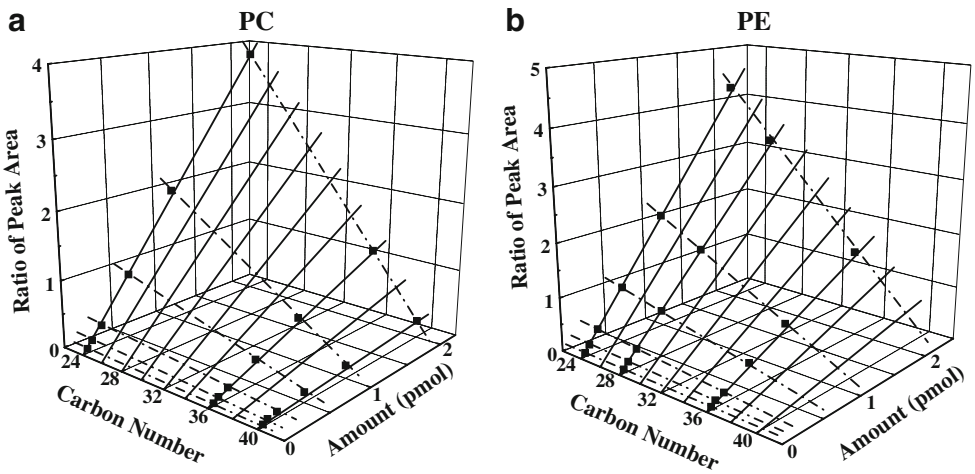

Fig. 3 Three-dimensional calibration curves established from standard (**a**) PCs and (**b**) PEs added to urine sample PL extracts. The *dashed lines* were obtained from a linear regression of data points to take into account the effect of acyl chain carbon number on MS intensity while the *solid lines* were used to calculate a concentration for each carbon number series

standard) for each PC molecule and the lengths of acyl chain by varying the concentration of the calibration standards that were added to the urine samples. Based on the observed relationship, a calibration curve for concentration dependency was deduced for each carbon length. The resulting calibration curves were constructed into two dimensions for each PL species as shown in Fig. 3. In this figure, the calibration curves plotted with dashed lines represent the dependency of peak intensity on the carbon numbers while the curves with solid lines represent the concentration dependency. The parameters for the linear regression of dashed lines are listed in Table 1. Importantly, the dependency of peak area on the acyl chain length appears to be significant at injection amounts up to 2 pmol (shown with slope values of −0.225 for both PCs and PEs); however, this dependency decreased significantly when the injection amount of PL species decreased to 0.05 pmol (as exhibited by a very small slope value of −0.002). The calibration parameters for the concentration dependency curves are listed in Table 2. With the exception of a slightly lower degree of linearity for the long-carbon-chain PC and PE series, the results exhibited an otherwise good linearity (>0.999) for injection amounts up to 40-fold of concentration range. Based on these calibration curves, the concentrations of the identified PC and PE species from the urine samples were calculated. We did not compensate for the effect of unsaturation in acyl chain on MS peak intensity, because this is minimal for injection of lipid molecules at or below 0.5 pmol [24]. In the present study, we minimized the injection amounts of urine sample such that the concentrations of urinary PCs and PEs were detectable at a regime where the effect of unsaturated acyl chains on peak intensity was minimized.

The identified PC and PE species from the control samples and samples from cancer patients before and after surgery are listed in Table 3, along with the concentrations calculated from Fig. 3. Twenty-one PCs and 12 PEs were identified by structural analysis from CID spectra for the

Table 1 Slope and intercept values obtained from the linear regression (dotted lines in Fig. 3) of the peak area ratio of PC and PE standards (relative to the internal standard) vs. carbon lengths of acyl chain

Amount injected (pmol)	Regression equation (x=carbon number, y=peak area ratio; n=5)	R^2
PC		
0.05	$y = -(0.002 \pm 0.001)x + (0.109 \pm 0.012)$	0.9999
0.1	$y = -(0.006 \pm 0.002)x + (0.305 \pm 0.034)$	0.9931
0.2	$y = -(0.015 \pm 0.003)x + (0.690 \pm 0.021)$	0.9984
0.5	$y = -(0.050 \pm 0.011)x + (2.144 \pm 0.028)$	0.9984
1	$y = -(0.114 \pm 0.018)x + (4.747 \pm 0.045)$	0.9962
2	$y = -(0.225 \pm 0.021)x + (9.251 \pm 0.022)$	0.9997
PE		
0.05	$y = -(0.002 \pm 0.001)x + (0.122 \pm 0.023)$	0.9666
0.1	$y = -(0.006 \pm 0.002)x + (0.339 \pm 0.015)$	0.9944
0.2	$y = -(0.020 \pm 0.007)x + (0.901 \pm 0.010)$	0.9996
0.5	$y = -(0.047 \pm 0.012)x + (2.164 \pm 0.021)$	0.9998
1	$y = -(0.100 \pm 0.002)x + (4.793 \pm 0.008)$	1.0000
2	$y = -(0.225 \pm 0.018)x + (9.693 \pm 0.029)$	0.9999

Table 2 Calibration slopes obtained for the plots (solid lines in Fig. 3) of peak area ratio vs. concentration of the added standard PC and PE species in a urine sample

Carbon number	PC		PE	
	Slope	R^2	Slope	R^2
24	1.937±0.025	0.9996	2.143±0.041	0.9999
26	1.713±0.043	0.9995	1.921±0.034	0.9999
28	1.490±0.011	0.9995	1.700±0.012	0.9999
30	1.266±0.038	0.9994	1.478±0.018	0.9999
32	1.043±0.016	0.9993	1.256±0.004	0.9999
34	0.819±0.021	0.9990	1.034±0.015	0.9997
36	0.596±0.018	0.9982	0.812±0.025	0.9991
38	0.372±0.036	0.9951	0.590±0.019	0.9975
40	0.148±0.023	0.9510	0.368±0.021	0.9902

control sample and were sorted according to an increasingly longer retention time. Two PCs (16:0/16:0 and 14:1/20:4) and one PE (18:3/18:0) were not detectable after surgery. Furthermore, Table 3 shows that some species exhibited a twofold to fourfold increase in patients before surgery, but this elevation was decreased after surgery. Relative changes in the concentrations of PCs and PEs from the urine of breast cancer patients collected before and after surgery are plotted in Fig. 4, where the bar graphs represent the relative ratio of concentrations of specific PL molecules to that of the control sample, where positive and negative ratios indicated an increased and decreased fold change, respectively. In the case of PCs, four species (16:1/16:0, 16:0/18:2, 18:1/16:1, and 16:0/16:0) were increased over twofold in patients

Table 3 Calculated concentrations of PC and PE species extracted from the control urine sample and breast cancer patients' urine before and after surgery using standard addition calibration

Class	Molecular species	*m/z*	t_r (min)	Control	Preoperation (PR)		Postoperation (PO)	
				Concentration (μg/g; *n*=5)	Concentration (μg/g; *n*=5)	Ratio to control	Concentration (μg/g; *n*=5)	Ratio to control
PC	18:3/18:4	776.1	18.04	0.30±0.01	0.32±0.14	1.07±0.47	0.25±0.02	0.82±0.07
	18:3/18:3	778.9	19.32	0.19±0.05	0.18±0.16	0.95±0.88	0.17±0.02	0.87±0.26
	18:2/16:1	756.6	23.92	0.84±0.04	0.61±0.55	0.72±0.65	0.53±0.02	0.63±0.04
	18:2/18:2	782.5	25.63	0.27±0.03	0.42±0.08	1.53±0.34	0.36±0.07	1.32±0.31
	18:2/14:3	724.4	26.97	0.62±0.27	0.68±0.29	1.10±0.67	0.78±0.29	1.25±0.72
	18:2/14:2	726.3	35.4	0.64±0.26	0.46±0.21	0.72±0.45	0.34±0.08	0.53±0.25
	16:1/16:0	732.6	38.82	0.22±0.15	0.86±0.55	3.89±0.37	0.23±0.02	1.05±0.69
	16:0/20:5	780.4	39.23	0.56±0.07	0.63±0.27	1.13±0.05	0.25±0.01	0.44±0.06
	16:0/20:4	782.4	40.59	0.71±0.20	1.08±0.27	1.52±0.58	0.36±0.22	0.51±0.34
	16:0/18:2	758.6	41.68	0.07±0.01	0.19±0.06	2.78±0.90	0.16±0.11	2.35±1.59
	18:1/16:1	758.6	41.76	1.45±0.25	3.18±1.31	2.19±0.98	2.18±0.12	1.50±0.27
	18:2/18:1	784.5	42.26	1.83±0.10	3.51±1.03	1.92±0.57	1.62±0.46	0.88±0.25
	16:0/16:0	734.5	45.22	0.43±0.19	1.30±0.58	3.03±1.89	ND	ND
	16:0/18:1	760.4	46.77	2.09±0.68	3.17±1.13	1.51±0.33	2.18±0.34	1.04±0.85
	18:1/18:1	786.5	48.69	2.32±0.69	3.12±1.05	1.35±0.60	2.26±0.66	0.97±0.41
	18:0/20:5	808.6	50.12	0.36±0.03	0.59±0.06	1.65±0.21	0.57±0.13	1.58±0.38
	14:1/20:4	752.4	53.24	0.46±0.20	0.41±0.37	0.90±0.09	ND	ND
	18:0/18:1	788.5	58.11	1.20±0.17	2.27±0.63	1.89±0.59	1.49±0.16	1.24±0.22
	18:0/20:4	810.7	62.35	0.78±0.19	0.92±0.41	1.18±0.60	0.29±0.01	0.37±0.09
	20:3/18:0	812.4	63.76	0.94±0.28	0.66±0.22	0.70±0.13	0.57±0.12	0.60±0.22
	20:2/18:0	814.5	64.23	1.27±0.46	1.19±0.16	0.94±0.20	0.15±0.13	0.12±0.06
PE	18:2/20:4	765.9	39.02	0.21±0.04	0.23±0.14	1.11±0.70	0.09±0.02	0.43±0.12
	18:2/18:2	740.4	42.69	0.39±0.01	1.70±0.27	4.33±0.70	0.43±0.02	1.09±0.05
	16:0/20:4	740.4	42.71	0.80±0.04	1.46±0.64	1.84±0.81	0.17±0.02	0.22±0.03
	18:1/20:4	766.4	43.2	1.58±0.55	2.90±1.11	1.83±0.95	0.70±0.07	0.44±0.16
	16:0/18:2	716.5	43.6	1.14±0.37	2.27±0.87	2.00±1.00	0.15±0.11	0.13±0.11
	18:3/18:0	742.4	44.17	0.72±0.19	0.57±0.76	0.79±0.28	ND	ND
	16:0/18:1	718.6	48.95	1.36±0.57	2.41±1.82	1.77±1.03	0.27±0.02	0.20±0.08
	18:1/18:1	744.4	49.18	1.08±0.13	1.62±0.57	1.50±0.56	0.22±0.02	0.20±0.03
	20:4/18:0	768.3	49.72	0.17±0.04	0.31±0.17	1.86±1.11	0.07±0.05	0.41±0.32
	20:0/18:4	768.3	49.79	1.88±0.19	1.77±0.43	0.94±0.25	0.27±0.11	0.15±0.06
	20:1/18:2	770.5	49.96	0.26±0.05	0.79±0.26	3.08±1.18	0.33±0.12	1.30±0.54
	18:2/20:0	772.9	64.12	0.20±0.05	0.32±0.25	1.60±1.33	0.13±0.05	0.67±0.30

ND not detected

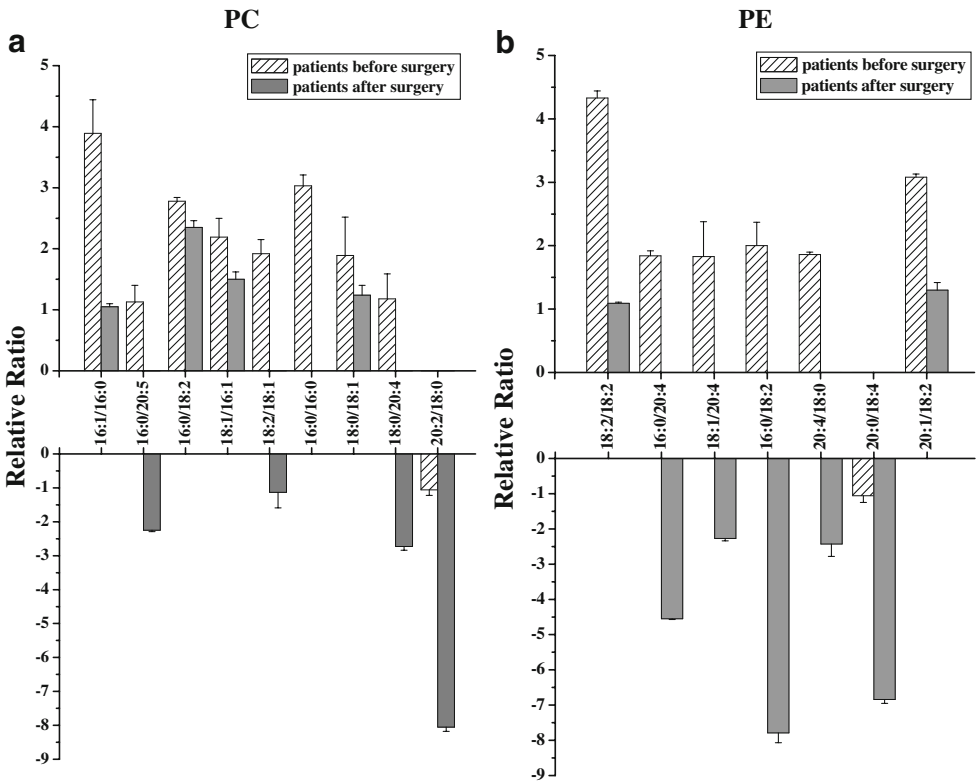

Fig. 4 Relative regulation of (**a**) PC and (**b**) PE species in urine samples showing concentration variations between patient groups before and after surgery

before surgery; however, most of these PCs decreased to the level of the control sample after surgery, with the exception of 16:0/16:0-PC which was not detected after surgery. Likewise, other species showed a significant decrease too after surgery. Similar patterns were observed for the PE species, although relatively more species exhibited a prominent decrease after surgery, including 16:0/20:4, 16:0/18:2, and 20:0/18:4.

In literature, it was reported that PCs in breast tissues with carcinoma have a higher linoleic acid (18:2) content and lower oleic acid (18:1) [5], as well as a lower stearic acid (18:0) content [6] when compared with normal breast tissue. In addition, StarD10, a protein overexpressed in breast cancer, preferentially selected for lipid species containing a palmitoyl (16:0) or stearoyl (18:0) chain via sn-1 chemistry and unsaturated fatty acyl chains (18:1 or 18:2) through the sn-2 position as determined by TLC followed by MALDI-TOF-MS [7]. While a previous study revealed exclusive selection of specific PCs by a lipid transfer protein [7], we provide more detailed information on the relative changes of PC and PE species from urine samples by using online nLC-ESI-MS-MS combined with structural verification. Among the nine PC species identified in our study, six consisted of 16:0 or 18:0 chains from sn-1 position and their levels were significantly changed following surgery, while four species consisted of 18:1 or 18:2 chains from sn-2 position. Similar observations were found for PE species, which showed significant changes in PEs of 18:2 chain species from sn-2 position when comparing urine from controls and urine after breast cancer surgery.

The relative changes in the overall concentration of PCs and PEs from patient urine samples are compared. The total concentration of PCs increased by 44% in breast cancer (26.70±1.68 μg/g) from the concentration in controls (18.55±1.31 μg/g) but was decreased by 20% (14.85± 0.60 μg/g) after surgery. However, the total concentration of PEs was 71% greater in breast cancer patient sample (16.75±0.69 μg/g) than the control sample (9.77±1.03 μg/g), although this decreased significantly by 78% (1.74± 0.12 μg/g) after surgery. The increased concentrations of PCs and PEs found in the urine of breast cancer patients is consistent with an earlier report that showed that breast cancer tissues contain significantly more PCs and PEs (7% to 26.7% increases) than corresponding reference tissues using TLC separation of lipids followed by GC/flame ionization detector analysis [27].

Conclusions

This study shows the utility of nLC-ESI-MS-MS for the profiling of human urinary PCs and PEs extracted from patient urine samples of breast cancer. While a number of studies on phospholipids in relation to breast cancer have focused on cancerous tissues, this is the first trial to demonstrate the possibility of analyzing urinary phospho-

lipids with breast cancer patients. Experiments showed that there was a correlation between the species of PC and PE identified in this study and those in the literature based on tissue analysis. It also carries an advantage of employing a small-volume (1 mL) handling method for extracting PL species from urine. Though the current work has not provided the multiple analysis of numerous samples to distinguish a significant difference among samples due to manual data processing, it shows a potential of the current analytical method that can be utilized for the clinical analysis once a more sophisticated software is provided. Further, such an analysis method would facilitate the intense and systematic investigation of phospholipid profiles of different physiological conditions using biological fluids.

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