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# Lipid Nanoparticle Compositional Analysis Using Charged Surface Hybrid Phenyl-Hexyl Separation With Evaporative Light Scattering Detection

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## Abstract

The composition of the lipids used to encapsulate drug products is critical to their structure-function properties. A reliable assay for quantifying their relative abundance in the lipid nanoparticle is important for formulation development or for quality control testing. Using a positive surface potential stationary phase and simpler mobile phases, a robust separation of the four lipids used in the patisiran (ONPATTRO) lipid nanoparticle formulation was developed. Utilization of a charged surface hybrid achieved narrow, symmetrical peaks for the ionizable lipid, DLin-MC3-DMA. The lower hydrophobicity phenyl-hexyl ligand facilitated the elution of extremely hydrophobic phospholipid, 1,2-distearoyl-sn-glycero-3-phosphocholine. Evaporative light scattering has been used as the

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detection mode, however, there is equivalent potential in applying charged aerosol detection and electrospray ionization MS (ESI-MS).

## Benefits

- Simple mobile phase composition that is compatible with both ELS and MS detection

- ACQUITY UPLC Premier CSH Phenyl-Hexyl Column Technology produces a sharp, narrow peak for the ionizable, cationic lipid, DLin-MC3-DMA

- Lower hydrophobicity phenyl-hexyl ligand facilitates the elution of extremely hydrophobic analytes such as DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine)

## Introduction

Research into and the pharmacology of lipid nanoparticles is exploding as a result of the proven effectiveness of encapsulated small interfering RNA (siRNA) and new mRNA-based vaccines, the success of which depends on the availability of a safe and efficient delivery vehicle. Lipids used in the particle formation can impart higher bioavailability of the packaged RNA and provide stability to the overall structure of formulated drug. The optimization of lipid nanoparticle formulations is best exemplified by the development of patisiran (ONPATTRO), the first siRNA-based drug approved by the US Food and Drug Administration (FDA) in 2018. The knowledge gained during its development has certainly contributed to the rapid development of COVID-19 mRNA vaccines.[1]

The increasing use of lipid nanoparticle technology for drug delivery brings with it the need for a fast, robust chromatographic analysis to determine the relative amounts of lipids in the formulation. In this application note, we demonstrate the advantage of a lower hydrophobicity stationary phase modified with a positive charged surface potential as a very useful starting point for the compositional analysis of lipid nanoparticles. A common challenge when developing a reversed-phase separation is achieving a desired level of retention. Often, this challenge is in encountered with small, polar acids that are difficult to retain. Conversely, this challenge can present itself with very hydrophobic compounds, like lipids, wherein it can be difficult to access enough eluotropic strength to elute the analyte. For this reason, we have evaluated six different reversed-phase stationary phases for their applicability to separate the compounds found in the lipid nanoparticles of patisiran. The analytical objective of this work was to separate the four lipid peaks within a six-minute gradient and to produce narrow, symmetrical peaks for each lipid used in the patisiran drug product (Figure 1). Evaporative light

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scattering was employed as the detection mode for the study, but with the volatile mobile phase that has been applied, there is equivalent potential in applying charged aerosol detection and electrospray ionization MS (ESI-MS).
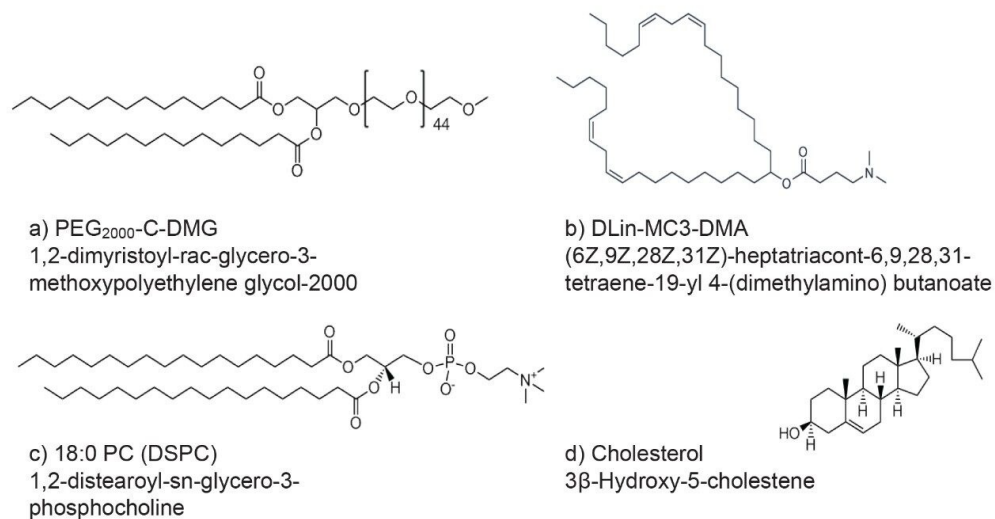


a) PEG$_{2000}$-C-DMG
1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000

b) DLin-MC3-DMA
(6Z,9Z,28Z,31Z)-heptatriacont-6,9,28,31-tetraene-19-yl 4-(dimethylamino) butanoate

c) 18:0 PC (DSPC)
1,2-distearoyl-sn-glycero-3-phosphocholine

d) Cholesterol
3β-Hydroxy-5-cholestene

*Figure 1. Chemical structure of the four lipid nanoparticle components.*

## Experimental

### Retention Factor Test Conditions

Retention factors were determined using the small molecule marker, acenaphthene, to establish the relative hydrophobicities for the columns. The mobile phase used was acetonitrile and water 70/30, (v/v), and the flow rate was 300 µL/minute. Sample concentration was thiourea 10 µL/mL (V$_o$) and acenaphthene 200 µg/mL.

| | |
|---|---|
| Data management: | Empower 3 Chromatography Data Software |
| | FR4 |
| LC system: | ACQUITY UPLC I-Class |

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| | |
|---|---|
| Detector: | ACQUITY UPLC Photodiode Array Detector |
| Wavelength: | 254 nm |
| Column temp.: | 30 °C |
| Sample temp.: | 24 °C |
| Injection volume: | 1.5 µL |
| Flow rate: | 0.3 mL/min |

## Lipid Analysis Test Conditions

The following experimental conditions were used to analyze the four lipids referenced in the ONPATTRO prescribing information document (Figure 1).

*Note: The ACQUITY UPLC Evaporative Light Scattering Detector does not employ high performance surfaces and is identified by the blue exterior panels. There are components in this detector which may impact recovery of metal-sensitive analytes such that an analyst may want to consider method conditions and sample passivation to mitigate losses and peak tailing.*

| | |
|---|---|
| Data management: | Empower 3 Chromatography Data Software FR4 |
| LC system: | ACQUITY Premier Quaternary System |
| Detector: | ACQUITY UPLC Evaporative Light Scattering |
| Gas pressure: | 40.0 psi |
| Nebulizer mode: | Heating |

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| | |
|---|---|
| Power level: | 70% |
| Drift tube temperature: | 48 °C |
| Column temp.: | 50 °C |
| Sample temp.: | 24 °C |
| Injection volume: | 3 µL |
| Flow rate: | 0.4 mL/min |

Linear gradients using 0.1% formic acid in acetonitrile were initially applied to evaluate which of the stationary phases provided the best starting point for further method optimization. Gradient 1, delivering 60% mobile phase B to 100% mobile phase B in six minutes, used 0.1% formic acid in 100% water as mobile phase A (MP A) and 0.1% formic acid in 100% acetonitrile as mobile phase B (MP B). A two-minute hold at 100% mobile phase B was programmed to elute lipids that were not eluting within the six-minute gradient window. The total run time was 12 minutes. Gradient 2 used mobile phases with a constant amount of isopropanol added to both MP A and MP B, replacing 10% of the acetonitrile. Mobile phase A was 0.1% formic acid (v/v) in isopropanol, acetonitrile, and 18MΩ water (10/50/40) (v/v/v). Mobile phase B was 0.1% formic acid (v/v) in isopropanol, acetonitrile, and 18MΩ water (10/80/10) (v/v/v) in 0.1% formic acid. Gradient 2 delivered 100% A to 100% B in six minutes with a two-minute hold at 100% B.

## Gradient Table - 1

| Time | mL/min | A | B | Curve |
|---|---|---|---|---|
| Initial | 0.4 | 40 | 60 | * |
| 6.00 | 0.4 | 0 | 100 | 6 |
| 8.00 | 0.4 | 0 | 100 | 6 |
| 8.50 | 0.4 | 40 | 60 | 6 |
| Run time = 12 minutes | | | | |

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## Gradient Table - 2

| Time | mL/min | A | B | Curve |
|---|---|---|---|---|
| Initial | 0.4 | 100 | 0 | * |
| 6.00 | 0.4 | 0 | 100 | 6 |
| 8.00 | 0.4 | 0 | 100 | 6 |
| 8.50 | 0.4 | 100 | 0 | 6 |
| Run time = 12 minutes | | | | |

The lipid nanoparticles used in the patisiran formulation are prepared from four main components: a neutral phospholipid, cholesterol, a polyethylene-glycol (PEG)-lipid, and an ionizable cationic lipid. The ionizable lipid, DLin-MC3-DMA, is important for particle formation, and for the complexation and releasing of the siRNA. PEG$_{2000}$-C-DMG helps the LNP stability in the circulation, achieving optimal circulation time, and enabling uptake of patisiran into the liver. DSPC and cholesterol imparts physicochemical stability to the LNP.[2] The four lipid structures are shown in Figure 1.

Lipid standards were purchased from multiple sources. DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) was purchased from Sigma (PN 1138), DLin-MC3-DMA (cationic lipid) (6Z,9Z,28Z,31Z)-heptatriacont-6,9,28,31-tetraene-19-yl 4-(dimethylamino)butanoate was purchased from Ambeed (Arlington Hts, IL 60004, USA), PEG$_{2000}$-C-DMG (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000) was purchased from Avanti Polar Lipids, and cholesterol USP was purchased from Sigma (PN 8667). Standards were stored at -80 °C.

Individual 1 mg/mL stock standards were prepared in 100% methanol and stored at -20 °C. To prepare dilutions, the stocks were brought to room temperature prior to dilutions that were made using acetonitrile. A mix of the four lipids was prepared with the final concentrations of 300 µg/mL PEG$_{2000}$-C-DMG, 100 µg/mL DLin-MC3-DMA, 50 µg/mL cholesterol, and 50 µg/mL DSPC. The sample was brought to volume using 0.1% formic acid in 100% acetonitrile. The concentrations in this sample were used for method development and serves as a starting point for a sample representing a dissociated LNP RNA drug product. The amounts used for the cationic lipid, DLin-MC3-DMA, and cholesterol were less than half the amounts used in the partisiran formulation, a nearly equivalent amount of DSPC was used. Since the PEG$_{2000}$-C-DMG was presenting as a broader peak under the chosen chromatographic conditions, a 10X concentration was used to ensure its identification.

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## Results and Discussion

Six different reversed-phase stationary phases were evaluated in this study for their resolving power, selectivity, and retentivity. These included stationary phases with various types of ligands and surface potentials, including ACQUITY Premier CSH $C_{18}$ (CSH $C_{18}$), ACQUITY UPLC Premier BEH $C_{18}$ (BEH $C_{18}$), Atlantis Premier BEH $C_{18}$ AX (BEH $C_{18}$ AX), ACQUITY UPLC BEH $C_8$ (BEH $C_8$), ACQUITY Premier CSH Phenyl-Hexyl (CSH Phenyl-Hexyl), and ACQUITY UPLC BEH Phenyl (BEH Phenyl) Columns. The column configuration for all stationary phases was 2.1 x 50 mm, 1.7 μm particle; CSH $C_{18}$, CSH Phenyl-Hexyl were evaluated in MaxPeak High Performance Surfaces (HPS) hardware and BEH Phenyl and BEH $C_8$ were evaluated in ACQUITY stainless-steel hardware. Previous evaluations have shown that on the more hydrophobic $C_{18}$-stationary phases lyso-glycerophosphocholine tended to elute in poorly shaped, broad peaks. Phospholipids were found to be the least retained on phenyl stationary phases.[3] Lower retention for lipids may reduce the issue of lipids building up on the column inlet causing deterioration of column performance.

Prior to evaluating the columns for their performance using the lipid analytes, they were evaluated using the small molecule marker, acenaphthene, to establish their relative hydrophobicities as shown in Table 1. Three columns, ACQUITY Premier CSH $C_{18}$ 1.7 μm, ACQUITY UPLC BEH $C_{18}$ 1.7 μm, and Atlantis Premier BEH $C_{18}$ AX,1.7 μm, produced retention factors for acenaphthene of 3.0 or greater. The remaining three columns, ACQUITY Premier CSH Phenyl-Hexyl 1.7 μm, ACQUITY UPLC BEH Phenyl 1.7 μm, and ACQUITY BEH $C_8$ 1.7 μm had lower retention factors for acenaphthene, approximately 1.5.

| Column | Retention factor |
|---|---|
| Atlantis Premier BEH $C_{18}$ AX Column | 4.66 |
| ACQUITY Premier CSH $C_{18}$ Column | 3.17 |
| ACQUITY UPLC BEH $C_{18}$ Column | 3.02 |
| ACQUITY Premier CSH Phenyl-Hexyl Column | 1.59 |
| ACQUITY BEH $C_8$ Column | 1.61 |
| ACQUITY UPLC BEH Phenyl Column | 1.40 |

*Table 1. Retention factor for acenaphthene using acetonitrile/water, 70/30, v/v*

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The detection method of evaporative light scattering can be used in either isocratic or gradient mode using a wide variety of mobile phases and additives. Recommended mobile phases are typically mass spectrometry (MS)-compatible and would provide a logical starting point to transfer the analysis to MS detection. Gradient 1 using only an acetonitrile in 0.1% formic acid gradient was used to conduct an initial screening of the CSH $C_{18}$ and BEH $C_8$ columns. For both columns, the DSPC peak eluted in the final 2-minute hold segment of the gradient, indicating that the gradient had little role in the elution. Additionally, the peak area was significantly lower on both columns as shown for the BEH $C_8$ column in figure 2, indicating that the acetonitrile/water in 0.1% formic acid mobile phase may not be strong enough to elute the phospholipid (peak 1) from the two stationary phases. For these two stationary phases, incomplete elution may cause the sample to accumulate on the column, degrading its performance over time.[3] Alternative mobile phases using other organic solvents such as isopropanol and methanol or combinations of solvents may be a better choice for these stationary phases and could be explored as an alternative method development option. For example, other researchers have eluted cholesterol with an isocratic method based on acetonitrile/methanol 60/40, v/v.[4] Nevertheless, with this initial result, further evaluation of the BEH $C_{18}$ and the BEH $C_{18}$ AX was not explored, out of a preference to find a retentivity that would match to a simpler mobile phase composition.

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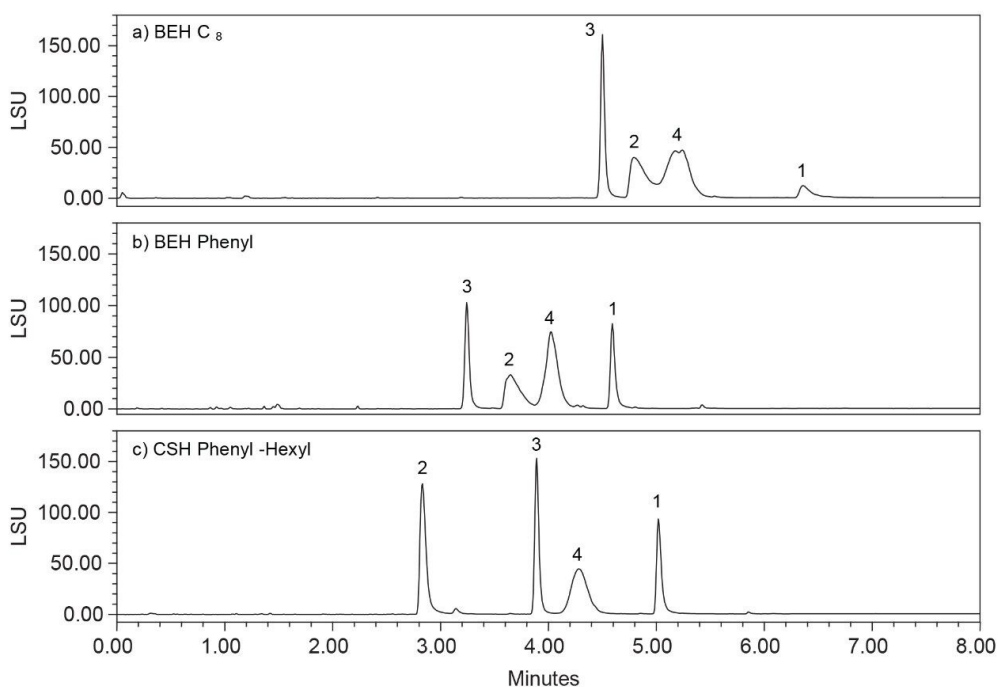
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*Figure 2. Comparison of three stationary phases using a six-minute gradient of 40% mobile phase A and 60% mobile phase B to 100% B with a two-minute hold at final conditions where mobile phase A is 0.1% formic acid in 100% water and mobile phase B is 0.1% formic acid in 100% acetonitrile. Peak Identification is 1.) DSPC, 2.) DLin-MC3-DMA, 3.) Cholesterol, and 4.) PEG2000-C-DMG.*

Since one of the goals for this study was to provide a fast analysis, increasing the run time at 100% organic was not a desirable option. The most promising chromatography using this mobile phase system was achieved using the CSH Phenyl-Hexyl and the BEH Phenyl columns shown in Figure 2. Narrow peaks were achieved for both cholesterol (peak 3) and for DSPC (peak 1). The differentiating performance was the narrower peak width and more symmetrical shape for the DLin-MC3-DMA (peak 2) that was achieved on the CSH Phenyl-Hexyl Column.

While both stationary phases are prepared using a phenyl-hexyl silane bonded to a BEH particle, the CSH Phenyl-Hexyl phase is prepared using a reproducible, low-level positive surface charge in acidic mobile phases.[5] This modification has been shown to give improvement in peak sharpness and symmetry for cationic analytes such as DLin-MC3-DMA, p$K_a$ of 6.44. Using low ionic strength acidic mobile phases, such as those used in both gradient 1 and gradient 2, causes ionic repulsion of the protonated DLin-MC3-DMA from the protonated

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stationary phase surface. The peak width for DLin-MC3-DMA measured at 13.4% of peak height was 6.4 seconds on the CSH Phenyl-Hexyl Column and 16.6 seconds on the BEH Phenyl Column packed with stationary phase prepared without a surface charge modifier. Herein, we also chose to apply the CSH Phenyl-Hexyl stationary phase in the form of an ACQUITY Premier Column that is constructed from hardware with hybrid inorganic organic surfaces. These surfaces can help reduce the need for column conditioning and thereby help an analyst obtain more symmetrical peaks even upon first-time use and even when dealing with potential metal-chelating compounds like the phosphate group containing DSPC lipid.

One other lipid component deserves discussion, namely the pegylated lipid. This species was separated as a relatively wide peak. For example, the 13.4% height peak width for $PEG_{2000}$-C-DMG was about 4 times wider the peak width for cholesterol on both the noted phenyl columns. The pegylated lipid was separated as a 17.5 second wide peak on the CSH Phenyl-Hexyl Column versus the same column producing a 4.5 second wide peak for cholesterol. The broader peak was unique to the $PEG_{2000}$-C-DMG and could not be attributed to the column efficiency.

## Conclusion

Lipids are garnering a significant amount of attention now that their use in lipid nanoparticles has proven to be effective for both the delivery of siRNA and mRNA vaccines. The composition of the lipids used in these two types of encapsulated drug products is critical to their structure-function properties. Accordingly, it is important to have a reliable assay for quantifying their relative abundance in the lipid nanoparticle, whether that be for formulation development or for quality control testing. The reliability for such an assay starts with chromatographic considerations.

With these results, we have demonstrated the advantage of a lower hydrophobicity stationary phase that bears a charged surface potential, namely the CSH Phenyl-Hexyl stationary phase. A column with this type of stationary phase is an effective starting point for the development of a compositional analysis method for lipid nanoparticles. The lower hydrophobicity of phenyl-hexyl stationary phases assisted in the complete elution of extremely hydrophobic analytes such as DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine). Higher hydrophobicity $C_{18}$ stationary phases did not afford complete elution of DSPC in either the gradient or within a high organic hold time. The CSH technology of the CSH Phenyl-Hexyl stationary phase also provided the added benefit of producing a sharp, narrow peak for the ionizable, cationic lipid, DLin-MC3-DMA. And in the end, it was

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possible to separate the 4 lipids of the patisiran formulation in a 6-minute method using a simple mobile phase composition amenable to both ELS and MS detection.

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