Application Note

Biopharmaceuticals

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Analysis of Lipid Nanoparticle Components Using an Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

The identity and quantity of lipid components are among the several critical quality attributes (CQAs) for lipid-based nanoparticle delivery systems. This application note proposes a liquid chromatography/mass spectrometry (LC/MS) method for the analysis of lipid components of mRNA encapsulated in lipid nanoparticles (mRNA LNPs). The method uses an Agilent 1290 Infinity II LC system and an Agilent 6545XT AdvanceBio LC/Q-TOF, and demonstrates high-resolution separation as well as lipid identification with high mass accuracy. The method performance was evaluated with mRNA LNPs to demonstrate simultaneous analysis of the lipid components.

Introduction

Drug development teams use lipid nanoparticles (LNPs)—a versatile formulation method—to deliver a range of therapeutics. Due to expanding research of LNP-encapsulated mRNA in the biopharmaceutical area[1], the need for analytical techniques to understand LNP properties has increased. Different physical aspects of LNPs, including size, morphology, zeta potential, polydispersity, composition, and stability, are part of the drug product CQAs. LNPs are composed of four different lipids: ionizable lipids, helper or neutral lipids, cholesterol, and polyethylene glycol (PEG) lipids. LNP composition is crucial to functionality and must be characterized in the formulation.[2,3] The lipid quantity, ratio, and purity are CQAs for manufacturing, and are needed to enable process and formulation development.

In this application note, we outline a lipid analysis procedure using LC/MS for LNPs used in potential vaccines. The method uses the 1290 Infinity II LC system and 6545XT AdvanceBio LC/Q-TOF.

Experimental

Materials

Heptadecan-9-yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (SM-102), 6-((2-hexyldecanoyl)oxy)-N-(6-((2-hexyldecanoyl)oxy)hexyl)-N-(4-hydroxybutyl)hexan-1-aminium (ALC-0315), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2k), dioleoylphosphatidylethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), and cholesterol were supplied from MedChemExpress. Methanol (MeOH) was obtained from Agilent Technologies. Acetonitrile (ACN) and formic acid was obtained from Fisher Chemicals. Sodium acetate and Tris were obtained from Sigma. Monarch RNA cleanup kit spin columns were obtained from New England Biolabs.

Instrumentation

The 1290 Infinity II LC system, which was coupled to the 6545XT AdvanceBio LC/Q-TOF, comprised the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 6545XT AdvanceBio LC/QTOF (G6549AA)

Software

The following software packages were used:

- Agilent MassHunter Workstation Data Acquisition software (version 11)
- Agilent MassHunter Qualitative Analysis software (version 10)

Standard and calibration curve

An 8 mM lipid standard was prepared in MeOH. Different working concentrations of each lipid standard or mixture of lipids were prepared in MeOH using the stock solutions. To generate calibration curves, a stock solution containing 2 mM SM-102, 2 mM DMG-PEG 2K, 2 mM DSPC, and 20 mM cholesterol was freshly prepared in MeOH. The calibration solution was then serially diluted in MeOH to the minimum concentration of 0.1 fmol SM-102, 0.1 fmol DMG-PEG 2K, 0.1 fmol DSPC, and 10 pmol cholesterol.

Liquid chromatography/mass spectrometry

LC/MS lipid separation was performed on an Agilent InfinityLab Poroshell 120 Phenyl-Hexyl column (2.1 × 50 mm, 1.9 µm) using a seven-minute gradient. LC/MS conditions are detailed in Table 1. The mRNA LNP samples were dissolved in water, and MeOH dilution aliquots were injected into the LC/MS system.

Table 1. LC/MS parameters. (Continued on next page).

Parameter	Value
Agilent 1290 Infinity II LC System	
Column	Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 50 mm, 1.9 µm
Sample Thermostat	25 °C
Mobile Phase A	90% MeOH in 10 mM ammonium acetate
Mobile Phase B	90% ACN in 10 mM ammonium acetate
Gradient	Time (min) %A %B 0.00 100 0 2.00 100 0 7.00 0 100
Stop Time	7 min
Column Temperature	55 °C
Flow Rate	0.4 mL/min

Table 1. LC/MS parameters. (Continued).

Parameter	Value
Agilent 6545XT AdvanceBio LC/Q-TOF	
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	250 °C
Drying Gas Flow	10 L/min
Sheath Gas Temperature	300 °C
Sheath Gas Flow	12 L/min
Nebulizer	35 psi
Capillary Voltage	3,500 V
Nozzle Voltage	500 V
Fragmentor Voltage	150 V
Skimmer Voltage	65 V
Octupole Ion Guide Voltage	750 V
Reference Mass	922.009798
Acquisition Mode	Data were acquired in Extended Dynamic Range (2 GHz)
MS Mass Range	110 to 1,700 *m/z*
Acquisition Rate	8 spectra/s
MS Range	350 to 3,200 *m/z*
MS Acquisition Rate	2 spectra/s

Preparation of mRNA LNPs

The mRNA LNPs were produced using the same composition as Spikevax—the COVID-19 vaccine pioneered by Moderna. The mRNA was in vitro transcribed from a PCR-amplified dsDNA template, purified using spin columns, then dissolved in 1 mM sodium acetate buffer (pH 4.7) to form the aqueous phase. For the Spikevax formulation[4], SM-102, DMG-PEG 2K, DSPC, and cholesterol were dissolved in ethanol at the molar ratio of 50:1.5:10:38.5 to form the organic phase. The mRNA was dispersed in 25 mM sodium acetate to form the aqueous phase. These two phases were mixed using the benchtop microfluidic device (NanoAssemblr platform, Precision NanoSystems) at the volume ratio 3:1, and the total flow rate was 12 mL/min. The N:P ratio was 5.67:1. Then, the formed mRNA LNPs were buffer exchanged with 20 mM Tris (pH 7.4) and concentrated by ultracentrifuge tubes with a molecular weight cutoff of 30 kDa at 4 °C and 2,500 g for 60 minutes to a total lipid concentration of ~ 4 mg/mL. The formed mRNA LNPs were subjected to lyophilization.

Results and discussion

Lipid components in the formulation of nanoparticles have been separated and quantified using RP-HPLC. Since most lipids lack a chromophore, they must be detected by alternative detection methods. HPLC coupled with MS detection has emerged as one of the most effective methods for identifying lipids. During the nanoparticle development phase, various lipid classes can be investigated to obtain the most effective LNPs. Table 2 shows the lipids used in this study. Separation and identification of lipids were performed on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF. Figure 1A shows the extracted ion chromatograms (EICs) from the simultaneous analysis of seven lipids with high-resolution separation using an InfinityLab Poroshell 120 Phenyl-Hexyl column. All the peak assignments were based on accurate mass measurements from Q-TOF analysis. The lipids were eluted in the order of: cholesterol, DOPE, DOTAP, DSPC, SM-102, ALC-0315, and DMG-PEG 2K. The mass spectrum corresponding to individually resolved lipids is depicted in Figure 1B. The most abundant charge states observed in the mass spectrum are indicated, and all spectra matched theoretical masses to within 2.5 ppm.

Table 2. LNP components employed in this study.

Lipid Role	Lipids	Formula	Molecular Weight
Ionizable Lipids	ALC-0315	$C_{48}H_{95}NO_5$	766.27
	SM-102	$C_{44}H_{87}NO_5$	710.17
	DOTAP	$C_{42}H_{80}ClNO_4$	698.54
Phospholipid	DSPC	$C_{44}H_{88}NO_8P$	790.15
Helper Lipid	Cholesterol	$C_{27}H_{46}O$	386.65
	DOPE	$C_{41}H_{78}NO_8P$	744.03
PEG-Lipid	DMG-PEG 2K	$(C_2H_4O)_nC_{32}H_{62}O_5$	2,526.00

The reproducibility of the method was evaluated with three consecutive LC/MS runs. Figure 2 shows the overlay EIC of the standard lipid mixture. The result shows excellent lipid separation with no observable shift in the chromatographic profile between the replicate injections. The retention time (Rt) and peak area relative standard deviation (RSD) for the lipids were less than 0.5 and 1.5% respectively, demonstrating the excellent reproducibility and precision of the method.

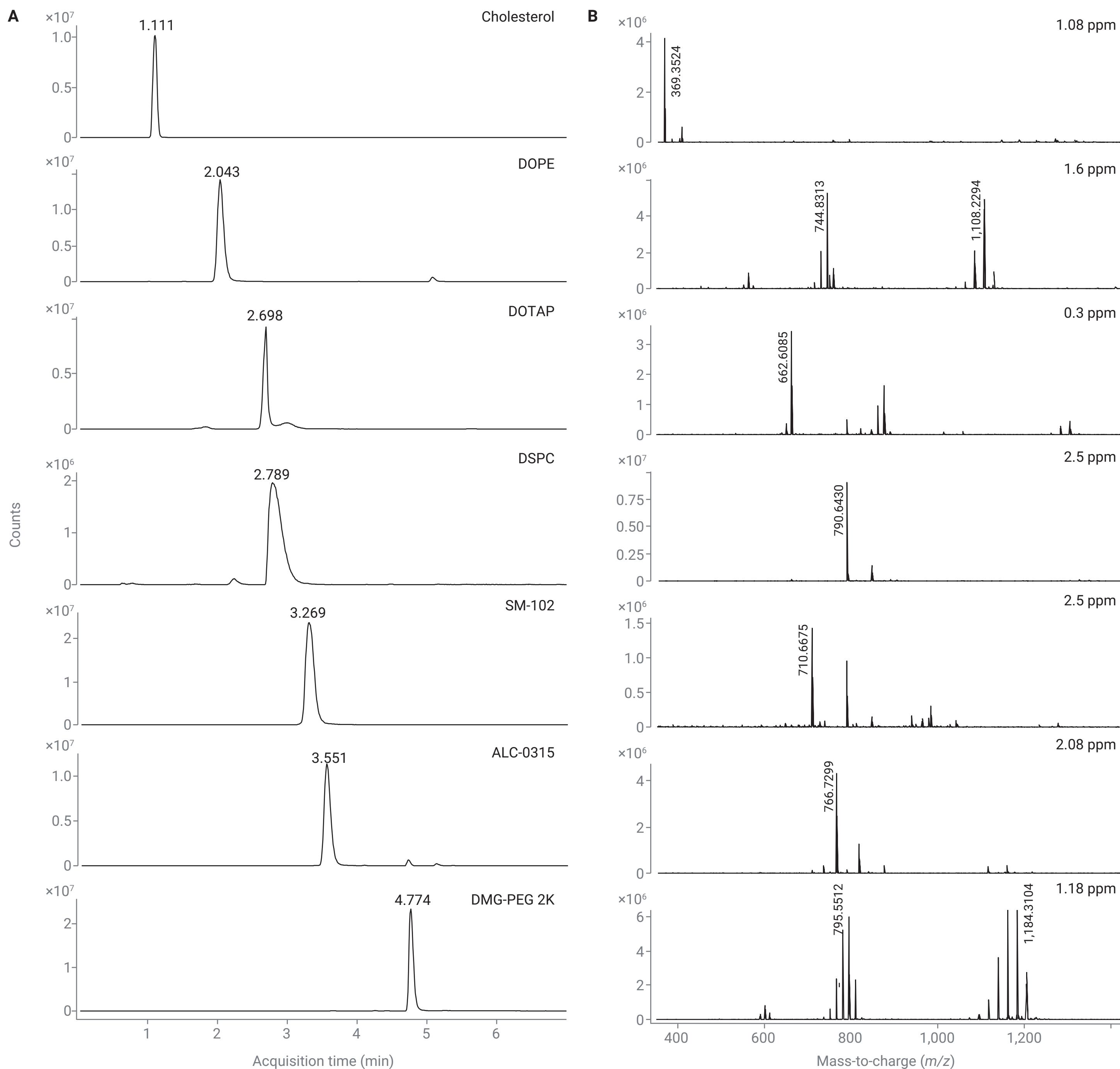
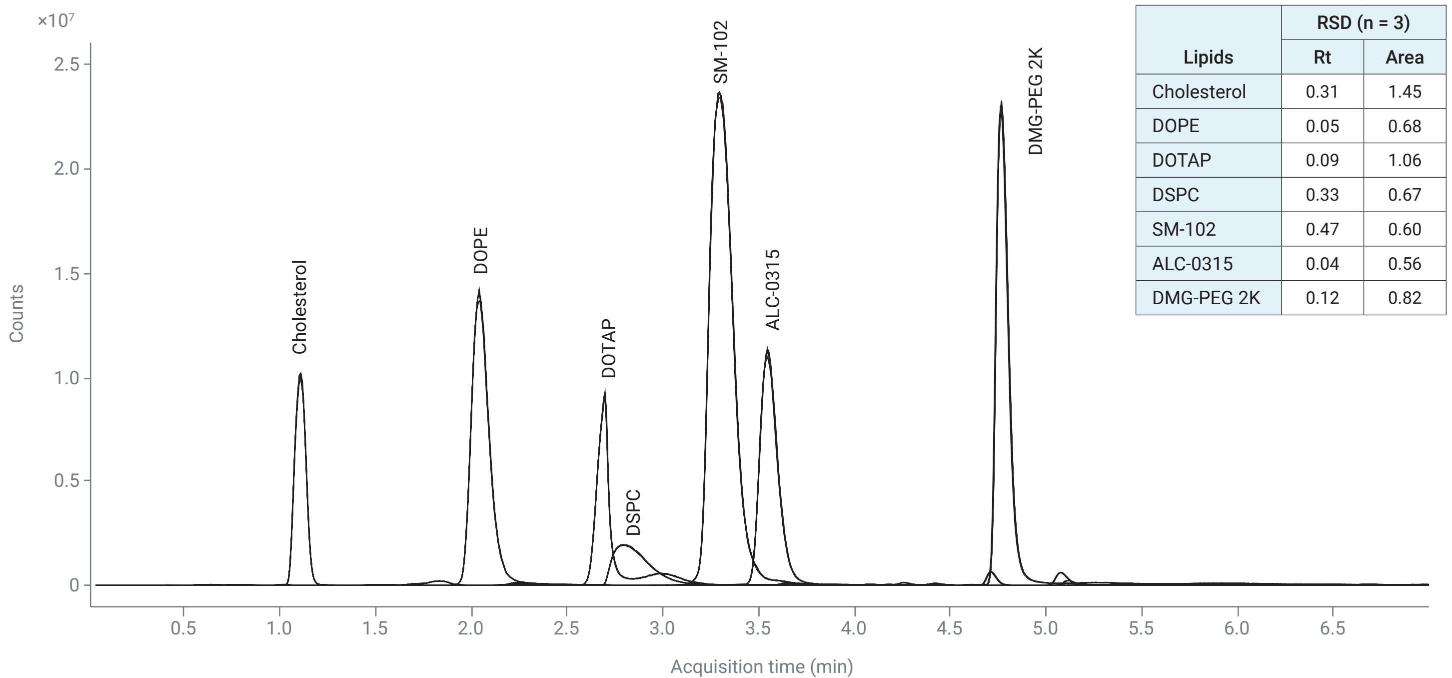


Figure 1. High-resolution and accurate mass identification of LNP components. (A) EICs and (B) mass spectra of the seven lipid components.



Lipids	RSD (n = 3)	
	Rt	Area
Cholesterol	0.31	1.45
DOPE	0.05	0.68
DOTAP	0.09	1.06
DSPC	0.33	0.67
SM-102	0.47	0.60
ALC-0315	0.04	0.56
DMG-PEG 2K	0.12	0.82

Figure 2. Overlay EIC of the lipid standards, demonstrating reproducibility.

The linearity of the method was assessed at a 0.1 to 1,000 pmol concentration range for four lipids: cholesterol, DMG, SM-102, and DSPC. Calibration curves (Figure 3) were established on a logarithmic scale by plotting log (peak area) versus log (lipid concentration) and fitted with a linear curve. The coefficient of determination $R^2 \geq 0.99$ demonstrated a strong linearity on the concentration ranges under study. The EIC overlay of the four lipids employed for the quantitative determination is displayed in Figure 4.

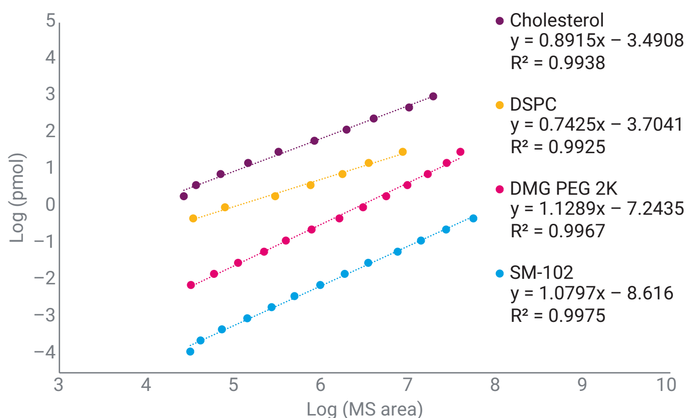


Figure 3. Calibration curve of lipid standards (n = 3).

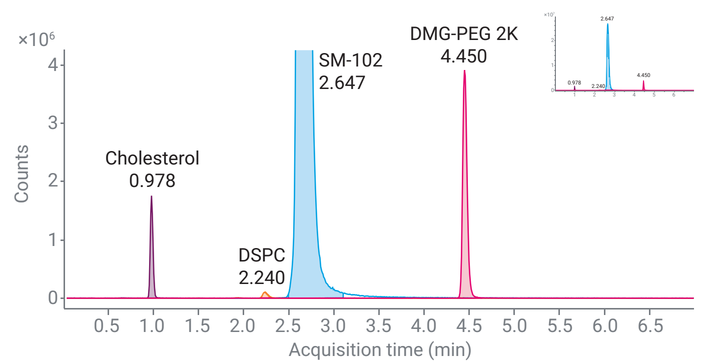
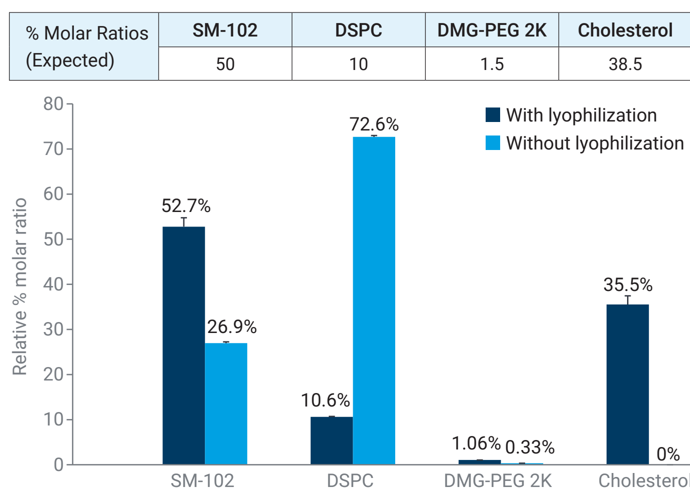


Figure 4. EIC of the four lipid standards used to prepare LNP.

Lipid stability observation is one of the important goals of mRNA LNP production. The applicability of the LC/MS method was evaluated as a quality control tool by examining the lipid content in different mRNA LNP formulation conditions. The mRNA LNP preparations (both with and without lyophilization storage conditions) are detailed in the "Experimental" section. The percent molar ratios of lipids under the two storage scenarios are displayed in Figure 5. The lipid content in lyophilized samples was consistent with the targeted mRNA LNP formulation (SM-102:DMG-PEG 2K:DSPC:cholesterol at 50:1.5:10:38.5), demonstrating the reliability of the LC/MS method. On the other hand, substantial changes in lipid concentration were noted in the nonlyophilized samples, suggesting inadequate lipid stability, which could be due to the chemical degradation of lipids. Overall, the LC/MS method highlighted the variations in lipid content over the various formulation conditions, which helps to improve the formulation process. Detailed LNP stability studies of different storage conditions (temperature and time) are currently under investigation.



% Molar Ratios (Expected)	SM-102	DSPC	DMG-PEG 2K	Cholesterol
	50	10	1.5	38.5

Figure 5. LC/MS analysis of lipid content of mRNA loaded in LNP samples stored at –20 °C for one month (n = 3).

Conclusion

In this application note, an LC/MS method using an Agilent 1290 Infinity II LC system and an Agilent 6545XT AdvanceBio LC/Q-TOF was developed for the identification and quantification of lipid components. The developed method enabled the separation of all lipids with good linearity and repeatability. The LC/MS method was applied to assess the stability of lipid excipients in mRNA LNP formulation preparations.

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