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Characterization of Glycerophospholipids and a Sphingolipid in the Cerebrospinal Fluid of Patients with Different Illnesses by LC-ESI-MS

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ABSTRACT

A sensitive and accurate reversed-phase liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) method for determining glycerophospholipids and a sphingolipid was developed and validated in order to profile and quantify the classes of lipids in the cerebrospinal fluids of patients with three different illnesses. The LC-ESI-MS method was optimized for the analysis, and an Inertsil 6 ODS-3 (4.6 × 150 mm) column was utilized with a mobile phase composed of acetonitrile/methanol/triethylamine in the ratio 550/1000/25 (w/w/w) eluted isocratically at a flow rate of 1.0 mL/min. The results demonstrated that both the accuracy and precision of the intra- and inter-day assays of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM) were within acceptable criteria. Clinical application demonstrated that the percentage and content of each class differed in the cerebrospinal fluids of patients with three different illnesses, with the richest lipid components being SM (848 ± 75.5 ng/mL, 40.7% ± 2.2%), PC (8042.5 ± 1214.4 ng/mL, 53.8% ± 4.2%) and PS (2124.2 ± 152.0 ng/mL, 43.3% ± 1.6%), respectively for patients with chordoma, trigeminal neuralgia and Parkinson's disease. It was concluded that this improved method can be used to directly detect glycerophospholipids and a sphingolipid, and applied to detect physiological changes in humans to discover preventive medicines or therapeutic medicines, or develop new drugs.

Key words: high-performance liquid chromatography and electrospray ionization mass spectrometry (HPLC-ESI-MS), cerebrospinal fluid, illness, glycerophospholipids, sphingolipid

INTRODUCTION

Phospholipids play important roles in mammalian cells and have a variety of biological functions. They provide structural integrity necessary for various protein functions and act like an energy reservoir. Lipidomics is known as the research of lipids in the academic community, and is a large research field concerned with the mechanisms of lipid actions and the development routes of lipids in cells of organisms[1-2] involving complete profiles of lipids and lipid development in cells, tissues and organs. Lipidomics is a recent research field and the analytical research of lipidomics has speedily developed with high-tech analytical instruments, such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and fluorescence spectroscopy. Research results indicate that there are some correlations between lipids and many current metabolic diseases such as obesity, stroke, diabetes, atherosclerosis and hypertension[3-8].

Lipids also play important roles in communicating messages between cells and are related to some neurological diseases. Central nervous system disorders such as bipolar disorders and schizophrenia may be related to lipid metabolism. On the other hand, neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are also related to lipid metabolism. Some results proved that changes in lipid metabolism are helpful in decreasing injury to the cerebrum[9-20]. Thus, analytical methods for glycerophospholipids and sphingolipids can be applied to detect physiological changes in humans in order to discover preventive medicines, therapeutic medicines and new drugs[21].

Phospholipids are separated into two classes, glycerophospholipids and sphingolipids, as shown in Figure

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1. Major classes of brain glycerophospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), while sphingolipids have one class of sphingomyelin (SM). Many differences in the structures of lipids originate from different biosynthesis mechanisms. In fact, it is hard to detect all probable structures in experiments because of the chain length of lipids and the limits of detection.

Many analytical methods are used to separate phospholipids. The traditional extraction method of lipids using a combination of chloroform and methanol is optimal for extracting physiological lipids[22-28]. The simplest analytical method for separating lipids is thin-layer chromatography (TLC)[22,23]. However, this traditional method is more inconvenient and laborious for the detection of lipids than other analytical methods. Currently, high-performance liquid chromatographic (HPLC) assays are more useful, selective and specific than traditional TLC-based assays for analyzing and separating lipid classes[29-31]. In recent years, HPLC techniques coupled with electrospray ionization (ESI) ion-trapping MS systems[32] and matrix-assisted laser desorption/ionization (MALDI)[33] are used to analyze several standard solutions of lipids and appropriate experimental conditions that allow the highest ionization into the ESI source are determined to achieve the best sensitivity. Optimized LC-ESI-MS methods are frequently applied to analyze lipids present in biological samples in order to determine the classes of lipid or the disease situation in a complex matrix.

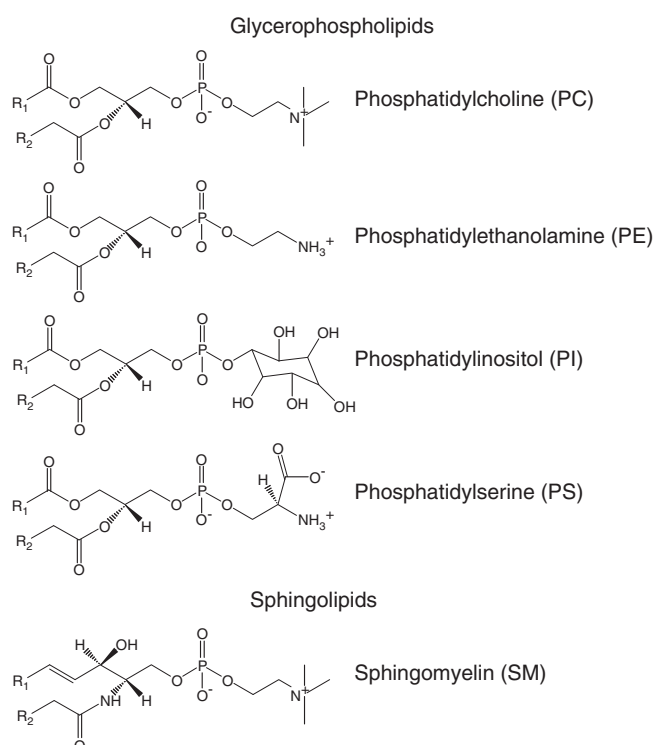Although several analytical methods were developed for application in the field of lipids[34], most focused on the molecular species of each class. Although Mulder *et al.* applied an ESI-MS technique to detect phospholipids in cerebrospinal fluid (CSF), the studies mainly focused on the decreased lysophosphatidylcholine/PC ratio in the CSF with Alzheimer's disease[35]. There are few studies documenting all classes and profiling the main lipids in the CSF with various illnesses. The aim of this study was to develop a useful, selective and specific method to analyze complex mixtures of lipids in the CSF of patients with various illnesses. We focused on five major phospholipid classes in the CSF, including PC, PE, PI, PS and SM. Although the instrumental response is also related to the acyl chain length and the degree of acyl chain unsaturation, except for the head group, the effect could be diminished and made nearly negligible with progressive dilution as previously reported[36]. In this study, the concentrations of several classes of phospholipid species examined were low, so the effect of acyl chain length and the degree of acyl chain unsaturation were not so prominent. Thus, this approach was acceptable. The quantified data were used to compare differences between the cases and controls, so the relative quantified data satisfied the estimation.

MATERIALS AND METHODS

I. *Reagent and Instruments*

Glycerophospholipids such as PC (egg yolk, ≥ 99%), PE (bovine brain, ≥ 98%), PI (soybean, ≥ 98%), PS (soybean, ≥ 98%) and SM (egg yolk, ≥ 98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol (HPLC-grade) was obtained from Merck (Darmstadt, Germany). Isopropanol was supplied by Mallinckrodt Inc. (St. Louis, MO, USA). Chloroform was purchased from Sigma (St. Louis, MO, USA).

LC-ESI-MS was performed using a Bruker Esquire 3000 instrument (Bruker Daltonics, Billerica, MA, USA). MS data of lipid mixtures were analyzed using Bruker Compass 1.0 (Bruker Daltonics). The condition of LC-ESI-MS was set to the positive ion mode, and nitrogen was used as the nebulizing agent (at 10 psi) and the drying gas (8.5 L/min at 300°C). Full-scan mass spectra in the range *m/z* 500-1000 were collected in the positive ion mode.

II. *Experimental Methods*

(I) *Analysis of PC, PE, PI, PS and SM Standards*

The ESI parameters for reverse-phase LC-ESI-MS were initially optimized to analyze glycerophospholipid (PC, PE, PI, and PS) and sphingolipid (SM) standards. The standards were respectively weighed and dissolved in a solvent mixture of chloroform and methanol at a ratio of 2 : 1 to produce stock solutions of 0.1 mg/mL. Further dilutions from the working stock solutions were made using the same solvent

Figure 1. Major classes of brain glycerophospholipids and a sphingolipid.

mixture to construct standard curves for PC, PE, PI, PS and SM in concentrations of 0.25, 0.5, 1.0, 2.5, 5.0 and 10 ng/mL. Six replicate injections were made at random. The accuracy (relative error of the mean; REM%) and precision (coefficient of variation; CV%) of the assay procedure for intraday and inter-day assays were assessed by evaluating six individual sets of PC, PE, PI, PS and SM standard samples on the same day and on six different days. Stock solutions were stored at -80°C and all working stock solutions were stored at -20°C. Before use, all working stock solutions were thawed and briefly sonicated at room temperature. The quantitative reversed-phase analysis of PC, PE, PI, PS and SM standards was optimized with the use of an Inertsil 6 ODS-3 column (4.6 × 150 mm) from GL Sciences (Vercopak, Taipei, Taiwan) with an isocratic solvent system of acetonitrile/methanol/triethylamine of 550/1000/25 (w/w/w) at a flow rate of 1.0 mL/min. The injection volume was set at 50 μL using an intelligent autosampler (Model 851-AS, Jasco, Tokyo, Japan). Abundance or total ion current for each phospholipid standards was measured by a full scan within m/z 500 to 1000 in the positive ion mode of ESI-MS and the area under the peak identified with the corresponding parent m/z value for each phospholipid was calculated. Detected species (R_1/R_2) and *m/z* values in each class of phospholipid in the standard were also monitored and summarized.

(II) *Collection of CSF of Patients with Different Illnesses and Extraction of CSF Lipids*

CSF samples for diagnostic purposes were taken from three patients in the Neurological Clinic of Wan Fang Hospital after they had signed informed consent forms. The final diagnoses of the three patients were chordoma, trigeminal neuralgia, and Parkinson's disease, respectively, and the CSF samples were obtained once from a single patient of each disease. The samples were subjected to vortex stirring at a 1 : 10 ratio with a solvent mixture of chloroform and methanol (2 : 1) as reported[37]. The mixture was filtered and the supernatant was collected for analysis.

(III) *Quantitation of PC, PE, PI, PS and SM in CSF Samples*

The classes of lipids were quantified and the amounts of PC, PE, PI, PS and SM in the CSF samples were calculated based on the standard calibration curves obtained. The abundance or total ion current for each phospholipid in each CSF sample and standard was also measured by a full scan from m/z 500 to 1000 in the positive ion mode of ESI-MS and the area under the peak, identified with the corresponding parent m/z value for each phospholipid, was calculateds. The percentage (%) and content (ng/mL) of each class of PC, PE, PI, PS and SM are reported. The detected species (R_1/R_2) and *m/z* values of each class of phospholipid in the CSF of the three patients with different illnesses were monitored and summarized.

RESULTS AND DISCUSSION

I. *Optimization of ESI-MS and Analytical Conditions*

ESI-MS is a common, simple and economical technique for the analysis of lipids. This method was used to identify each class of lipid including PC, PE, PI, PS and SM and the molecular species of each class, and was directly applied in the detection of the lipid contents in samples of organisms. First, the ionization conditions of ESI were optimized so that it was capable of analyzing and separating fragments formed by each class of lipid. Then, this optimized LC-ESI-MS method was validated for directly profiling and quantifying lipids in the CSF of patients with three different illnesses in order to monitor the progress of the illness and the course of the disease. If the progress of the illness or course of the disease correlated with changes in the lipid contents in the CSF of these patients by LC-ESI-MS in one run, this analytical method would prove to be more convenient, labor-saving and economical than other analytical methods.

II. *Analysis and Validation of PC, PE, PI, PS and SM Standards*

The results of the LC-ESI-MS analysis of the PC, PE, PI, PS and SM standards by reversed-phase elution are shown in Figure 2. Calibration graphs were constructed for six levels in the range 0.25 - 10 ng/mL in the chloroform/methanol (2 : 1, v/v) solvent. A linear regression was performed by plotting the peak areas of the PC, PE, PI, PS and SM standards versus concentration. The linear regression results for PC, PE, PI, PS and SM yielded the equations $y = (1.6364E+06 \pm 1.0048E+04)x + (7.7530E+04 \pm 4.7231E+04)$, $y = (6.3776E+06 \pm 4.6403E+04)x - (1.4738E+05 \pm 2.1811E+05)$, $y = (3.1009E+06 \pm 1.3772E+04)x - (6.8998E+04 \pm 6.4734E+04)$, $y = (2.0978E+06 \pm 9.5433E+03)x - (2.9611E+03 \pm 4.4857E+04)$, and $y =$
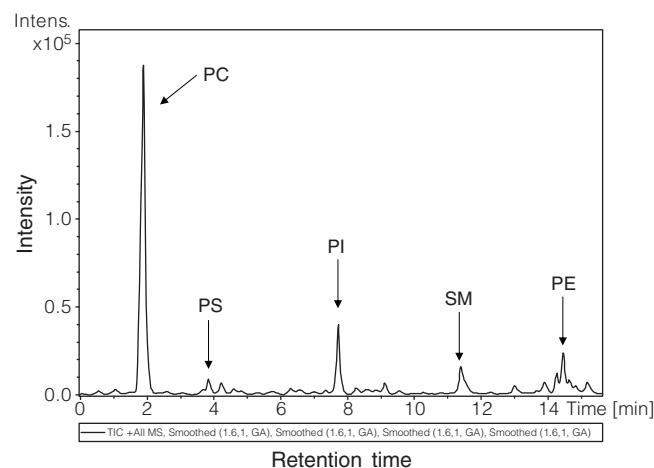

Figure 2. LC-ESI-MS chromatograms of major classes of brain glycerophospholipids standards (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS)), and a sphingolipid standard (sphingomyelin (SM)).

$(2.5327\text{E}+06 \pm 1.4186\text{E}+04)x - (1.6979\text{E}+04 \pm 6.6680\text{E}+04)$, respectively. R^2 values for the regression of the calibration curves were 0.9998, 0.9997, 0.9999, 0.9999 and 0.9998, respectively. The linearity was also confirmed by an analysis of variance (ANOVA).

Intra-day and inter-day (n = 6) analytical accuracies and

Table 1. Intra-day and inter-day (n = 6) analytical accuracies and precisions for phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM).

Phosphatidylcholine (PC) concentration (ng/mL)	Intra-day			Inter-day		
	Mean (SD)	CV (%)	Rel. error (%)	Mean (SD)	CV (%)	Rel. error (%)
0.25	0.248 (0.004)	1.85	-0.72	0.249 (0.005)	2.18	-0.40
0.50	0.507 (0.019)	3.80	1.52	0.493 (0.009)	1.95	-1.36
1.00	1.004 (0.026)	2.65	0.42	1.007 (0.029)	2.95	0.72
2.50	2.498 (0.009)	0.38	-0.07	2.498 (0.013)	0.52	-0.08
5.00	5.009 (0.082)	1.63	0.18	5.002 (0.009)	0.19	0.05
10.0	9.997 (0.110)	1.10	-0.02	10.029(0.065)	0.65	0.29

Phosphatidylethanolamine (PE) concentration (ng/mL)	Intra-day			Inter-day		
	Mean (SD)	CV (%)	Rel. error (%)	Mean (SD)	CV (%)	Rel. error (%)
0.25	0.249 (0.004)	1.66	-0.32	0.251 (0.003)	1.57	0.72
0.50	0.505 (0.011)	2.33	1.00	0.499 (0.002)	0.53	-0.04
1.00	0.998 (0.027)	2.80	-0.20	1.007 (0.020)	2.01	0.76
2.50	2.509 (0.003)	0.15	0.38	2.513 (0.011)	0.46	0.53
5.00	5.036 (0.064)	1.28	0.73	5.003 (0.014)	0.29	0.06
10.0	10.035(0.060)	0.60	0.35	10.026(0.043)	0.43	0.26

Phosphatidylinositol (PI) concentration (ng/mL)	Intra-day			Inter-day		
	Mean (SD)	CV (%)	Rel. error (%)	Mean (SD)	CV (%)	Rel. error (%)
0.25	0.251 (0.005)	2.11	0.56	0.250 (0.002)	1.11	0.16
0.50	0.513 (0.020)	4.06	2.60	0.510 (0.019)	3.73	2.04
1.00	0.992 (0.013)	1.37	-0.76	0.997 (0.006)	0.62	-0.26
2.50	2.506 (0.018)	0.73	0.24	2.508 (0.013)	0.52	0.32
5.00	5.011 (0.054)	1.09	0.23	5.021 (0.045)	0.90	0.42
10.0	9.999 (0.022)	0.22	-0.01	10.020(0.031)	0.31	0.20

Phosphatidylserine (PS) concentration (ng/mL)	Intra-day			Inter-day		
	Mean (SD)	CV (%)	Rel. error (%)	Mean (SD)	CV (%)	Rel. error (%)
0.25	0.252 (0.003)	1.35	0.02	0.246 (0.004)	1.62	-1.60
0.50	0.501 (0.010)	2.15	0.24	0.506 (0.011)	2.23	1.24
1.00	1.014 (0.029)	2.86	1.46	0.973 (0.014)	1.44	-2.66
2.50	2.500 (0.023)	0.92	0.03	2.470 (0.034)	1.40	-1.20
5.00	5.019 (0.049)	0.99	0.38	5.003 (0.079)	1.58	0.06
10.0	10.030(0.062)	0.62	0.30	10.022(0.043)	0.43	0.22

Sphingomyelin (SM) concentration (ng/mL)	Intra-day			Inter-day		
	Mean (SD)	CV (%)	Rel. error (%)	Mean (SD)	CV (%)	Rel. error (%)
0.25	0.250 (0.003)	1.30	0.32	0.249 (0.002)	1.07	-0.32
0.50	0.500 (0.021)	4.37	0.16	0.511 (0.020)	3.95	2.24
1.00	1.010 (0.031)	3.16	1.06	0.985 (0.029)	2.94	-1.44
2.50	2.510 (0.024)	0.98	0.40	2.504 (0.022)	0.91	0.19
5.00	4.974 (0.055)	1.11	-0.50	5.001 (0.088)	1.76	0.02
10.0	9.958 (0.092)	0.92	-0.42	9.973 (0.083)	0.83	-0.26

precisions for PC, PE, PI, PS and SM were summarized in Table 1. The accuracy expressed as the REM% for the intra- and inter-day assays with six replicate determinations for six consecutive days at working concentrations of 0.25 - 10.0 ng/mL were in the range of -0.72 - 1.52% and -1.36 - 0.72% for PC, -0.32 - 1.00% and -0.04 - 0.76% for PE, -0.76 - 2.60% and -0.26 - 2.04% for PI, 0.02 - 1.46% and -2.66 - 1.24% for PS, and -0.05 - 1.06% and -1.44 - 2.24% for SM. The precision expressed as the CV% for the intra- and inter-day assays with six replicate determinations for six consecutive days at working concentrations of 0.25 - 10.0 ng/mL were in the range of 0.38 - 3.80% and 0.19 - 2.95% for PC, 0.15 - 2.80% and 0.29 - 2.01% for PE, 0.22 - 4.06% and 0.31 - 3.73% for PI, 0.62 - 2.86% and 0.43 - 2.23% for PS, and 0.92 - 4.37% and 0.83 - 3.95% for SM. Both the accuracy and precision of the intra- and inter-day assays of PC, PE, PI, PS and SM were within acceptable criteria for analyzing their concentrations in CSF samples.

The molecular mass peaks of the PC, PE, PI, PS and SM standards were detected using a full-scan ESI-MS analysis. In order to determine the species of each phospholipid class, ion-trap MS^n was used to detect the fragments. The molecular mass peaks obtained from these analyses are shown in Figure 3. Table 2 presents the major detected species (R_1/R_2) and the corresponding *m/z* values for the PC, PE, PI, PS and SM standards. The results demonstrated that main mass peaks were C34(16 : 1/18 : 1)/C36(18 : 1/18 : 1; 18 : 0/18 : 1), C38(18 : 0/20 : 1)/C40(18 : 0/22 : 5; 18 : 0/22 : 4), C36(16 :

0/20 : 3; 18 : 0/22 : 5)/C38(18 : 3/20 : 5), C36(16 : 0/20 : 3)/C38(16 : 1/22 : 6)/C38(18 : 1/22 : 6), and C34(18 : 1/16 : 0)/C40(18 : 1/22 : 0)/C42(18 : 1/24 : 0), respectively, for the PC, PE, PI, PS and SM standards.

III. *Limits of Detection (LOD) and Limits of Quantitation (LOQ)*

The LOD, defined as the concentration at which the signal-to-noise ratio was 3 (S/N = 3), for PC, PE, PI, PS and SM under the optimized HPLC conditions were 2.0, 4.0, 4.0, 5.0 and 5.0 pg/mL, respectively. The LOQ (S/N = 10) for PC, PE, PI, PS and SM were 6.0, 12.0, 12.0, 15.0 and 15.0 pg/mL, respectively.

IV. *Analysis of PC, PE, PI, PS and SM in the CSF of Patients*

The CSF from a single patient with either one of three different illnesses, chordoma, trigeminal neuralgia and Parkinson's disease, was obtained and extracted with a solvent mixture of chloroform and methanol at a 2 : 1 ratio. The extracted lipids were analyzed by reversed-phase LC-ESI-MS, leading to a separation of the PC, PE, PI, PS and SM classes. It turned out that the five classes, PC, PE, PI, PS and SM, for all three types of illnesses were well-separated as demonstrated in Figure 4. This method was found to be more convenient than the traditional TLC method. Each class of PC, PE, PI, PS and SM was identified by LC-ESI-MS. Figure
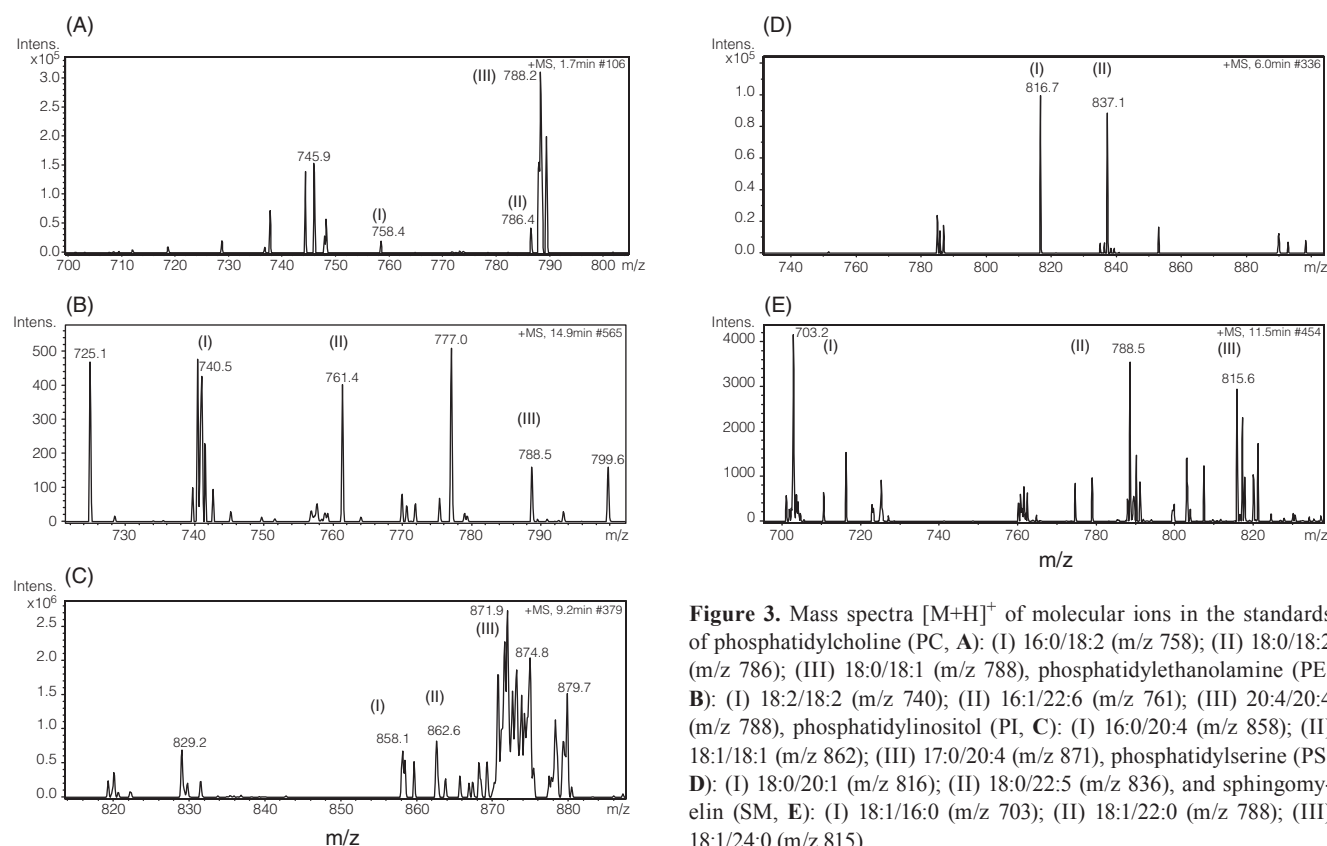

Figure 3. Mass spectra $[M+H]^+$ of molecular ions in the standards of phosphatidylcholine (PC, **A**): (I) 16:0/18:2 (m/z 758); (II) 18:0/18:2 (m/z 786); (III) 18:0/18:1 (m/z 788), phosphatidylethanolamine (PE, **B**): (I) 18:2/18:2 (m/z 740); (II) 16:1/22:6 (m/z 761); (III) 20:4/20:4 (m/z 788), phosphatidylinositol (PI, **C**): (I) 16:0/20:4 (m/z 858); (II) 18:1/18:1 (m/z 862); (III) 17:0/20:4 (m/z 871), phosphatidylserine (PS, **D**): (I) 18:0/20:1 (m/z 816); (II) 18:0/22:5 (m/z 836), and sphingomyelin (SM, **E**): (I) 18:1/16:0 (m/z 703); (II) 18:1/22:0 (m/z 788); (III) 18:1/24:0 (m/z 815).

Table 2. Detected species (R_1/R_2) and *m/z* values of the phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and sphingomyelin (SM) in the standards and the cerebrospinal fluid of three illnesses by LC-ESI-MS

Class	Standard			Chordoma			Trigeminal neuralgia			Parkinson's disease		
	Molecular Species (R_1/R_2)	*m/z*	Carbon number	Molecular Species (R_1/R_2)	*m/z*	Carbon number	Molecular Species (R_1/R_2)	*m/z*	Carbon number	Molecular Species (R_1/R_2)	*m/z*	Carbon number
Phosphatidylcholine (PC)	16:1/18:1	758	34	16:0/16:1	732	32	16:0/16:0	734	32	16:0/16:1	732	32
	18:1/18:1	786	36	16:1/18:1	758	34	16:1/18:1	758	34	16:0/16:0	734	32
	18:0/18:1	788	36	18:1/18:2	784	36	16:0/18:0	762	34	16:0/18:0	762	34
				18:1/18:1	786	36	18:0/18:1	788	36	18:0/20:4	810	38
				16:1/22:6	804	38	16:1/22:6	804	38	18:0/20:2	814	38
				18:0/20:4	810	38	18:0/20:2	814	38	18:0/22:4	838	40
				18:0/22:4	838	40	18:0/22:4	838	40			
Phosphatidylserine (PS)	18:0/20:1	816	38	18:1/18:1	786	36	16:0/18:1	760	34	16:0/18:1	760	34
	18:0/22:5	836	40	16:0/22:6	806	38	18:0/20:4	810	38	18:1/18:1	786	36
	18:0/22:4	838	40	18:0/20:4	810	38	18:0/20:3	812	38	16:0/22:6	806	38
				18:0/22:6	834	40	18:0/20:2	814	38	18:0/20:2	814	38
				18:0/22:5	836	40	18:0/20:1	816	38	18:0/22:6	834	40
				18:0/22:4	838	40	18:0/22:3	840	40	18:0/22:5	836	40
				18:0/22:3	840	40				18:0/22:4	838	40
										18:0/22:3	840	40
Phosphatidylinositol (PI)	16:0/20:3	859	36	16:0/18:1	835	34	16:0/18:2	833	34	16:0/18:2	833	34
	18:0/18:1	863	36	16:0/18:0	837	34	16:0/18:1	835	34	16:0/18:1	835	34
	18:3/20:5	877	38	16:0/20:4	857	36	16:0/18:0	837	34	16:0/18:0	837	34
				16:0/20:3	859	36	16:0/20:4	857	36	18:3/20:5	877	38
				18:0/18:1	863	36	16:0/20:3	859	36	18:1/20:4	883	38
				18:3/20:5	877	38	18:1/20:4	883	38	18:3/22:6	903	40
				18:1/20:4	883	38	18:3/22:6	903	40	18:0/22:6	909	40
				18:0/20:3	887	38	18:0/22:6	909	40	18:0/22:3	915	40
				18:0/22:3	915	40						
Phosphatidylethanolamine (PE)	16:0/20:3	740	36	16:1/20:4	736	36	16:1/20:4	736	36	18:1/18:1	742	36
	16:1/22:6	761	38	16:1/22:6	761	38	16:0/20:3	740	36	18:0/18:1	744	36
	18:1/22:6	788	40	18:1/20:4	764	38	18:1/18:1	742	36	16:1/22:6	761	38
				18:0/20:4	766	38	18:0/18:1	744	36	18:1/20:4	764	38
				18:0/20:3	768	38	16:1/22:6	761	38	18:0/20:4	766	38
				18:0/20:1	772	38	18:1/20:4	764	38	18:0/20:3	768	38
				18:3/22:6	784	40	18:0/20:4	766	38	18:3/22:6	784	40
				18:1/22:6	788	40	18:0/20:3	768	38	18:0/24:1	828	42
				18:0/22:6	790	40	18:0/22:6	790	40	18:0/24:0	830	42
				18:0/24:1	828	42	18:0/24:0	830	42			
Sphingomyelin (SM)	18:1/16:0	703	34	18:1/16:1	701	34	18:1/16:1	701	34	18:1/18:1	729	36
	18:1/22:0	788	40	18:1/16:0	703	34	18:1/16:0	703	34	18:0/18:0	733	36
	18:1/24:0	815	42	18:1/20:0	759	38	18:1/18:1	729	36	18:1/20:0	759	38
				18:1/22:0	788	40	18:1/20:1	757	38	18:1/22:0	788	40
				18:1/24:2	811	42	18:1/20:0	759	38	18:1/24:2	811	42
				18:1/24:1	813	42	18:1/22:0	788	40	18:1/24:1	813	42
				18:1/24:0	815	42	18:1/26:1	841	44	18:1/24:0	815	42
				18:1/26:1	841	44				18:1/26:1	841	44

5 presents the detected species (R_1/R_2) and the corresponding *m/z* values of the five classes in the CSF of patients with the three types of illnesses. A summary was shown in Table 2, which further demonstrated that alkyl chain lengths between C16 and C22, C16 and C22, C16 and C22, C16 and C24, and C16 and C26 were detected, corresponding to the fatty esters of PC, PE, PI, PS and SM. It seemed to demonstrate that more species were detected in the CSF samples. The detected species (R_1/R_2) within each class of phospholipid for three illnesses varied to a minor extent.

V. *Profiles and Contents of PC, PE, PI, PS and SM in the CSF of Patients with Three Different Illnesses*

The percentage (%) and content (ng/mL) of each class of PC, PE, PI, PS and SM in the CSF of patients with the three different illnesses are presented in Table 3. The results demonstrated that the percentage and content of each class differed in the CSF of patients with the different illnesses. In the CSF of the patients with chordoma, SM was the richest lipid component (848 ± 75.5 ng/mL, 40.7 ± 2.2%), and PC, PE, PI and PS were 28.3 ± 7.9 ng/mL and 1.3 ± 0.4%, 329.0 ± 22.7 ng/mL and 15.8 ± 1.3%, 437.2 ± 67.7 ng/mL and 21.0 ± 3.2%, and 438.2 ± 47.8 ng/mL and 21.0 ± 2.4%, respectively. In the CSF of the patients with trigeminal neuralgia, PC was the richest lipid component (8042.5 ± 1214.4 ng/mL, 53.8 ± 4.2%), and PE, PI, PS and SM were 5353.0 ± 392.6 ng/mL and 36.1 ± 3.4%, 664.8 ± 50.5 ng/mL and 4.5 ± 0.7%, 442.3 ± 9.2 ng/mL and 2.9 ± 0.3%, and 367.4 ± 8.7 ng/mL and 2.4 ± 0.2%, respectively. In the CSF of the patients with Parkinson's disease, PS was the richest lipid component (2124.2 ± 152.0 ng/mL, 43.3 ± 1.6%), and PC, PE, PI and SM were 199.7 ± 19.2 ng/mL and 4.0 ± 0.5%, 346.4 ± 26.0 ng/mL and 7.0 ± 0.7%, 662.1 ± 51.1 ng/mL and 13.5 ± 1.1%, and 1564.9 ± 132.6 ng/mL and 31.9 ± 2.2%, respectively. Therefore, this study established a reversed-phase LC-ESI-MS method for the separation of glycerophospholipids and a sphingolipid and the detection of all classes of glycerophospholipids and a sphingolipid (PC, PE, PI, PS and SM) in CSF in a single run.
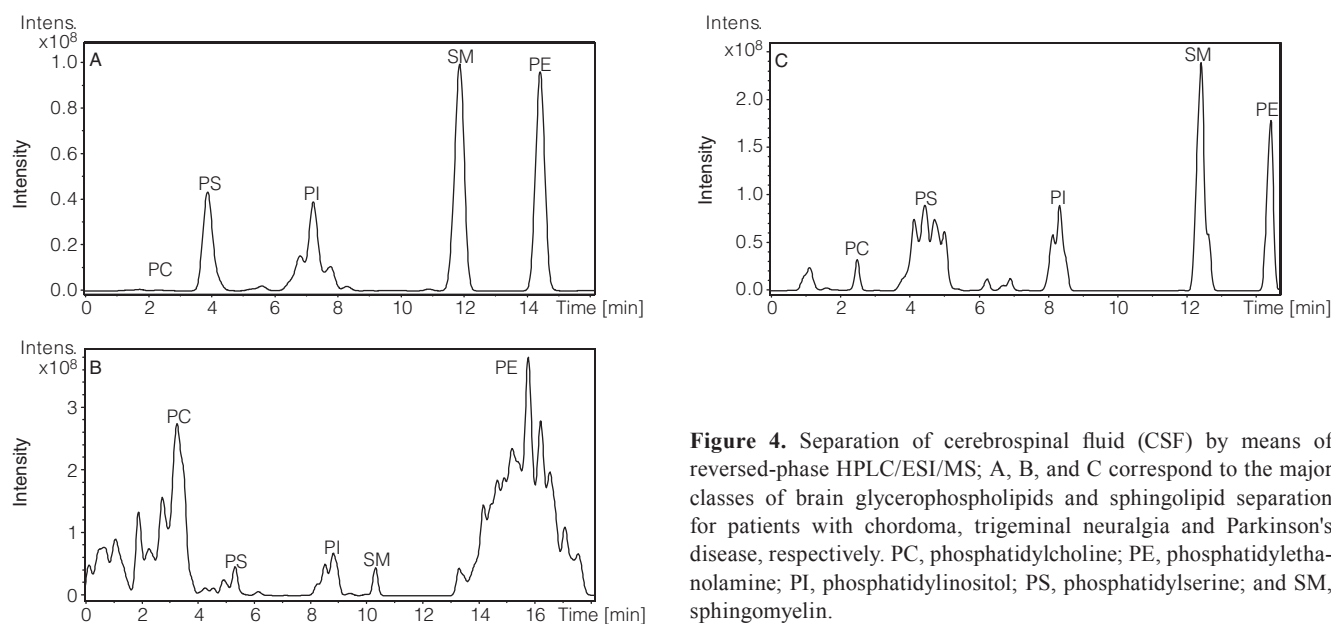

Figure 4. Separation of cerebrospinal fluid (CSF) by means of reversed-phase HPLC/ESI/MS; A, B, and C correspond to the major classes of brain glycerophospholipids and sphingolipid separation for patients with chordoma, trigeminal neuralgia and Parkinson's disease, respectively. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; and SM, sphingomyelin.

Table 3. The percentage (%)[a] and content (ng/mL)[b] of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and sphingomyelin (SM) in Chordoma, Trigeminal neuralgia and Parkinson's disease patients' cerebrospinal fluids by LC-ESI-MS

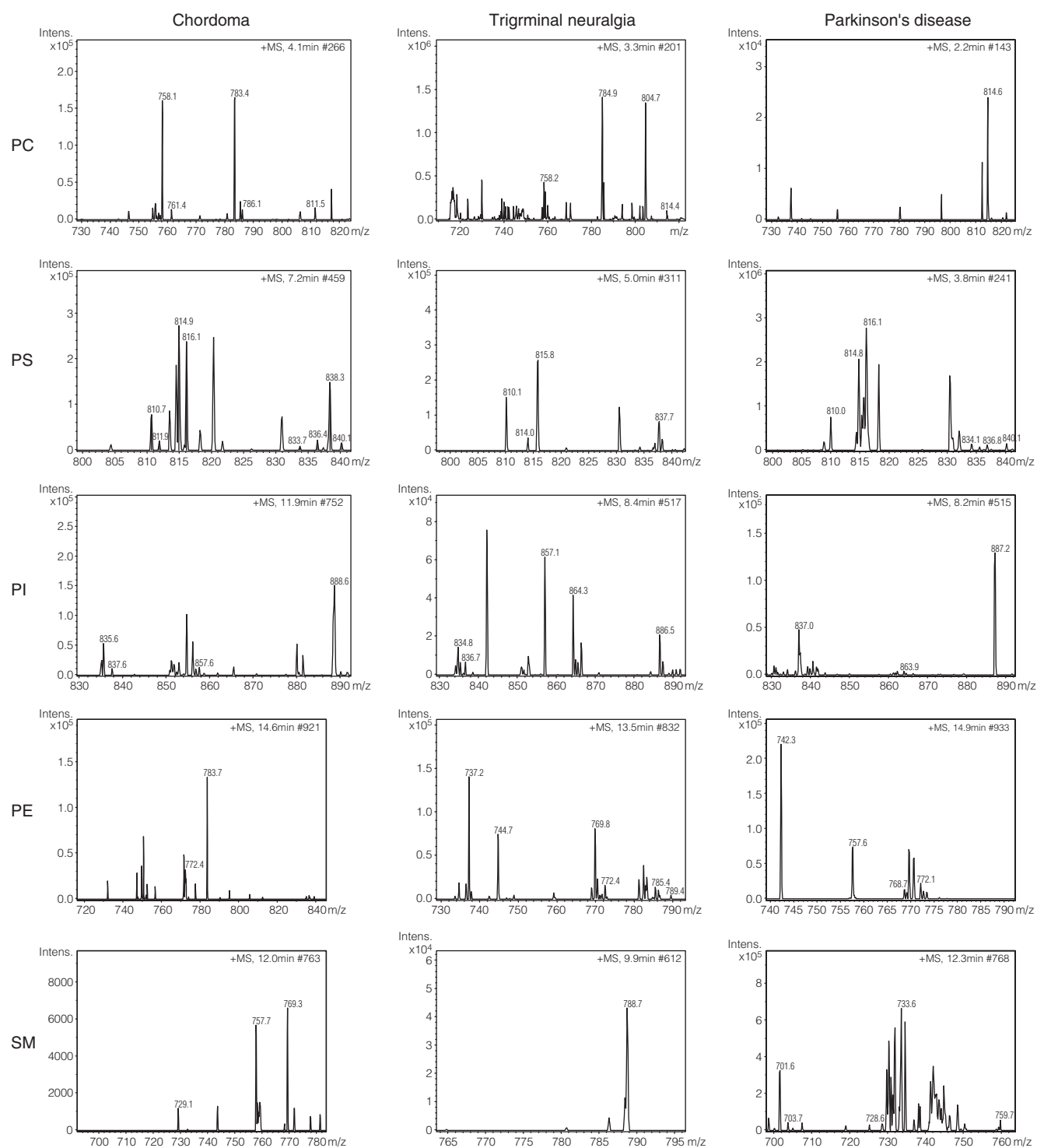| Disease	PC	PS	PI	PE	SM
Chordoma	1.3 ± 0.4[a] (28.3 ± 7.9)[b]	21.0 ± 2.4 (438.2 ± 47.8)	21.0 ± 3.2 (437.2 ± 67.7)	15.8 ± 1.3 (329.0 ± 22.7)	40.7 ± 2.2 (848.1 ± 75.5)
Trigeminal neuralgia	53.8 ± 4.2 (8042.5 ± 1214.4)	2.9 ± 0.3 (442.3 ± 9.2)	4.5 ± 0.7 (664.8 ± 50.5)	36.1 ± 3.4 (5353.0 ± 392.6)	2.4 ± 0.2 (367.4 ± 8.7)
Parkinson's disease	4.0 ± 0.5 (199.7 ± 19.2)	43.3 ± 1.6 (2124.2 ± 152.0)	13.5 ± 1.1 (662.1 ± 51.1)	7.0 ± 0.7 (346.4 ± 26.0)	31.9 ± 2.2 (1564.9 ± 132.6)

Figure 5. Mass spectra of molecular ions of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanol-amine (PE) and sphingomyelin (SM) in the cerebrospinal fluids of three patients with different illnesses by LC-ESI-MS.

CONCLUSIONS

In this report, a reversed-phase LC-ESI-MS method was established to analyze glycerophospholipids and a sphingolipid. It further provided a valuable and useful tool to analyze all classes of lipids in the CSF of patients with different illnesses. The developed method was used to profile five classes of PC, PE, PI, PS and SM and their contents in CSF. This method can be used to directly detect the classes of lipids in the CSF and is more convenient and labor-saving than the traditional TLC method.

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