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RP-HPLC-CAD method for the rapid analysis of lipids used in lipid nanoparticles derived from dual centrifugation

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ABSTRACT

The use of lipids as suitable excipients for drug carrier systems has been established for years. Liposomes or lipid nanoparticles (LNPs) in general have been shown capable of delivering both hydrophilic and hydrophobic drugs. The Covid-19 pandemic and the resulting vaccines have significantly increased interest in the potential for these lipid-based systems, which can carry different types of therapeutic RNAs. LNPs used for the transfection of RNA are usually a multi-component mixture of phospholipids and other lipids. Essential components are positively charged or ionizable lipids such as DOTAP or SM-102, but also uncharged helper lipids such as cholesterol, DOPE, DSPC, DMG-PEG_{2000} or DSPE-PEG_{2000}. Due to the differences in charge, simultaneous detection is a challenge. Here, we present a reversed-phase high-performance liquid chromatography charged-aerosol-detector method (RP-HPLC-CAD method) using a C-18 column for the simultaneous determination of charged and uncharged lipids. Our method has been validated according to the ICH-Q2 (R2) guideline for accuracy, precision, specificity and working range, including the limit of detection (LOD) and quantification (LOQ), as well as the calibration range. We were able to show satisfactory results in both precision and accuracy. The working range also shows great potential with a calibration range from 9.375 to 1000 µg/ml, LODs <1.85 µg/ml and LOQs <6.16 µg/ml. This method represents a fast and reproducible procedure for quantifying the lipids mentioned. In combination with the novel approach for the production of LNPs using dual centrifugation (DC), it offers the possibility of extremely rapid production of RNA-loaded LNPs, and the immediate analysis for their lipid components.

1. Introduction

Since the COVID-19 pandemic and the launch of the messenger RNA (mRNA) vaccines Comirnaty® and Spikevax®, the development of ribonucleic acid (RNA) therapeutics has become of great interest. mRNA codes for proteins and can be used for a number of different objectives through simple modifications to the nucleotide sequence. (Damase et al., 2021; Chaudhary et al., 2021) In addition to the relatively large mRNA molecules and their potential, RNA therapeutics have become a suitable approach for further therapeutic applications since the first FDA approved RNA interference (RNAi) therapeutic Onpattro® in 2018. The active pharmaceutical ingredient (API) of the Onpattro® formulation is a small interfering RNA (siRNA), but similar uses could also be applied to microRNA (miRNA). Both siRNA and miRNA involve RNAi and therefore can be used as gene regulators at the posttranscriptional level. While each are approximately 22 nucleotides in length and comparable in size, siRNA and miRNA show differences in their specificities. (Moraes et al., 2021)

Although the therapeutic mechanisms behind mRNA vaccines and RNAi therapeutics are fundamentally different, the hurdles in formulating these RNA therapeutics are primarily the same. The relatively large and manifold negatively charged molecules are poorly internalized by cells. Rapid degradation by ubiquitous RNases and excretion via the kidneys also pose a challenge. (Damase et al., 2021; Chaudhary et al., 2021; Sajid et al., 2020; Hald Albertsen et al., 2022) For this reason, special drug delivery systems (DDS) are being developed, with a particular focus on the formulation of lipid nanoparticles (LNPs).

LNPs usually consist of cationic or ionizable lipids, helper lipids and PEGylated lipids. Cationic or ionizable lipids help to improve the association as well as the transfection efficiency of RNA. Cationic lipids such as DOTAP have a permanently charged headgroup. Ionizable lipids are charged at lower pH ranges with a pKa of about 6 to 7, and uncharged at a physiological pH of about 7.4 e.g. SM-102. The positively charged headgroups of these lipids interact electrostatically with the negatively

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charged phosphate backbone of the RNA, thus improving the association efficiency of the RNA with the LNP. In the case of ionizable lipids, manufacturing therefore takes places under acidic conditions (pH ~ 4 commonly used in microfluidic systems). In addition, ionizable lipids have the advantage of improving transfection efficiency at least in part to improved endosomal escape. Helper lipids such as cholesterol, DSPC or DOPE influence rigidity and stability, as well as cellular mechanisms such as endocytosis of the LNPs. PEGylated lipids influence many different physiological properties, such as prolonged blood circulation, circulation half-life and in vivo distribution, as well as having an impact on size characteristics, encapsulation efficiency, and LNP aggregation. (Hald Albertsen et al., 2022; Li et al., 2022; Jayesh et al., 2018; De and Ko, 2023)

Because of the advantages described above for using lipids and their compositions in developing RNA formulations, we require appropriate analytical methods that can be used in drug development for both the quantification of the respective components and stability studies. We present a reversed-phase high-performance-liquid-chromatography (RP-HPLC) method for quantification of lipids commonly used in RNA LNP formulations. The method is suitable for the simultaneous quantification of cationic and ionizable lipids, as well as for the uncharged helper lipids and PEGylated lipids using a C-18 column for separation. UV quantification of lipids is of limited use, however, the mass-based Charged-Aerosol-Detector (CAD) method is an established means for lipid quantification. (Hazotte et al., 2007; Kiełbowicz et al., 2013; Nair and Werling, 2009) The aim of this work was to establish a suitable, rapid method for lipid quantification and to validate it according to the ICH Q2(R2) guideline with regards to accuracy, precision, specificity and working range, including the limit of detection and quantification, as well as the calibration range.

Combining this quick and reproducible RP-HPLC-CAD method with the novelty and advantages of LNP production by dual centrifugation (DC) gives us the possibility of an extremely rapid approach from formulating RNA loaded LNPs to studying and quantifying lipid behavior of the sterile and small-sized batch products.

2. Material and methods

2.1. Material

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and N-(carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-mPEG$_{2000}$) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Carl Roth GmbH+Co KG (Karlsruhe, Germany), 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG$_{2000}$) from Avanti Polar Lipids (Alabaster, USA) and 9-Heptadecanyl 8-{(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino}octanoate (SM-102) from Cayman Chemical Co. (Ann Arbor, USA). Methanol (MeOH) HPLC ultra gradient grade as well as Ethanol (EtOH) HPLC gradient grade were purchased from Carl Roth GmbH+Co KG (Karlsruhe, Germany). Trifluoroacetic acid (TFA) 99% Extra Pure was purchased from Acros Organics | Thermo Fisher Scientific GmbH (Dreieich, Germany). For buffer preparation, purified water of an Arium pro system from Sartorius AG (Göttingen, Germany), HEPES and Sucrose from Carl Roth GmbH+Co KG (Karlsruhe, Germany) were used. Hydrochloric acid for film preparation was purchased from Carl Roth GmbH+Co KG (Karlsruhe, Germany). hsa-miR-100-5p (mature sequence: 5′-AACCCGUAGAUCCGAACUUGUG-3′) from Thermo Fisher Scientific GmbH (Dreieich, Germany) was used for loading LNPs with RNA.

2.2. HPLC

2.2.1. HPLC-CAD

An Ultimate 3000 HPLC system (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used for sample quantification. The HPLC was set up with a quaternary pump (LPG-3400SD), an autosampler (WPS-3000TSL), and a thermostatted column compartment (TCC-3000SD). A corona charged-aerosol-detector (CAD) from Dionex Softron GmbH (Germering, Germany) was used for lipid detection. The CAD was set to an upper limit of 500 pA, a data acquisition rate of 10 Hz, evaporation temperature was factory fixed to 30 °C and an inlet pressure of 35 psi.

2.2.2. Sample separation

The lipid-containing samples were separated using a Luna C18(2) column (150 mm × 4.6 mm) with a particle size of 3 µm from Phenomenex Ltd. (Aschaffenburg, Germany). The column oven temperature was set to 50 °C and the autosampler temperature to 20 °C. The sample injection volume used for all samples was 10 µl. A two mobile phase composition was used for the HPLC-Method, consisting of Eluent A (0.15% TFA in H_2O *V/V*) and Eluent B (0.1% TFA in MeOH V/V). The HPLC-Method started with a composition of 15% Eluent A and 85% Eluent B, followed by a linear gradient over 7.5 min to 100% Eluent B. Eluent B was held at a plateau of 100% for 4.5 min and then returned to the initial composition of 15% Eluent A and 85% Eluent B. The initial composition was held for further 3 min until the end (total HPLC duration of 15 min). The flow rate was set to 2 ml/min, and the upper pressure limit was 345 bar. Before each new daily run, the HPLC was equilibrated with the original mobile phase composition for a minimum of 20 min, followed by two blank runs. Blank runs were also performed after each change in lipid composition.

2.2.3. Working range - lipid calibration and sample preparation

The lipids for the calibration were dissolved in EtOH and determined over a nine-point calibration range from 9.375 µg/ml to 1000 µg/ml. According to the non-linearity of the CAD detector at high concentrations, we used a quadratic fit for the calibration curves for all lipids in the entire calibration range. (Nair and Werling, 2009; Liu et al., 2008) In addition, for the PEGylated lipids, a linear regression was calculated over a range from 9.375 µg/ml to 150 µg/ml.

For measurement of precision, accuracy, specificity and working range, lipids were simply dissolved in EtOH. For the recovery of LNP excipients, the samples were diluted 1:9 (V:V) with EtOH for formulations 1 and 2. Because of the high molar percentage of SM-102 in formulation 3, this formulation was diluted 0.75:9.25 (V:V) to remain within the calibration range. All samples were gently vortexed until each solution appeared clear.

2.3. Validation

2.3.1. Precision

Precision was calculated by measuring retention time [min] and peak area [pA*min] for each lipid at three different concentrations according to the ICH Q2(R2) guideline. (European Medicines Agency ©, 2021) These two parameters were determined for the intra-laboratory repeatability (ILR), with three measurements on the same day, as well as for inter-day precision (IP), with three measurements for each concentration on three different days.

2.3.2. Accuracy

Accuracy was determined by measuring recovery according to the ICH Q2(R2) guideline. The recovery is calculated by measuring the concentration of the sample and relating to the expected concentration. For all non-PEGylated lipids, a quadratic fit was used. However, we used a linear fit for the PEGylated lipids, since the calibration samples including concentrations of 60, 100, and 140 µg/ml are in the linear calibration range. According to ICH Q2(R2) guideline, a linear fit should

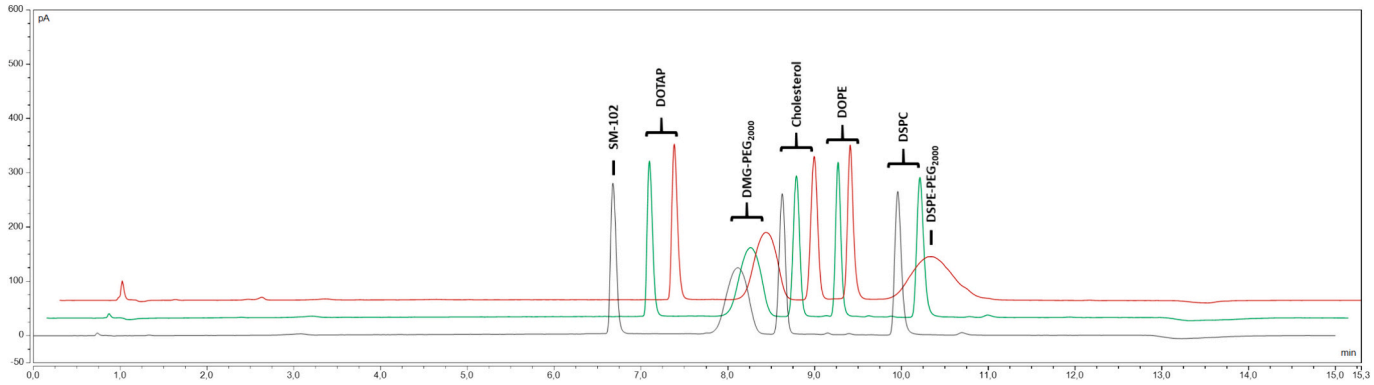

Fig. 1. Overlaid chromatogram of exemplary lipid compositions with the same lipid content. Red (DOTAP/DMG-PEG$_{2000}$/Chol/DOPE/DSPE-PEG$_{2000}$), Green (DOTAP/DMG-PEG$_{2000}$/Chol/DOPE/DSPC), Grey (SM-102/DMG-PEG$_{2000}$/Chol/DOPE/DSPE-PEG$_{2000}$).

be preferred if not specified. (European Medicines Agency ©, 2021)

$$Recovery\ [\%] = \frac{measured\ concentraion\ \left[\frac{\mu g}{ml}\right]}{expected\ concentration\ \left[\frac{\mu g}{ml}\right]} * 100$$

2.3.3. Working range - limit of detection and quantification

The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were quantified based on the signal-to-noise ratio. A signal-to-noise ratio of 3:1 is considered acceptable for the detection limit, and a signal-to-noise ratio of 10:1 for the quantification limit. (European Medicines Agency ©, 2021) The Chromeleon™ software was used to calculate the signal-to-noise ratio based on the signal of each lipid at a concentration of 9.375 µg/ml and related to an appropriate baseline range from 14:00–15:00 min. LOD and LOQ were calculated 3 times for each lipid.

2.3.4. Specificity

Forced degradation was performed for specificity investigations. (European Medicines Agency ©, 2021) Lipids were treated with 0.1 M NaOH for 2 h. The resulting degradation products were observed with regard to interferences related to retention times with other lipids.

2.4. Lipid nanoparticle (LNP) formulation

2.4.1. RNA loaded LNP formulation by dual centrifugation

LNPs were prepared by dual centrifugation (DC), which was first described by Massing et al. (Koehler et al., 2023a; Massing et al., 2017; Massing et al., 2008) To prepare a dry lipid film, each lipid was dissolved in EtOH and combined in a 2 ml polystyrene screwcap vial (Sarstedt AG & Co. KG Nümbrecht, Germany) at the desired molar ratio. We used three different formulations with different molar ratios, as listed in Table 4. Preliminary experiments showed an improvement in encapsulation and transfection efficiency by adding 100 µl 0.1 M HCl to the organic solvent in formulation 3. The organic solvent was removed using a Concentrator plus (Eppendorf AG, Hamburg, Germany) to obtain a dry lipid film. For the homogenization procedure during DC, 600 mg of SiLibeads type ZY-P Pharma (Sigmund Lindner GmbH, Warmensteinach, Germany) were added to each vial. The weight of the lipid film was adjusted to 10 mg and an additional 40 µl of 1 mg/ml micro-RNA-100-5p dissolved in water was added. With a lipid content of 20%, small unilamellar liposomes can be achieved. (Koehler et al., 2023b) DC was carried out with a ZentriMix 380 R (Andreas Hettich GmbH & Co. KG). The first centrifugation step was performed under DC conditions of 2350 rpm, 4 °C and 5 min. Redispersion of the resulting vesicular phospholipid gel (VPG) took place after adding 460 µl of SH-buffer (270 mM sucrose, 30 mM HEPES, pH 7.6) and a second DC step under conditions of 1500 rpm, 4 °C and 2 min. As a result, the final lipid concentration in the DC-vial was 20 mg/ml. All LNPs formulations were freshly prepared before analytical investigations.

2.4.2. Size measurements

Size and size distribution were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS Malvern Analytics (Kassel, Germany). Freshly sterile-filtered SH-buffer was used as the dispersion solvent, and 10 µl of each sample was diluted in it. Average size was determined by hydrodynamic diameter and the size distribution by polydispersity index (PDI). Formulation 1 showed a size of 190 nm and a PDI of 0.19, formulation 2 a size of 147 nm and PDI of 0.23 and formulation 3 a size of 159 nm and PDI of 0.26.

3. Results and discussion

3.1. HPLC-Method

3.1.1. Development

A challenge for the simultaneous separation of LNP formulations containing partially charged lipids is the fact that these polar head groups interact poorly with the reversed-phase C-18 column. (Zhong et al., 2010) For this reason, using a different column or adjusting the mobile phase may produce better results. Zhong et al. firstly published a suitable method for the detection and separation of DOTAP using a C18-column by adding TFA to the mobile phase. TFA can cover the positively charged headgroups of cationic and ionizable lipids, thereby prolonging the interaction of these lipids with the stationary phase. (Zhong et al., 2010) In this study, 0.15% of TFA was used in Eluent A because lower TFA concentrations showed incomplete separation of cationic and ionizable lipids in the form of two separate peaks with high lipid concentrations, which could indicate insufficient coverage of the polar headgroups. The flow rate was set quite high with 2 ml/min, according to the resulting pump pressure (up to 230 bar), but lower flow rates resulted in tailing of some of the lipid peaks, as well as overlaying lipid peaks.

The HPLC-Method shows clear peaks and a baseline separation of all lipids. Fig. 1 presents exemplary overlaid histograms of the lipid compositions which can be used. The PEGylated lipids show broader peaks, which can be explained by the molecular weight distribution of these lipids, thereby presenting a challenge for HPLC analytics, in contrast to the clear sharp peaks of all other lipids. (Shibata et al., 2013) Only DSPC and DSPE-PEG$_{2000}$ have similar retention times and cannot be detected at the same time. Mousli et al. published a method with an appropriate setting for this purpose. (Mousli et al., 2022) We aware, that other methods are described for detection and quantification of some of the lipids used in this paper and also for LNP formulations. However, the method presented here is validated for lipids that are used as excipients in recently approved formulations. Combined this method with the novel approach of LNP formulations by DC is therefore a truly

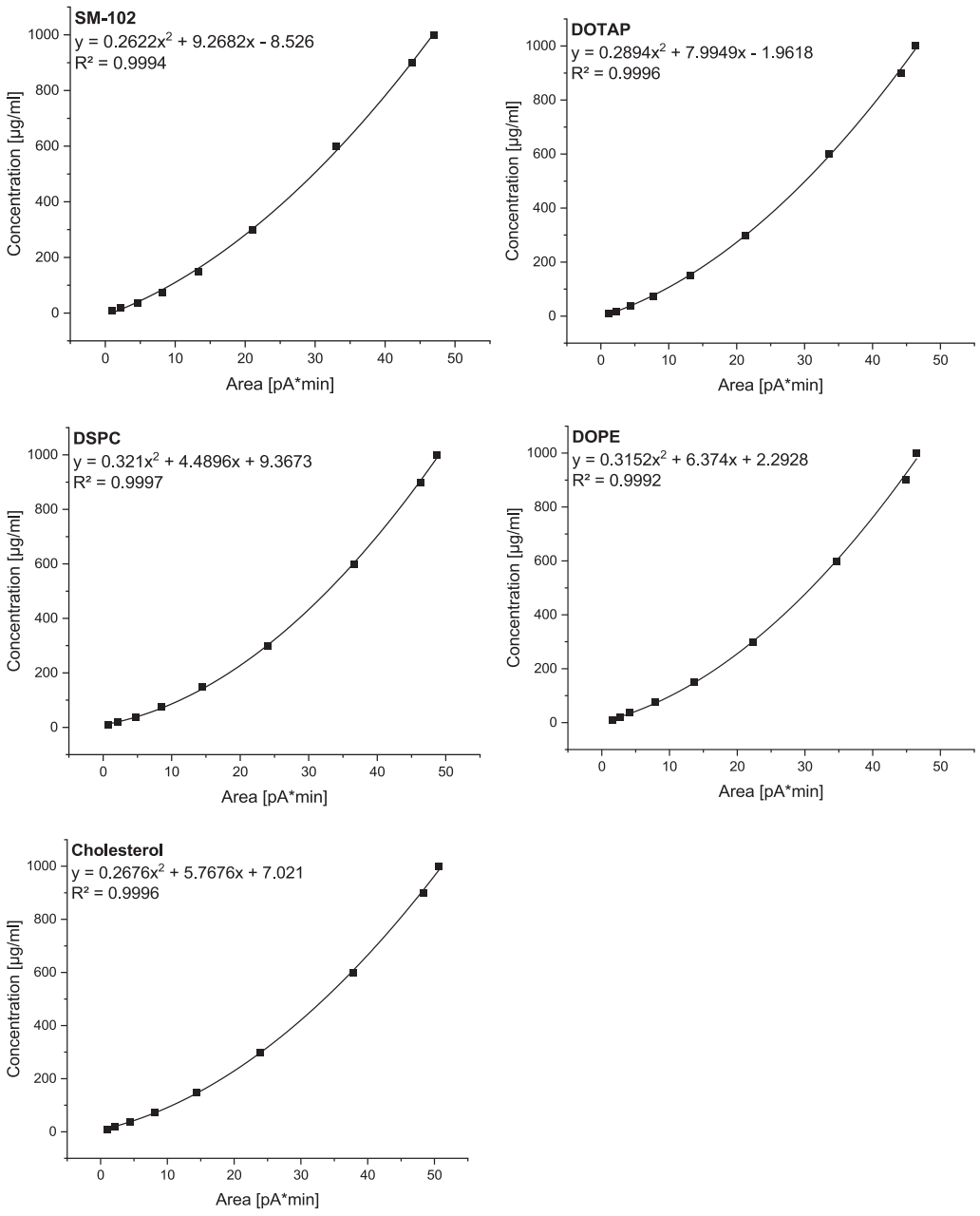

Fig. 2. Calibration curves for all non-PEGylated lipids (DOTAP/SM-102/DOPE/DSPC/Cholesterol). Calibration curves are shown as quadratic fits with 9 point calibrations (9.375–1000 µg/ml) and an overall correlation factor of $R^2 \geq 0.9992$.

breakthrough for fast LNP formulation and analysis.

3.1.2. Calibration curves

Due to the fact that the CAD exhibits a non-linear response at higher concentrations, a quadratic fit was created for calibration curves. (Nair and Werling, 2009; Liu et al., 2008) For all lipids, a high correlation coefficient $R^2 \geq 0.9992$ was achieved throughout the entire calibration range (Fig. 2 and Fig. 3). The PEGylated lipids also show high linearity even in the lower concentration range (9.375–150 µg/ml) with $R^2 \geq 0.9995$ (Fig. 3). All other lipids showed a linear correlation in the calibration range from 9.375 µg/ml to 75 µg/ml with a correlation factor of $R^2 \geq 0.99$ (data not shown). Unless otherwise stated, a linear fit should be applied to the calibration curves according to ICH Q2(R2) guideline. (European Medicines Agency ©, 2021) The wide calibration range allows quantification of lipid compositions for lipid or liposomal formulations with smaller molar ratios, as well as major lipid components, without additional dilution steps.

3.2. HPLC-Method validation

3.2.1. Precision

The samples used for the precision and accuracy measurements were adjusted to three different concentrations to cover a wide range, because lipids can occur in a wide variety of compositions. For all non-PEGylated lipids 300, 500 and 900 µg/ml were chosen (Table 1). Since PEGylated lipids are typically used in a low molar ratio range, we chose three concentrations at the lower range of 60, 100 and 140 µg/ml (Table 2) for precision and accuracy measurements. (Hald Albertsen et al., 2022) Measurements of intra-laboratory repeatability (ILR) as well as inter-day precision (IP) were carried out to analyse the precision of this analytical method. The ILR showed excellent results with a relative standard deviation (RSD) < 2% for retention time [min], as well as for the peak area

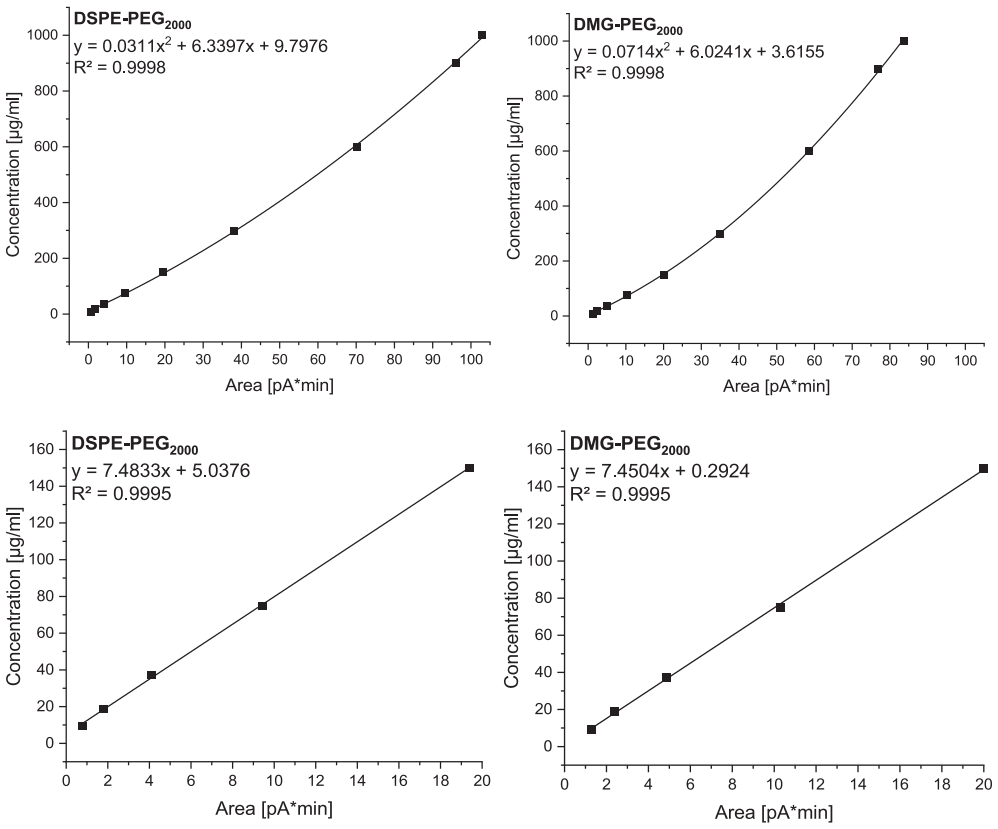

Fig. 3. Calibration curves for the PEGylated lipids show great correlation factors $R^2 \geq 0.9998$ for the whole calibration range (9.375–1000 µg/ml) with a quadratic fit, and the linear fit for the lower concentration range (9.375–150 µg/ml) with $R^2 \geq 0.9995$ indicates great linearity.

[pA*min] for all lipids. For inter-day reproducibility, the method also showed remarkable values, with RSD < 2.5% again for both retention time [min] and peak area [pA*min].

3.2.2. *Accuracy*

To validate the accuracy of the analytical method, we also tested recovery with the same concentrations as listed above. We were able to demonstrate that the recovery for all lipids was within the range of 100 ± 10%, as shown in Fig. 4. These results confirm that the HPLC-Method is suitable.

3.2.3. *LOD and LOQ*

To determine the limit of detection and quantification, a concentration at the lower limit of the calibration curve was measured three times. Since there is a linear dependence in the lower range, the concentrations determined were related to the signal-to-noise ratio and then extrapolated. A signal-to-noise ratio of 3:1 was used for LOD and 10:1 for LOQ. The obtained values for both LOD and LOQ show satisfactory results for the sensitivity of this analytical method. The slightly higher values for the PEGylated lipids can be explained by the width of the peaks, since in this case the noise is more pronounced compared to the sharper peaks (Table 3).

3.2.4. *Specificity*

All lipid components were analysed according their degradation products. Forced degradation was carried out under alkaline conditions using 0.1 M NaOH for 2 h to promote hydrolysis. Fig. 5 shows an overlaid chromatogram of all lipids and their degradation products. DOTAP and DOPE degradation products appeared in additional oleic acid peaks. DOPE was completely degraded, while DOTAP still showed an undegraded peak. The degradation products of DSPC and DSPE-PEG_{2000} appeared in additional stearic acid peaks. Both lipids were completely degraded. SM-102 was also not completely degraded and appeared in two additional peaks, representing degradation products. DMG-PEG_{2000} was also completely degraded and did not appear in additional peaks. No additional degradation products occurred for cholesterol. Under acidic conditions the appearance of lyso lipids is also described. We therefore tested reference substances of Lyso PC 18:1, Lyso PC 18:0 and Lyso PC 14:0 which occurred in retention times of 2.0 to 4.5 min and are comparable to prior literature. (Zhong et al., 2010; Mousli et al., 2022)

All occurring degradation products showed no interference with the non-degraded lipids regarding their retention time. The method is therefore suitable in terms of specificity.

3.3. *HPLC-Method for LNP formulations*

The combination of DC and our RP-HPLC-CAD method shows the potential for an enormously fast, small batch sized, sterile and resource-saving realization for pharmaceutical LNP development based on its net time of 22 min (DC preparation process of 7 min and sample separation in HPLC of 15 min). To show that the presence of RNA does not affect lipid analysis, LNPs were prepared with miRNA-100-5p as described above. The resulting miRNA-LNPs formulations were dissolved directly in EtOH after the second DC centrifugation step. The duration of the analytical method can thus be further shortened. The selected lipid compositions have already shown good transfection results in previous cell experiments (data not shown). The first two formulations contain the cationic lipid DOTAP. Cholesterol and DOPE were used as helper lipids. The differences in the two formulations lies in the use of DSPE-PEG_{2000} in formulation 1 and DMG-PEG_{2000} in formulation 2, as well as in the molar ratios of DOTAP and DOPE. Formulation 3 reflects the composition of Spikevax® from Moderna and contains the ionizable lipid SM-102.

Table 1
Table shows all non-PEGylated lipids and their retention times as well as their peak areas. The data shows the lipids at three different concentrations for intra-laboratory repeatability (ILR) as well as inter-day precision (IP). To verify the suitability of the precision of the method for lipid analysis the relative standard deviation was calculated and shown with an overall RSD < 2.5 promising results. Data shown as mean ($n = 3$).

Lipid	Concentration [µg/ml]		Retention time [min]	RSD [%]	Peak Area [pA*min]	RSD [%]
DOTAP	900	IP	6.92	0.94	44.88	1.54
		ILR	6.94	0.11	45.60	0.01
	500	IP	6.93	1.12	30.85	1.43
		ILR	7.00	0.07	31.38	0.22
	300	IP	6.91	1.13	22.02	2.33
		ILR	6.98	0.09	22.69	0.48
SM-102	900	IP	6.55	0.49	41.82	0.84
		ILR	6.57	0.12	41.91	0.82
	500	IP	6.53	0.44	29.35	1.25
		ILR	6.55	0.13	29.36	1.49
	300	IP	6.51	0.38	21.44	1.31
		ILR	6.52	0.15	21.43	1.85
Cholesterol	900	IP	8.70	0.08	48.36	1.45
		ILR	8.69	0.01	49.26	0.08
	500	IP	8.70	0.10	34.60	1.42
		ILR	8.69	0.02	35.21	0.19
	300	IP	8.69	0.11	24.71	2.37
		ILR	8.69	0.03	25.45	0.36
DOPE	900	IP	9.17	0.31	43.75	1.29
		ILR	9.16	0.02	44.36	0.17
	500	IP	9.16	0.39	30.82	1.17
		ILR	9.13	0.07	31.25	0.09
	300	IP	9.17	0.37	22.48	1.97
		ILR	9.14	0.00	23.05	0.27
DSPC	900	IP	10.03	0.25	46.26	0.67
		ILR	10.00	0.04	46.11	0.33
	500	IP	10.04	0.26	33.41	0.58
		ILR	10.02	0.02	33.18	0.35
	300	IP	10.06	0.28	24.26	0.83
		ILR	10.03	0.09	24.11	0.51

Table 2
PEGylated lipids with three concentrations in a lower range were used to calculate the intra-laboratory repeatability (ILR) as well as inter-day precision (IP). With a RSD < 2% satisfactory results can be shown. Data shown as mean (n = 3).

Lipid	Concentration [µg/ml]		Retention time [min]	RSD [%]	Peak Area [pA*min]	RSD [%]
DMG-PEG_{2000}	140	IP	8.17	0.35	18.56	1.74
		ILR	8.13	0.10	18.55	1.32
	100	IP	8.17	0.30	13.57	1.49
		ILR	8.14	0.04	13.63	1.26
	60	IP	8.17	0.28	8.17	1.46
		ILR	8.14	0.04	8.20	0.70
DSPE-PEG_{2000}	140	IP	9.98	0.40	18.26	0.85
		ILR	9.95	0.02	18.34	0.40
	100	IP	10.01	0.32	12.88	1.49
		ILR	9.99	0.15	13.04	0.97
	60	IP	10.04	0.31	7.28	1.11
		ILR	10.02	0.15	7.33	1.48

Formulations 1 and 2 showed satisfactory results in terms of lipid recovery. For both formulations, the recovery was in the range of 100 ± 10%. Formulation 3 showed a loss of cholesterol and SM-102, which could be explained by the fact that a slight residue was still visible on the inside of the DC vials after the second centrifugation step. Lipid loss can affect the morphology of LNPs and therefore needs to be taken into account. One reason for the losses could be the oily consistence of SM-102, which is not available as a powder like the other lipids. Our results clearly indicate that the formulation needs to be further optimized

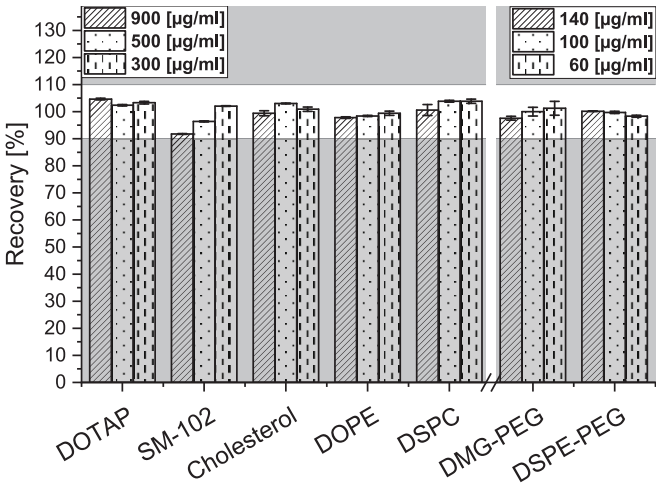

Fig. 4. Recovery of all lipids calculated by the measured concentration and related to the expected concentration. For all non-PEGylated lipids three concentrations (900, 500 and 300 µg/ml) were used. For the two PEGylated lipids three concentration ranges at a lower concentration range were used (140, 100 and 60 µg/ml). Data shown as mean ± SD ($n = 3$).

Table 3
Verifying the working range at the lower limit of detection (LOD) and limit of quantification (LOQ) for every lipid. Data shown as mean ± SD (n = 3).

Lipid	LOD [µg/ml]	LOQ [µg/ml]
DOTAP	0.21 ± 0.06	0.69 ± 0.20
SM-102	0.39 ± 0.14	1.30 ± 0.46
Cholesterol	0.24 ± 0.10	0.79 ± 0.34
DOPE	0.13 ± 0.10	0.43 ± 0.35
DSPC	0.61 ± 0.23	2.02 ± 0.76
DMG-PEG_{2000}	1.85 ± 0.51	6.16 ± 1.68
DSPE-PEG_{2000}	1.14 ± 0.36	3.81 ± 1.20

Table 4
Recovery of all LNP formulations was calculated by the measured concentration and related to the expected concentration. Data shown as mean ± SD ($n = 3$).

	Lipid	Molar ratio [%]	Recovery [%]
Formulation 1	DOTAP	20	99.3 ± 0.5
	Cholesterol	40	101.6 ± 0.7
	DOPE	38	98.6 ± 1.4
	DMG-PEG_{2000}	2	92.4 ± 2.0
Formulation 2	DOTAP	30	101.1 ± 3.2
	Cholesterol	40	103.8 ± 3.8
	DOPE	28	96.5 ± 3.6
	DSPE-PEG_{2000}	2	95.3 ± 1.5
Formulation 3	SM-102	50	89.4 ± 4.4
	Cholesterol	38.5	72.1 ± 5.3
	DSPC	10	102.8 ± 6.8
	DMG-PEG_{2000}	1.5	92.8 ± 3.9

and is under current improvement investigations. A possible approach would be to obtain the ionizable lipids, which are mostly found as oily components, as their chloride salts. Alternatively, HCl could be used for film formation, as shown in our example, to further optimize this process. The addition of HCl also brings up the opportunity of not working in acidic conditions (pH ~ 4) as the ionizable lipid is already protonated in the dry lipid film. The electrostatic interactions can take place without the addition of an additional acidic component. Nevertheless, the factors that affect RNA LNP formulation development by DC do not challenge the HPLC method as itself.

In addition to lipid quantification, it is also necessary to establish a

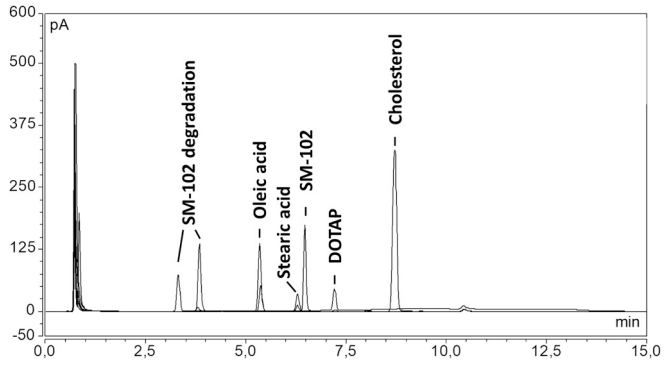

Fig. 5. Overlaid chromatogram shows forced degradation of all lipid components using 0.1 M NaOH over 2 h. Lipid degradation products showing no interference with other lipid retention times.

suitable method for RNA quantification. Hirsch et al. were able to show that siRNA remains intact in formulations produced with dual asymmetric centrifugation (DAC), but this would need to be tested under our new conditions. (Hirsch et al., 2009) It has also been shown that mRNA in a hybrid biopolymer LNP complex can be processed by DAC. (Siewert et al., 2020) It was possible to detect signals at a wavelength of 260 nm using a UV-DAD detector, but these signals came at the same time as the injection peak and therefore were overlaid with the peaks from HEPES and sucrose, which were present in the buffer. A clear quantification was not possible to date. For this purpose, a further optimization of this method could be done or, alternatively, already established methods for the quantification of RNA using other methods and columns could be considered, such as anion-exchange (AEX-HPLC) or reversed-phase ion pair (RP-IP-HPLC) HPLC methods. (Seiffert et al., 2011; Patel et al., 2023; Packer et al., 2021)

4. Conclusion

We present a RP-HPLC-CAD method for the simultaneous quantification of both charged and uncharged lipids. We tested lipids that are contained in approved formulations and therefore have high current relevance. The approach showed excellent results in terms of accuracy, precision, specificity and working range (LOD, LOQ and calibration range) in agreement with the requirements of the ICH Q2(R2) guidelines. With a wide calibration range, a large number of possible LNP formulation compositions can be tested (supplementary data showing a potential lipid screening). Combined with the first described preparation of small batches of LNPs using DC, these combined methods open up the potential for a tremendous fast approach for LNP screenings. Due the simplicity of both methods, patient individual bedside gene therapy is a step closer.

CRediT authorship contribution statement

Valentin Bender: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Leon Fuchs:** Writing – review & editing, Software, Methodology, Investigation. **Regine Süss:** Writing – review & editing, Supervision, Project administration, Investigation.

Declaration of competing interest

Valentin Bender, Leon Fuchs and Prof. Dr. Regine Süss report no conflict of interest.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpx.2024.100255.

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