

ADVANCES IN LIPID PURIFICATION FOR THERAPEUTIC NANOPARTICLES



RotaChrom
Purified Solutions

Abstract

Lipid nanoparticles (LNPs) have emerged as a key platform in recent therapeutic advancements, particularly in mRNA-based vaccines and gene therapy products. However, the synthetic production of lipid components often results in structurally similar and chemically labile impurities that pose challenges for conventional chromatographic purification. This collection of studies carried out

by the Test Laboratory of RotaChrom Technologies demonstrates how Centrifugal Partition Chromatography (CPC), a gentle, scalable, and solid-phase-free technique, can be effectively applied to purify a variety of lipid classes. By minimizing degradation and improving process robustness, CPC offers a valuable purification strategy for industrial lipid manufacturing.

Introduction

Lipid-based drug delivery systems, particularly lipid nanoparticles (LNPs), represent one of the most actively researched (**Figure 1**) and rapidly advancing platforms in modern therapeutics. Their significance has become especially prominent in the context of mRNA-based vaccines and gene therapy products^{1,2}.

These nanostructured formulations typically consist of four key lipid components^{3,4}:

- a PEG-conjugated lipid, which enhances particle stability and circulation time;
- an ionizable (cationic) lipid, which facilitates cellular uptake and promotes endosomal escape;
- a structural lipid, which stabilizes the lipid bilayer architecture;
- and cholesterol, which stabilizes lipid membranes by modulating fluidity and reducing permeability, contributing to overall structural integrity.

During the synthetic production of lipid raw materials, by-products are often formed *in situ*. Such by-products can be generated from a variety of sources, including unreacted starting materials, over-alkylated derivatives, or degradation products. The efficient removal of these impurities poses a considerable challenge, especially in the case of ionizable lipids, which tend to be unstable under conventional normal-phase chromatographic conditions.

CPC is a liquid-liquid chromatographic technique in which separation occurs between two immiscible liquid phases. Both the mobile and stationary phases are liquids, with the stationary phase retained in the rotor by centrifugal force. The retention of solutes is governed by the partition coefficient (K_D), which reflects the relative affinity of a compound for the stationary phase versus the mobile phase. Practically, it is defined as the ratio of the solute concentration in the stationary phase to that in the mobile phase. The absence of a solid stationary phase eliminates the risks of irreversible adsorption and chemical degradation, making CPC a gentle and effective method for isolating molecules with labile or reactive functional groups.

CPC does not require expensive solid stationary-phase and allows the separation of compounds in gram-scale quantities, making it a highly suitable method for the purification of lipids.

The use of CPC technology yielded the following key advantages:

- significantly improved isolation yield compared to traditional column chromatography,
- and the ability to perform purifications on a preparative (gram or kilogram-scale) level.

Analytical documentation is typically available for the material to be purified, enabling prior identification of the target components. Following the CPC run, fractions are identified based on retention time and merged accordingly. Subsequent evaporation is guided by the following considerations:

- the purity of the compound of interest (Col),
- and the maximization of overall yield.

In this collection of applications, CPC was used to purify various lipid materials, including DMG-PEG 2000, ALC-0315, a silica-based cationic lipid 23B1, and a silica-based lipid 19B28.

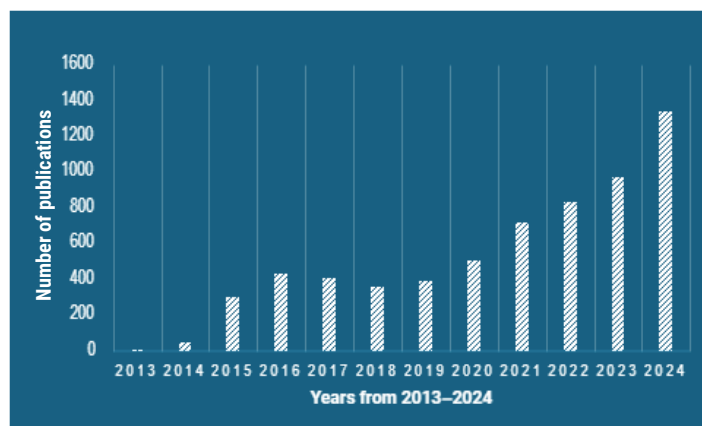


Fig.1.: Trends in the number of scientific publications applying the keywords 'lipid nanoparticles' and 'lipid nanoparticles (LNPs)' from 2013 to 2024, according to PubMed.

Analytical and Preparative Parameters

HPLC Analysis

For method development and analytical characterization of the crude materials, an HPLC system was used. The setup included a quaternary pump, an autosampler, a diode array detector, and an evaporative light scattering detector (ELSD). Due to the poor UV activity of lipid-based compounds, an ELSD detector was employed to enable reliable detection and quantification, as conventional UV-VIS detection would provide limited or no response for many of the target molecules.

CPC Experiments

Laboratory-scale CPC experiments were carried out using a benchtop CPC purification system equipped with a 250 mL rotor and operated via dedicated control software. During each run, the rotor was first filled with the stationary phase, followed by equilibration with the mobile phase. Sample injection was performed using the built-in 10 mL injection loop of the instrument. Detection was carried out using a TOYDAD 600 scanning UV-PDA detector, which enabled simultaneous acquisition at four wavelengths and full spectral scanning in the 200–600 nm range, with a scanning speed of 5 Hz.

Preparative Isolation and Purification of Lipids

Types of Lipids Investigated

In this study, four different lipid components were separated using CPC. These compounds differ in chemical structure and function, yet each plays a critical role in the structural integrity or function of lipid nanoparticles (LNPs). The purification of each lipid required an individualized approach, taking into account their chemical stability, polarity, and impurities.

19B28 and 23B1 Lipid

19B28 – Silica-Based Lipid

19B28 is a synthetic, silicate-based neutral lipid used as a structural component in lipid nanoparticle (LNP) formulations. The main impurity in the crude material was an over-alkylated byproduct with a structure closely resembling the Col as illustrated in **Figure 2**, making purification particularly challenging using traditional chromatographic techniques.

The structure of 19B28 exhibits a pronounced amphipathic character due to its unique molecular architecture: two long hydrophobic alkyl chains are attached via Si–O bonds to a central ionizable tertiary amine group. The presence of the silyl ether function increases the polarity and rigidity around the polar domain, while the extended hydrophobic regions contribute to strong apolar character. This combination makes the molecule more amphipathic than conventional structural lipids. Due to its amphipathic nature, normal-phase column chromatography posed a risk such as irreversible adsorption or degradation. Switching to CPC enabled a solid support-free separation, thereby significantly reducing the risk of decomposition or sample loss. The biphasic solvent system applied was based on an alkane/alcohol/water mixture with salt additive, offering sufficient polarity contrast and stability for CPC operation.

Method development revealed that satisfactory chromatographic resolution between the Col and its impurities could be maintained up to an injection amount of 1380 mg. The present investigation identified this load as the upper limit for attaining both high purity and acceptable yield, as higher loads resulted in overlapping peaks (**Table 1**).

Purification was performed using a Multiple-Dual Mode (MDM) configuration. This continuous switching approach, known as

multiple-dual mode CPC (MDM-CPC), maximizes separation efficiency by leveraging both elution modes sequentially. This switching mechanism, along with changes in flow direction and elution order, allows for the effective separation of binary mixtures and compounds with similar partition coefficients that have limited selectivity in standard chromatography. In practical applications, the flow direction of the liquid phases is altered by switching a valve in the CPC system. This original elution mode is thus a semi-continuous process with a classical sample injection, and which only requires a single CPC column. The optimized CPC conditions resulted in good separation and high purity, as shown by the representative chromatogram in **Figure 3**. The strategy significantly improved the separation of the target compound, the results of which are shown in **Table 1**.

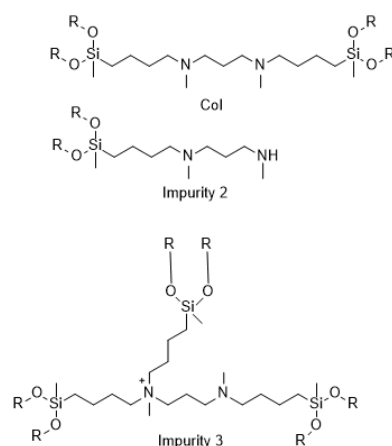


Fig.2.: 19B28 and the chemical structures of its main impurities

Lipid Type	Injected Amount (mg)	CPC Mode	Purity (%)	Comment
19B28	460	MDM	96.4	Good resolution, well separated peaks
19B28	920	MDM	97.6	Peaks approaching
19B28	1380	MDM	97.7	Peak overlap
23B1	460	EE	97	-
23B1	920	EE	95.5	Lower Col content

Table 1.: Summary of CPC Conditions and Results for Lipid 19B28 and 23B1 Compounds

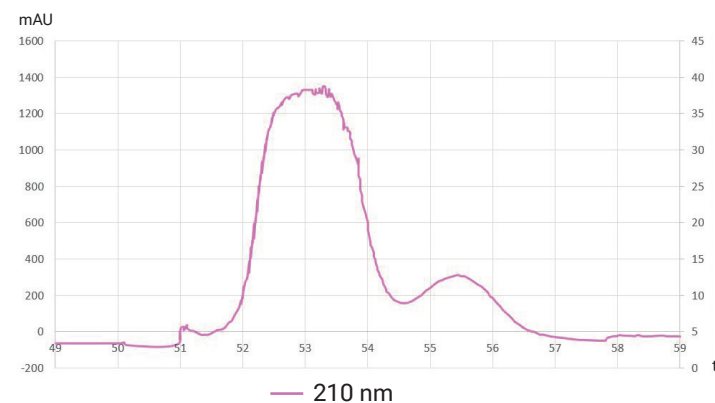


Fig.3.: Representative CPC chromatogram of 19B28 isolation

Analytical and Preparative Parameters

23B1-silica based cationic lipid

Similar to 19B28, 23B1 (**Figure 4**) is a silicate-based lipid. Its ionizable functional group and amphipathic character present additional challenges for purification, making conventional methods suboptimal.

CPC was carried out using an elution-extrusion (EE) mode (**Figure 5**). The separation resulted in high-purity isolation, with the chromatographic outcome shown in **Figure 4** and summary data in **Table 1**. The process demonstrated scalability and reproducibility across different injected amounts.

DMG-PEG 2000

DMG-PEG 2000 (**Figure 6**) also known as 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000, is a lipid excipient specifically designed for the formulation of lipid nanoparticles (LNPs) intended for RNA delivery. It is derived from dimyristoyl glycerol (DMG) and exhibits phospholipid-like properties in terms of membrane association.

The crude reaction mixture used for synthesis contained a variety of process-related impurities. These included methoxypolyethylene glycol 2000 monoglyceride (MPEG2000 MG), myristic acid (14:0 FA), methoxypolyethylene glycol 2000 myristate (14:0 MPEG2000), 14:0 monoglyceride (14:0 MG), 14:0 diglycerides (14:0 DG), and 14:0 triglyceride (14:0 TG), as well as residual coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC-HCl) and its urea-based byproduct.

The objective of this work was to isolate the Col using CPC, while minimizing precipitation events throughout the process in order to improve overall yield. The resulting chromatogram is shown in **Figure 7**, and performance data are summarized in **Table 2**.

DMG-PEG 2000 is a PEGylated lipid, the purification of which via CPC presented technical difficulties due to its amphiphilic nature and tendency to cause turbidity, foaming, and precipitation in aqueous-organic biphasic systems.

Several ether/alcohol/water-based and alkane/aromatic/polar aprotic solvent systems were evaluated for CPC. While some of these systems initially showed promising phase behavior and partition characteristics, they ultimately proved unsuitable for preparative purification due to issues such as column bleeding, pressure drop, or phase collapse under CPC operating conditions.

Based on these observations, an alternative approach was evaluated using a cyrene-based solvent system (**Table 2**). While this system poses technological challenges, it provided stable phase behavior and enabled successful separation for PEGylated components.

An injected amount of 100.5 mg of crude material was dissolved in 10 mL of the lower phase.

ALC-0315

ALC-0315 (**Figure 8**) is an ionizable cationic aminolipid that plays a pivotal role in mRNA-based lipid nanoparticle (LNP) vaccine formulations, such as in the Pfizer-BioNTech COVID-19 vaccine. It is commercially available in research-grade purity (~98%) for laboratory and preclinical development purposes.

ALC-0315 condenses nucleic acids and becomes protonated at endosomal pH, facilitating endosomal escape and subsequent cytoplasmic release of the mRNA cargo. Comparative preclinical studies have demonstrated that LNP formulations prepared with ALC-0315 elicit stronger immune responses (both humoral and cell-mediated) compared to earlier ionizable lipids.

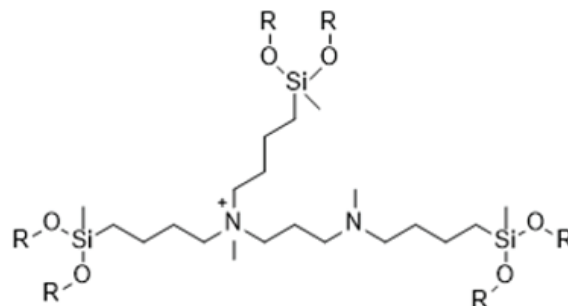


Fig.4.: Structure of 23B1

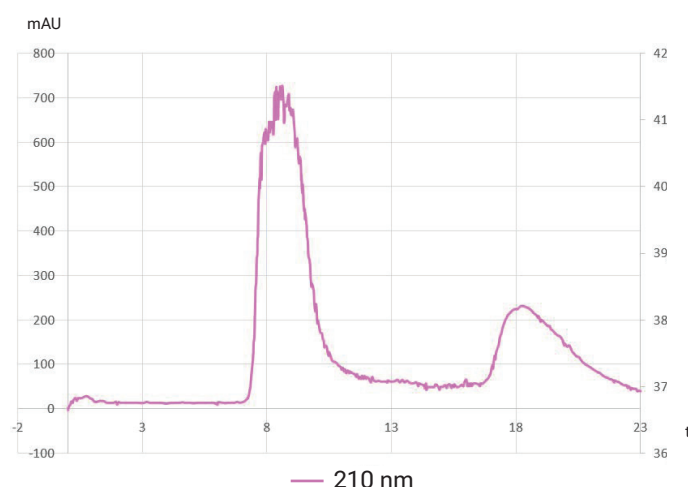


Fig.5.: Representative CPC chromatogram of 23B1

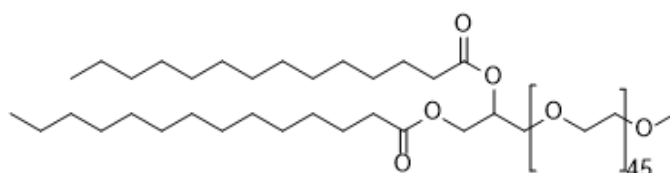


Fig.6.: Structure of DMG-PEG2000

Type of solvent system	Result	Purity (%)	Yield (%)	Comment
Ether/alcohol/water with salt; alkane/aromatic/polar aprotic	Failure of the separation	-	-	Severe column bleeding
Cyrene based solvent system	Successful isolation	98.9	89.7	Removing cyrene is challenging (high boiling point, risk of decomposition)

Table 2: Summary of CPC Conditions and Results for Lipid Compounds

ALC-0315 has been widely utilized in the development of lipid nanoparticle-based delivery systems for mRNA therapeutics and vaccines, where its ability to promote efficient intracellular delivery is considered essential to formulation success⁵.

The compound of interest is a synthetically produced, relatively soluble lipid, but its crude form contains a mixture of structurally related contaminants with similar solubility characteristics. Two major impurities (**Figure 8**) closely resemble polarity and partition behavior posed a significant challenge for purification. Achieving efficient separation required a carefully selected biphasic solvent system composed of an alkane/halogenated alkane/alcohol solvent system, which provided a favorable K_d for the target compound.

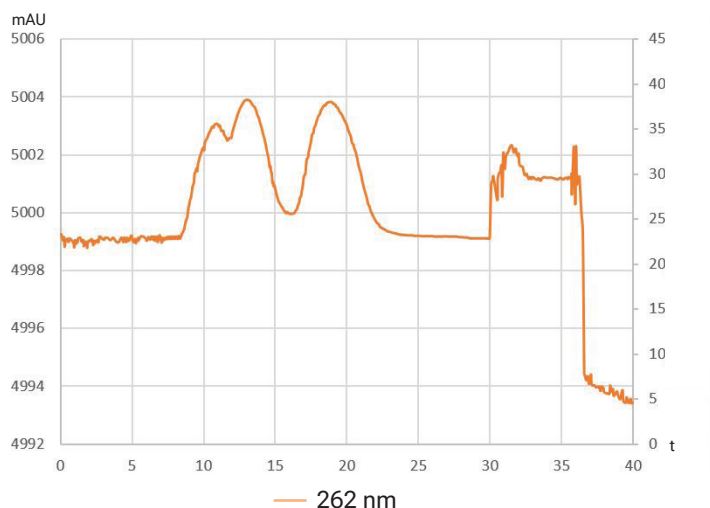


Fig.7.: Representative CPC chromatogram of DMG-PEG 2000

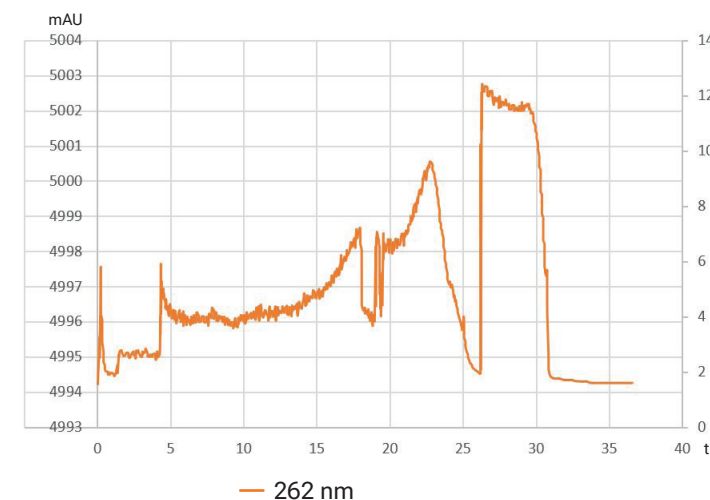


Fig.9.: Representative CPC chromatogram of ALC-0315

500 mg of crude material was dissolved in the upper phase and injected into the CPC system. The separation was carried out under optimized elution and extrusion conditions, which enabled effective resolution of the compound of interest from the co-eluting impurities. The process yielded the compound in excellent purity, as confirmed by analytical results shown in **Table 3** and the representative CPC chromatogram in **Figure 9**.

Lipid type	Purity (%)	Yield (%)
ALC-0315	>99	89.63

Table 3: Summary result of lipid compounds

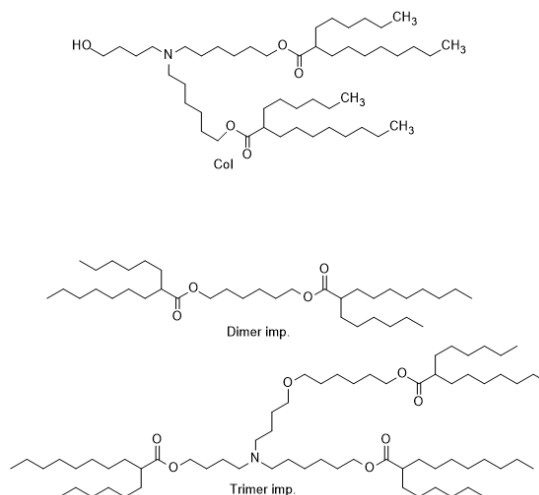


Fig.8.: Structure of ALC-0315 and its impurities

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