LIPID PROFILING OF SERUM SAMPLES FROM CALCIFIC CORONARY ARTERY DISEASE PATIENTS USING UPLC/TOF-HDMSE AND MULTIVARIATE DATA ANALYSIS

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INTRODUCTION

Traditionally, lipids are only associated with cellular roles involving energy storage and used as structural building blocks. Recent developments in lipid research have identified the important role of lipids in modulating cellular signaling and cellular trafficking. Alterations in lipid metabolites are associated with various human diseases including obesity, heart disease, and diabetes mellitus. Hence, there is a need to develop comprehensive analytical approaches that allow for the automatic analysis and identification of lipids in complex biological mixtures.

The discovery of novel alterations in lipid levels related to human diseases could lead to the development of novel biomarkers and future diagnostic testing.

Waters new instrumentation allows to accurately and rapidly measure hundreds of individual molecular species providing the opportunity to use more complex lipid profiles for disease diagnostics.

The challenge with global lipid analysis — lipidomics — is the chemical complexity and the large range of concentrations of thousands of lipid species that are present in biological samples.

In this study, we present a robust workflow for global lipid profiling, which employs a combination of ultra performance LC coupled to ion mobility TOF MS (Figure 1) with novel informatics tools (MarkerLynx®, MS^E Data Viewer®, and SimLipid®) for high throughput and automated identification of lipid species from serum lipid extract to maximize the amount of information gained from MS analysis. Serum was obtained from control and patients diagnosed with calcific coronary artery disease (CCAD). CCAD is a predictor of myocardial infarction (heart attack) and an early diagnosis using a panel of potential biomarkers may play an increasing role in cardiovascular disease risk stratification. Calcific coronary artery (CCA) is measured using echocardiography (ultrasound imaging of the heart). It is defined as the presence of a dense band of calcification that could be clearly seen when the valve was open and closed. CCA is measured in millimeter (mm) segments.

These study contains three groups: non calcification (coronary artery calcium score 0), mild calcification (coronary artery calcium score 1-250) and severe calcification (coronary artery calcium score >250).

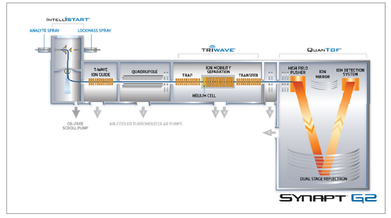

Figure 1. Schematic of the Synapt™ HDMS instrument configuration.

METHODS

Sample preparation. CCAD serum (100 µL) was mixed with dichloromethane/methanol (3/1, 600 µL), vortexed for 30 s and allowed to stand for 5 min followed by vortexing for other 30 s. Sample was centrifuged at 13,000 x g for 10 min. The lower organic phase containing lipids was collected to a new vial. The organic phase was dried under vacuum. Prior to injection, the dried extract was reconstituted to a final volume of 10x the original volume of serum in isopropanol/acetonitrile/water (50/25/25).

UPLC Conditions

Instrument	Waters ACQUITY UPLC System
Column	ACQUITY CSH™ C_{18}, 1.7µm, 2.1 x 100 mm
Column temp	55 °C
Mobile phase	A) 10mM NH_4HCO_2 in ACN/H_2O (60/40) and 0.1%FA B) 10mM NH_4HCO_2 in IPA/ACN (90/10) and 0.1%FA
Injection	3 µL (+ve) and 7µL (-ve)
Flow rate	0.4 mL/min
Gradient	

Time (min)	% A	% B	Curve
Initial	60.0	40.0	Initial
2.0	57.0	43.0	6
2.1	50	50	1
12.0	46	54	6
12.1	30.0	70.0	1
18.0	1.0	99.0	6
18.1	60.0	40.0	6
20.0	60.0	40.0	1

MS Conditions

MS System	Waters Xevo G2 and Synapt™ HDMS
Mode of operation	TOF MS^E and TOF HDMS^E
Ionization	ESI +ve and –ve
Capillary voltage	2.0 KV (+ve) and 1.5 KV (-ve)
Cone voltage	30.0 V
Trap CE	Ramp 35-55 V (+ve) and 30-50V (-ve)
Source temp.	120.0
Desolvation temp.	550.0
Cone gas	50 L/hr
Desolvation gas	900 L/hr (Nitrogen)
Mobility gas	90.0 mL/min (Nitrogen)
Acquisition range	50-1200

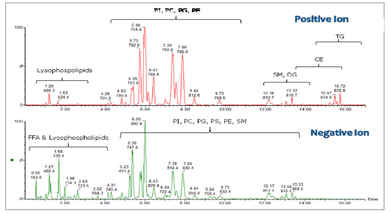

Figure 2. Representative UPLC/MS^E in positive mode (top) and negative mode (bottom) from CCAD serum lipid extract.

RESULTS

A mixture of total lipids extracted from CCAD serum patients was analyzed using UPLC/HDMS^E acquisition mode with Charged Surface Hybrid (CSH) C_{18} column (Figure 2). CSH provided excellent separation, peak shape and reproducibility, offering an alternative chromatographic solution over existing Waters methods for lipid analysis (Figure 3) [1-4]. Data was collected on Xevo G2 and SYNAPT G2 HDMS™ system operated in both negative and positive mode using the MS^E and HDMS^E data acquisition technique.

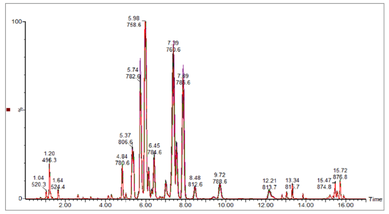

Figure 3. The ACQUITY UPLC CSH C_{18} column has excellent retention time reproducibility with RSD values of < 0.023 (n=15).

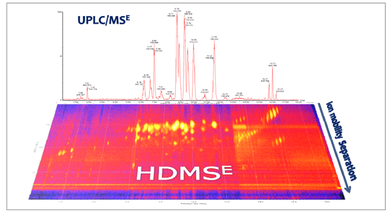

Figure 4. Co-eluting chromatographic peaks are further separated using ion mobility which simplifies lipid identification.

Lipids were separated according to acyl chain length and number of double bonds. In positive mode UPLC/MS^E, we identified the major lipid classes including lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), phosphatidylcholines (PC), sphingomyelins (SM), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), cholesterol esters (CE), diacylglycerols (DG) and triacylglycerols (TG). In negative mode, we characterized fatty acids (FA), phosphatidylinositols (PI), phosphatidylserines (PS), phosphatidylethanolamines (PE) and phosphatidylglycerols (PG).

After chromatographic separation, lipids were ionized and entered in the mass spectrometer, where they passed through the Ion Mobility Separation (IMS) cell. A T-Wave mobility separator uses a repeating train of DC pulses to propel ions through the gas-filled cell in a mobility dependent manner. Lipids migrate with characteristic mobility times (drift times) according to their size and shape. For example difference in acyl chain length or number and position of double bonds affect the shape and size of lipid molecules, resulting in characteristic drift times. Therefore, IMS provides an additional degree of separation besides chromatography, improving peak capacity over conventional UPLC. This leads to a better separation of lipid species and increased selectivity (Figure 4).

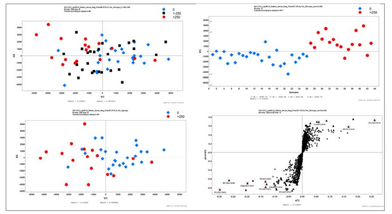

Figure 5. MarkerLynx interrogates low energy negative mode UPLC/MS^E data applying multivariate analysis for potential biomarker discovery (Coronary artery calcium score, Blue=0, Black 1-250 and Red >250).

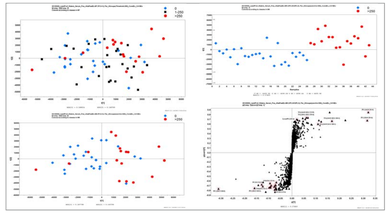

Figure 6. MarkerLynx interrogates low energy positive mode UPLC/MS^E data applying multivariate analysis for potential biomarker discovery (Coronary artery calcium score, Blue=0, Black 1-250 and Red >250).

Finally, the lipid ions exiting the IMS cell were fragmented in the transfer T-Wave cell in MS^E mode, which utilizes parallel low and elevated collision energy to acquire both precursor and product ion information in a single analytical run. The combination of IMS and MS^E — known as HDMS^E — provides greater specificity and hence confidence for lipid identification in complex biological mixtures over regular MS^E, reducing false-positive identifications. Therefore, HDMS^E acquisition is ideal for the rapid analysis of unknown lipid mixtures in biological samples. The UPLC/MS datasets can be further processed using Waters MarkerLynx application manager that provides automatic peak detection followed by multivariate statistical analysis (Figures 5 and 6).
The data generated by UPLC/HDMS^E was extracted using Waters® MS^E Data Viewer, software developed for visualization, processing, and interpretation of multi dimensional MS or HDMS data (Figure 7) . MS^E Data Viewer uses a Waters proprietary algorithm, Apex 4D, to assign a unique retention time, drift time, m/z, and intensity to each individual lipid ion in the mixture. Precursor and product spectra are then aligned according to retention and drift times and linked together , generating an exportable text file format, which was used for lipid identification through SimLipid Software (Figure 8) [5].

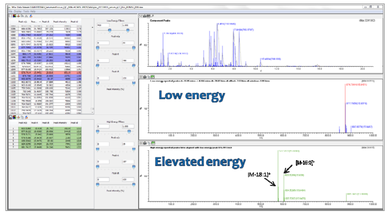

Figure 7. MS^E Data Viewer aligns the data for precursor and corresponding fragment ion spectra by retention and drift times.

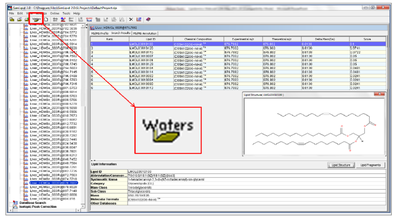

Figure 8. Identification of lipid species using accurate mass precursor and product ion information exported from the MS^E Data Viewer.

The dataset processed in MS^E Data Viewer can be imported into SimLipid, a powerful lipid identification software from Premier Biosoft [5]. SimLipid accepts the experimental UPLC/MS^E and UPLC/HDMS^E data (retention time, m/z, drift time, and intensity values) in their native file format. Then the software matches the exact masses of the precursor and product ions of unknown lipids with those on an *in silico* database containing over 22,000 lipid species belonging to the major lipid classes. SimLipid assigns a probability score to the unknown lipid structure according to the best fit of the experimental *m/z* values with the theoretical *m/z* values of both precursor and product ions of the SimLipid database. By matching the exact masses of the characteristic fragment ions, in addition to precursor ions, SimLipid is able to identify isomers with the same m/z value, reducing the misidentification of lipid structures (Figure 8).

CONCLUSIONS

- A simple and robust solution for global lipid profiling and automated identification of lipid molecular species using ultra performance LC with ion mobility TOF MS and novel informatics tools such as MarkerLynx, MS^E Data Viewer and SimLipid (Figure 9).
- UPLC coupled to HDMS^E provides multiple degrees of orthogonal separation, delivering unprecedented peak capacity required for the confident identification of lipid species in a biological mixture.
- MarkerLynx allows details of the samples to be processed so that important markers of variance can be measured as shown on the S-plot.
- MS^E Data Viewer aligns the precursor low energy and corresponding high energy fragment ion spectra by retention and drift times.
- Automated lipid identification is performed using SimLipid. The major lipid molecular species that contribute to the variance between biological groups or treatments are automatically identified using SimLipid.

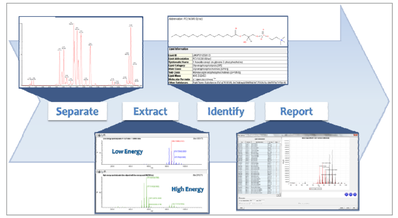

Figure 9. Summary of the UPLC/HDMS^E global lipidomics analysis workflow.

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