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응용 자료

Application of a Rapid (UPLC™-MS/MS) Targeted Lipidomics Assay (LipidQuan™) to a Rodent Toxicology Study

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Abstract

Measuring the changes in the lipidome in early rodent safety studies in drug discovery holds the potential to detect off/on target pharmacology and markers of toxicity. A rapid (8-minute) HILIC-MS/MS (MRM) method, LipidQuan, using ACQUITY™ Premier System and Xevo™ TQ Absolute Triple Quadrupole Mass Spectrometer was used to detect and quantify over 430 bioactive plasma lipids following repeat oral administration of the model hepatotoxin methapyrilene to male rats. Confirmation of identity and concentration measurement of a broad range of lipid classes was facilitated by using stable labelled isotopes authentic standards, eliminating the need for time consuming structural elucidation. The method displayed excellent reproducibility and accuracy through the course of the analytical batch with CVs ranging from 1.5–12%. Multivariate statistical analysis of the resulting data allowed for facile identification and visualization of the lipids dysregulated following dosing.

Benefits

- LipidQuan provides a simple, rapid (eight minute) approach for the detection and quantification of over 430 bioactive lipids facilitating the visulation of changes in lipid concentration due to toxicity or on/off target pharmacology

- The method can be downloaded directly to the LC-MS system eliminating transcription errors via a Quanpedia™ library
- Commercially available stable labelled isotopes simplified feature annotation and quantification

Introduction

Lipids play a key role in mammalian biology including energy storage, cell membrane architecture, cellular signalling, and cell–cell interactions and are thus important to cellular survival, growth, proliferation, interaction, and cell death.[1] They are present in cells, tissues, biofluids, membranes, brain ***etc.***, understanding the changes in lipid abundance can allow insight into basic biology, mechanism, toxicology, and disease progression, as well as facilitate treatment evaluation and patient stratification. To extract this information from tissues and biofluids, a robust, reliable, accurate method with a wide dynamic range and broad lipid class coverage is required.

Previously we have demonstrated and validated the use of a rapid (eight minute) HILIC-MS/MS (MRM) method for the quantification of over 430 bioactive lipids using positive and negative ESI switching.[2] In the study presented here, this methodology was applied to the quantification of the plasma lipidome following the repeat (five days) oral administration of the antihistamine drug methapyrilene to the male Wistar rat. The data derived was processed using multivariate analysis (MVA) to determine which lipids were dysregulated and to what extent following dosing. Also, the impact of dose and repeat administration of the plasma lipidome were investigated.

Experimental

Sample Description

The rats included in the study were housed at Evotec France SAS Animal facility. This facility is accredited by the French Ministry of Agriculture and by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). This study is compliant with the corresponding project APAFIS #32640–2021101419119467 v5. This project underwent full ethical and managerial review by Evotec France Ethical Committee (CEPAL: CE 029) and authorized by the French Ministry of Education, Advanced Studies, and

Research.

Plasma samples were derived from the repeat oral administration of N,N-dimethyl-N'-pyridin-2-yl-N'-(2-thienylmethyl)ethane-1,2-diamine, methapyrilene, Figure 1, to the male Wistar rat at 0, 50, and 150 mg/kg.[3] The samples were collected 24 hours after dosing on days one, three, and five. Full particulars of the animal study and sample preparation are detailed in Waters application note "Tandem Quadrupole Acquisition Modes in DMPK Studies", EN 720008016.[4] Control Wistar rat plasma was obtained from BioIVT, NY, USA.

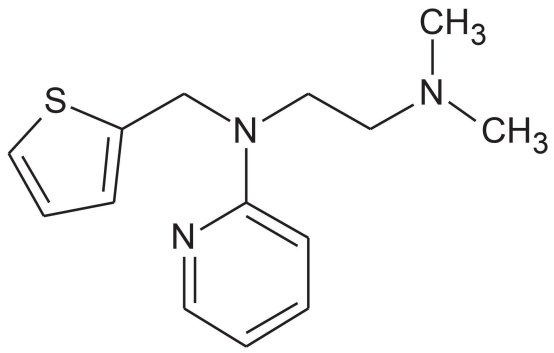


Figure 1. Methapyrilene (N,N-dimethyl-N'-pyridin-2-yl-N'-(2-thienylmethyl)ethane-1,2-diamine).

Sample Preparation

Aliquots of plasma (25 µL) were transferred to low protein binding Eppendorf tubes, the samples were subjected to protein precipitation by the addition of 125 µL IPA/ACN containing Equisplash Lipidomix stable labelled isotope mix (1:2, v/v). Samples were vortex mixed for one minute prior to refrigeration at −20 °C for 1 h. The samples were then shaken at 500 rpm at 5 °C for two hours to ensure complete protein precipitation. The extracted samples were then centrifuged at 10,300 g for 10 minutes at 5 °C before transferring the supernatant to total recovery glass vials for LC-MS/MS analysis. A calibration line was constructed by spiking a known concentration of stable labelled lipid isotopic standards into control rat plasma, a pool batch QC was also created by combining an equal volume of plasma from each sample. The calibration standards and batch QC were processed in an identical manner to the samples. The calibration standards were analysed at the beginning and end of the analytical batch, the batch QC samples were evenly distributed throughout the analysis, each

sample was analysed in duplicate.

Method Conditions

The resulting rat plasma extracts (n=2) from the three dose groups on days two, four, and six were analysed by HILIC-MS/MS (MRM) methodology in both positive and negative ion ESI mode using the MRM transitions detailed in the reference.[2]

The LC-MS/MS data was acquired in MassLynx™ and processed using either TargetLynx™ or Skyline.[5] The processed dataset was further interrogated using MetaboAnalyst.[6] Multivariate statistical analysis was conducted using unsupervised PCA to determine the group differences. Pareto scaling was used, in which each variable was centred and multiplied by $1/\sqrt{SK}$, where SK is the standard deviation of the variable. Hierarchical clustering and Pearsons R coefficient were used for the correlation analysis and pattern searching.

LC Conditions

LC system:	ACQUITY Premier System
Vials:	Total recovery vials (p/n: 186002805)
Column(s):	2.1 × 100 mm, 130 Å, 1.7 µm ACQUITY BEH Amide Column (p/n: 186009505)
Column temperature:	45 °C
Sample temperature:	8 °C
Injection volume:	1 µL for positive and 2 µL for negative ESI mode
Flow rate:	0.6 mL/min
Mobile phase A:	95% ACN, 5% 10 mM ammonium acetate
Mobile phase B:	50% ACN, 50% water, 10 mM ammonium acetate

Gradient Table

Time (min)	Flow rate (mL/min)	%A	%B	Curve
initial	0.6	99.9	0.1	6
2	0.6	80	20	6
5	0.6	20	80	6
5.1	0.6	99.9	0.1	6

MS Conditions

MS system:	Xevo TQ Absolute Triple Quadrupole Mass Spectrometer
Ionization mode:	Positive and negative ion mode
Acquisition range:	MRM Acquisition
Capillary voltage:	1.0 kV
Desolvation temperature:	500 °C
Source temperature:	120 °C
Collision energy:	Compound dependent[2]
Cone voltage:	Compound dependent[2]

Data Mangement

MS software:	MassLynx v4.2, TargetLynx v4.2

Informatics:	Skyline 23.1.0.380, MetaboAnalyst 6.0

Results and Discussion

The rat plasma extracts (n=2) were analysed by HILIC-MS/MS in positive and negative ESI mode using 450 MRM transitions to quantify 430 lipid species. The resulting LC-MS/MS data was subject to peak detection, analyte quantification followed by statistical analysis, Figure 2.

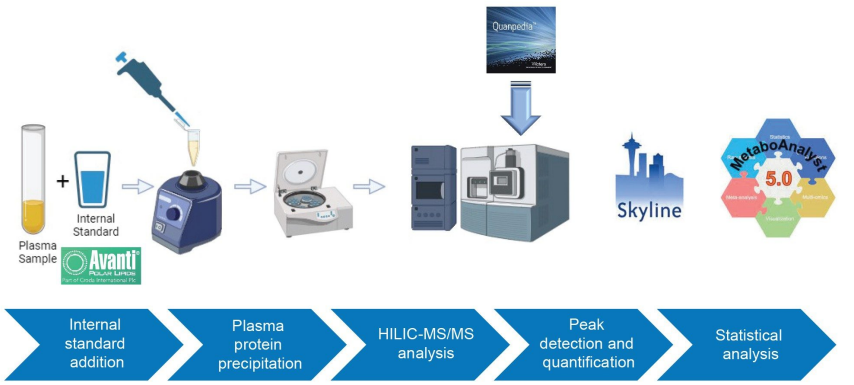


Figure 2. LipidQuan workflow summary.

Representative chromatograms for the batch QC in positive and negative ion mode are given in Figure 3A and B, respectively. The data showed good separation between the lipid classes with the neutral lipids ***e.g.***, mono-, di-, triglycerides, FFA's, eluting just after the solvent front, followed by the ceramides, hexosyl ceramides, Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylglycerol (PG), Phosphatidylinositol (PI), Phosphatidic acid (PA), Lysophosphatidylcholine (LPC), Lysophosphatidylethanolamine (LPE), Lysophosphosphatidylinositol (LPI), Cholesterol Ester (Chol Ester), Monoacylglycerols (MG), Diacylglycerols (DG), Triacylglycerols (TG), Sphingomyelins (SM), Cholesterol (Chol) lipids, and bile acids. Whilst many lipids were detected in only one ionization polarity, ***e.g.*** carnitines and ceramides in positive and FFA and bile acids in negative, some lipids were detected using both polarities. In this case, these lipids were quantified using the ionization mode which produced the most abundant and reproducible signal.

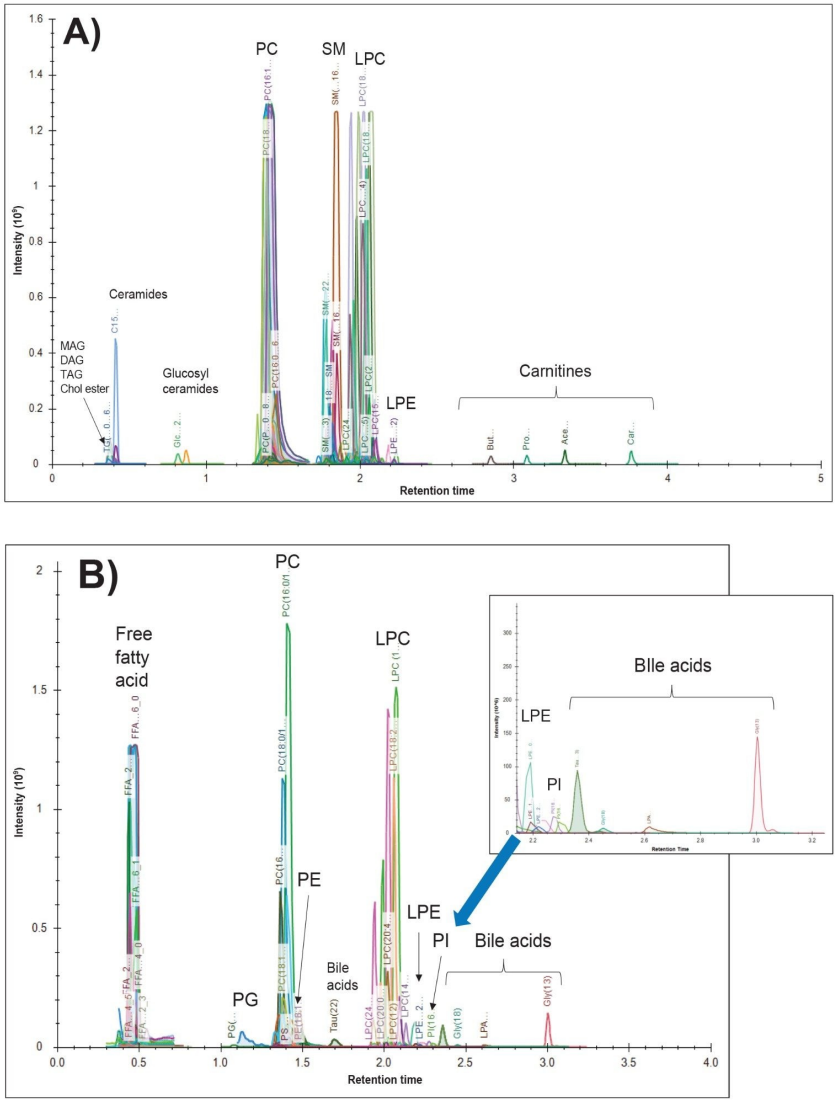


Figure 3. HILIC-MS/MS analysis of batch QC in A) positive ESI and B) negative ESI mode.

The rapid MRM data acquisition capability of the Xevo TQ Absolute Triple Quadrupole Mass Spectrometer allowed for accurate and reproducible acquisition of over 200 MRM channels in both positive and negative ESI mode. The method showed excellent stability and reproducibility over the course of the batch, with %CV for the Equisplash mix in the batch QC ranging from 1.5 to 12%, for the 16:1 d7 LPC and 15:0_18:1 d7 PS, respectively, Figure 4.

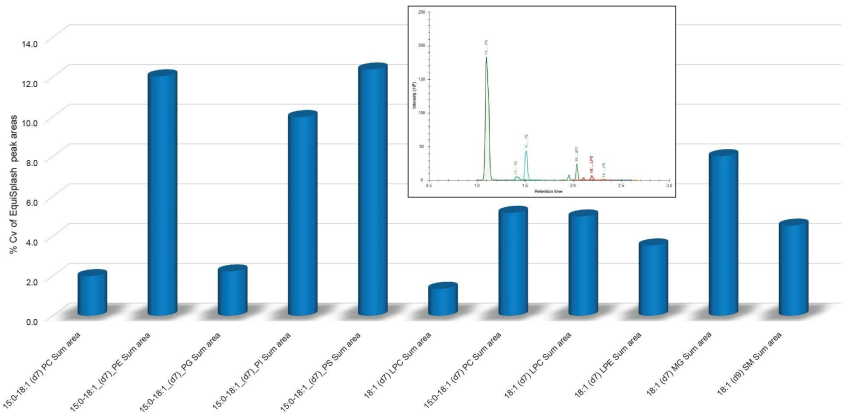


Figure 4. Summary of Equisplash stable labelled isotope lipid response in batch QC samples.

Statistical analysis of the data showed that there was no observable difference in the vehicle only samples on the three sampling occasions (days one, three, and five). The day one 24 h samples from both the 50 and 150 mg/kg dose group clustered with the vehicle only samples (day one, three, and five) as did the day three 50 mg/kg samples. However, the day three 150 mg/kg samples were clearly separated from the vehicle samples suggesting a significant dysregulation of the plasma lipidome in the day three 150 mg/kg samples. The day five 50 and 150 mg/kg dose group samples also separated from the vehicle samples and the day three 50 mg/kg, however, these two groups did not cluster together suggesting that there was a difference in the lipid profile of the 50 and 150 mg/kg dose group samples on day five. An example of the principal components analysis (PCA) scores plots for the negative ESI data is given in Figure 5A (a similar result was obtained for the positive ion data).

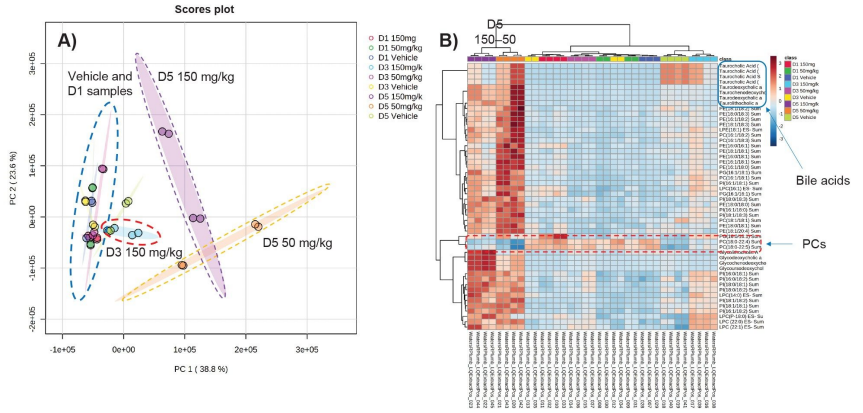
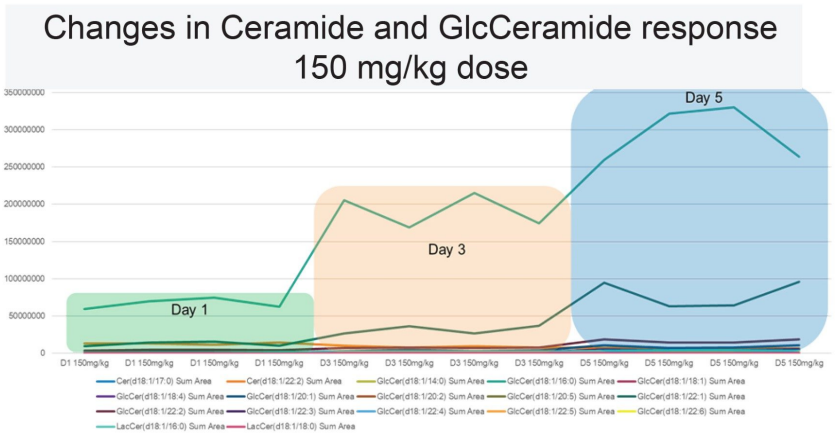


Figure 5. Scores (A) and VIP (B) plots obtained from the statistical analysis of the -ve ESI HILIC-MS/MS analysis of rat plasma.

The variable importance plot (VIP) of the derived data showed that in the positive ESI data ceramides and carnitines contributed most significantly to the observed variance. Whilst in negative ion mode FFAs, bile acids, PIs, Ps, PCs, and LPEs all showed some degree of dysregulation and contributed to the variation observed in the statistical analysis, Figure 5B.

To illustrate these changes the abundance of GlcCeramides and Carnitines were compared between the dose groups and sampling occasion. Figure 6A illustrates the increase in GlcCereamides 18:1_22:1 and 18:1_16:0 abundances relative to other GlcCereamides for the 150 mg/kg dose group on days one, three, and five. Similarly, hexadeconyl carnitine C_{16} and oleoyl carnitine 18:1 showed a significant increase on day five compared to day one and day three, whereas stearoyl-L-carnitine C_{18} and linoleoyl-L-carnitine C_{18} showed almost no changes in relative abundances on the three sampling occasions, Figure 6B.

6A



6B

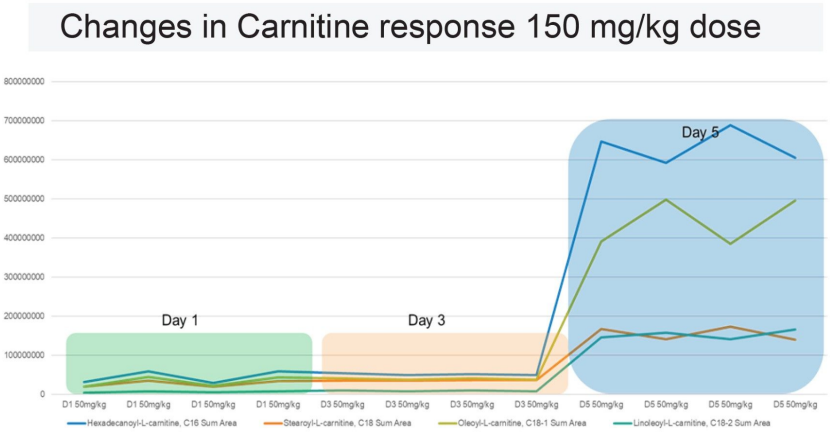


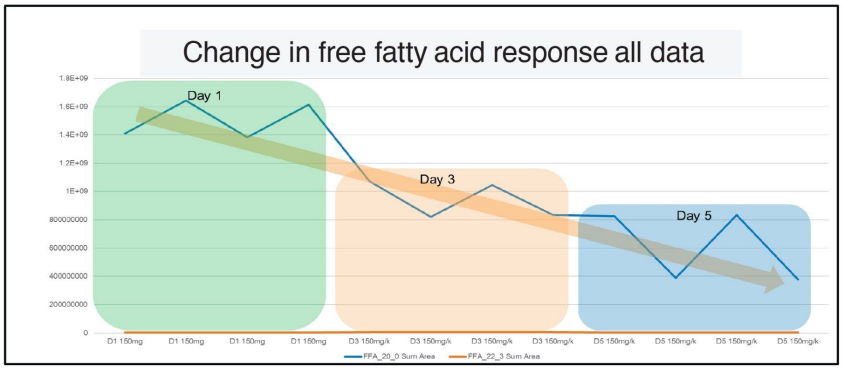
Figure 6A. Variation in Ceramide and GlcCeramide abundance following dosing with methapyrilene at 150 mg/kg.

Figure 6B. Variation in Carnitine abundance following dosing with methapyrilene at 150 mg/kg.

This change in lipid abundance observed in the positive ion data was also demonstrated in the negative ESI data, for example FFA 20_0 exhibited a reduction in abundance compared to other free fatty acids, (such as FFA 22_3) from day one to day five, with this observed downward trend being independent of dose, Figure 7A. Conversely the bile acids glycochendexoycholic acid, glycoursodeoxycholic acid, glycodeoxycholic acid, and glycolithocholic acid showed a significant increase on day five in both the 50 and 150 mg/kg dose group

samples. There was also an observable dose related response with the 150 mg/kg samples showing a greater increase in bile acid abundance compared to the 50 mg/kg samples, Figure 7B. The lipid data acquired in this study suggested that the repeat administration of methapyrilene to male rats resulted in significant changes in the plasma lipidome with selected lipids, ***e.g.*** FFA and PE, reducing following dosing and bile acids, LPE, and ceramides increasing after dosing. The dysregulation in lipid abundance varied with both dose level and sampling occasion with the greatest variation observed on day five with the 150 mg/kg dose.

7A



7B

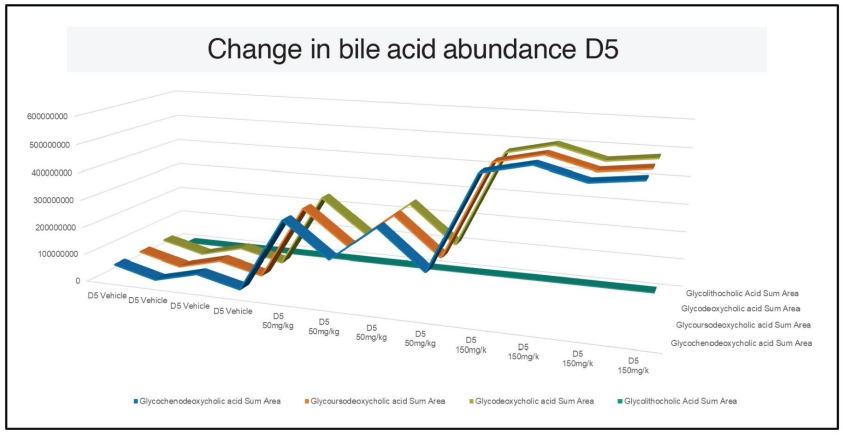


Figure 7A. Variation in FFA abundance following dosing with methapyrilene at 150 mg/kg.

Figure 7B. Variation in bile acid abundance following dosing with methapyrilene at 50 and 150 mg/kg.

Conclusion

Monitoring the changes in lipid abundance (concentration) can provide understanding into biological processes such as disease mechanism, response to treatment, age, and gender variation in response and drug toxicity. LipidQuan is a rapid, sensitive HILIC-MS/MS (MRM) method for the quantification of over 430 bioactive lipids in plasma, serum, and tissue. This assay was employed using the ACQUITY Premier System coupled to Xevo TQ Absolute Triple Quadrupole Mass Spectrometer to analyse plasma following the oral administration of the model hepatotoxin methapyrilene to male rats. The method showed excellent reproducibility, dynamic range, and accuracy. The derived data showed lipids, such as selected ceramides, carnitines, FFAs, bile acids, PIs, Pes, and LPEs all underwent some degree of dysregulation. There was also an observable temporal and dose related response from these lipids.

References

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