Profiling of human urinary phospholipids by nanoflow liquid chromatography/tandem mass spectrometry

Hanna Kim, Eunjeong Ahn and Myeong Hee Moon*

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Nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nanoLC-ESI-MS-MS) was used for the first time in a comprehensive analysis of human urinary phospholipids (PL). PL mixtures from human urine were separated with a reversed phase LC capillary column coupled to ESI-MS-MS. This study used the dual scan method in which two consecutive LC-ESI-MS-MS runs were done in both positive ion mode to detect phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and in negative ion mode to detect phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylglycerol (PG). We focused on identifying the maximum number of PLs from a healthy human urine sample by varying the extracted volume of urine along with the evaluation of extraction efficiency for urinary PLs. We found that 22 PCs, 14 PEs, 15 PIs, 13 PSs, 7 PAs, and 4 PGs were identified during nLC-ESI-MS-MS when phospholipids in urine were extracted by ultracentrifugation. The efficiency of lipid extraction by ultracentrifugation *versus* lyophilization was evaluated by reducing the initial urine volume. We found that lyophilization was more efficient than ultracentrifugation for extracting lipids from small volumes (1 mL) of urine.

Introduction

Lipidomics, a growing field of metabolomics research, examines the extensive classes of lipid molecules, both qualitatively and quantitatively, in relation to changes in lipid metabolism, signal transduction, and apoptosis.[1] Among the complicated lipid molecules, phospholipids (PLs) are a major group of lipids that constitute cell membranes. They are classified into many molecular species based on differences in the length and degree of unsaturation of acyl chains and in polar head groups.[1–3] Due to the potential for developing biomarkers helpful to drug development, there is great interest in monitoring global changes in the distribution and concentration of lipids.

Conventionally, intact phospholipids are studied by analytical techniques such as the simple thin layer chromatography (TLC) with various detection ways: phosphorous detection method,[4,5] matrix-assisted laser desorption and ionization time-of flight mass spectrometry (MALDI-TOF MS),[6,7] and densitometry.[8,9] High performance liquid chromatography (HPLC) is utilized with UV detection,[10,11] and diode array detection (DAD).[12] Recent advances in mass spectrometry (MS) coupled with electrospray ionization (ESI) have allowed the rapid and highly sensitive analysis of PLs[13–15] as well as quantitative analysis,[16] but ESI-MS alone can not bypass the ion suppression effect caused by the complexity of PL molecules. Therefore, chromatographic separation of PL species prior to MS is necessary for the complicated PL mixtures found in biological samples[17] and has expanded this method's capacity to characterize PL species by LC-ESI-MS.[18–23] Recently capillary LC with tandem mass spectrometry (LC-ESI-MS-MS), introduced to enhance identification of PL molecules with a lower detection limit,[24–26] was applied to the simultaneous qualititative and quantitative analysis of PL mixtures from rat liver.[27] Most previous studies have focused on PL species from cellular membranes or tissues, with only a few reports including urine analysis. Nonetheless, urine samples are convenient for noninvasive diagnosis and reflect a variety of clinical and physiological states. TLC with densitometric analysis has documented increased urinary excretion of lipids by patients with kidney stones.[5] Total PLs in patient urine have been quantified by detecting phosphate after digesting PL with a strong acid to produce evidence of elevated urinary PL concentration during treatment with gentamicin.[28] A direct ESI-MS-MS with multiple reaction monitoring (MRM) method facilitated calculation of the total concentration of phosphatidylcholine (PC) species from urine of patients with metachromatic leukodystrophy[29] and Fabry disease.[30] In a recent report, rats treated with gentamicin exhibited increased concentrations of several urinary PLs (9 phosphatidylcholines (PCs), 4 phosphatidylethanolamines (PEs), and 2 phosphatidylinositols (PIs)) based on LC-MS peak area.[31] However, to our knowledge, there has been no comprehensive study profiling PL species from urine samples in detail.

In this study, a shotgun method based on nLC-ESI-MS-MS was employed to separate and characterize various PL molecules in human urine. Urinary PLs were separated using a pulled tip capillary column for nanoflow separation, followed by data-dependent collision induced dissociation (CID) for structural identification of PL molecules. MS detection of PL molecules was carried out in both positive and negative ion modes as reported in our previous study[26] during gradient RPLC runs. Studies were focused to compare the numbers and types of identified PL species from a urine sample of a healthy person by reducing the volume of urine sample that was needed for

Department of Chemistry, Yonsei University, Seoul, 120-749, South Korea. E-mail: mhmoon@yonsei.ac.kr; Fax: 82 2 364 7050; Tel: 82 2 2123 5634

ultracentrifugation method prior to solvent extraction. Results from ultracentrifugation were further compared with those from lyophilization method at minimized volume of urine sample.

Experimental

Materials and reagents

The following PL standards were used to check nLC-ESI-MS-MS experimental conditions: 14:0/14:0-PC, 18:0/18:0-PC (phosphatidylcholine) and 14:0/14:0-PE (phosphatidylethanolamine) from Avanti Polar Lipids (Alabaster, AL, USA), and 16:0/18:2-PI (phosphatidylinositol), 16:0/16:0-PS (phosphatidylserine), and 14:0/14:0-PG (phosphatidylglycerol) from Sigma-Aldrich (St. Louis, MO, USA). Capillary RPLC columns were prepared in the laboratory by packing RP resin (Magic C18, 5 μm-100Å) from Michrom Bioresources Inc. (Auburn, CA, USA) into silica capillary tubes (16.0 cm, 75 μm id and 360 μm od) purchased from Polymicro Technology LLC (Phoenix, AZ, USA). Before packing, one end of each silica capillary was pulled by flame to make a needle with an inner diameter (id) of about 10 μm. The detailed procedure for preparing pulled tip capillary columns is described elsewhere.[27] Other capillaries (20 and 50 μm id) were also used for tubing connections between the HPLC pump and column and for on-off valve connections. HPLC grade solvents (H_2O, CH_3CN, CH_3OH, $CHCl_3$, HCOOH, and isopropanol) were used for nLC-ESI-MS-MS and lipid extraction.

Extraction of lipids from human urine

A urine sample from a healthy individual was obtained with consent and stored at −20°C until use. Two methods were compared for concentrating urinary lipids prior to solvent extraction: ultracentrifugation and freeze-drying. For ultracentrifugation, 10 mL of the urine sample was centrifuged at 4 °C using an Optima L-100XP ultracentrifuge from Beckman Coulter Inc. (Fullerton, CA, USA) at 100 000 *g* for 60 min. After recovering the sediment, urinary lipids were extracted by a procedure slightly modified from the Folch method.[32] Sediments of urine samples were dispersed with 0.90 mL of 2 : 1 (v/v) $CHCl_3$: CH_3OH, vortexed, and then left to stand at room temperature for 1 h. The solution mixture was mixed with H_2O (*e.g.* 0.18 mL for an initial sample volume of 10 mL) and agitated by vortexing. The mixture was then centrifuged at 13 000 rpm for 5 min at room temperature, and only the lower phase containing lipids was recovered for drying with SpeedVac. The final lipid extracts were re-dispersed in CH_3OH/CH_3CN (1 : 1) for storage at −20 °C. When the initial volume of the urine sample was reduced to 5 mL or 1 mL, all volumes of solvent used for the extraction procedure were reduced accordingly. The final lipid solutions in storage were diluted with CH_3OH/CH_3CN (9 : 1) for nLC-ESI-MS-MS experiments at a concentration of 5.0 μg μL^{-1}.

nLC-ESI-MS-MS

Nanoflow RPLC separation was carried out with a pulled tip capillary column (160 mm × 75 μm id, 360 μm od) prepared in our laboratory. RPLC was carried out with a binary gradient elution using a model 1200 capillary pump system from Agilent Technologies (Palo Alto, CA, USA) equipped with an autosampler.

The analytical column was in electrical contact using a Pt wire through a PEEK microcross where the on-off valve and the binary pump were connected together using capillary tubings. Mobile phase compositions were 50 : 50 (v/v) H_2O/CH_3CN for solution A and 90 : 10 isopropanol/CH_3CN for B. To help ionization of lipid molecules during ESI, 0.1% formic acid was added to both mobile phase solutions when used in the positive ion mode of nLC-ESI-MS-MS, and 0.05% NH_4OH was added to both solutions in the negative ion mode. Gradient elution conditions used for nLC separation in the positive ion mode began at 100% mobile phase A, ramped to 55% mobile phase B over 1 min after sample loading, and then linearly increased to 70% of B over 30 min, to 90% of B over 20 min, and to 100% B over 10 min. For the negative ion mode, a modified gradient elution mode was utilized with mobile phases containing ammonium hydroxide, and it 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. The sample was directly loaded in the capillary column using mobile phase A at a flow rate of 300 nL min^{-1} for 10 min. After sample loading, a binary gradient elution for separation at a high speed flow (7 μL min^{-1}) was started, but only a portion of flow was delivered to the capillary column at 300 nL min^{-1} with the on-off valve opened. This restriction reduced dwell time during gradient elution. Pump flow was divided at a PEEK microcross, which was connected with capillary tubing (50 μm id, 360 μm od) to an HPLC pump, an on-off valve for both venting (20 μm id, 360 μm od) and pressurizing, and to a Pt wire that applied electric voltage for ESI. The detailed configuration of the column connection and Pt wire can be found in an earlier report.[27] When the valve was opened, only 300 nL min^{-1} of the flow rate was allowed to pass through the analytical column by controlling the length of the capillary vent tubing.

For MS analysis, an LCQ Deca XP MAX ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) was used. PCs and PEs were identified in the positive ion mode, and PIs, PSs, PGs, and PAs were detected in the negative ion mode. ESI voltages applied to the Pt wire of the PEEK microcross were 2.0 kV for the positive ion mode and 3.0 kV for the negative ion mode. The capillary temperature was fixed at 200 °C. Ions were detected first with precursor scans from 600 to 900 u (positive ion mode) and from 700 to 1000 u (negative ion mode), followed by three data dependent collision induced dissociation (CID) runs (40% normalized collision energy) from 200 to 900 u.

Results & discussion

nLC-ESI-MS-MS of urinary phospholipids

We began our profiling of urinary lipids from humans with an examination of the minimum urine volume needed to enhance the number of identified urinary PL species. Urinary lipid samples were prepared by concentrating different initial volumes of urine (10.0, 5.0, and 1.0 mL) with ultracentrifugation followed by solvent extraction. Fig. 1 shows the nLC-ESI-MS-MS base peak chromatograms (BPCs) of a sample extracted from 1 mL of

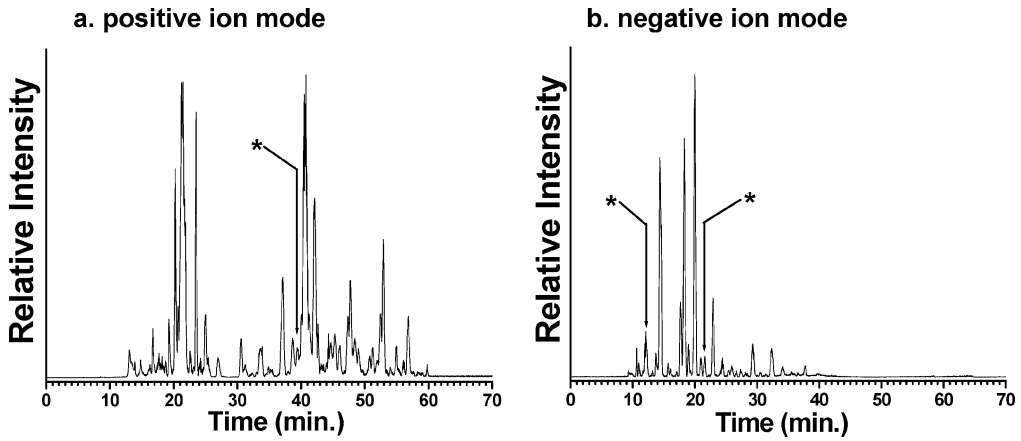

Fig. 1 Base peak chromatograms of phospholipids extracted from 1 mL of human urine in (a) positive ion mode and (b) negative ion mode of nanoflow LC-ESI-MS-MS.

urine. Each PL sample was run twice at each different binary gradient condition for both positive and negative ion modes of MS precursor scans. Since not all PL types (PC, PE, PI, PS, PG, and PA) can be simultaneously detected in a single ion mode of MS, it is necessary to detect them separately at both positive and negative ionization mode.[16] In this work, nLC separation and selective detection of different PL classes at each ion mode of MS detection (PC and PE by positive ion mode and PI and PS by negative ion mode) are based on the dual scan method reported by the previous study,[26] however in this work PG and PA species are newly added for simultaneous identification. Therefore, at positive ion mode in Fig. 1a, nLC of the PL sample extracts show the separation of PC and PE depending on their chain lengths and degree of unsaturation. At negative ion mode in Fig. 1.b, a slightly modified gradient nLC run exhibited the separation of four PL categories: PI, PS, PG, and PA. Injection amounts of urinary lipid extract (containing complicated lipid mixtures) were 15 μg for the run in positive ion mode and 5 μg for the negative ion mode. In this experiment, injection amounts of lipid extract were increased to a level that provided sufficient MS peak intensity for the identification of PLs. The injected amount of urinary lipid extract shown in Fig. 1 was much higher than that needed for PL mixture samples (25–100 ng) from rat liver tissue described in earlier studies.[27,33] This difference is likely due to the fact that urine contains complicated lipid mixtures such as cholesterols and neutral fats that may also be extracted in our extraction procedure. The difference in injection amounts for the samples in Fig. 1a and b originates from the sensitivity difference between positive and negative ion modes of MS for PLs. The nLC run of lipid mixtures in the positive ion mode (Fig. 1a) yielded separation of mostly PCs and PEs up to 60 min, while a similar gradient elution in the negative ion mode (Fig. 1b) resulted in separation of PIs, PSs, PGs, and PAs up to 30 min.

The identification of PLs from human urine by nLC-ESI-MS-MS was carried out with a precursor MS scan, followed by data-dependent CID for structural identification of PLs. For example, a precursor MS scan of the urine sample at 39.85 min in Fig. 1a revealed three prominent ions detected between *m*/*z* 700–850 (Fig. 2a). The three ions *m*/*z* 740.5, 758.5, and 784.6 were then selected for CID. The CID spectra of the first two ions are shown in Fig. 2b and c, respectively. The positive ion *m*/*z* 740.5 was identified as 16:0/20:4-PE based on characteristic fragment ions during CID: *m*/*z* 599.5 $[M + H - 141]^+$ for the loss of ethanolamine ($HPO_4(CH_2)_2NH_3$, 141 u), *m*/*z* 502.6 and 484.4 for the loss of a fatty acid in the form of a ketene ($[M + H - R_1'CH{=}C{=}O]^+$) and a carboxylic acid ($[M + H - R_1COOH]^+$). Similarly, the ion *m*/*z* 758.5 was identified as 18:1/16:1-PC with fragment ions such as *m*/*z* 575.6 for $[M + H - 183]^+$ ($HPO_4(CH_2)_2N(CH_3)_3$, 183 u), *m*/*z* 494.5 and 476.8 for the loss of a fatty acid (sn-1) in the form of a ketene and a carboxylic acid, respectively. The ion *m*/*z* 784.6 was identified in the same manner as 18:2/18:1-PC, but CID results are not shown in Fig. 2. In this experiment, the ion intensities of acyl chain fragments that were lost from PE (enlarged view in Fig. 2b) were relatively weak, while similar ions of acyl chain fragments appeared as intense as the fragment ions that had lost a choline head group (*m*/*z* 183). This change happens when PE and PC are under CID at a fixed collision energy, since PE requires a higher collision energy than PC. If the collision energy is increased to a level which produces fragment ions with increased intensity for PE, it is too strong for PC molecules. The sample measurements were carried out with two more urine samples that were extracted from different volumes of urine. Table 1 lists the identified PC and PE molecules expressed relative to retention time data depending on the initial volume. In the positive ion mode of nLC/ESI/MS/MS, 22 PCs and 14 PEs were identified from the urinary lipid sample extracted from 10 mL of urine. When the urine volume was reduced to 5 mL, 16:0/14:1-PC, 22:0/14:5-PE, and 16:0/22:6-PE were not detected. When 1 mL of urine was used, 16:0/20:5-PC, 16:0/18:2-PC, 20:4/18:0-PE were not found. Consequently, the total numbers of PC and PE molecules detected were 19 and 8, respectively.

In the negative ion mode nLC-ESI-MS-MS shown in Fig. 1b, four different kinds of PLs (PS, PI, PA, and PG) were identified with smaller amounts of injected sample. Precursor MS scans at retention times of 12.05 and 21.46 min (marked with arrows in Fig. 1b) along with CID spectra are shown in Fig. 3 and 4, respectively. From the precursor MS scan at 12.05 min in Fig. 3a, two ions (*m*/*z* 788.5 and 835.6) were selected for CID, which identified them as 18:0/16:1-PS and 16:0/18:1-PI, respectively.

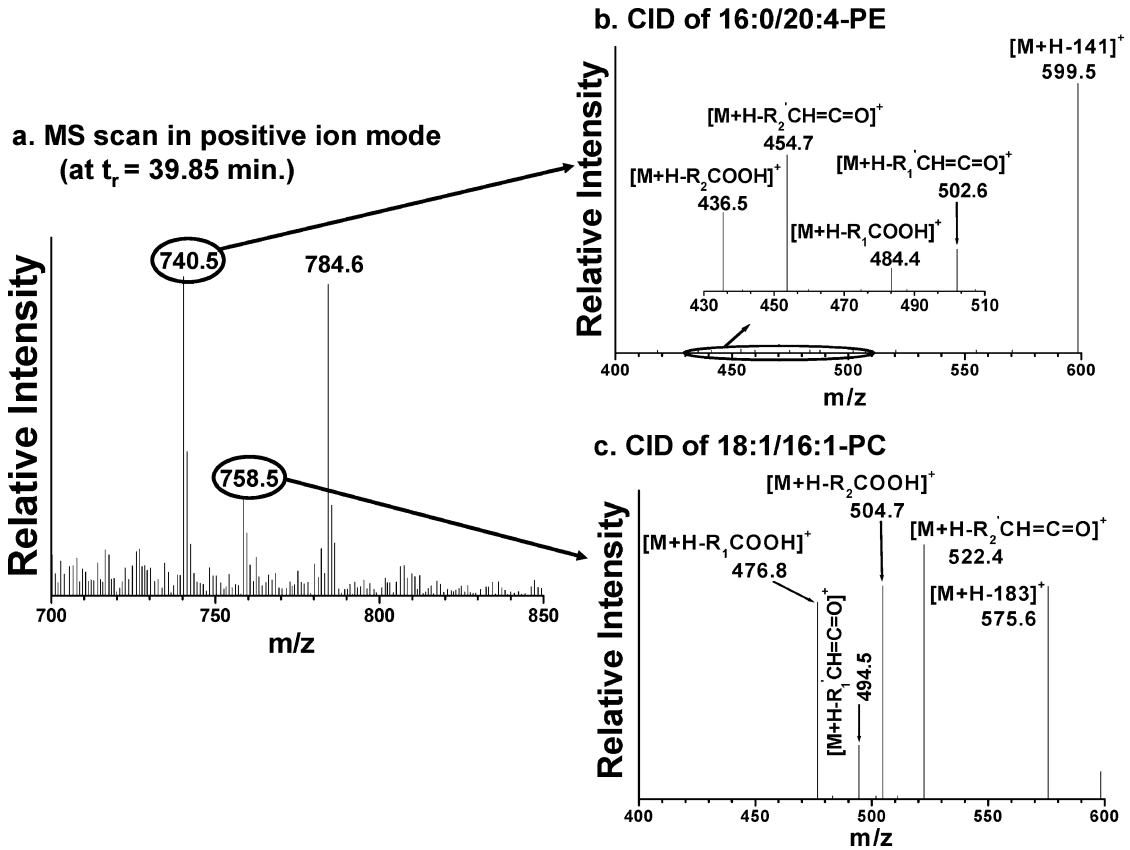

Fig. 2 (a) Precursor MS scan at a retention time of 39.85 min (Fig. 1a) in positive ion mode. Data dependent MS/MS spectra of (b) *m/z* 740.5 (identified as 16:0/20:4-PE) and of (c) *m/z* 758.5 (identified as 18:1/16:1-PC).

Fig. 3b shows a characteristic base peak of $[M - H - 87]^-$ (*m/z* 701.2) that represents the loss of serine ($C_3H_5NO_2$, 87 Da), two carboxylate anions (*m/z* 283.1 for $[R_1COO]^-$ and *m/z* 281.5 for $[R_2COO]^-$), and *m/z* 435.5 and 437.8, which reflect the loss of a fatty acid in the form of ketene as $[M - H - 87 - R_1'CH{=}C{=}O]^-$ and $[M - H - 87 - R_2'CH{=}C{=}O]^-$, respectively. In the case of the PI shown in Fig. 3c, CID of the *m/z* 835.6 ion produced various fragment ions that reflected the loss of a fatty acyl chain as *m/z* 579.5 for $[M - H - R_1COOH]^-$ and *m/z* 553.5 for $[M - H - R_2COOH]^-$, *m/z* 417.3 for $[M - H - 162 - R_1COOH]^-$, and *m/z* 391.1 for $[M - H - 162 - R_2COOH]^-$. The loss of *m/z* 162 represented the loss of an inositol head group (162 Da). We also noted that the $R_1CO_2^-$ anion (*m/z* 255.5) was more abundant than the $R_2CO_2^-$ anion (*m/z* 281.2) in Fig. 3c, which is the same phenomenon observed in earlier experiments.[26] A precursor scan of the 21.46 min peak in the negative ion mode detected two distinct ions (Fig. 4a), which were identified as 18:0/20:4-PA for *m/z* 723.6 and 18:0/16:2-PG for *m/z* 745.5 (Fig. 4b and c, respectively).

Ultracentrifugation *vs.* lyophilization for extraction of urinary PLs

The PL species identified from human urine are listed in Table 1. These species were compared to those identified when the initial volume of urine for extraction was decreased from 10 to 5 and 1 mL. When 10 mL of urine was concentrated by ultracentrifugation, a total of 75 PLs (22 PCs, 14 PEs, 13 PSs, 15 PIs, 7 PAs, and 4 PGs) were identified. The number of identified species decreased to 67 when 5 mL of urine was used, and it dropped further to 62 when 1 mL of urine was used. As noted earlier, the required injection amount of urinary lipid extract was unusually high compared to previous studies using tissue lipid extracts. In this study, a precise quantitative calculation of PL species was not a goal, but the limit of detection was calculated using several standards in the negative ion mode by lowering the injection amount from 3.0 fmol to 0.5 fmol (BPC not shown). Based on calibration of the peak area *vs.* concentration (r^2= 0.9998), the limit of detection (LOD) was calculated as 0.68 fmol (0.45 pg) at a signal-to-noise ratio of 3. To the best of our knowledge, the LOD value observed in this study is the lowest so far reported. Table 1 also lists the PL molecules identified in 1 mL of urine concentrated by lyophilization prior to solvent extraction. A total of 69 PL species (21 PCs, 12 PEs, 13 PIs, 13 PSs, 4 PGs, and 6 PAs) were found when lyophilization was employed, which is slightly greater than the total number of PLs found from 5 mL of urine with ultracentrifugation. To compare the extraction efficiency of the two concentration methods (ultracentrifugation and lyophilization), 1 mL of urine was spiked with 3 pmol of 18:0/18:0-PC standard, and the two lipid extraction procedures were carried out. To compensate for spectral variances, a fixed amount (4 pmol) of internal standard (IS, 14:0/14:0-PC) was

Table 1 Phospholipids identified from 10, 5, and 1 mL of human urine in positive ion (PC and PE) and negative ion (PS, PI, PG, and PA) modes of nLC-ESI-MS-MS

			t_r/min				
			Urine volume for ultracentrifuge			Volume for lyophilization	
Class	Molecular species	*m/z*	10 mL	5 mL	1 mL	1 mL	Relative peak area (%) ($n = 3$)
(a) Positive ion mode							
PC	16:0/14:1	704.5	15.34	N.D.	N.D.	N.D.	N.D.
	18:3/18:4	776.1	18.16	18.02	18.63	18.12	0.91 ± 0.14
	18:3/18:3	778.9	18.72	18.54	19.22	18.63	0.58 ± 0.16
	18:2/16:1	756.6	21.27	21.14	21.73	21.08	3.61 ± 0.55
	18:2/18:2	782.5	24.26	24.01	24.97	24.03	0.82 ± 0.08
	18:2/14:3	724.4	25.86	25.32	26.21	25.30	3.38 ± 0.89
	18:2/14:2	726.3	30.47	30.13	30.81	30.08	3.46 ± 0.81
	16:1/16:0	732.6	35.92	35.75	36.35	35.71	1.16 ± 0.55
	16:0/20:5	780.4	39.58	38.56	N.D.	38.46	1.43 ± 0.27
	16:0/20:4	782.4	39.62	38.72	39.70	38.69	2.08 ± 0.27
	16:0/18:2	758.6	39.69	39.02	N.D.	38.98	0.29 ± 0.06
	18:1/16:1	758.6	39.73	39.10	39.78	39.12	6.18 ± 1.31
	18:2/18:1	784.5	39.85	39.61	39.99	39.50	5.51 ± 1.03
	16:0/16:0	734.5	43.09	42.78	43.13	42.71	2.30 ± 0.58
	16:0/18:1	760.4	43.34	43.16	43.47	43.12	8.17 ± 5.13
	18:1/18:1	786.5	44.93	44.78	45.06	44.68	7.17 ± 3.05
	18:0/20:5	808.6	46.76	46.44	46.86	46.52	0.65 ± 0.06
	14:1/20:4	752.4	48.01	47.79	47.94	47.77	1.88 ± 0.47
	18:0/18:1	788.5	48.41	48.34	48.83	48.38	3.57 ± 0.63
	18:0/20:4	810.7	49.27	49.21	49.72	49.25	1.42 ± 0.41
	20:3/18:0	812.4	51.76	51.52	52.27	52.03	1.66 ± 0.22
	20:2/18:0	814.5	52.75	52.41	52.94	52.69	4.00 ± 0.16
PE	22:0/14:5	738.8	34.56	N.D.	N.D.	N.D.	N.D.
	16:0/22:6	765.9	35.96	N.D.	N.D.	N.D.	N.D.
	18:2/20:4	765.9	38.85	38.46	38.94	38.42	0.63 ± 0.14
	18:2/18:2	740.4	39.81	39.57	39.85	39.47	1.70 ± 0.27
	16:0/20:4	740.4	40.04	39.91	40.42	39.80	3.46 ± 0.64
	18:1/20:4	766.4	40.38	40.27	40.69	40.24	4.90 ± 2.11
	16:0/18:2	716.5	40.92	40.74	41.06	40.65	6.27 ± 0.87
	18:3/18:0	742.4	44.89	44.71	44.98	44.50	3.07 ± 0.76
	16:0/18:1	718.6	45.07	44.84	45.15	44.84	7.41 ± 1.82
	18:1/18:1	744.4	45.33	45.20	45.37	45.07	4.62 ± 0.57
	20:4/18:0	768.3	45.44	45.39	N.D.	45.28	0.51 ± 0.17
	20:0/18:4	768.3	45.51	45.43	45.58	45.39	5.77 ± 1.43
	20:1/18:2	770.5	57.34	56.90	57.47	57.16	0.79 ± 0.26
	18:2/20:0	772.9	57.96	57.59	58.07	57.59	0.62 ± 0.25
(b) Negative ion mode							
PS	16:0/18:2	758.7	9.24	9.36	9.60	9.34	0.85 ± 0.17
	16:0/20:4	782.6	9.26	9.39	9.67	9.38	1.25 ± 0.19
	18:1/18:2	784.6	9.50	9.63	9.84	9.58	0.38 ± 0.08
	18:0/20:5	808.6	9.84	9.92	N.D.	9.90	0.26 ± 0.07
	16:0/18:1	760.5	10.22	10.33	10.59	10.32	9.81 ± 1.22
	18:1/18:1	786.6	10.41	10.48	10.74	10.45	1.31 ± 0.35
	18:0/20:4	810.5	10.57	10.66	10.95	10.58	9.64 ± 2.04
	18:2/18:0	786.6	10.59	10.70	11.02	10.66	6.17 ± 1.63
	18:0/20:3	812.5	11.02	N.D.	N.D.	11.18	0.61 ± 0.07
	18:1/18:0	788.5	11.51	11.59	N.D.	11.56	0.59 ± 0.09
	18:0/18:1	788.5	11.63	11.72	11.94	11.68	39.55 ± 1.11
	18:1/20:0	816.7	13.05	13.52	13.67	13.35	2.36 ± 0.44
	22:1/18:1	842.6	14.89	N.D.	N.D.	14.91	0.51 ± 0.40
PI	16:0/20:4	857.6	10.74	10.79	11.12	10.76	0.48 ± 0.37
	16:0/18:2	833.7	10.82	11.02	11.16	10.94	0.31 ± 0.06
	16:0/18:1	835.6	11.70	11.79	12.09	11.78	0.80 ± 0.07
	18:0/20:4	885.6	12.00	12.07	12.40	12.05	2.65 ± 0.49
	18:0/18:2	861.6	12.06	12.15	12.47	12.13	0.54 ± 0.10
	18:1/18:1	861.6	12.10	12.19	12.53	12.14	0.11 ± 0.01
	18:0/20:3	887.6	12.43	12.54	12.93	12.46	0.51 ± 0.05
	18:0/16:0	837.6	12.85	N.D.	N.D.	N.D.	N.D.
	18:0/18:1	863.6	13.09	13.57	13.69	13.42	0.58 ± 0.23
	16:1/20:2	859.6	15.37	15.44	15.72	15.40	6.29 ± 4.44
	18:6/22:0	909.8	16.04	16.06	16.93	16.04	0.41 ± 0.22
	20:1/18:0	891.8	16.72	N.D.	N.D.	N.D.	N.D.
	20:2/20:2	913.6	19.22	19.28	19.69	19.24	1.75 ± 0.80
	20:2/20:1	915.7	19.55	19.73	19.92	19.60	0.87 ± 0.12
	20:5/22:1	937.6	19.58	19.91	20.00	19.72	0.78 ± 0.36

Table 1 (*Contd.*)

			t_r/min				
			Urine volume for ultracentrifuge			Volume for lyophilization	
Class	Molecular species	*m/z*	10 mL	5 mL	1 mL	1 mL	Relative peak area (%) ($n = 3$)
PG	18:0/14:3	715.4	17.56	17.73	18.29	17.68	3.80 ± 2.26
	18:0/16:2	745.5	19.64	19.98	20.72	19.92	0.59 ± 0.57
	16:3/18:1	741.8	22.11	22.48	23.80	22.44	0.47 ± 0.24
	16:2/20:4	767.2	22.27	23.02	24.08	23.01	0.54 ± 0.30
PA	18:0/20:4	723.6	20.03	20.05	21.58	20.06	1.73 ± 0.85
	20:2/20:4	747.5	20.26	20.22	21.71	20.70	1.15 ± 0.68
	18:0/18:2	699.7	20.48	N.D.	N.D.	N.D.	N.D.
	20:1/20:4	749.5	24.08	24.11	25.91	24.20	0.77 ± 0.26
	20:1/18:2	725.6	24.71	24.84	26.25	24.79	0.36 ± 0.31
	18:0/18:1	701.7	24.81	25.06	26.51	24.89	0.63 ± 0.00
	18:1/20:1	727.4	28.00	28.04	30.06	28.10	0.60 ± 0.05

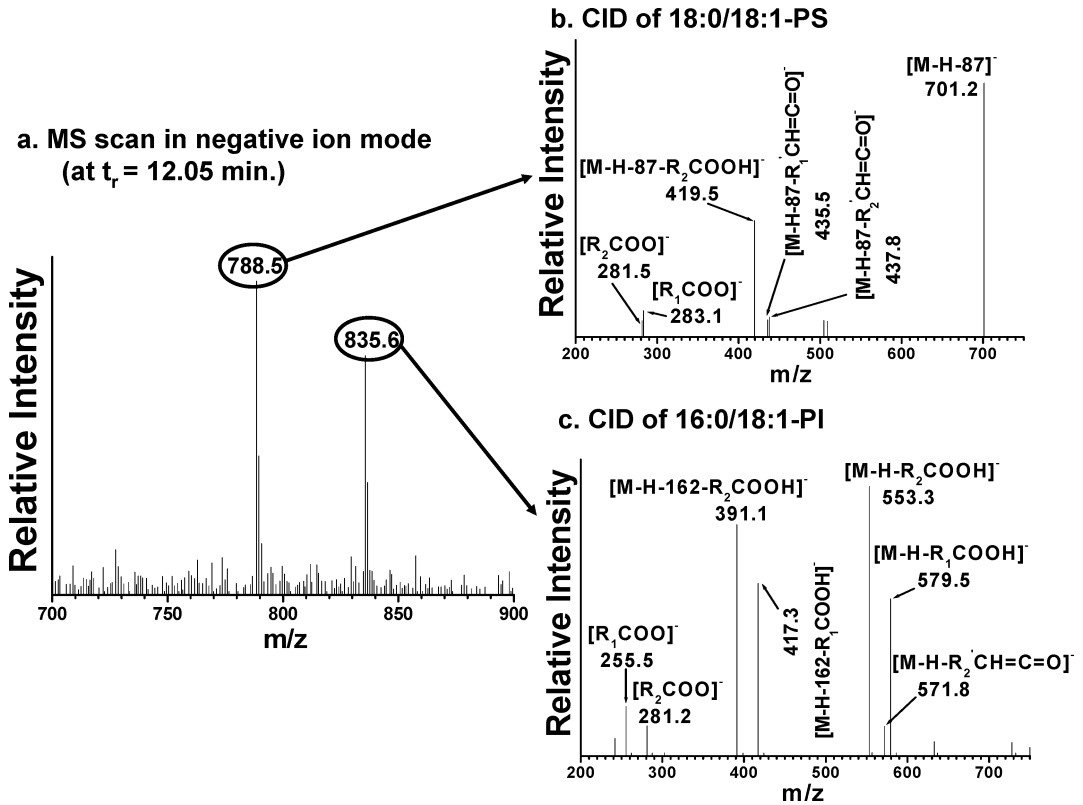

Fig. 3 (a) Precursor MS scan at a retention time of 12.05 min (Fig. 1b) in negative ion mode. MS/MS spectra of (b) *m/z* 788.5 (identified as 18:0/18:1-PS) and of (c) *m/z* 835.6 (identified as 16:0/18:1-PI).

added to both urine samples. The peak area ratio (relative to IS) was calculated as 53.3 ± 4.6% ($n = 5$) when ultracentrifugation was used. The extraction efficiency increased to 63.2 ± 8.8% ($n = 5$) following lyophilization. This increase in extraction efficiency allowed us to successfully identify seven additional species not found when ultracentrifugation was used, including 16:0/20:5-PC, 16:0/18:2-PC, 20:4/18:0-PE, 18:0/20:5-PS, 18:0/20:3-PS, 18:1/18:0-PS, and 22:1/18:1PS. The relative peak area of each of these species was mostly less than 1% (Table 1), indicating that these molecules were present in the urine at low abundance. Although the lyophilization method was more effective for extracting urinary lipids than the ultracentrifuge method, the recovery of the phospholipid standard was still low (~63%). Systematic examination of sample preparation methods is needed to improve recovery and extraction efficiency.

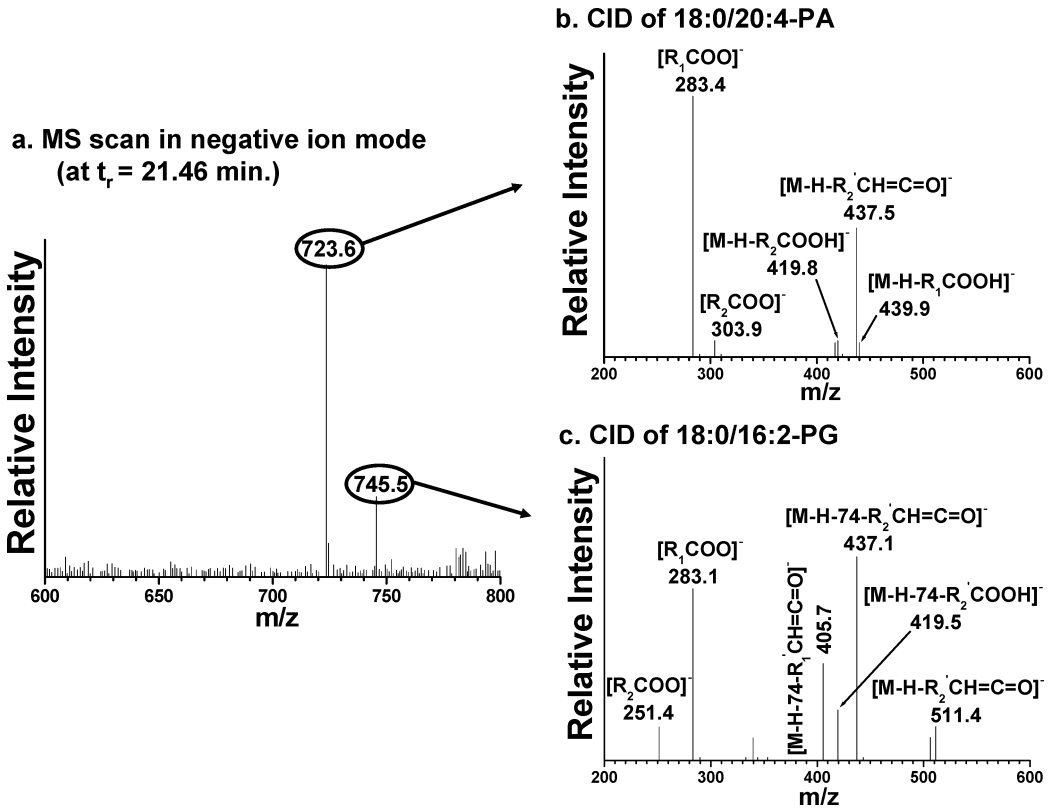

Fig. 4 (a) Precursor MS scan at a retention time of 21.46 min (Fig. 1b) in negative ion mode. MS/MS spectra of (b) *m/z* 723.6 (identified as 18:0/20:4-PA) and of (c) *m/z* 745.5 (identified as 18:0/16:2-PG).

Conclusions

It is demonstrated that nLC-ESI-MS-MS method with dual scan method can be utilized as a highly sensitive tool for the separation and the simultaneous identification of complex PL mixtures from human urine. In concentrating small volume (1 mL) of urine samples prior to solvent extraction, it is found that lyophilization method provides a higher recovery of phospholipids from urine which leads to enlarge the identification number of PLs than ultracentrifugation. Though the current study is focused to demonstrate the separation and selective identification of six (PC, PE, PI, PS, PG, and PA) families of PL species from urinary extracts, it will provide the potential of nLC-ESI-MS-MS for pattern analysis and examination of the relative regulation of urinary PL species or biological fluids. Such information may contribute to the development of potential biomarkers related to disease status.

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References

1 C. Wolf and P. J. Quinn, *Prog. Lipid Res.*, 2008, **47**, 15–36.
2 J. F. H. M. Brouwers, E. A. A. M. Vernooji, A. G. M. Tielens and L. M. G. van Golde, *J. Lipid Res.*, 1999, **40**, 164–169.
3 M. M. Wright, A. G. Howe and V. Zaremberg, *Biochem. Cell Biol.*, 2004, **82**, 18–26.
4 G. Rouser, S. Fkeischer and A. Yamamoto, *Lipids*, 1970, **5**, 494–496.
5 S. R. Khan and P. A. Gleton, *Br. J. Urol.*, 1996, **77**, 506–511.
6 B. Fuchs, J. Schiller, R. Süβ, M. Schürenburg and D. Suckau, *Anal. Bioanal. Chem.*, 2007, **389**, 827–834.
7 A. Rohlfing, J. Muthing, G. Pojlentz, I. Distler, J. Peter-Katalinc, S. Berkenkamp and K. Dreisewerd, *Anal. Chem.*, 2007, **79**, 5793–5808.
8 F. Helmy, F. Rothenbacher, L. Nosavanh, J. Lowery and A. Juracka, *J. Planar Chromatogr.—Modern TLC*, 2007, **20**, 209–215.
9 K. E. Murray, B. Fried and J. Sherma, *Acta Chromatogr.*, 2007, **18**, 190–198.
10 T. L. Kaduce, K. C. Norton and A. A. Spector, *J. Lipid Res.*, 1983, **24**, 1398–1404.
11 Z. Guan, J. Grunler, S. Piao and P. J. Sindelar, *Anal. Biochem.*, 2001, **297**, 137–143.
12 F. Q. He, C. T. Wang, X. K. Li and X. Q. Liu, *J. Anal. Chem.*, 2007, **62**, 369–372.
13 X. Han and R. W. Gross, *J. Am. Soc. Mass Spectrom.*, 1995, **6**, 1202–1210.
14 H.-Y. Kim, T.-C. L. Wang and Y.-C. Ma, *Anal. Chem.*, 1994, **66**, 3977–3982.
15 M. Koivusalo, P. Haimi, L. Heikinheimo, R. Kostiainen and P. Somerharaju, *J. Lipid Res.*, 2001, **42**, 663–672.
16 B. Brugger, G. Erben, R. Sandhoff, F. T. Wieland and W. D. Lehmann, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 2339–2344.
17 B. L. Peterson and B. S. Cummings, *Biomed. Chromatogr.*, 2006, **20**, 227–243.
18 R. Taguchi, J. Hayakawa, Y. Takeuchi and M. Ishida, *J. Mass Spectrom.*, 2000, **35**, 953–966.
19 G. Isaac, D. Bylund, J.-E. Mansson, K. E. Markides and J. Borgquist, *J. Neurosci. Methods*, 2003, **128**, 111–119.
20 M. Hermansson, A. Uphoff, R. Käkelä and P. Somerharju, *Anal. Chem.*, 2005, **77**, 2166–2175.
21 T. Houjou, K. Yamatani, M. Imagawa, T. Shimizu and R. Taguchi, *Rapid Commun. Mass Spectrom.*, 2005, **19**, 654–666.

22 D. Oursel, C. Loutelier-Bourhis, N. Orange, S. Chevalier, V. Norris and C. M. Lange, *Rapid Commun. Mass Spectrom.*, 2007, **21**, 1721–1728.
23 D. Pacetti, E. Borselli, P. Lucci and N. G. Frega, *J. Chromatogr., A*, 2007, **1150**, 242–251.
24 R. Taguchi, T. Houjou, H. Nakanishi, T. Yamazaki, M. Ishida, M. Imagawa and T. Shimizu, *J. Chromatogr., B*, 2005, **823**, 26–36.
25 D. Y. Bang, D. Kang and M. H. Moon, *J. Chromatogr., A*, 2006, **1104**, 222–229.
26 D. Y. Bang, E. Ahn and M. H. Moon, *J. Chromatogr., B*, 2007, **852**, 268–277.
27 E. Ahn, H. Kim, B. C. Chung and M. H. Moon, *J. Sep. Sci.*, 2007, **30**, 2598–2604.
28 D. A. Saunders, E. J. Begg, C. M. J. Jirkpatrick, J. Yeo, G. G. Graham and R. R. Bailey, *Br. J. Clin. Pharmacol.*, 1997, **43**, 435–440.
29 P. D. Whitfield, P. C. Sharp, D. W. Johnson, P. Nelson and P. J. Meikle, *Mol. Gen. Metab.*, 2001, **73**, 30–37.
30 M. Fuller, P. C. Sharp, T. Rozaklis, P. D. Whitfield, D. Blacklock, J. J. Hopwood and P. J. Meikle, *Clin. Chem.*, 2005, **51**, 688–694.
31 E. T. Baronas, J.-W. Lee, C. Alden and F. Y. Hsieh, *Toxicol. Appl. Pharmacol.*, 2007, **218**, 72–78.
32 J. Folch, M. Lees and G. H. S. Stanley, *J. Biol. Chem.*, 1957, **226**, 497–509.
33 E. Ahn, H. Kim, B. C. Chung, G. Kong and M. H. Moon, *J. Chromatogr., A*, 2008, **1194**, 96–102.