Quantitative profiling of phosphatidylcholine and phosphatidylethanolamine in a steatosis/fibrosis model of rat liver by nanoflow liquid chromatography/ tandem mass spectrometry

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ABSTRACT

Quantitative analysis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was carried out using a steatosis/fibrosis model of rat livers, which were induced by a chronic administration of carbon tetrachloride (CCl_4). Intact phospholipid mixtures from each liver sample (from rats fed with 0.5 mL CCl_4/kg three times a week for 30, 60, and 90 days) were analyzed by nanoflow liquid chromatography–electrospray ionization tandem mass spectrometry (nLC/ESI/MS/MS), and identifications of 37 PC and 19 PE species were made by collision-induced dissociation. The quantitative analysis utilized a multiple standard addition method with an internal standard, and the relationship between the MS peak intensities of different PC species and their carbon chain length was included for calibration. It was found that the total amount of PC and PE species decreased significantly with administration of CCl_4. While concentrations of most PC and PE species decreased to a great extent, three PEs and seven PCs were up-regulated more than twofold.

1. Introduction

Lipids are not only major components of biological membranes and energy storage, but also participate in sensing, transducing, and executing cellular proliferation and death [1–3]. Lipidomics, a prominent area of metabolomics, utilizes novel analytical methodologies to study the extensive classes of lipid molecules, changes in lipid metabolism, and lipid-mediated signaling processes [3]. Lipidomics holds promise for biomedical research, with a variety of applications in drug and biomarker development. In particular, phospholipids (PLs), a major group of lipids, are classified into several classes by differences in the structure of polar head groups, acyl chain length, and the degree of unsaturation. The composition and level of PLs in biological tissue are altered by physiologic and pathologic cellular states related to diseases like diabetes or brain diseases [4,5]. However, due to the complexity of PLs in cells or biological fluids, a complete quantitation of PL composition is not easily accomplished. Accordingly, separation of PLs using HPLC is critical for the characterization of PLs in biological samples,

and the hyphenation of electrospray ionization mass spectrometry (ESI/MS) with HPLC enhances identification of PL species, since the combination of HPLC and ESI/MS reduces the ionization suppression effect of low abundance PL species compared to direct ESI/MS alone [6–9].

The quantitative analysis of PLs is not straightforward, since MS signals of PLs are influenced by acyl chain length and unsaturation, preventing the application of a simple calibration method. More sophisticated and computerized analysis methods are not yet available. A few attempts have been made to study the composition and levels of PLs using HPLC/ESI/MS by calculating the relative MS ion intensity of bacterial PLs in comparison to that of an internal standard [10] or the peak area of an extracted ion chromatogram [11], and by utilizing multiple internal standards along with the development of software tools to correct for overlap in lipid isotopic patterns [8]. The latter approach showed potential for high throughput scanning of complicated lipid species, but the assignment of lipid species still mostly relied on mass measurement alone. Recently, a quantitative analysis method with the simultaneous identification of lipid structures was reported by our laboratory [12], in which the shotgun identification of PLs was achieved with nanoflow LC (nLC)/ESI/MS/MS [13], followed by scanning MS peak intensities of identified PL species only with nLC/ESI/MS. In this

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method [12], addition of multiple standards to a PL mixture sample from rat liver was used to compensate the dependency of MS signals on acyl chain carbon number, and the effect of the degree of acyl chain unsaturation on MS peak intensity was minimized by keeping the injection amount of PLs lower than 1 pmol.

In this work, a qualitative and quantitative profiling of intact phospholipids using nLC/ESI/MS/MS was demonstrated for the first time using PL mixtures extracted from a steatosis/fibrosis model of rat livers induced by the chronic administration of carbon tetrachloride (CCl_4). CCl_4 is known to affect lipid homeostasis [14] and known to induce mild and severe fatty liver/steatosis up to 60 days of injection and induce fibrosis/cirrhosis at 90 days of injection by histopathological and biochemical analyses [15]. This study uses the same rat liver tissue samples which were utilized to study differential gene expression profiles following chronic administration of CCl_4 [15]. In this study, nLC/ESI/MS/MS with the developed calibration method demonstrates, for the first time, its capability of examining changes in both the amount and composition of PC and PE species in rat liver tissue depending on the chronological development of hepatic steatosis/fibrosis. Experiments were carried out to compare the relative change in the concentrations of PC and PE species during chronic injury of rat livers.

2. Experimental

2.1. Materials and reagents

The following phosphatidylcholine (PC) and phosphatidylethanolamine (PE) standards were used to establish calibration curves: 12:0/12:0-PC, 20:0/20:0-PC, 12:0/12:0-PE, 14:0/14:0-PE, and 18:0/18:0-PE from Avanti Polar Lipids (Alabaster, AL, USA) and 14:0/14:0-PC from Sigma–Aldrich (St. Louis, MO, USA). For an internal standard, 14:0/14:0-phosphatidylglycerol (PG) from Sigma–Aldrich was added to PL mixture samples in order to correct the peak recovery variation of each PC component from every LC separation. Capillary reversed-phase liquid chromatography (RPLC) columns were prepared with 75 μm I.D. and 360 μm O.D. silica capillaries purchased from Polymicro Technology (Phoenix, AZ, USA). Silica capillaries of smaller I.D. but the same O.D. as the above were used for tubing connections (50 μm I.D.) and for pressure regulation to obtain nanoflow (20 μm I.D.). RP resin (Magic C18, 5 μm, 100 Å) from Michrom Bioresources (Auburn, CA, USA) was used for column packing. All solvents (H_2O, CH_3CN, CH_3OH, $CHCl_3$, HCOOH, and isopropanol) used were HPLC grade.

2.2. Lipids from rat liver

The rat liver tissue samples used were the same ones studied for differential gene expression profiling in the steatosis/fibrosis model of rat livers [15]. Sprague–Dawley male rats (Orient, Seoul, Korea), approximately 5 weeks of age, were repeatedly injected with 0.5 mL/kg CCl_4 diluted 1:1 in maize oil or maize oil alone three times per week at Hanyang University (Seoul, South Korea). Three rats were killed at 0, 30, 60, and 90 days after initial treatment of CCl_4. Seven control tissue samples and three tissue samples for each time point (16 samples total) were provided by Professor Kong's laboratory at Hanyang University.

Tissue samples from each time point were minced and mixed thoroughly using surgical blade. Approximately 0.1 g of the tissue mixture was taken and mixed with 0.75 mL of $CHCl_3/CH_3OH$ (1:2), then the mixture was homogenized to extract lipids according to the Bligh and Dyer method [16]. The tissue mixture was

added with 0.25 mL of $CHCl_3$ and then vortexed again. After adding 0.25 mL of water to the mixture, it was centrifuged at 13,000 rpm for 5 min at room temperature, the supernatant discarded, and the pellets evaporated by Speedvac. Dried lipid powder of each sample was weighed: 3.7, 18.7, 9.5, and 8.3 mg for the liver samples obtained at days 0, 30, 60, and 90, respectively. Each lipid powder sample was reconstituted in 1 mL of $CHCl_3/CH_3OH$ (1:1) and stored in refrigerator. For HPLC separation, a small portion of lipid solution was taken and dispersed with CH_3OH/CH_3CN (9:1) solution at a concentration of 0.5–1% (v/v).

2.3. nLC/ESI/MS/MS

Nanoflow RPLC separation of lipid mixture samples was accomplished with a model 1200 capillary pump system from Agilent Technologies (Palo Alto, CA, USA) and on-line hyphenation of nLC to MS was made via electrospray ionization directly without flow splitting. The RPLC column was prepared in our laboratory by packing a methanol slurry of 5 μm 100 Å Magic C18 resin at a constant pressure (1000 psi) of He into a pulled tip capillary column. Detailed procedures to prepare a pulled tip capillary column embedded with sol–gel frit have been described [11,12,17]. The pulled tip capillary column was connected to a polyether ether ketone (PEEK) microcross, which was connected with capillary tubing (50 μm I.D., 360 μm O.D.) to an HPLC pump, an on–off valve for venting, and to a Pt wire, which applied electric voltage for electrospray ionization. The detailed configuration of the column connection and Pt wire can be found in an earlier report [12]. The lipid sample was loaded to the capillary column from the autosampler of the HPLC system at 300 nL/min for 10 min with the on–off valve closed. During separation, a high speed flow (7 μL/min) was delivered from the pump with the on–off valve open so that most of the flow exited through the on–off valve, which was connected with capillary tubing (20 μm I.D., 360 μm O.D.) to apply pressure. In this way, only a small portion of flow at 300 nL/min was delivered to the column. Flow rate adjustment was made by controlling the length of the vent tubing, which allowed the dwell time to be minimized.

nLC separation was carried out through binary gradient elution with mobile phase compositions of 50/50 CH_3CN/H_2O for solution A and 90/10 isopropanol/CH_3CN for solution B. Both phases were added with 0.1% formic acid. The binary gradient elution used for the separation of PL species began at 100% mobile phase A, ramped to 55% mobile phase B over 1 min after sample loading, and then linearly increased to 90% B over 90 min. After separation was complete, the mobile phase condition was returned to 100% A, and the column was reconditioned at 100% A for at least 20 min. Eluting PLs were directly fed into a LCQ Deca XP MAX ion trap mass spectrometer from Thermo Finnigan (San Jose, CA, USA) via ESI voltage of 2.0 kV applied to a Pt wire in positive ionization mode and at a capillary temperature of 200 °C. To identify PC and PE species in ESI/MS/MS, a precursor MS scan was made first and followed by three data-dependent collision-induced dissociation (CID) runs (40% normalized collision energy). Mass ranges of MS scanning were set from 577 to 900 m/z for each precursor run and from 200 to 900 m/z for CID fragment spectra. Identification of PC and PE species was manually made with the parent mass information and their characteristic fragment ions in the CID spectrum by using lists of PL species prepared in our laboratory. After PC and PE species were identified for each sample, the peak area of the extracted MS chromatogram of each identified species was monitored in separate nLC/ESI/MS runs obtained repeatedly, but without CID runs. For all calculations, the ratio of peak area of each PC or PE species to the peak area of the internal standard (14:0/14:0-PG) was used.
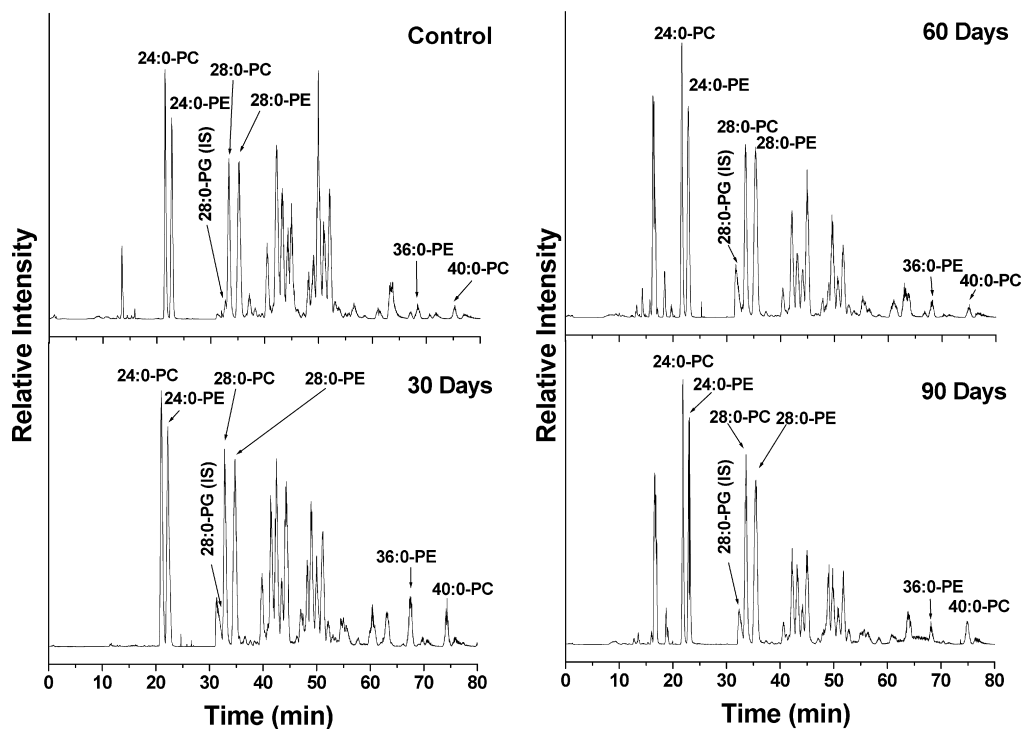
Fig. 1. Base peak chromatograms (BPCs) of an equimolar mixture of rat liver PL extracts added with an internal standard (I.S., 14:0/14:0-PG, 1.0 pmol) and calibration standards (two PCs and PEs) by nLC/ESI/MS/MS in positive ionization mode. Binary gradient elution conditions are described in the text.

3. Results and discussion

We began quantitative profiling of PLs of rat livers at different stages of steatosis/fibrosis with structural identification of the PE and PC species in the control sample (lipid extracts from control rat liver) by nLC/ESI/MS/MS based on previous reports [12,13]. Next, we measured the peak area of the identified PE and PC species in separate nLC/ESI/MS analyses with the addi-
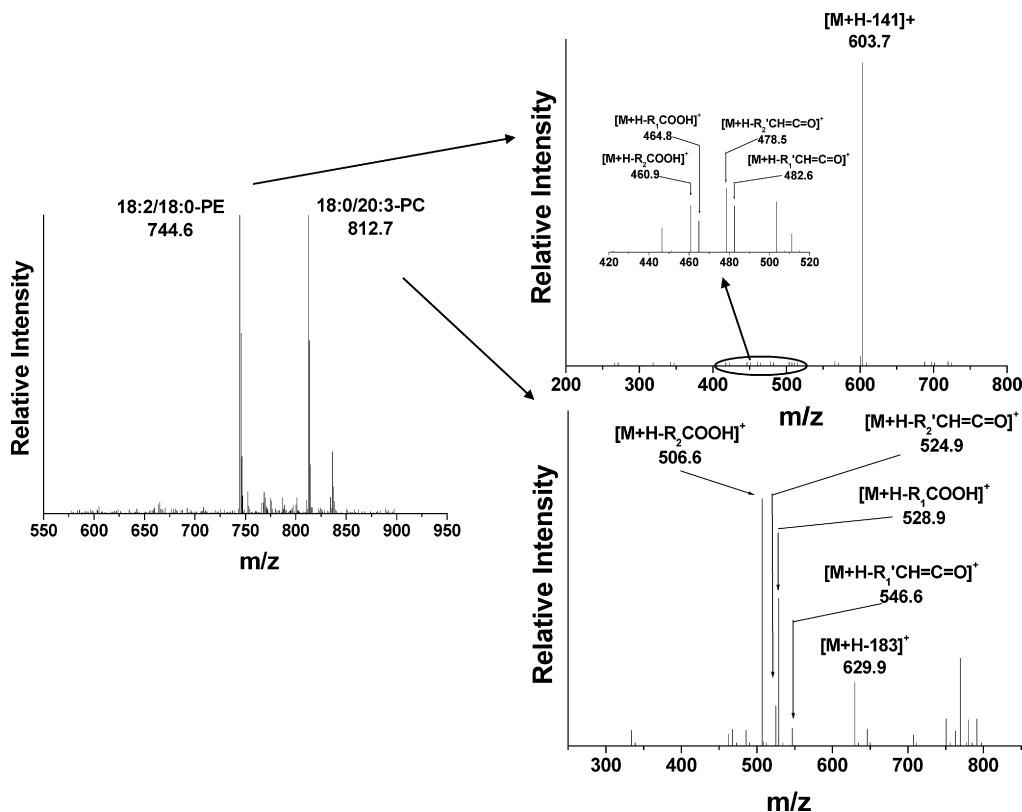

Fig. 2. Precursor ESI/MS scan of t_R = 53.04 min obtained from the nLC/ESI/MS/MS run of the control sample shown in Fig. 1 and the two CID spectra of ions of m/z 744.6 and 812.7 by data-dependent MS/MS.
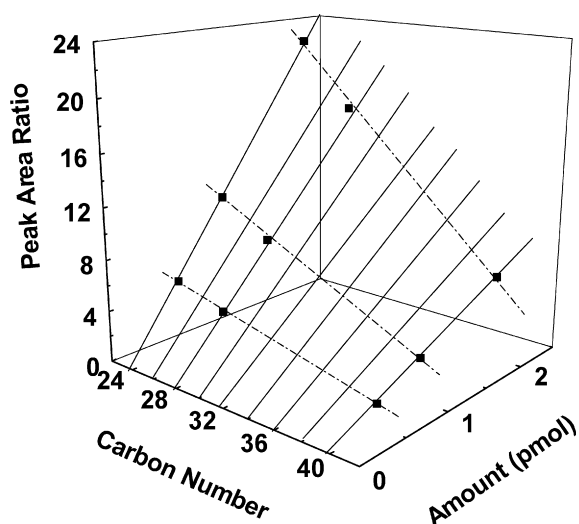
Fig. 3. Three-dimensional calibration curves established from standard PEs added to rat liver PL extracts. The broken lines were obtained from linear regression of data points to consider the effect of acyl chain carbon numbers on MS intensity and the solid lines were obtained to calculate concentrations for each series of carbon number. Peak area ratio represents the ratio of peak area of the extracted chromatogram of PE standard to that of the I.S.

Table 1
Calibration slopes and intercepts obtained for the calibration of PL standards with the internal standard dependent upon acyl chain length

Amount injected (pmol)	Regression equation ($n = 12$)	R^2
PE		
0.5	$y = -(0.33 \pm 0.01)x + (13.67 \pm 0.24)$	0.999
1.0	$y = -(0.67 \pm 0.02)x + (27.34 \pm 0.48)$	1.000
2.0	$y = -(1.34 \pm 0.03)x + (54.68 \pm 0.96)$	1.000
PC		
0.5	$y = -(0.30 \pm 0.02)x + (12.48 \pm 0.55)$	0.998
1.0	$y = -(0.61 \pm 0.04)x + (25.17 \pm 1.27)$	0.998
2.0	$y = -(1.09 \pm 0.02)x + (45.61 \pm 0.52)$	1.000

(y = peak area ratio and x = acyl chain carbon number).

tion of calibration standards to each lipid sample, where the concentrations of standards were varied but there was a fixed amount of an internal standard (I.S.). Fig. 1 shows the four base peak chromatograms (BPCs) of the lipid samples (control and 30, 60, and 90 days of CCl$_4$ addition) added with an equimolar (0.5 pmol each) mixture of three PC standards (12:0/12:0, 14:0/14:0, and 20:0/20:0) and three PE standards (12:0/12:0, 14:0/14:0, and 18:0/18:0) as well as an I.S. (14:0/14:0-PG, 1 pmol) obtained by nLC/ESI/MS/MS. In Fig. 1, the number of acyl carbons of each standard is expressed as the total carbon number of acyl chains. Each lipid extract sample of rat liver was injected as 25 ng and the gradient elution condition used for the four runs in Fig. 1 was identical. An MS scan of the control sample at 53.04 min obtained in positive ion mode is shown in the left of Fig. 2. The two ions of m/z 744.6 and 812.7 were identified with subsequent data-dependent MS/MS experiments. The CID spectra of the molecular ion at m/z 744.6 is shown in the upper right side of Fig. 2 with characteristic fragment ions identified as 18:2/18:0-PE: m/z 603.7 [M+H−141]$^+$ for the loss of ethanolamine (HPO$_4$(CH$_2$)$_2$NH$_3$, 141 amu), and m/z 482.6 and 464.8 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$_1$COOH]$^+$), respectively. A similar procedure for the molecular ion m/z 812.7 resulted in the identification of 18:0/20:3-PC with characteristic fragment ions such as m/z 629.9 for [M+H−183]$^+$ (HPO$_4$(CH$_2$)$_2$N(CH$_3$)$_3$, 183 amu), among others. The fragment ion intensities of the PE molecule (except [M+H−141]$^+$) were less intense than those for the PC molecule. This is typically observed when CID is carried out for both PC and PE at the same collision energy due to the difference in the dissociation energies required for each of the molecules [13]. In this experiment, the collision energy was optimized to detect fragment ions of both species in positive ion mode. We identified 37 PC and 19 PE species from the control sample to use as target molecules to compare the relative concentrations at different stages of steatosis/fibrosis of rat liver samples. The entire list of these species will be quantitatively discussed later.

The four BPCs in Fig. 1 show a clear difference in the intensities of peaks observed at 10–20 min of retention time interval for samples after 60 and 90 days of CCl$_4$ injection when compared with the

peak profiles observed with the control and 30-day sample. Later, we found that the species observed in the 10–20 min of retention time interval did not belong to the PC and PE species considered for comparison in this report. We also observed lower peak intensities at 40–50 min as CCl$_4$ was added to the rats for longer periods.

For a systematic comparison of the PL species among the four samples, the concentrations of the liver PC and PE species were calculated using the calibration method developed in our earlier study [12] that incorporates the effect of carbon numbers on MS intensity. Calibration curves were established for each different length of acyl chain of PC and PE standards. In order to compensate the variation of MS intensity due to the length of carbon chain, the MS peak area of the three standards of PC and PE, respectively, was measured from extracted chromatograms of each nLC/ESI/MS run with varying concentrations (2, 1, and 0.5 pmol). Fig. 3 shows the three-dimensional calibration curves established from standard lipids added to rat liver PL extracts. The data points in Fig. 3 were first plotted as the peak area ratio (relative to the peak area of I.S.) vs. carbon number of acyl chain of PE standards, followed by linear regression of the data from each injection amount. Results are shown as three broken lines in Fig. 3 and the parameters of each linear regression curve are listed in Table 1. Based on the first regression lines, secondary regressions were carried out for each carbon number of PE species for concentration calibration (shown with solid lines) by selecting the peak area ratio corresponding to each length of acyl chain (the slope value of each concentration calibration curve is listed in Table 2). From the solid line calibration in Fig. 3, concentrations of identified liver PE species from rat liver sample could be calculated. We calibrated PC standards with the same method used in Fig. 3; calibration parameters are listed in Table 2 together with those obtained for PE. In this calibration procedure, however, the effect of lipid unsaturation on MS peak response was not considered, since our earlier study [12] demonstrated that this effect can be minimized when the injection amount of lipid molecules is kept at as low as 0.5 pmol. While the present calibration method is not complete, since the effect of acyl chain unsaturation on MS peak

Table 2
Calibration slopes obtained for the plot of peak area ratio vs. concentration of the added standard PC and PE species in liver PL extracts of the control rat sample

Carbon number	PE		PC	
	Slope	R^2	Slope	R^2
24	11.32 ± 0.001	1.000	9.88 ± 0.33	0.998
26	9.98 ± 0.33	0.999	8.64 ± 0.002	0.999
28	8.65 ± 0.24	0.999	7.65 ± 0.20	0.999
30	7.31 ± 0.10	0.999	6.26 ± 0.18	0.999
32	5.97 ± 0.13	0.999	5.25 ± 0.15	0.999
34	4.64 ± 0.13	0.999	4.36 ± 0.11	1.000
36	3.30 ± 0.001	1.000	3.15 ± 0.08	0.999
38	1.97 ± 0.001	1.000	2.15 ± 0.05	0.999
40	0.63 ± 0.03	0.999	0.46 ± 0.02	0.998

Table 3
Regulation of PE and PC composition and concentration in rat livers after 30, 60, and 90 days of intoxicating CCl$_4$ compared to the control sample

	m/z	t_R (min)	Concentration (μg/g) ± SD ($n = 5$)		Relative ratio ($n = 5$)	
			Control	30 days	60 days	90 days
PE						
20:3/20:4	781	35.58	986.7 ± 46.6	0.34 ± 0.02	1.20 ± 0.06	2.02 ± 0.24
22:6/16:0	765	38.96	268.1 ± 13.5	0.35 ± 0.02	9.89 ± 0.54	8.95 ± 0.75
16:0/20:5	739	39.49	141.2 ± 3.7	0.35 ± 0.03	16.24 ± 0.81	12.38 ± 1.11
18:2/20:3	767	42.49	490.0 ± 17.7	1.31 ± 0.05	0.28 ± 0.01	0.30 ± 0.03
20:6/18:0	765	42.51	956.0 ± 23.5	1.64 ± 0.11	0.35 ± 0.03	0.39 ± 0.05
18:1/22:6	791	42.71	569.9 ± 73.5	0.64 ± 0.09	0.23 ± 0.03	0.32 ± 0.04
18:0/18:4	741	44.37	2059.7 ± 25.0	0.73 ± 0.01	0.04 ± 0.00	0.02 ± 0.00
16:1/18:1	717	44.52	670.7 ± 3.8	1.20 ± 0.12	0.44 ± 0.05	0.33 ± 0.02
20:5/18:0	767	44.58	1887.5 ± 13.8	0.51 ± 0.06	0.28 ± 0.00	0.33 ± 0.03
14:0/22:6	737	44.78	258.7 ± 8.3	0.92 ± 0.08	0.88 ± 0.04	0.91 ± 0.07
16:0/20:3	743	45.53	374.9 ± 10.5	0.73 ± 0.02	0.34 ± 0.01	0.59 ± 0.06
18:0/20:5	767	47.16	306.7 ± 5.3	1.54 ± 0.05	0.16 ± 0.02	0.20 ± 0.03
18:0/22:6	793	50.35	1149.7 ± 13.7	2.39 ± 0.03	0.06 ± 0.01	0.08 ± 0.02
16:0/18:1	719	50.83	119.4 ± 2.8	2.68 ± 0.27	4.79 ± 0.17	7.45 ± 0.99
20:1/18:3	769	52.08	5396.8 ± 126.5	0.95 ± 0.02	0.02 ± 0.00	0.03 ± 0.00
18:0/22:5	795	52.42	524.1 ± 17.6	1.50 ± 0.07	0.56 ± 0.05	1.64 ± 0.22
18:2/18:0	745	53.18	483.8 ± 8.7	1.45 ± 0.35	2.18 ± 0.07	2.09 ± 0.14
20:4/20:0	797	56.98	260.5 ± 1.4	1.54 ± 0.08	10.20 ± 2.99	0.16 ± 0.02
18:0/18:1	747	58.45	138.4 ± 7.2	2.04 ± 0.33	0.44 ± 0.02	0.33 ± 0.02
PC						
16:1/22:6	805	33.80	194.4 ± 15.5	0.62 ± 0.08	1.86 ± 0.17	1.35 ± 0.45
14:0/20:4	755	34.95	110.8 ± 2.9	0.47 ± 0.02	0.43 ± 0.02	0.35 ± 0.02
20:4/20:4	831	35.00	1266.9 ± 20.6	0.40 ± 0.01	0.70 ± 0.06	1.03 ± 0.09
18:4/16:5	743	35.13	17.8 ± 3.2	0.69 ± 0.16	0.67 ± 0.16	0.42 ± 0.08
22:2/18:6	831	35.44	1306.1 ± 16.2	0.38 ± 0.01	0.16 ± 0.01	0.22 ± 0.02
18:2/16:1	757	36.62	129.6 ± 12.0	0.81 ± 0.08	0.37 ± 0.04	0.30 ± 0.03
18:2/18:2	783	38.23	364.0 ± 12.8	0.51 ± 0.04	0.11 ± 0.01	0.09 ± 0.01
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18:5/22:4	829	40.41	586.0 ± 73.2	0.85 ± 0.14	0.31 ± 0.05	0.27 ± 0.04
16:0/22:6	807	40.44	3153.4 ± 119.3	0.89 ± 0.05	0.08 ± 0.00	0.05 ± 0.00
20:0/18:5	809	40.48	472.3 ± 12.6	0.69 ± 0.03	8.40 ± 0.35	6.86 ± 0.24
20:3/20:4	833	40.68	1101.5 ± 102.0	0.30 ± 0.03	0.77 ± 0.11	0.90 ± 0.12
14:2/18:2	725	41.17	25.8 ± 4.6	0.96 ± 0.21	1.01 ± 0.19	0.67 ± 0.12
16:1/16:0	733	41.72	250.9 ± 14.8	1.28 ± 0.10	0.11 ± 0.01	0.05 ± 0.00
20:4/18:3	805	42.17	313.1 ± 37.4	0.80 ± 0.13	0.22 ± 0.03	0.16 ± 0.02
16:0/20:4	783	42.24	5284.0 ± 146.3	0.74 ± 0.04	0.009 ± 0.00	0.005 ± 0.00
18:2/22:6	831	42.47	244.8 ± 22.8	0.93 ± 0.09	2.07 ± 0.25	2.65 ± 0.37
18:1/20:4	809	42.64	2569.6 ± 73.7	0.30 ± 0.01	0.14 ± 0.01	0.12 ± 0.00
16:0/18:2	759	43.33	2727.7 ± 38.5	1.26 ± 0.04	0.01 ± 0.00	0.01 ± 0.00
18:2/18:1	785	43.55	1008.8 ± 23.3	0.36 ± 0.03	0.09 ± 0.00	0.06 ± 0.00
18:2/20:3	809	44.28	328.7 ± 16.0	0.31 ± 0.02	2.62 ± 0.17	2.27 ± 0.21
20:3/18:2	809	45.14	265.8 ± 36.1	1.04 ± 0.15	1.87 ± 0.26	1.86 ± 0.27
16:3/18:5	747	45.21	65.9 ± 2.5	0.77 ± 0.17	0.66 ± 0.03	0.55 ± 0.11
16:0/20:3	785	45.21	916.5 ± 46.4	0.43 ± 0.06	0.76 ± 0.06	0.67 ± 0.09
18:2/20:2	811	45.46	1378.7 ± 39.0	0.36 ± 0.02	0.10 ± 0.01	0.09 ± 0.01
18:6/20:5	797	46.15	622.4 ± 19.5	0.49 ± 0.02	4.84 ± 0.32	5.15 ± 0.49
18:0/22:6	835	48.21	4198.5 ± 136.3	0.89 ± 0.04	0.14 ± 0.01	0.18 ± 0.01
16:0/18:1	761	49.10	1288.4 ± 94.1	1.46 ± 0.12	0.04 ± 0.00	0.04 ± 0.00
22:3/18:4	833	49.96	9324.5 ± 203.1	0.60 ± 0.09	0.67 ± 0.12	0.65 ± 0.11
18:0/20:4	811	49.96	555.8 ± 73.4	0.72 ± 0.02	0.03 ± 0.00	0.05 ± 0.00
18:0/18:2	787	50.94	3177.9 ± 81.6	0.82 ± 0.03	0.03 ± 0.00	0.02 ± 0.00
18:0/20:5	809	51.19	174.5 ± 29.8	0.89 ± 0.16	0.42 ± 0.08	0.30 ± 0.06
18:0/20:3	813	53.04	1632.6 ± 89.0	0.41 ± 0.03	1.19 ± 0.07	0.82 ± 0.06
20:0/20:4	839	55.37	321.4 ± 28.3	1.33 ± 0.17	1.57 ± 0.15	2.11 ± 0.20
18:1/20:3	811	56.43	188.5 ± 21.4	1.97 ± 0.43	5.98 ± 0.68	5.08 ± 0.59
18:0/18:1	789	56.56	639.5 ± 121.4	1.77 ± 0.35	0.05 ± 0.01	0.07 ± 0.02
18:0/20:2	815	57.74	248.2 ± 41.1	0.42 ± 0.07	2.81 ± 0.47	2.37 ± 0.40
18:0/18:0	791	63.75	1212.0 ± 32.0	0.02 ± 0.004	0.03 ± 0.001	0.03 ± 0.01

intensity can be significant for the highly unsaturated PL species of larger concentrations, the current approach using the minimum injection amount of total PL species offers an alternative until a proper method to include the unsaturation effect is developed.

Identified PE and PC species of the control sample and their concentrations are listed in Table 2 and the relative changes of concentrations are described for each stage of CCl$_4$ administration. Quantitative comparison of each PC and PL species shows that the concentration of most species decreased as the number of days of CCl$_4$ administration increased. Some species dropped to less than 5% of the concentration of the control sample, including two PEs (18:0/18:4 and 20:1/18:3) and seven PCs (16:0/22:6, 16:1/16:0, 16:0/20:4, 16:0/18:1, 18:0/20:4, 18:0/18:2, and 18:0/18:0). This decline was expected, since administration of CCl$_4$ is known to induce death of mammalian cells due to a perturbation of the PC homeostasis. However, interesting phenomena were observed with a few species. Two PEs (16:0/18:1–50.83 min and 18:2/18:0–53.18 min) and three PCs (20:3/18:2; 45.14 min,
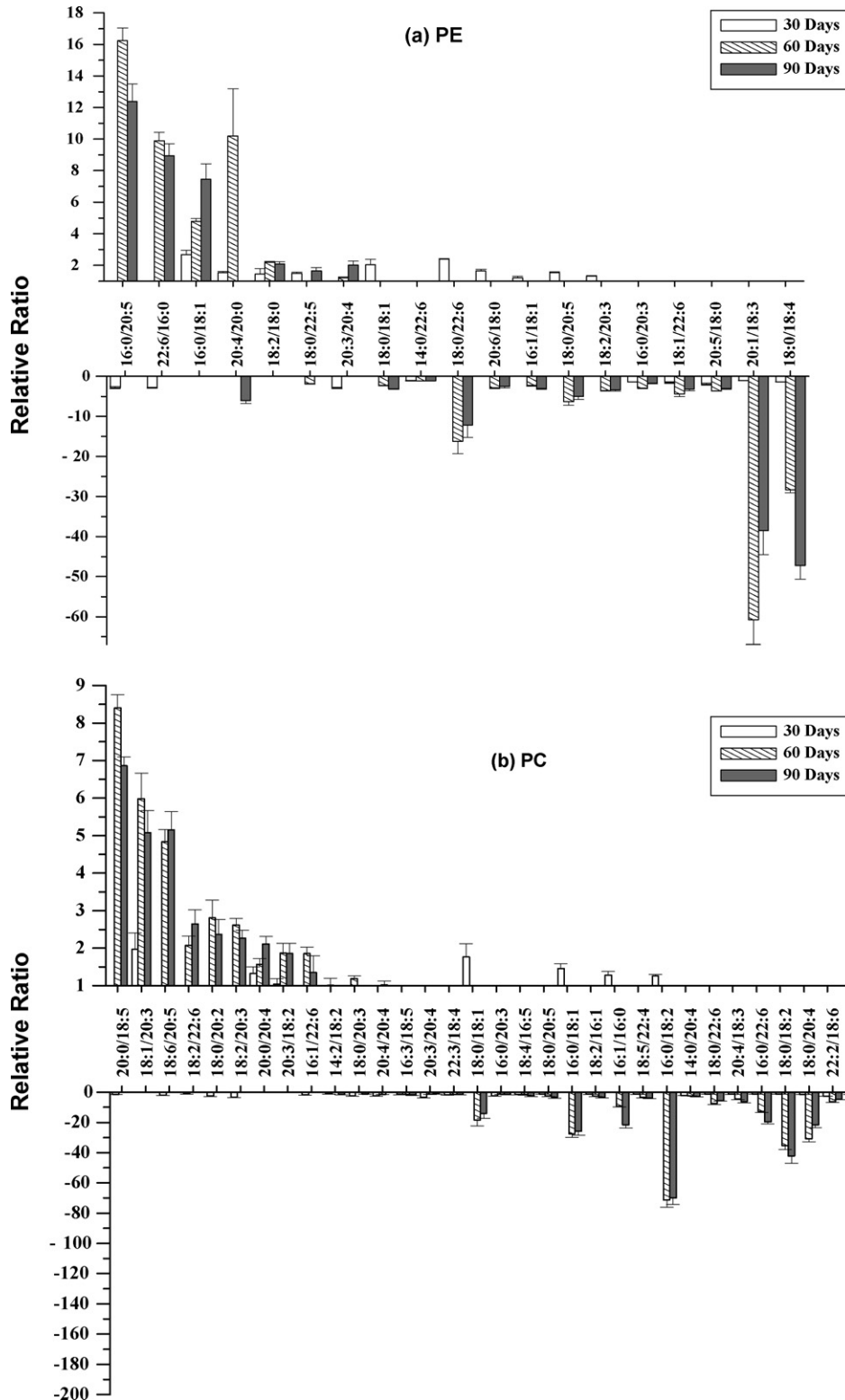
Fig. 4. Relative regulation of (a) PE and (b) PC species in rat liver samples showing concentration variations during the process of steatosis/cirrhosis induced by CCl$_4$. A positive ratio represents the increase of concentration by that multiplication factor and a negative ratio represents a decrease.

20:0/20:4; 55.37 min, and 18:1/20:3; 56.43 min) species were found to increase about 2–7-fold over the time course of CCl$_4$ injection. In addition, concentrations of some species decreased at 30 days of CCl$_4$ administration (moderate steatosis) but increased at later stages (severe steatosis/cirrhosis), including

three PEs (20:3/20:4, 22:6/16:0, and 16:0/20:5) and seven PCs (16:1/22:6, 20:4/20:4, 20:0/18:5, 18:2/22:6, 18:2/20:3, 18:6/20:5, and 18:0/20:2) (Fig. 4). The vertical scale of Fig. 4 is expressed as the relative ratio of concentration to the control sample; the positive scale measures an increase of concentration by that multiplication
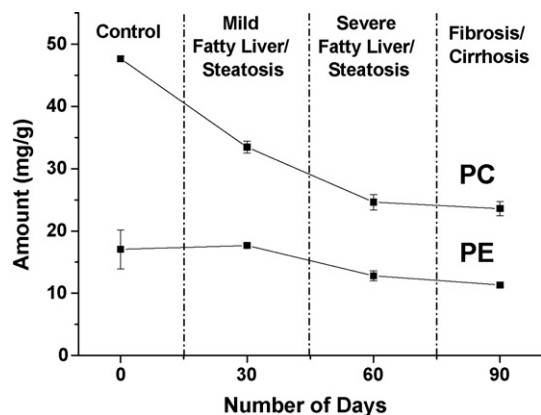
Fig. 5. Decrease of total amount of PC and PE species vs. the number of days of CCl$_4$ administration in rats.

factor and the negative scale measures a decrease. For instance, a relative ratio of -10 represents a concentration decrease of 10 times compared to the control sample. Fig. 4a shows that majority of PE species, except the first four, exhibited decreases in concentration with the administration of CCl$_4$ over 3 months. The concentrations of eight PC species increased more than twofold, but the rest decreased significantly (Fig. 4b). The concentration variations of PE and PC species identified in this study are plotted in Fig. 5 as the total amount (expressed with milligram of lipids per gram of liver tissue) vs. number of days. Total amounts of PE and PC were calculated by summing concentration values of all PE and PC species, respectively, as listed in Table 3. The total PC concentration decreased by $\sim$51% (from 47.7 to 23.6 mg/g), and the PE concentration decreased by only about 34% (from 17.0 to 11.3 mg/g).

4. Conclusions

In this study, nLC/ESI/MS/MS was powerfully utilized for the characterization and quantitative analysis of PC and PE species in a steatosis/fibrosis model of rat livers, induced by chronic administration of CCl$_4$. Since PC constitutes a major portion of cellular PLs, a significant change in PC concentration during the development of cirrhosis induced by CCl$_4$ demonstrates critical damage to liver tissues. While this study accomplishes a quantitative analysis of PE and PC species from rat livers, it does not discriminate which

species of PC or PE are related to the methylation pathway of PE to produce PC, which is known to be the predominant pathway in the liver [18]. However, the current work demonstrates that PLs can be quantified with nLC/ESI/MS/MS, and the relative regulation of PL species under different physiologic and pathologic cellular states can be investigated. Moreover, this method can be utilized for the development of PL biomarkers of diseases. The present technique has a limitation, in that the effect of acyl chain unsaturation on MS peak intensity can be minimized only when sample concentration is kept at a very low level. Further study is needed to determine how to include this effect in calibration by developing a proper method such as a scale factor to compensate for the additional increase in MS intensity of highly unsaturated PL species.

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