POSTER NOTE

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Identification of Phospholipid Species Implicated in Dementia by Untargeted LC/HRMS and Data Dependent MS/MS

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ABSTRACT

Purpose: Determine the identity of molecular lipid species implicated in Alzheimer's Disease.

Methods: LC/MS and data dependent MS/MS was employed using a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer and automated lipid identification with Thermo Scientific™ LipidSearch™ software.

Results: Fatty acids comprising the molecular phospholipid species were unambiguously identifie

INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia in the elderly. Recently, Mapstone et al. reported a panel of plasma phospholipids (Table 1) that predicted cognitively normal adults who lat progressed to either mild cognitive impairment or dementia due to AD. This study utilized a targete metabolomics p180 kit (Biocrates, Life Science AG, Austria) that measures phospholipids by infusi and selected reaction monitoring (SRM) with a triple quadrupole mass spectrometer. Since phospholipids have many isomers and isobars comprised of different fatty acid and alkyl/alkenyl etl combinations, it is not possible to assign unequivocally the phospholipid species using a low resolution approach.

Identification of fatty acid constituents in phospholipids implicated in AD is critical for two reasons: (to determine the functions of these lipids and their contributions to the pathophysiology of disease; and (b) to determine the molecular identities so one can develop quantitative assays to measure these particular phospholipids in human plasma.

The objective of this study is to determine which fatty acids comprise phosphatidylcholine (PC) lipi including lyso-PC 18:2; PC 36:6, 38:0, 38:6, 40:1, 40:2, 40:6 and 40:6e (ether) in human plasma using liquid chromatography and high-resolution accurate mass and tandem mass spectrometry (L MS/MS).

These results demonstrate that high resolution accurate mass (HRAM) LC-MS/MS is an excellent method for identifying isomeric lipid species from human plasma and which is essential for discovering the most meaningful changes between normal and diseased states.

MATERIALS AND METHODS

Sample Preparation

Aliquots of human plasma (30µL) from 5 normal, 5 MCI (mild cognitive impairment) and 5 AD Alzheimer's-type dementia patients were extracted using the method of Bligh and Dyer. The organi phase was evaporated to dryness and the lipid extract was reconstituted in 100µL of 50:50 IPA/ methanol. A pooled sample was prepared and injected in duplicate for positive and negative ion rur

LC-MS and dd-MS2 Method

An LC/MS system was employed consisting of a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system coupled to an Orbitrap Fusion Lumos mass spectrometer. HPLC separation (Table 2 was achieved with a 2.1 x 150mm, 3.0µm, Thermo Scientific™ C30 Acclaim™ column[2] and MS analysis (Table 3) was performed at 120K resolution (FWHM at m/z 200) and untargeted data dependent HCD MS^2 at 15K resolution. When a phosphocholine (m/z 184.0733) product ion was observed during the profiling experiment, a single CID experiment was also performed on the same precursor ion[3].

Data Analysis

LipidSearch 4.1 software was used for lipid identification using the workflow in Figure 1. The LC-MS data (4 raw files, 2.3 GB total) containing high resolution MS and data dependent MS^2 were searche using the parameters in Table 4. For each MS^2 spectrum, results are obtained for those lipid species matching the accurate m/z of the precursor ion and its predicted fragment ions. Lipid annotations from all 4 files were correlated within ±0.1 min and search results were merged into a single table. I this study, molecular lipid annotations were reported only if the ID's correlated in both positive and negative ion modes.

Table 1. Lipid Species in Previous Studies That May Be Potential Indicators of Dementia Stat

Lipid Class	Species	Composition	m/z $[M+H]^+$	m/z $[M+HCO_2]^-$
LPC	18:2	$C_{26}H_{50}NO_7P$	520.3398	564.3306
PC	36:6	$C_{44}H_{76}NO_8P$	778.5381	822.5291
PC	38:6	$C_{46}H_{80}NO_8P$	806.5694	850.5604
PC	38:0	$C_{46}H_{92}NO_8P$	818.6633	862.6543
PC	40:6e	$C_{48}H_{86}NO_7P$	820.6215	864.6124
PC	40:6	$C_{48}H_{84}NO_8P$	834.6007	878.5954
PC	40:2	$C_{48}H_{92}NO_8P$	842.6633	886.6543
PC	40:1	$C_{48}H_{94}NO_8P$	844.6790	888.6700

RESULTS

The LipidSearch results for PC 40:6 illustrates the identification process (Figure 2). Three different adducts were observed for the PC 40:6 species including $M+H^+$ (m/z 834.6007), $M+Na^+$ (m/z 856.5827) and $M+HCO_2^-$ (m/z 878.5916). Mass chromatograms (Figure 2a and 2b) are displayed for formate and protonated adduct ions at retention time 15.1 min.

The negative ion HCD MS^2 spectrum (Figure 2c) matches PC containing 22:6 and 18:0 fatty acids: neutral loss (NL) of 18:0 and 22:6 ketene from the $M-CH_3$ anion (m/z 818.5705) are observed at m/z 552.3096 and 508.3409, respectively, in addition to the fatty acid anions. A choline containing head group in negative ion is inferred by NL of methyl formate.

The positive ion HCD and CID MS^2 spectra combined give the phospho-choline fragment ion (Figure 2d, m/z 184.0733) and NL of fatty acid / ketene: -22:6 (m/z 506.3605 / 524.3711); -18:0 (m/z 524.3711 / 552.3102) confirming the negative ion results. The underscore (18:0_22:6) is used to denote the fatty acyl position on the glycerol is not assigned in this case.

Similarly, all species in Table 1 were investigated to determine the fatty acid compositions and the identification results are summarized in Table 5. Although some of the previously identified sum composition species are present, there were instances of incorrect assignments, based on the SRM data, and mixtures of multiple species including false positives.

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Figure 3 shows that three different PC 38:6 isomers are observed in the negative ion mass chromatogram of the formate adduct, m/z 850.5604. The MS^2 spectrum of isomer (1) matches the fragment ions predicted for 18:2_20:4; isomer (2), 18:1_20:5; and isomer (3), 16:0_22:6. NL of 20:4, 20:5 and 22:6 ketene from the M-CH_3 anions, and formation of a pair of fatty acid anions provide unambiguous annotations.

Table 2. HPLC Method and Operating Conditions

Time, min	% A	% B	HPLC Parameter	Conditions
0.0	70	30	Mobile phase A	60:40 CH_3CN / H_2O
2.0	57	43	Mobile phase B	90:10 IPA / CH_3CN
2.1	45	55	buffer (A & B)	10mM NH_4HCO_2 + 0.1% HCO_2H
12	35	65	Flow rate	260 µL/min
18	15	85	Column Temp	45 ° C
20	0	100	Injection vol.	2 µL
25	0	100	Mobile phase A	60:40 CH_3CN / H_2O
25.1	70	30	Mobile phase B	90:10 IPA / CH_3CN
30	70	30	buffer (A & B)	10mM NH_4HCO_2 + 0.1% HCO_2H

Table 3. High Resolution LC-MS Profiling Method

HESI Source	Orbitrap Fusion Lumos MS
Sheath Gas: 40	Pos. Ion (250–1200 amu); Neg. Ion (200–1200 amu)
Aux Gas: 3	MS Resolution: R = 120K (FWHM m/z 200
Spray Voltage: 3.5 kV (2.5 kV neg. ion)	Top-Speed dd-MS^2: 1.0 sec at 15K (FWHM m/z 200)
RF-Lens: 50	MS^2 Isolation Width: 1.0 Da
Cap. Temp: 320 C	Stepped NCE – Pos. 27 ± 3; Neg. 30 ± 10
Heater Temp: 300 C	AGC Target: 4.0 E+5 MS, 50 msec max. 5.0 E+4 MS^2, 35 msec max. for 1.0 sec HCD m/z 184.0733 triggered CID MS^2 (30%)

Figure 1. LipidSearch Workflow

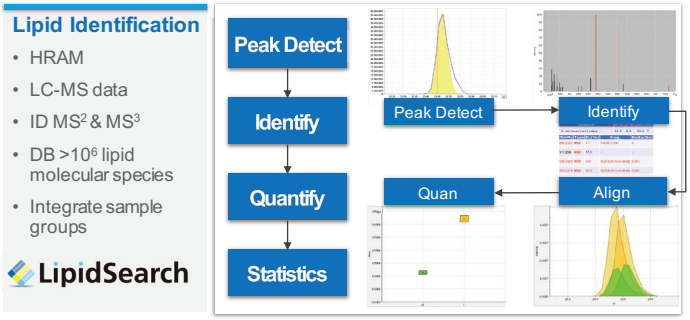
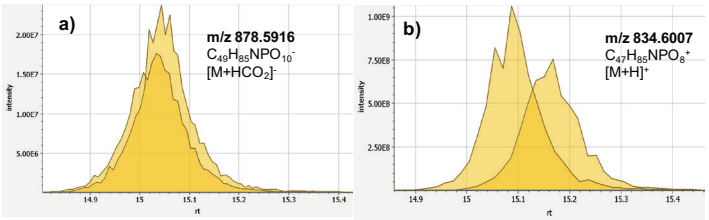


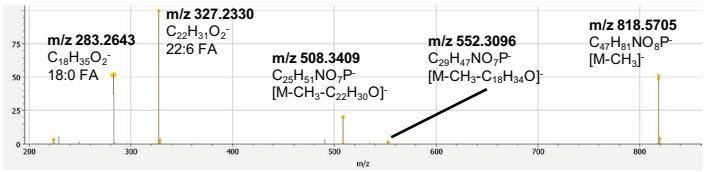
Table 4. LipidSearch 4.1 SP1 Parameters

Parameter	Setting
Prec. mass tolerance	5 ppm
Prod. mass tolerance	5 ppm
Phospholipids	LPC, PC, LPE, PE, LPS, PS, LPG, PG, LPI, PI, LPA, PA, CL
Sphingolipids	So, LSM, SM, Cer, CerG1, CerG2, CerG3
Glycerolipids	MG, DG, TG
Neutral lipids	ChE, CoQ
Other lipids	Acylcarnitine

Figure 2. LipidSearch Identification of 18:0_22:6 PC



c) Negative Ion HCD MS^2 Spectrum



d) Positive Ion HCD and CID MS^2 Spectra Combined

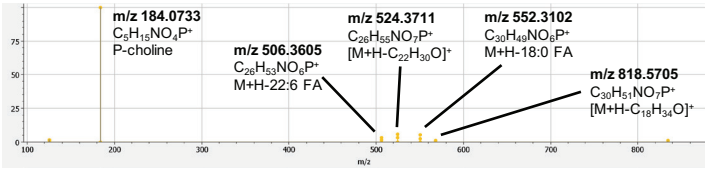
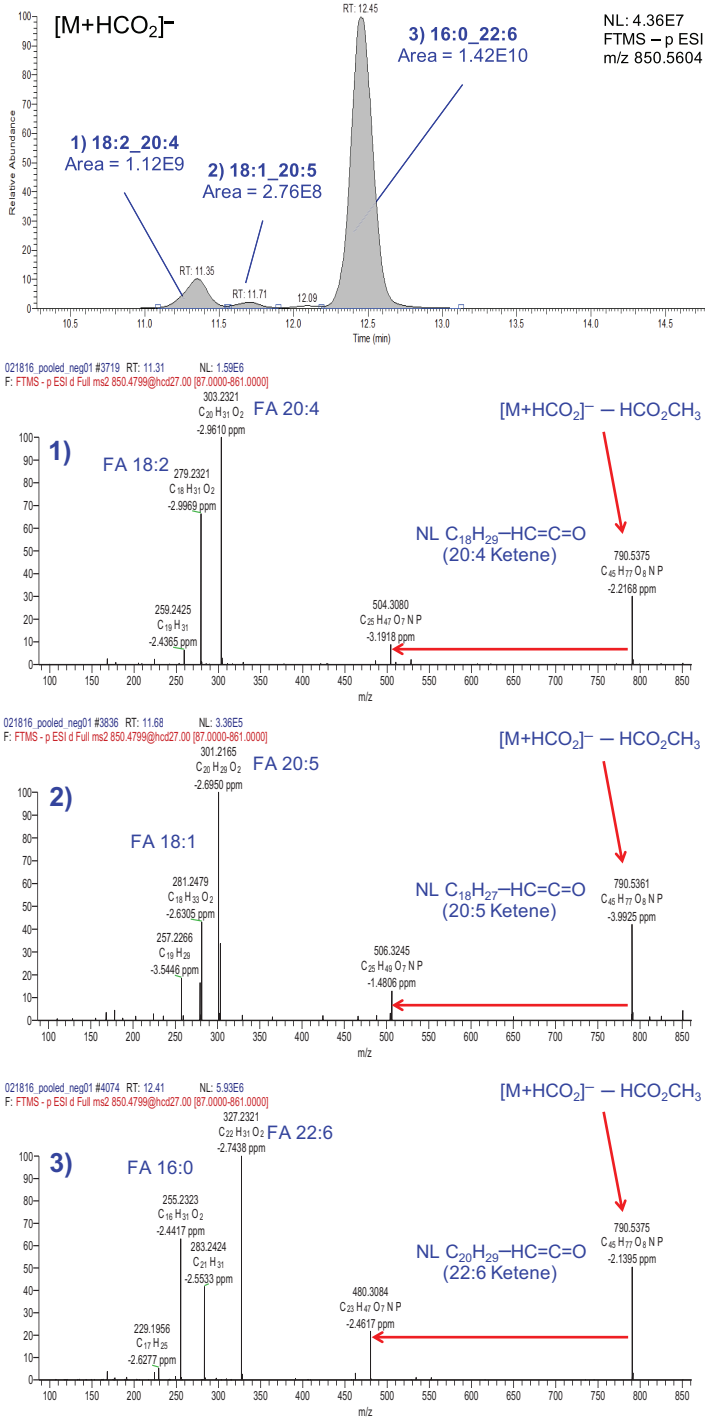


Table 5. PC Molecular Species Identified by High Resolution LC-MS and dd-MS^2

Rt, min	PC	Molecular Species	m/z $[M+H]^+$	m/z $[M+HCO_2]^-$	Confirmed? – Comments
3.8	Lyso 18:2	Lyso 18:2	520.3398	564.3307	Yes; HCD - / CID +
10.2	36:6	14:0_22:6	778.5381	822.5291	Yes; HCD - / CID +
11.4	38:6	18:2_20:4	806.5694	850.5604	Yes; HCD - / CID +
11.7	38:6	18:1_20:5	806.5694	850.5604	Yes; HCD - / CID +
12.5	38:6	16:0_22:6	806.5694	850.5604	Yes; HCD - / CID +
19.5	38:0	38:0	818.6633	862.6543	No; HCD - mix; + m/z 184
15.9	40:7e	18:1E_22:6	818.6058	862.5967	Yes; HCD - / CID +
15.0	40:6e	18:1E_22:5	820.6215	864.6124	Yes; HCD -
16.3	40:6e	20:2E_20:4	820.6215	864.6124	Yes; HCD - / CID +
15.1	40:6	18:0_22:6	834.6007	878.5917	Yes; HCD - / CID +
18.9	40:2	22:0_18:2	842.6633	886.6543	Yes; HCD - / CID +
19.6	40:1	40:1	844.6790	888.6699	No; No MS^2 data

Figure 3. Identification of Three Main 38:6 PC Isomers



DISCUSSION

The fatty acids in a total of 23 Lyso PC and 67 PC molecular species were confirmed in this study by both positive and negative ion MS/MS) including the species of interest from previous AD studies (highlighted in red, Table 5) and the additional species listed here.

- ✓ **Lyso PC:** 14:0, 15:0, 16:1 (3 iso), 16:0, 17:1, 17:0, 18:3, 18:2, 18:1 (2 iso), 18:0, 19:0, 20:5, 20:4, 20:3, 20:2, 20:1 (2 iso), 22:6, 22:5, 22:4
- ✓ **PC diacyl:** 32:2 (14:0_18:2), 32:1 (16:0_16:1), 32:0 (16:0/16:0), 33:2 (15:0_18:2), 33:1 (15:0_18:1; 16:0_17:1), 34:4 (14:0_20:4), 34:3 (16:0_18:3; 16:1_18:2), 34:2 (16:0_18:2), 34:1 (16:0_18:1), 34:0 (16:0_18:0), 35:4 (15:0_20:4), 35:3 (17:1_18:2), 35:2 (17:0_18:2, 2 iso), 35:1 (17:0_18:1), 36:6 (14:0_22:6), 36:5 (18:3_18:2; 16:1_20:4; 16:0_20:5), 36:4 (16:0_20:4; 18:2/18:2), 36:3 (16:0_20:3, 2 iso; 18:1_18:2; 18:1_18:3), 36:2 (18:1/18:1; 18:0_18:2), 36:1 (18:0_18:1), 37:6 (15:0_22:6), 37:4 (17:0_20:4, 2 iso), 37:3 (17:0_20:3), 37:2 (19:0_18:2), 38:6 (16:0_22:6, 90%; 18:2_20:4, 8%; 18:1_20:5, 2%), 38:5 (18:1_20:4, 2 iso; 18:2_20:3), 38:4 (16:0_22:4; 18:1_20:3; 18:0_20:4), 38:3 (18:0_20:3, 2 iso), 38:2 (18:0_20:2; 18:1_20:1), 39:6 (17:0_22:6), 39:4 (19:0_20:4), 40:7 (18:1_22:6, 2 iso; 20:3_20:4), 40:6 (18:0_22:6; 20:2_20:4), 40:5 (18:0_20:5, 2 iso; 18:1_22:4), 40:4 (18:0_22:4), 40:2 (22:0_18:2)
- ✓ PC **PC acyl/ether**: 34:2e (16:0e_18:2), 36:2e (18:0e_18:2), 36:4e (16:0e_20:4; 16:1e_20:3), 38:5e (18:1e_20:4, 2 iso), 38:4e (18:0_20:4, 2 iso), 40:7e (18:1E_22:6), 40:6e (18:1E_22:5, 17%; 20:2E_20:4, 83%)
- ✓ PC Previously miss-identified 38:0 PC was found to be instead 40:7e; a low mass resolution SRM experiment (m/z 818.6 > 184.1) does not distinguish 38:0 from 40:7e PC. However, high resolution accurate mass measurements of precursor (m/z 818.6633 pos. ion, 862.5967 neg. ion) and product ions from LC-MS^2 data unequivocally assigns 40:7e as the species of interest.
- ✓ PC 40:1 was not confirmed due to extremely low abundance.

CONCLUSIONS

- Orbitrap-based LC-MS/MS untargeted lipidomics and LipidSearch software enables reliable and comprehensive lipid identification. This method was successfully applied to identification of lipid species implicated in AD patients.
- High mass accuracy and specificity of the LC-MS (120K) and MS^2 HCD/CID identification (15K) obtained with the Fusion Lumos MS allows confident lipid identification from human plasma extracts.
- Profiling of lipids in human plasma by flow injection SRM may lead to false positives or incorrect assignments.

REFERENCES

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2. R Kiyonami, *et al.*, Large-scale lipid profiling of a human serum lipidome using a high-resolution, accurate-mass LC/MS/MS approach, ThermoFisher Scientific Application Note 647, **2016**.
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