Characterization and quantification of lipid nanoparticle components and their degradants in vivo using an LC-HRAM MS platform

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ABSTRACT

Purpose: Develop a highly sensitive and selective LC-MS/MS method to simultaneously characterize the metabolites of the LNP lipid components and quantify the LNP components in the biological matrix samples..

Methods: Five commonly used LNP lipid components (DOTMA, DMG PEG 2000, Cholesterol, DSPC & DSPE) and a bovine liver total lipid extract were purchased from Avanti® Polar Lipids. To mimic the biological matrix samples at different time points after the LNP administration, the five lipids were spiked in the bovine liver total lipid extract at nine concentration levels (0.1ng/mL, 0.25ng/mL, 0.5ng/mL, 1ng/mL, 5ng/mL,10ng/mL, 50ng/mL, 100ng/mL, 1µg/mL). A Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon HPLC pump was used for all experiments. A full MS experiment was followed by a data dependent MS/MS experiment first, then followed by a targeted MS/MS experiment for data collection. Data were processed with small molecular specific software.

Results: The developed LC-MS/MS method enabled LNP lipid components quantification with high sensitivity and high selectivity and confident lipid metabolite identification and profilin.

INTRODUCTION

Lipid nanoparticles (LNPs) have emerged across the pharmaceutical industry as promising vehicles to deliver a variety of therapeutic agents including the mRNAs. A key aspect to design and optimize a LNP formulation is the development of biodegradable ionizable lipids which improve LNPs clearance and reduce toxicity in vivo while maintaining the structural features required for lipid potency. In order to rapidly measure the clearance rate of administrated lipid components of LNP and monitor the degradants/metabolites of these lipid components in vivo with minimum consumption of biological samples, we developed a HPLC MS-MS/MS method which enables simultaneous targeted quantification of ionizable lipid/PEG-lipid and unknown lipid metabolite characterization within a single HPLC MS run. The results are reported here.

MATERIALS AND METHODS

Sample Preparation

Five commonly used LNP lipid components (DOTMA, DMG PEG 2000, Cholesterol, DSPC & DSPE) and a bovine liver total lipid extract were purchased from Avanti™ Polar Lipids.

A mixture of five lipid standards was prepared at the 1 µg/mL concentration in methanol.

The five lipid standards were further spiked in the 0.1 mg/mL concentration of bovine liver total lipid extract as a dilution series at nine concentrations: 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, 1 µg/mL to make a mimic biological matrix sample series.

HPLC conditions

The Vanquish Horizon UHPLC system performed separations. Mobile phase A was 60% ACN/40% H2O containing 10 mM ammonium formate and 0.1% difluoroacetic acid. Mobile phase B was 90% IPA/10% ACN containing 10 mM ammonium formate and 0.1% difluoroacetic acid. The column was a Thermo Scientific™ Accucore™ C30 column (2.1 x 150 mm, 2.6 µm) that operated at 50 °C and a flow rate of 350 µL/min. The gradient condition used was listed in Table 1. The injection volume was 2 µL. Each sample was analyzed in triplicate.

Table 1. HPLC gradient condition

Time	Flow (ml/min)	%B
0	0.35	30
2	0.35	43
2.1	0.35	55
10	0.35	65
13	0.35	85
14	0.35	100
16.5	0.35	100
16.6	0.35	30
22	0.35	30

Mass spectrometry

The Orbitrap Exploris 120 mass spectrometer was used for MS data collection. The instrument was operated with Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System (CDS) software and controlled by Orbitrap Exploris Series 3.1 instrument control software. A data-dependent MS/MS experiment, followed by a targeted MS/MS experiment, was carried out. Table 2 shows the detail settings for the two alternate experiments.

Data Analysis

The lipid identification, impurity/degradant characterization, and relative quantification were carried out using Thermo Scientific™ Compound Discoverer™ 3.3 software and Thermo Scientific™ LipidSearch™ 5.0 software. The calibration curve generation was carried out using Chromeleon 7.2.10 Chromatography Data System (CDS) software.

Table 2. ESI source and MS parameter set ups

MS source setting	Value	Experiment #1: MS full MS /dd MS2 (top3) setting	Value	Experiment #2: targeted MS/MS setting	Value
Sheath gas	40	General		tMS/MS	
Aux gas	8	Application mode	small molecule	Resolution	30,000 at m/z 200
Sweep gas	1	Pressure mode	Standard	Isolation window (m/z)	2
Spray voltage (+V)	3400	RF lens (%)	50	AGC target value (%)	100
Capillary temp. (°C)	300	Full MS		Max inject time (ms)	200
Vaporizer temp. (°C)	350	Scan range (m/z)	300 - 1400	Fixed first mass (m/z)	60
		Resolution	120,000 at m/z 200	Collision Energy Mode	Stepped
		AGC target value (%)	300	HCD collision energy (%)	15,45
		Max inject time (ms)	100		
		dd-MS/MS (top3)		Targeted precursor mass list	
		Resolution	30,000 at m/z 200	m/z 836.5353, +3	RT window: 4 - 5.5 min
		Isolation window (m/z)	1.5	m/z 634.6508, +1	RT window: 9 -10.5 min
		AGC target value (%)	100		
		Max inject time (ms)	100		
		Fixed first mass (m/z)	60		
		Targeted mass exclusion	On		
		Collision Energy Mode	Stepped		
		HCD collision energy (%)	15, 30, 50		

RESULTS

Lipid components confirmation using high-resolution, accurate mass MS and MS/MS data

Figure 1 shows the representative extracted ion chromatography of the five lipid standards at the 1 µg/mL concentration. The five lipid components were efficiently separated using the C30 column and detected by the high resolution and accurate MS and MS/MS with threat mass accuracy. DMG PEG-2000 exists as a non-uniform polydisperse polymer and contains uneven PEG chain lengths. In this study, the identified PEG chain length was from 34 to 54 (CH2CH2O).

Less than 3 ppm mass accuracy for the precursor ions was observed across all lipid peaks, enabling confident identification of all lipid components. In addition to the accurate MS data, the MS/MS data provides more structure-related information of the lipid components. Figure 2 shows the 18:0 fatty side chains of the DSPE were unambiguously confirmed using the MS/MS fragment ions information.

Figure 1. Extracted precursor ion chromatograms of the five lipid components

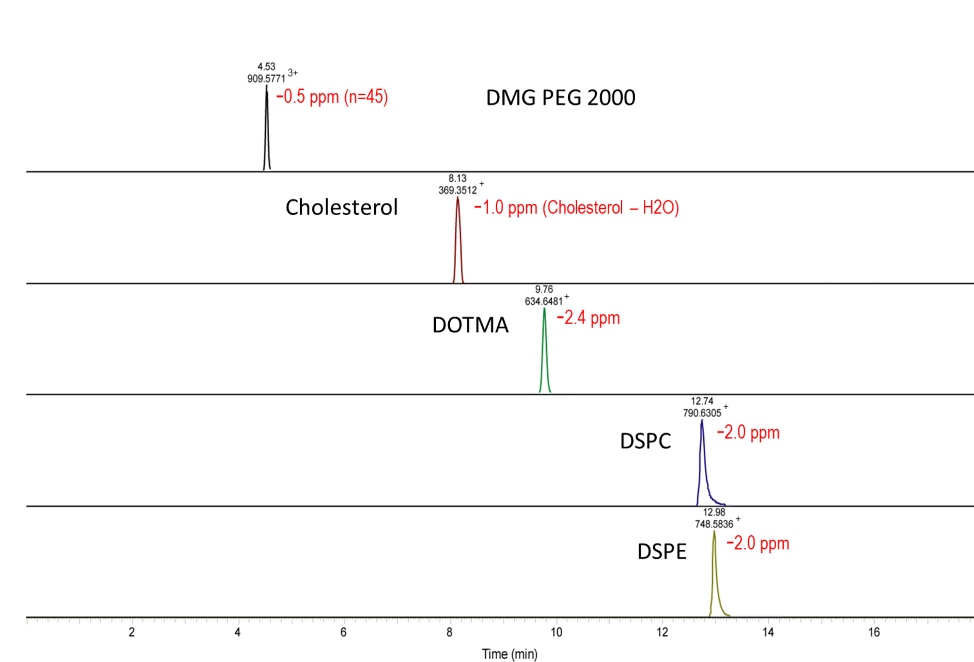
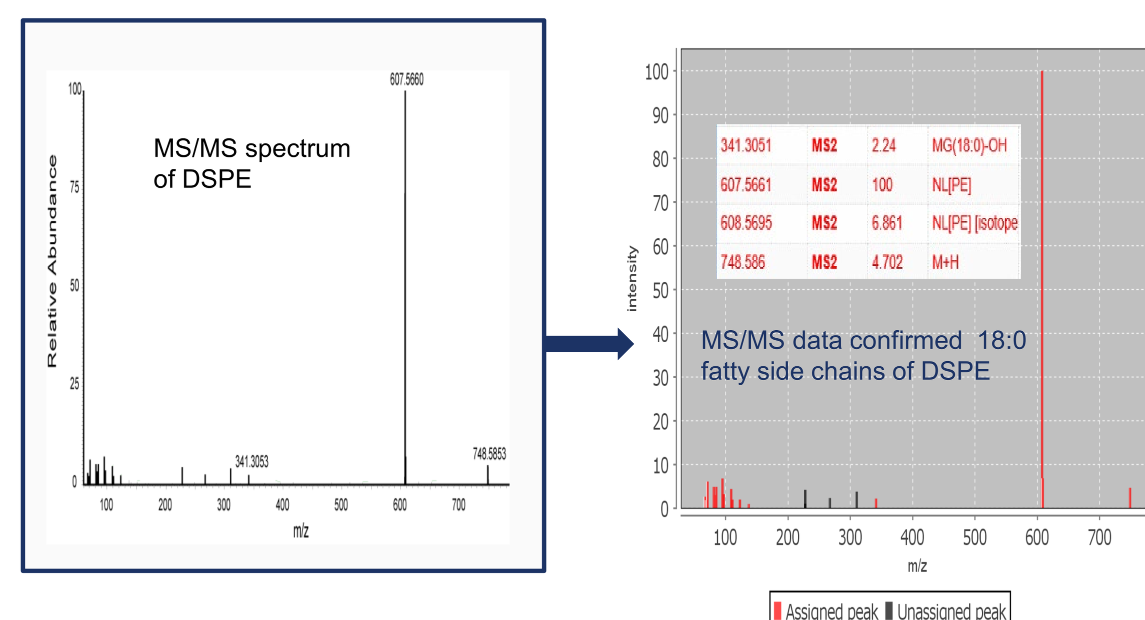


Figure 2. Structure confirmation of the DSPE by fragment ion matches



Simultaneous targeted lipid quantification and unknown lipid metabolite analysis in biological matrix using high-resolution, accurate mass MS and MS/MS data

It is challenging to rapidly monitor the clearance rate and biodegradation pathway of the LNP lipid components in vivo with limited volume of biological matrix (such as tissues, serum, plasma) samples. We were able to develop a single LC-MS method for both metabolite profiling and targeted lipid quantification. After a full MS scan, MS/MS spectra on the three most intense precursor ions are collected for unknown metabolite identification. In the same cycle, MS/MS spectra on targeted precursor ions (DOTMA and PEG-lipid) are collected only when they are eluted, maximizing the duty cycle. The specific fragment ions from the collected targeted MS/MS data were used for quantification. This hybrid approach enables rapid analysis of clearance rate and biodegradation pathway in vivo using minimum amount of samples.

For proof of concept, we applied the method to analyze a series of liver total lipid extract samples that have spiked-in lipid standards at different concentrations to mimic the biological matrix samples across different time points after a LNP administration. The targeted MS/MS (tMS/MS) quantification approach uses integrated peak areas of extracted unique fragment ion(s) for quantification which increases ion trapping efficiency and selectivity and significantly improve the LOD/LOQ of targeted components. Figure 3 shows that the peak of DMG PEG 2000 spiked in the bovine liver extract at 0.25 pg/µL (0.25 ng/mL; 0.5 pg on column) was not detected using full MS scan, but clearly detected using tMS/MS. Great sensitivity and wide dynamic range were achieved for DOTMA using the tMS/MS approach. Figure 4 shows the calibration curve of DOTMA generated from the dilution series (0.1 ng/mL,0.25 ng/mL,0.5 ng/mL,1 ng/mL, 5 ng/mL,10 ng/mL, 50 ng/mL,100 ng/mL,1 µg/mL). Excellent linearity was observed with R^2 = 0.9995 over four orders of dynamic range.

Figure 3. The LOD comparison of DMG PEG 2000 using full MS vs. tMS/MS approaches

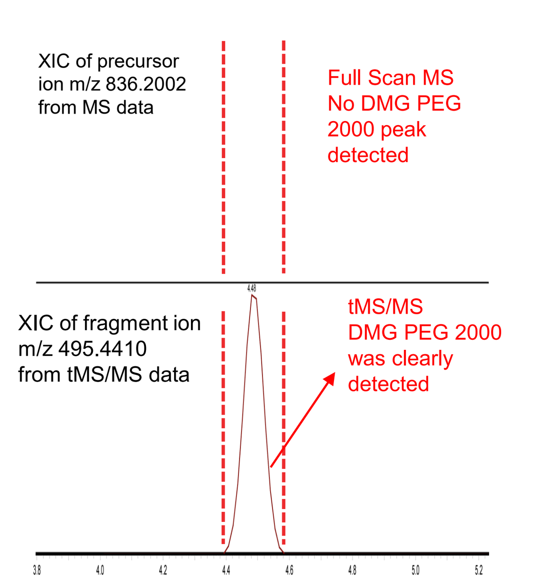
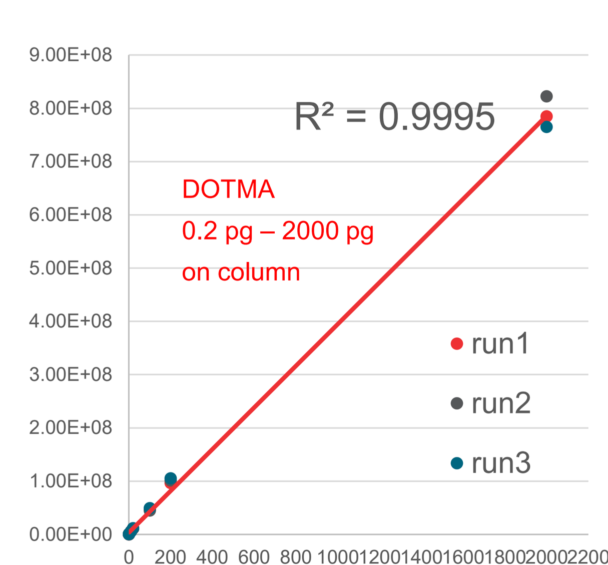
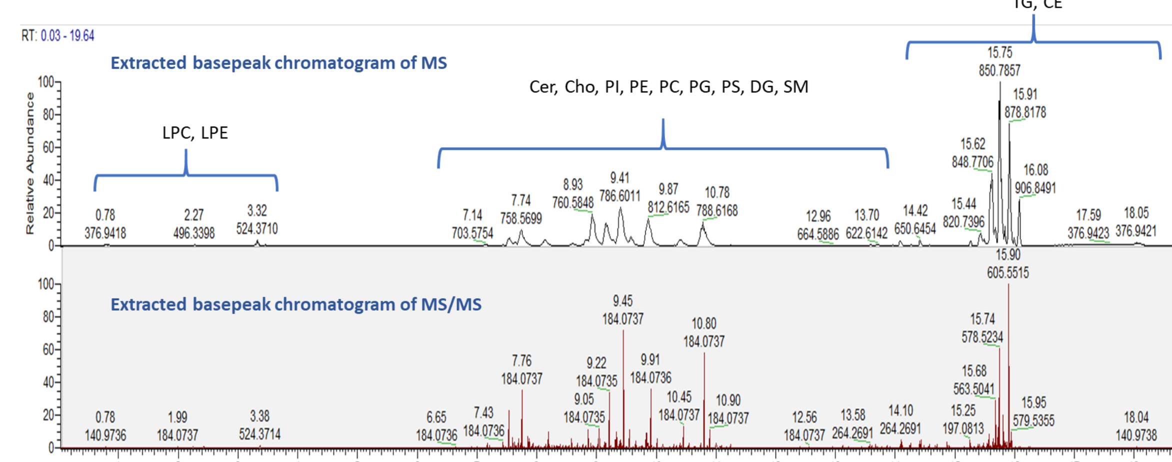


Figure 4. Calibration curve of DOTMA generated using the tMS/MS approach



The data collected from data-dependent MS/MS were used for unknown metabolites characterization using either Compound Discoverer 3.0 software or LipidSearch 5.0 software. Figure 5 shows a representative base peak chromatogram of MS and MS/MS from the liver total lipid extract with spiked-in lipid standards at 50 ng/mL concentration. Multiple classes of lipid species were identified using both full MS and MS/MS data using the default search parameters of LipidSearch software.

Figure 5. Representative base peak chromatogram of the liver total lipid extract with spiked-in lipid standards at 50 ng/mL concentration



Of the five lipid components studied, cholesterol, 18:0 PC, and 18:0 PE are endogenous lipid components. A previous study has shown that 18:0 Lyso PC could be generated by the hydrolysis of an 18:0 PC molecule.9 Since 18:0 lyso PC is also an endogenous lipid molecule, our approach should be able to detect it from the bovine liver total lipid matrix sample (0.1 mg/mL). As expected, the endogenous 18:0 Lyso PC was identified confidently by the great quality of MS and MS/MS data using LipidSearch 5.0 software (Figure 6). The measured molecule weight error was 1.3 ppm.The fragment ion information enabled confident confirmation of the 18:0 Lyso PC structure. To mimic the 18:0 Lyso lipid concentration changes across different time points for a biological sample, the bovine liver total lipid matrix (0.1 mg/mL) sample was injected with 1 µL, 2 µL, and 3 µL, in triplicate. The quantification trend of the endogenous 18:0 Lyso PC across three samples having different injecting volumes is shown in Figure 7.

Figure 6. Confident identification of 18:0 Lyso PC using LipidSearch 5.0 software

ID	Formula	ObsMz	CalcMz	TopRT	Height	Area
LPC(18:0)+H	C26 H55 O7 N1 P1	524.3718	524.3711	3.33	1.227E07	4.094E07

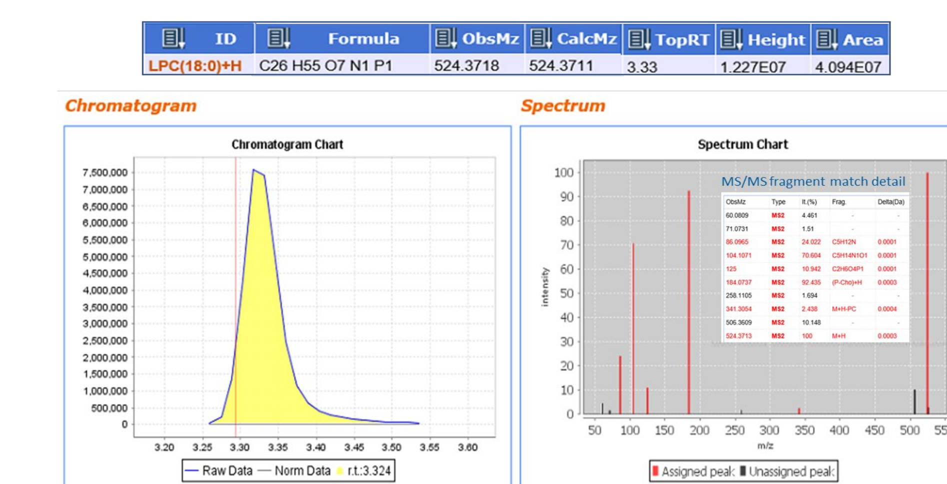
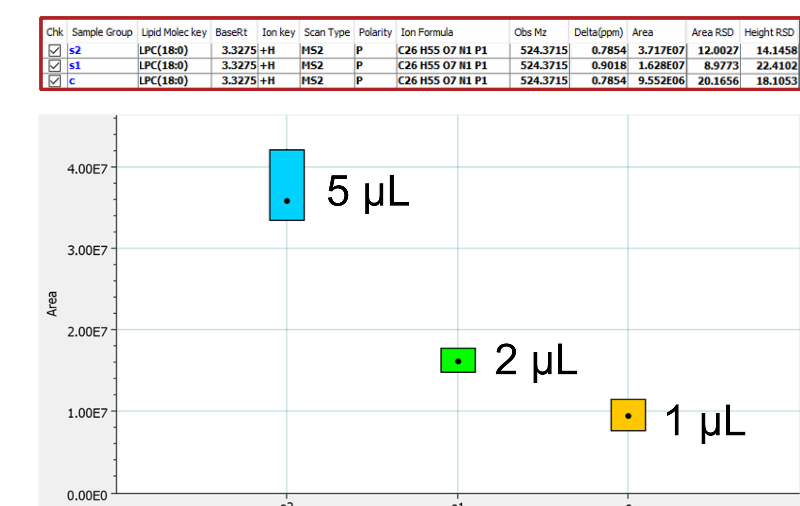


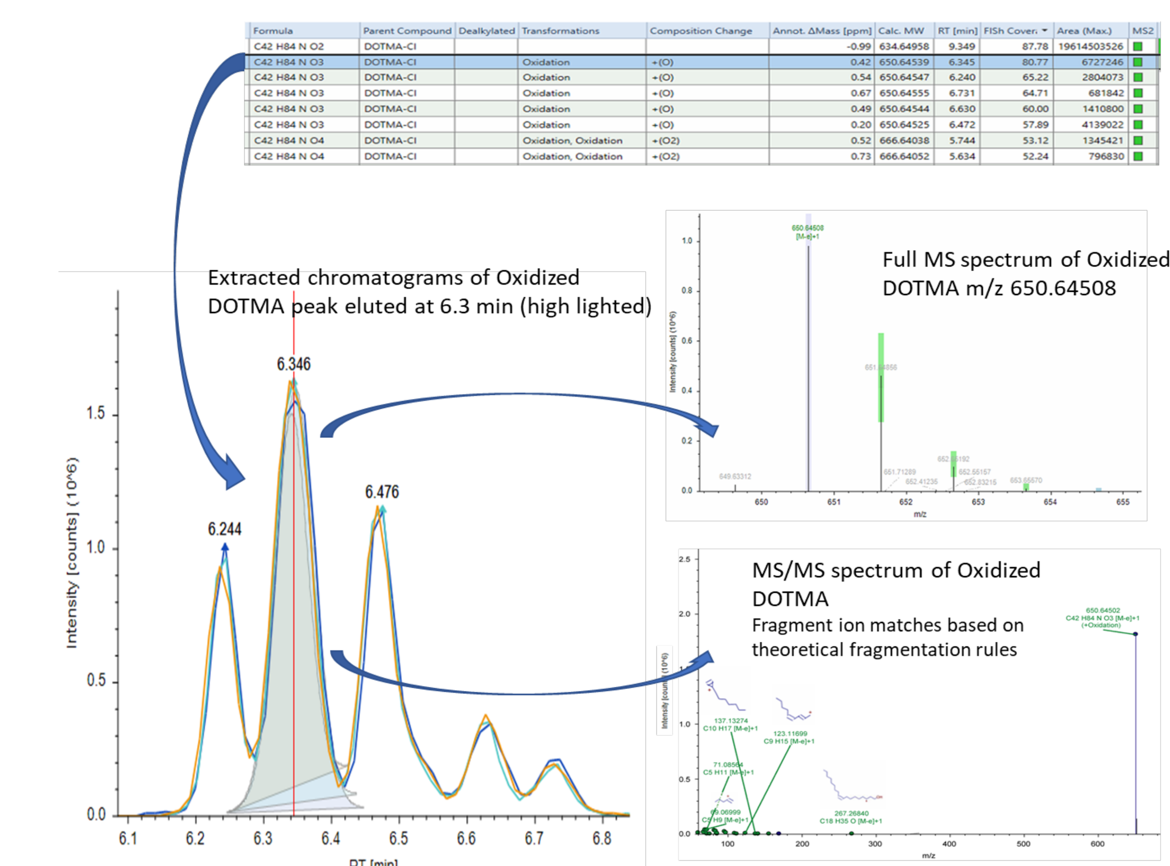
Figure 7. Endogenous 18:0 Lyso PC quantitation trend observed from the bovine total lipid extract (0.1 mg/mL) with different sample injection volumes (1 µL, 2 µL and 5 µL, in triplicate)

Chk	Sample Group	Lipid Molec key	BaseRt	Ion key	Scan Type	Polarity	Ion Formula	Obs Mz	Delta(ppm)	Area	Area RSD	Height RSD
☑	s2	LPC(18:0)	3.3275	+H	MS2	P	C26 H55 O7 N1 P1	524.3715	0.7854	3.717E07	12.0027	14.1458
☑	s1	LPC(18:0)	3.3275	+H	MS2	P	C26 H55 O7 N1 P1	524.3715	0.9018	1.628E07	8.9773	22.4102
☑	c	LPC(18:0)	3.3275	+H	MS2	P	C26 H55 O7 N1 P1	524.3715	0.7854	9.552E06	20.1656	18.1053



As the data shown in the previous session revealed, the DOTMA standard includes very low abundant (<0.1%) oxidized degradants. These low abundant DOTMA degradants with oxidization in multiple sites were confidently identified using the predefined metabolite workflow template “MetID w Stats Expected w FISh Scoring” included in the Compound Discoverer 3.3 software. The software detects the metabolites from the expected metabolite list, which was generated based on the common metabolic pathways list. Subsequently, the FISh Scoring node (FISh = fragment ion search) automatically annotates the MS/MS fragments that match and compare with the parent MS/MS and color codes/annotates the fragment ions. Figure 8 shows oxidized DOTMA degradants identification using MS and MS/MS data in the complex bovine liver total lipid extract matrix. The measured molecule weight errors for the oxidized metabolites were less than 1 ppm.

Figure 8. Confident identification of oxidized metabolites of DOTMA in the bovine total lipid extract matrix using Compound Discoverer 3.3 software.



CONCLUSIONS

In summary, the Orbitrap Exploris 120 mass spectrometer coupled with the Vanquish Horizon UHPLC system and Accucore C30 column provides excellent analytical solutions for rapid and confident characterization of LNP lipid components and degradants and can be applied to support LNP product development and manufacturing, including raw material testing, formulation stability study, in process testing, scale-up, and DMPK.

- The developed HPLC MS-MS/MS methods are platform methods and can be applied to different types of LNP formulations, offering excellent analytical solutions for quality control and quality assurance of raw materials and LNP formulations.
- The high resolution, high sensitivity, and the instrument acquisition versatility offered by Orbitrap MS enables rapid lipid quantification and unknown metabolite identification in vivo, helping to speed up the LNP optimization and pre-clinical studies.
- Comprehensive software tools enable rapid and confident characterization and quantification of lipid components and their metabolites/degradants.

TRADEMARKS/LICENSING

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