Metabolic Profiling of Phospholipids in Rat Plasma utilizing Ultra Pressure Liquid Chromatography and oa TOF Mass Spectrometry

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INTRODUCTION

Metabolic profiling of endogenous metabolites within biological matrices may provide insight into gene functions, environmental stimuli, and disease states of organisms. Global separation and identification of all analytes in a complex matrix such as blood plasma can be a daunting analytical challenge because there is currently no single best analytical technique that works well for all analytes simultaneously. Hence, most metabolic profiling experiments target certain chemical classes that are suitable for the chosen analytical technique. In this study, we target our analytical analysis towards phospholipids. Phospholipids are an essential part of biomembranes and participate in biological processes and cellular signaling systems. Phospholipids have also been shown to be associated with metabolic diseases such as diabetes.[1] Traditional chromatographic methods employed in the analysis of lipid species have been accomplished through the use of normal-phase chromatography due to the ready solubility of lipids in weak normal phase mobile phases such as hexane or chloroform. This form of normal phase methodology is compatible with mass spectrometry and allows for direct identification of lipids. However, these normal phase methods lack high resolving power. Reversed-phase chromatography methods have been successfully developed to improve chromatographic separation of phospholipids over normal phase methods. Unfortunately, the mobile phases utilized in these reversed phase methods typically make use of nonvolatile salts, such as phosphates which are incompatible with mass spectrometry. In contrast, we have developed a reversed-phase UPLC method that is both compatible with mass spectrometry and has high chromatographic resolution. This is accomplished by using columns packed with 1.7µm particles and by making use of mobile phases fully compatible with mass spectrometry. Here we show the utilization of this method in the separation and direct identification of phospholipids (PL) and lysophospholipids (LPL) species from rat plasma.

METHODS

SAMPLE PREPARATION

Phospholipid standards were obtained as a dry powder from Avanti Polar Lipids (AL, USA) and dissolved in chloroform/methanol 2:1. Lipids were extracted from Zucker rat plasma using a modified Folch[2] extraction and evaporated until dry. The lipids were then reconstituted in chloroform/methanol 2:1 and injected into the UPLC/MS system.
Phospholipids from rat plasma Equitech-Bio Inc. (TX, USA) were obtained by protein precipitation using a 2:1 ratio of acetorintrile/rat plasma. The sample was then centrifuged, evaporated until dry, and reconstituting in a lower volume of acetonitrile. The mixture was then injected into the UPLC/MS system.

Ultra Performance LC™ (UPLC) / MS
Figures 1,3

Column: ACQUITY UPLC™ BEH C_8 1.7 µm 2.1 x 100 mm
Column temperature: 60.0 °C
Flow rate: 600 µL/min
Mobile phase A: 20 mM ammonium acetate pH 5.0
Mobile phase B: 90/10 acetonitrile/acetone
Gradient elution: 35-100 %B/10 min
Injection volume: 5 µL
Waters LCT Premier™
ESI+ Capillary voltage: 3200 V Cone voltage: 30 V
ESI– Capillary voltage: 2500 V Cone voltage: 45 V
Desolvation temperature: 350 °C
Source temperature: 120 °C
Desolvation gas: 800 L/Hr
Acquisition range: 100-1000 m/z
Scan time: 0.095 sec

UPLC / MS^E
Figures 2, 4, 5

Waters Q-Tof Premier™
ESI+
Capillary voltage: 3200 V
Cone voltage: 30 V
Desolvation temperature 350 °C
Source temperature: 120 °C
Desolvation gas 800 L/Hr
Collision Energy 1: 5 ev
Collision Energy 2: 25 ev
Acquisition range 100-1000 m/z
Scan time 0.095 sec

CHROMATOGRAPHY

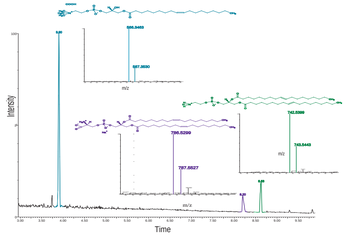


Figure 1: ESI- UPLC/MS separation of phospholipids standards shown with corresponding MS spectra. Shown are:
1-oleoyl-2- hydroxy-sn-Glycero-3-Phosphocholine, 3.9 min;1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine], 8.20 min;
and 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine, 8.63 min.
The separation generated peak widths at base of 3 seconds (average) thus yielding a peak capacity of 200 in 10 minutes.

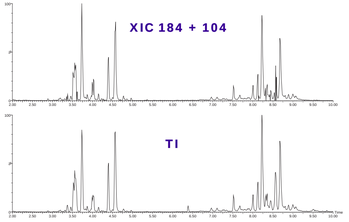


Figure 2: ESI+ UPLC/MS^E separation of PL and LPL from protein precipitated rat plasma. Extracted ion chromatogram (XIC) of high energy scan showing most peaks from protein precipitated rat plasma contain 184 and 104 m/z fragments (top). This is consistent with the fragmentation pattern of PL and LPL containing a phosphotidylcholine head group.[3,4]

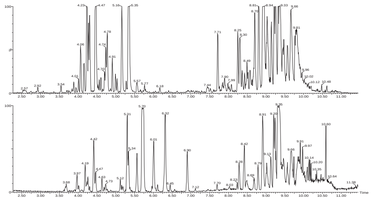
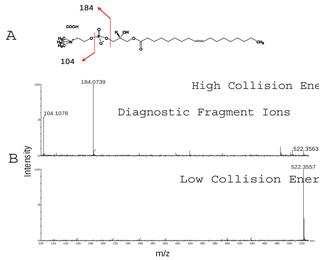


Figure 3: Zoomed BPI of UPLC/MS separation of lipids extracted from Zucker rat plasma using modified Folch extraction. A) electospray positive BPI and B) electrospray negative BPI.

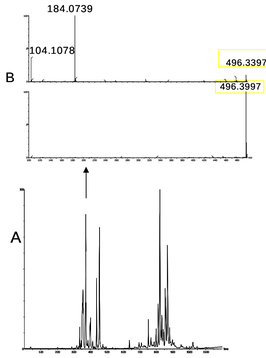
MASS SPECTROMETRY

MS^E is a technique whereby both precursor and fragment mass spectra are simultaneously acquired by alternating between high and low collision energy during a single chromatographic run.[5]



Measured m/z (High CE)	Measured m/z (Low CE)	Theoretical m/z	Percent ppm error error (High CE)	Percent ppm (Low CE)
522.3557	522.3563	522.3560	0.6	0.6
184.0739	-	184.0739	0.0	-
104.1078	-	104.1075	-2.8	-

Figure 4:(A) Chemical structure with known losses and (B) Mass spectra of 1-oleoyl-2- hydroxy-sn-glycero-3-phosphocholine during MS^E experiment.



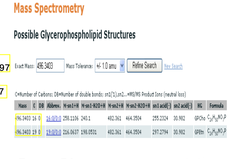


Figure 5A shows a representative separation of LPL and PL in the protein precipitated rat plasma sample. Figure 5B shows the MS^E spectra from a peak eluting with a retention time of 3.9 minutes and a precursor mass of 496.3397. High collision energy scans show major fragments of 104.1078 and 184.0739 m/z arising from the phospholipid head group. A database search of the monoisotopic precursor mass was done using the LIPID MAPS website and produced two possible matches within an error of 1.3 ppm: 2-lyso glycerophospho-ethanolamine (2-lyso GPEtn)

DATA ANALYSIS

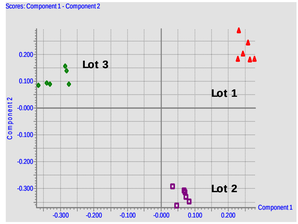


Figure 6: Multivariant data analysis using MarkerLynx software, Principal Component Analysis (PCA) scores plot showing phospholipid variability between lots of protein precipitated rat plasma.

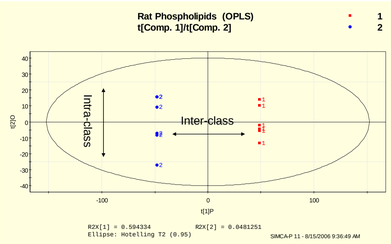


Figure 7: Orthogonal Partial Least Squares (OPLS) showing Inter and intra-class variability between phospholipids from protein precipitated rat plasma lots 1 and 2.

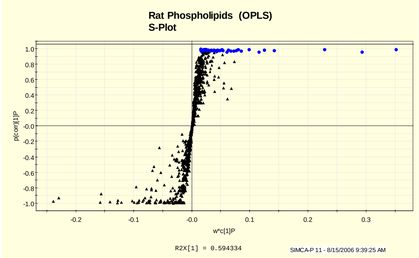


Figure 8: OPLS S-Plot, data point in blue indicate phospholipids that are responsible for variation between lots 1 and 2 of protein precipitated rat plasma.

m/z	R.T.	Weight	Confidence
496.3307	3.88	0.350881	0.992827
524.3601	4.78	0.292548	0.965764
520.3317	3.65	0.227726	0.99346
522.3512	4.2	0.141613	0.980016
521.3377	3.657	0.124123	0.987388
544.3345	3.72	0.114852	0.960234
496.6135	3.88	0.0983533	0.996497
802.5972	7.96	0.0842536	0.97337
774.5697	7.36	0.079154	0.997217
722.604	10.1	0.0763954	0.982884
722.6034	10.15	0.0714419	0.975662
803.5998	7.96	0.0663829	0.977822

Table 1: Top 12 data points determined by OPLS S-Plot for variation between protein precipitated rat plasma lots 1 and 2.

CONCLUSIONS

Lipids from zucker rat plasma and protein precipitated rat plasma were successfully analyzed by UPLC/MS.

Peak widths of 3 seconds during a 10 minute gradient yielded a peak capacity of 200 for a separation of phospholipid standards.

MS^E allows both high and low collision energy mass spectra during a single acquisition, yielding both structural and parent mass information.

The MS^E approach provided unique structural fragment information needed to definitively confirm the correct structural identity from the database without further LC/MS analysis.

Phospholipds containing phosphotidylcholine are the most abundant species found in protein precipitated rat plasma.

Multivariant data analysis using PCA and OPLS showed statistical variation of phospholipids between lots of protein precipitated rat plasma.

REFERENCES

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5. RH Bateman, R Carruthers, JB Hoyes, C Jones, JI Langridge, A Millar, JP Vissers. *Journal of The American Society for Mass Spectrometry*, 2002;13(7):792-803.

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