Identification of Phospholipid Molecular Species in Porcine Brain Extracts Using High Mass Accuracy of 4.7 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Received February 4, 2006

Key Words : Fourier-transform ion cyclotron resonance mass spectrometer (FTICR-MS), Phospholipid (PL), Lipid, Lipidomics, High mass accuracy

Lipids play a critical role in many metabolic and biological processes, including energy production and storage, the formation and functioning of cellular membranes, and signal transduction.[1-3] The biological importance of lipids has drawn extensive attention to the analysis of lipid molecular species. In particular, mass spectrometry (MS) has been shown to be a powerful tool in elucidating the diversity of lipid species and their participation in regulating cellular functions.[4-12] In recent years, Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) has been increasingly utilized, particularly in profiling global lipid distribution.[7,13-18] Its unprecedented mass accuracy enables the discernment of mass differences of less than 0.01 mass unit, such as those often observed in lipid analyses. Under the assumption that the cation species are known, the minimum accuracy for assigning the chemical composition of lipid species is 21.38 ppm.[14] With the current FTICR-MS technology, this mass accuracy is achievable even in a broad band mass analysis in the range *m/z* 400-1,000. In addition, databases(DBs) with extensive entries are now available and play an essential role in identifying individual lipid molecular species among an enormous number of lipids.[20-22] For example, the group of Taguchi has demonstrated that low magnetic field (4.7 T) FTICR MS can be applied to identify the molecular species of phophatidylethanolamine (PE) of *Caenorhabditis elegans* and their oxidized metabolites.[15] Electrospray ionization (ESI) FTICR-MS of total lipid extracts has also been shown to be useful in detecting differences between the distributions of highly heterogeneous mixtures of lipids found in eukaryotic cells.[7]

In the present study, 4.7 T ESI-FTICR-MS was performed on porcine brain extracts to demonstrate its lipid profiling capability, and these results were rigorously checked with ESI-MS/MS using a linear ion-trap mass spectrometer. To our knowledge, this is the first study to rigorously validate the preliminary assignments of lipid species based on high accuracy mass values of FTICR MS by comparison with MS/MS results. In addition, a recently publicized web DB, "Lipidsearch", with extensive lipid entries (advertised to have more than 100,000 entries) is fully utilized to obtain a lipid profile of the porcine brain sample.[20,21]

Experimental Section

Experiments were performed on a commercial 4.7 T FTICR mass spectrometer (Ionspec Inc., Lake Forest, CA, USA). The detailed experimental setup is described elsewhere.[23-25] A total lipid extract from *porcine* brain tissue was purchased from Avanti polar lipids, inc. (Alabaster, AL, USA) in the form of chloroform:methanol extracts. Sample preparation was performed using the procedure described previously.[26] For positive ESI MS, a 200-μL aliquot of the sample was mixed with 10 μL acetic acid. Phospholipids can ionize in both positive and negative ion modes, but they are more efficiently analyzed in the positive ion mode. The prepared sample solution was infused directly through a fused silica capillary (I.D.= 75 μm) emitter at a flow rate of 0.5 μL/min using a syringe pump (Harvard Apparatus 22, Holliston, MA, USA). The sprayed ions were externally accumulated for 2 s in a hexapole ion trap in order to achieve maximum abundance of lipid species. The ions were detected at 1 MHz with 128 k data points taken for a total observation time of 6.3 s. The ESI MS spectrum was obtained by co-adding 100 time-domain data sets. The time-domain data sets were Hanning apodized and zero filled once before a fast Fourier transform procedure was performed. Accurate mass measurements were made through an internal calibration procedure. The theoretical masses of the following protonated molecular species $(M+H)^+$ present at high abundance were used as references: 734.5699 (PC, 1-acyl 16:0/16:0), 760.5856 (PC, 1-acyl 16:0/18:1), 782.5699 (PC, 1-acyl 16:0/20:4), 798.5437 (PE, 1-alkyl 42:10), 810.6012 (PC, 1-acyl 18:0/20:4), and 813.6849 (SM, 2-amido 24:1), where PC, PE, and SM refer to phosphatidylcholine, phosphoethanolamine, and sphingomyelin, respectively (Scheme 1). In order to confirm the preliminary identifications of lipid molecular species obtained with 4.7 T FTICR-MS, tandem mass spectrometry was also performed using a linear ion trap mass spectrometer (LTQ, Thermo Electron Corp., San Diego, CA, USA). In MS/MS, the ion

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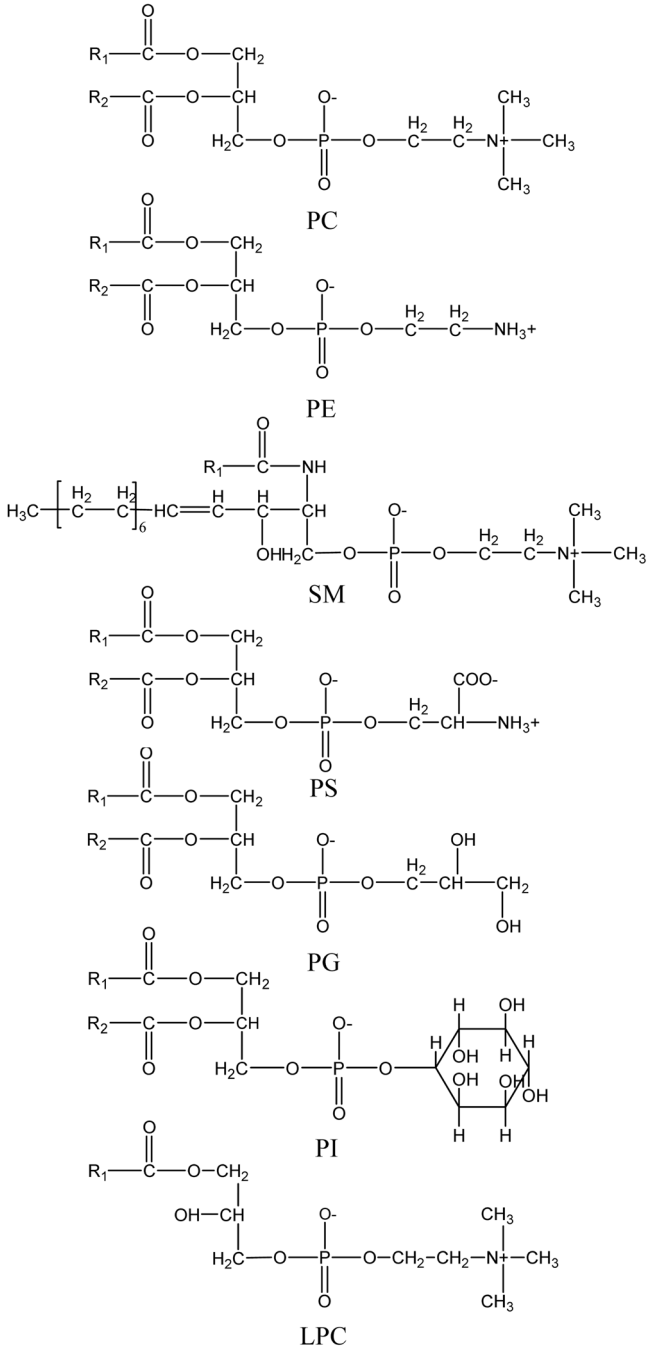

Scheme 1. Representative molecular structures of PC (phosphatidylcholine), PE (phosphoethanolamine), SM (sphingomyelin), PS (phosphoserine), PG (phosphoglycerol), PI (phosphoinositol), and LPC (lysophosphatidylcholine).

peaks observed in the ESI spectrum were manually selected one by one, and each one was then subjected to collisionally activated dissociation (CAD).

Results and Discussion

Figure 1(a) shows a positive ESI mass spectrum, displaying the region of *m/z* 150-1,000. While only a small number of ion peaks are observed in the region between 150 and 700

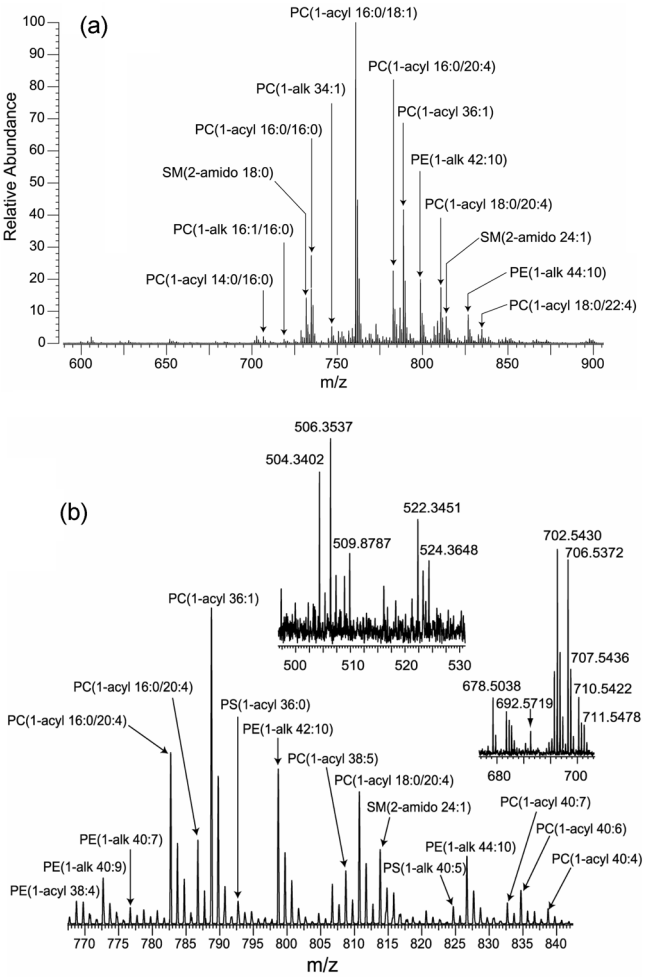

Figure 1. (a) ESI-FTICR MS spectrum in the region of m/z 580-910 obtained for porcine brain extracts in a chloroform:methanol (2 : 1) solution. (b) An enlarged mass spectrum in the region of m/z 768-842. Identified ion peaks are labeled with the class and constituent acyl (or alkenyl, alkyl) groups.

m/z, a large number of ion peaks appear in the region m/z 700-900. This implies that major components of the extracts are phospholipids with a mass range of 700-900 Da. Phospholipids include the following classes: PC, PE, SM, PS (phosphoserine), PG (phosphoglycerol), PI (phosphoinositol), LPC (lysophosphatidylcholine), etc (see Scheme 1). This result shows some contrast to the component analysis table provided by the commercial sample provider, in which other lipid species are shown to be included in a substantial amount (> 50%). This discrepancy is due largely to the abundance bias in ESI MS measurements originating from much higher ionization efficiency of phopholipids in comparison with other lipid species.

As shown in Figure 1(b), the ion peaks observed in this experiment shows a good mass resolution that surpasses that of other types of mass spectrometer, such as a time-of-flight mass spectrometer and triple quadrupole mass spectrometer. High mass resolution of the ion peaks observed resulted in high mass accuracy. The accuracy is further improved with an internal calibration. As a result, an average mass error of

Table 1. Lipid species found within 30 ppm mass tolerance assigned using the theoretical masses of the lipid species available in web database "Lipidsearch". The species identified as a false positive are marked with X in parenthesis and those with ambiguous identification with in parenthesis. The reference lipid species used in internal calibration are denoted in *italic* **bold** letters

Class	Observed m/z	Theoretical m/z	ppm	Assignment
PC	678.5040	678.5068	4.14	1-acyl 28:0 (△)
	692.5720	692.5588	19.0	1-alk 30:0 (△)
	706.5373	706.5381	1.14	1-acyl 14:0/16:0 (O)
	716.5536	716.5588	7.32	1-alk 32:2 (△)
	718.5796	718.5745	7.12	1-alk 16:1/16:0 (O)
	720.5928	720.5901	3.70	1-alk 32:0 (O)
	734.5712	734.5694	2.45	1-acyl 16:0/16:0 (O)
	744.5897	744.5901	0.59	1-alk 34:2 (O)
	746.6056	746.6058	0.95	1-alk 34:1 (O)
	756.5521	756.5538	2.19	1-acyl 16:0/18:3 (O)
	758.5706	758.5694	1.58	1-acyl 16:0/18:2 (O)
	760.5846	760.5851	0.60	1-acyl 16:0/18:1 (O)
	770.6109	770.6058	18.3	1-alk 36:3 (O)
	774.6275	774.6371	12.4	1-alk 16:1/20:0 (O)
	782.5676	782.5694	2.31	1-acyl 16:0/20:4 (O)
	786.6008	786.6007	0.12	1-acyl 36:2 (O)
	788.6174	788.6164	1.33	1-acyl 36:1 (O)
	794.5849	794.6058	26.3	1-alk 18:2/20:3 (O)
	796.6164	796.6214	6.34	1-alk 38:4 (O)
	804.5649	804.5538	13.9	1-acyl 16:0/22:7 (O)
	806.5782	806.5694	10.9	1-acyl 38:6 (O)
	808.5805	808.5851	5.64	1-acyl 38:5 (O)
	810.5973	810.6007	4.21	1-acyl 18:0/20:4 (O)
	832.5886	832.5851	4.26	1-acyl 40:7 (O)
	834.6056	834.6007	5.87	1-acyl 18:0/22:6 (O)
	838.6368	838.6320	5.72	1-acyl 18:0/22:4 (O)
	848.5334	848.5225	12.9	1-acyl 42:13 (O)
	850.6731	850.6684	5.54	1-alk 42:5 (O)
	854.5533	854.5694	18.9	1-acyl 42:10 (O)
	856.5916	856.5851	7.65	1-acyl 42:9 (O)
	858.5965	858.6007	4.90	1-acyl 42:8 (O)
	860.6532	860.6164	3.77	1-acyl 18:1/24:6 (O)
	862.6532	862.6320	24.6	1-acyl 42:6 (O)
	864.6449	864.6477	3.19	1-acyl 42:5 (O)
	866.6599	866.6633	3.93	1-acyl 42:4 (O)
	892.6703	892.6790	9.71	1-acyl 44:5 (O)
PE	700.5248	700.5275	3.92	1-alk 34:3 (△)
	702.5431	702.5432	0.13	1-alk 34:2 (△)
	724.5249	724.5275	3.65	1-alk 36:5 (O)
	740.5021	740.5225	27.5	1-alk 36:3 (△)
	750.5405	750.5432	3.59	1-alk 38:6 (O)
PE	752.5595	752.5588	0.88	1-alk 38:5 (O)
	766.5225	766.5381	20.4	1-acyl 38:5 (O)
	768.5580	768.5538	5.53	1-acyl 38:4 (O)
	772.5278	772.5275	0.34	1-alk 22:0/18:9 (O)
	776.5543	776.5588	5.85	1-alk 40:7 (O)
	778.5730	778.5745	1.92	1-acyl 36:6 (O)
	780.5808	780.5901	12.0	1-alk 40:5 (O)
	794.5849	794.5694	19.5	1-acyl 40:5 (O)
	798.5446	798.5432	1.77	1-alk 42:10 (O)
	818.5532	818.5694	19.8	1-acyl 42:7 (O)
	826.5776	826.5745	3.77	1-alk 44:10 (O)
	862.6532	862.6684	17.6	1-alk 46:6 (O)
	874.6155	874.6320	23.5	1-acyl 46:7 (△)
SM	***813.6896***	813.6844	6.43	2-amido 24:1 (O)
	841.7416	841.7157	30.8	2-amido 26:1 (O)
PS	740.5021	740.5222	27.5	1-alk 34:5 (△)
	766.5225	766.5017	27.1	1-alk 36:6 (△)
	772.5278	772.5487	27.0	1-alk 36:3 (△)
	792.5773	792.5749	3.05	1-acyl 36:0 (O)
	798.5446	798.5643	24.7	1-alk 38:4 (△)
	818.5532	818.5330	24.7	1-alk 40:8 (O)
	820.5233	820.5487	30.9	1-alk 40:7 (O)
	824.5568	824.5800	28.1	1-alk 40:5 (O)
	844.5289	844.5487	23.5	1-alk 42:9 (△)
	846.5435	846.5643	24.6	1-alk 42:8 (O)
	870.6223	870.6218	0.54	1-acyl 42:3 (O)
	872.5773	872.5800	3.06	1-alk 44:9 (O)
	874.6115	874.5956	18.2	1-alk 44:8 (O)
PEt	683.5061	683.5010	7.49	1-alk 20:0/14:4 (O)
	871.7129	871.7150	2.42	1-alk 48:3 (X)
	881.7538	881.7357	20.5	1-acyl 46:1 (X)
	895.7154	895.7150	0.44	1-acyl 48:3 (X)
	897.7320	897.7307	1.50	1-acyl 48:2 (X)
	899.7479	899.7463	1.77	1-acyl 48:1 (X)
	901.7743	901.7620	13.7	1-acyl 48:0 (X)
	923.7509	923.7463	4.98	1-acyl 50:3 (X)
LPC	496.3315	496.3397	16.6	1-acyl 16:0 (O)
	504.3404	504.3448	8.79	1-acyl 18:3 (△)
	506.3539	506.3605	13.0	1-alk 18:2 (△)
	522.3453	522.3554	19.4	1-acyl 18:1 (△)

2.73 ppm was achieved for these six reference molecules, which is quite reasonable given the magnetic strength of 4.7 Tesla used in the present study. For the mass values with high accuracy, the ion peaks observed were assigned using the web DB "Lipidsearch". The DB search was initially performed with a mass tolerance of 0.3 Da, and then stricter criteria of 30, 20, and 10 ppm mass tolerances were applied. With the mass accuracy criteria of 10, 20, and 30 ppm, 50, 67, and 82 lipid molecular species were found, respectively. These lipid species are listed in Table 1, where they are categorized based on their classes. The number of the lipid species found within 30 ppm tolerance was 82, and is comparable to that identified in other studies performed with different types of mass spectrometer in combination with liquid chromatography.[10,21,27,28]

Identification of the lipid species listed in Table 1 was confirmed using ESI-MS/MS investigations with a linear ion trap mass spectrometer. An example of a representative MS/

MS spectrum can be found in Supporting Figure 1. Out of 82 lipid species found within 30 ppm mass tolerance, 60 were positively identified (Table 1: marked with O in parenthesis), while 7 were found to be a different species and represent false positives (X). Fifteen species could not be verified (△) due to low abundance, but are expected, on the statistical basis, to show a similar ratio of positive identification, *i.e.* 90%, when compared with those clearly confirmed with MS^n. This value should be compared with our DB statistical analysis results, which reveal that 10, 20, and 30 ppm mass tolerance can lead to false positive identifications with the probability of 3.26, 17.55, and 43.65%, respectively. Considering that in our experiments, most ion peaks are within 20 ppm mass tolerance range, our results are consistent with the DB statistical analysis. The false positive ion peaks are by and large the ones with low abundance, and close inspection of these ion peaks revealed that they do not show a sharp peak shape due in part to the low abundance. For example, the ion peak observed at m/z 692.5719 (preliminarily assigned as PC 1-alkyl 30 : 0; see the inset in Figure 1(b)) has a relative abundance of 0.274, which corresponds to 4 : 1 signal-to-noise (S/N) ratio.

On the other hand, the false positive ion peaks between m/z 504 and 523 appear to arise from loss of an acyl group from a larger PC species; LPC 1-acyl 18:3 (m/z 504.3404), LPC 1-alkyl 18:2 (m/z 506.3539), and LPC 1-acyl 18:1 (m/z 522.3453), [see the insets in Figure 1(b)]. In addition to the protonated lipid species, 21 lipid species are found to exist in a sodiated adduct form $(M+Na)^+$. None of these exists solely in a sodiated form, and they are all present at low levels than their protonated counterparts. Thus, interference due to alkali metal adducts was not a significant factor in the analysis based on the protonated form of lipid species.

In global profiling of lipidomes, reproducibility of ESI mass spectra is essential. A product moment analysis, which plots the abundances of the ion peaks observed in one experiment versus those observed in another experiment, shows an excellent linear regression coefficient of R=0.997. This suggests that our method is very useful in discerning differences between a control lipidome and the one of interest.

In summary, analysis of lipid molecular species in porcine brain extracts was achieved using 4.7 T FTICR MS. On the basis of high mass accuracy provided by FTICR MS, 82 lipid species could be found within 30 ppm mass tolerance. Verification with the linear ion trap MS/MS results shows a 90% positive identification ratio. To the best of our knowledge, this is the first report of a specific positive identification ratio for the lipid mass values obtained with high resolution FTICR MS. This test was possible due largely to the development of an extensive lipid DB, "Lipidsearch". Further investigation is ongoing to provide more generalized results.

Acknowledgments. This study was supported by grant 2005-02926 from the Korea Ministry of Science & Technology (MOST).

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