Formulation of peptide-based cancer vaccines

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CHAPTER 5

Quantification of lipid and peptide content in antigenic peptide-loaded liposome formulations by reversed-phase UPLC using UV absorbance and evaporative light scattering detection

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Adapted from J Pharm Sci. 2022 Apr;111(4):1040-1049

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ABSTRACT

Antigenic peptide-loaded cationic liposomes have shown promise as cancer vaccines. Quantification of both peptides and lipids is critical for quality control of such vaccines for clinical translation. In this work we describe a reversed phase ultra-performance liquid chromatography (RP-UPLC) method that separates lipids (DOTAP, DOPC and their degradation products) and two physicochemically different peptides within 12 minutes. Samples were prepared by dilution in a 1:1 (v/v) mixture of methanol and water. Peptide quantification was done via UV detection and lipids were quantified by an evaporative light scattering detector (ELSD), both coupled to the RP-UPLC system, with high precision (RSD < 3.5%). We showed that the presence of lipids and peptides did not mutually influence their quantification. Limit of detection (LOD) and limit of quantification (LOQ), as determined in the ICH guidelines, were 6 and 20 ng for DOTAP, 12 ng and 40 ng for DOPC, 3.0 ng and 8.0 ng for peptide A and 2.4 ng and 7.2 ng for the more hydrophobic peptide B. Finally, lipid degradation of DOTAP and DOPC was monitored in peptide loaded DOTAP:DOPC liposomes upon storage at 4 °C and 40 °C.

INTRODUCTION

Cationic liposomes have shown to be an efficient delivery vehicle for peptide-based cancer vaccines (1-7). Moreover, we have shown that positively charged liposomes, composed of 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), can accommodate a wide range of physicochemically diverse antigenic peptides (1). This enables the use of these liposomes in personalized cancer vaccines, where each patient will receive a personalized vaccine containing a unique set of tumor antigens (1, 8-10). Chemical quantification of both lipids and peptides in such personalized liposomal cancer vaccines is essential for further formulation development, mechanistic immunological studies and clinical application (11, 12). The FDA identified multiple critical quality attributes for liposomal drug products, including the identity and quantity of lipid species and active pharmaceutical ingredient(s) (11, 13, 14).

Earlier studies have made use of a lipid extraction prior to peptide quantification (3, 4, 15). However, extraction of different peptides requires different extraction media, depending on the peptide characteristics, and extraction efficiency can vary strongly (4, 15, 16). Ideally, a quantification method for liposomal peptide-based vaccines should not require extraction and should be suitable for any kind of peptide, whereas at the same time also enables the quantification of the lipids. This implies that the presence of lipids and peptides should not mutually influence each other's quantification. Reversed phase ultra-performance liquid chromatography (RP-UPLC) methods have been extensively used to separate peptides and lipids based on their hydrophobicity (1, 5, 11, 12, 16). Upon separation, peptides are detectable by ultraviolet (UV) detection of the peptide bond at a wavelength of 214 nm, where the $\pi \rightarrow \pi^*$ transition leads to light absorption (17, 18). Detection of lipids can be done by an evaporative light scattering detector (ELSD) in which the non-volatile lipids are nebulized when the volatile mobile phase (containing the lipids) is heated. The mobile phase is removed and a mist of lipid particles are passed through an optical cell in which the lipids scatter the light. The scattered light can then be used to quantify the lipid masses in the analyte based on appropriate calibration curves (12, 19). In general ELSD responses are nonlinear due to a correlation in-between solute concentration and aerosol particle size distribution upon nebulization and evaporation. The ELSD responses are therefore fitted by non-linear regression (20-22).

In this study we describe the development of a rapid and simple RP-UPLC method that does not require an extraction in sample preparation and is able to quantify both peptides and lipids in antigenic peptide-loaded cationic liposome formulations. Peptides were quantified by UV detection and an ELSD was used to quantify the two lipids, DOTAP and DOPC. For both lipids and two physiochemically different peptides the accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) were determined based on the ICH guidelines (23, 24). No matrix effects were observed between lipids and peptides, also when mixed in different ratios. Finally, lipid degradation was studied

in an (accelerated) stability study in which peptide-loaded liposome samples were stored at 4 °C and 40 °C.

2. MATERIALS & METHODS

2.1 Materials

Chloroform ($CHCl_3$), methanol (MeOH) and acetonitrile (ACN) were obtained from Biosolve BV (Valkenswaard, the Netherlands). Ammonium hydroxide 25% (w/v) was purchased from Brocacef BV (Maarssen, the Netherlands) and trifluoracetic acid (TFA) from Sigma Aldrich (Zwijndrecht, the Netherlands). Two physicochemically different antigenic peptides, A and B, containing the ovalbumin derived SIINFEKL epitope were synthesized at the peptide facility at the Department of Immunology at the Leiden University Medical Center (LUMC) (1). Both DOTAP and DOPC were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Vivaspin 2 centrifuge membrane concentrators were bought from Sartorius Stedim Biotech GmbH *(*Göttingen, Germany*)*. Phosphate buffers were composed of 7.7 mM $Na_2HPO_4 \cdot 2\ H_2O$ and 2.3 mM $NaH_2PO_4 \cdot 2\ H_2O$, pH 7.4 (10 mM PB, pH 7.4) in deionized water with a resistivity of 18 MΩ•cm, produced by a Millipore water purification system (MQ water).

Table 1. Physicochemical characteristics of the two antigenic peptides used in this study. Hydropathicity (GRAVY) index and theoretical isoelectric point (pI) of the peptides were calculated by making use of the sequence analysis tools from bioinformatics.org (25).

Peptide ID	Amino acid sequence	Theoretical pI	Hydropathicity
Peptide A	DAKHDHLLHAASIINFEKLAAAK	7.02	-0.104
Peptide B	GSAAESASGSASIINFEKLAAAK	6.14	0.126

2.2 Peptide quantification by RP-UPLC-UV

The recovery of the peptide in the liposomal formulations was determined by RP-UPLC-UV analysis (Waters Acquity UPLC^{å} combined with an Acquity UV detector and a Waters BEH C18 – 1.7 mm (2.1 ´ 50 mm) column). An ACN/MQ with 0.1% TFA gradient with a flow rate of 0.5 ml/min was used. The run was initiated with 95% solvent A (MQ water with 0.1% TFA) and 5% solvent B (ACN with 0.1% TFA) followed by a linear gradient to 100% solvent B in 7 minutes staying at 100% B until 9 minutes and back to the initial 5% solvent A at 9.1 minutes. Peptides were detected by measuring the UV absorbance at λ = 214 nm. Peptide containing liposomal samples were diluted 20-fold in 1:1 (v/v) MeOH:MQ water prior to injection on the UPLC system. Calibration curves for both peptides were prepared by automated injections of increasing volumes (5-50 μl) from a 50 μg/ml peptide solution in 1:1 (v/v) MeOH:MQ water. Quantification was done by integration of the peptide peaks to obtain the area under the curve (AUC) of all calibration samples, resulting in linear calibration curves. The peptide content in the samples was determined by interpolation of the AUC values of the peptide on the calibration curve. Peak integration was done by

using MassLynx 4.2 software (Waters.) and linear regression followed by interpolation by using Graphpad Prism 8 (Graphpad Software).

2.3 Lipid quantification by RP-UPLC-ELSD

Lipid recovery was determined by using the same RP-UPLC-UV system in-line (tandem) coupled to a Waters ACQUITY UPLC® ELS Detector. The same gradient method was used as described under 2.2. Peptide containing liposomal samples were injected in the same sequence as for the peptide quantification, but needed a 100-fold dilution in 1:1 (v/v) MeOH:MQ water prior to injection of 10 μl to prevent overloading of the ELSD signal. Calibration curves for DOTAP and DOPC were prepared by automated injections of increasing volumes of 5-50 μl from a 50 μg/ml DOTAP/DOPC solution in 1:1 (v/v) MeOH:MQ water solution. Lipid calibration curves were prepared by second order polynomial regression of the AUCs of lipid calibration samples. The concentration of DOTAP and DOPC in the lipid samples were determined by interpolation of the AUC values on the calibration curves. Peak integration was done by using MassLynx software (Waters, software 4.2.) and interpolation by using Graphpad Prism 8 (Graphpad Software).

2.4 Automated and manual calibration curve preparation

Increasing volumes (1, 5, 10, 20, 30, 40 and 50 μl) of DOTAP, DOPC and peptide stock solutions were injected by the autosampler of the UPLC system. Both DOTAP and DOPC stock solutions were 50 μg/ml in 1:1 MeOH:MQ (v/v) and the peptides (table 1, section 2.9) were dissolved in 0.04% (w/v) NH_4OH or in $CHCl_3$:MeOH:MQ water (60:36:4, v/v). The correlation between lipid mass and the ELSD response was determined by second order polynomial regression (Eq. 1). The correlation between peptide mass and UV response was linear and determined by linear regression (Eq. 2). In both equations χ depicts analyte mass (ng) and γ the integrated peak area (arbitrary units). Manual calibration curves were prepared by a serial dilution of lipid and peptide stock solutions to obtain a range of 250 – 10 μg/ml, and 10 μl of each dilution was injected on the UPLC system.

Equation 1. $\gamma = a\chi^2 + b\chi + c$

Equation 2. $\gamma = a\chi + b$

2.5 Storage of lipid calibration curve standards

Stock solutions of DOTAP and DOPC, both 50 μg/ml, were prepared in $CHCl_3$ and subsequently aliquoted in 1 ml portions in UPLC vials. Chloroform was evaporated under a stream of N_2 and the dry calibration samples, containing 50 μg DOTAP and 50 μg DOPC per vial, were stored at -80 °C.

2.6 Liposome formulation

Antigenic peptide-loaded and empty liposomes were prepared as described previously (1, 4). In brief, lipids (DOTAP:DOPC) were dissolved in $CHCl_3$ and mixed in a 1:1 molar ratio in a round bottomed flask followed by rotary evaporation to obtain a dry lipid film. The peptide was added to the dry lipid film as a 1 mg/ml solution in 0.04% (w/v) ammonium hydroxide or MQ water was added when no peptide was encapsulated. The resulting dispersion was snap frozen in liquid nitrogen and followed by overnight freeze-drying in a Christ alpha 1–2 freeze-dryer (Osterode, Germany). The lipid-peptide cake was rehydrated in three consecutive steps with 10 mM phosphate buffer to reach the initial volume. Next, the liposomes were down-sized by extrusion through polycarbonate filters (Nucleopore Milipore, Kent, UK) of 400 and 200 nm (four cycles through each filter). In order to remove free peptide from the antigenic peptide-loaded liposomes, the formulations were purified by centrifugation (931 G) in Vivaspin 2 centrifugation concentrators (molecular weight cut-off: 300 kDa). Liposomal dispersion were concentrated five-fold by centrifugation and re-diluted with PB to its initial volume after which purification was repeated.

2.7 Analytical method validation

2.7.1 Limits of detection and quantification

For both peptides the slope method, as described in the ICH guideline *Validation of analytical procedures: Text and Methodology*, was used to determine the LOD (Eq. 3) and LOQ (Eq. 4) (23, 24). In equation 3 and 4, σ represents the standard deviation of the slope and *s* is the slope of the calibration line.

Equation 3. $LOD = \frac{3.3\ \sigma}{s}$

Equation 4. $LOQ = \frac{10\ \sigma}{s}$

Since the calibration curves for both DOTAP and DOPC are not linear, the slope method cannot be used. The LOD and LOQ were determined based on the signal-to-noise (S/N) ratio, as defined in the ICH guideline (23). In brief, the S/N ratio was calculated by dividing peak height by the height between upper and lower limits of the noise signal. The LOD is defined as a S/N of 3 and for the LOQ a S/N of 10 (23). Calculations were performed by making use of equations 5 and 6.

Equation 5. $LOD = mass\ injected * \frac{3}{\left(\frac{S}{N}\right)}$

Equation 6. $LOQ = mass\ injected * \frac{10}{\left(\frac{S}{N}\right)}$

2.7.2. Accuracy and precision

The influence of the peptides on DOTAP and DOPC responses were determined by the spiking of lipid calibration standards, containing both lipids, with a hydrophilic or

a hydrophobic peptide (peptide A and B, table 1). A total of four different mass ratios of lipid : peptide and peptide : lipid were prepared to cover a wide range of potential mass ratios of peptide-loaded liposomes (table 2) (1, 4, 5). Similar experiments were performed by spiking both peptides with empty liposomes (table 2). All samples were measured in triplicate to determine intraday variability. Interday variability was determined by measuring the same sample on 5 different days in triplicate.

Table 2. Mass ratios to determine a potential effect of peptides on the DOTAP and DOPC response (left) and the ratios used to determine a potential effect of both lipids on the peptide response (right). The DOTAP:DOPC ratio was 1:1 (molar) in all experiments.

Lipids spiked with peptide Lipid:peptide ratio (*w/w*)	**Peptide spiked with empty liposomes Peptide:lipid ratio (*w/w*)**
20 : 0	1.0 : 0
20 : 0.5	1.0 : 10
20 : 1.0	1.0 : 20
20 : 1.5	1.0 : 30

2.8 Identification lipid degradation products

Lipid analysis was performed using a LC-MS/MS based lipid profiling method using a Shimadzu Nexera X2 system consisting of LC-30 pumps, a SIL30AC autosampler and a CTO-20AC column oven kept at 50 °C (Shimadzu, 's Hertogenbosch, The Netherlands). A gradient consisting of solvent A (water/acetonitrile 80:20 v/v) and solvent B (water/acetonitrile/2-propanol 1:9:90 v/v), with a flow of 300 µl/min, was: 0 min 40% solvent B, 10 min 100% solvent B, 12 min 100% solvent B. Both eluents contained 5 mM ammonium formate and 0.05% formic acid. A Phenomenex Kinetex C18, 2.7 µm particles, 50 × 2.1 mm (Phenomenex, Utrecht, The Netherlands) was used as column. The MS was a Sciex TripleTOF 6600 (AB Sciex Netherlands B.V., Nieuwerkerk aan den Ijssel, The Netherlands) operated in positive (ESI+) and negative (ESI–) ESI mode, with the following conditions: Ion Source Gas 1 45 psi, Ion Source gas 2 50 psi and Curtain gas 30 psi, temperature 350 °C, acquisition range *m/z* 100–1200, IonSpray Voltage 5500 V (ESI+) and –4500 V (ESI-), declustering potential 80 V (ESI+) and –80 V (ESI–). An information dependent acquisition (IDA) method was used to identify lipids, with the following conditions for MS analysis: collision energy ±10 eV, acquisition time 250 ms and for MS/MS analysis: collision energy ±45 eV, collision energy spread 25 eV, ion release delay 30 ms, ion release width 14 ms, acquisition time 40 ms. The IDA switching criteria were set as: for ions greater than *m/z* 300, which exceed 200 cps, exclude former target for 2 s, exclude isotopes within 1.5 Da, max. candidate ions 20. Before data analysis, raw MS data files were converted with the Reifycs Abf Converter (v4.0.0) to the Abf file format. MS-DIAL (v4.18), with the FiehnO (VS66) database, was used to align the data and identify the different lipids [10.1038/nmeth.3393]. TAP lipids were manually added to the database with a home developed R (v3.6.3) script.

3. RESULTS

3.1 Baseline separation of lipids and peptides by RP-UPLC

Both lipids, DOTAP and DOPC, were separated by the RP-UPLC method, and eluted at 8 minutes and 8.25 minutes, respectively. Peptide A and B eluted in-between 2 and 3 minutes (Fig. 1).

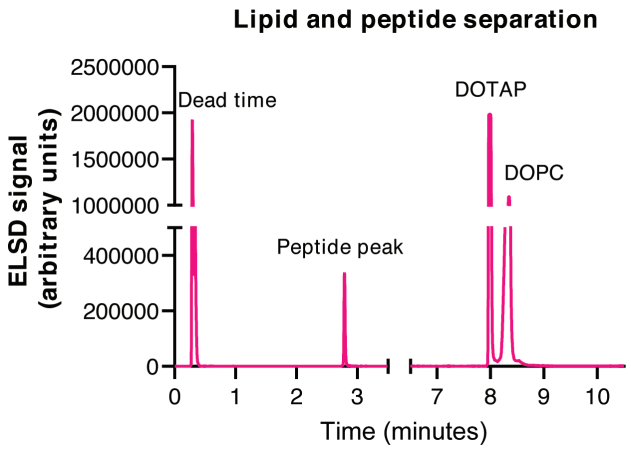

Figure 1. Representative ELSD chromatogram of the separation of the peptide, DOTAP and DOPC by the RP-UPLC method in a peptide-standard mixed with empty liposomes, diluted 20 times in 1:1 (v/v) meOH:MQ water. The DOTAP ELSD signal is saturated in this dilution.

3.2 Calibration curves

The lipid calibration curves for both DOTAP and DOPC were prepared by automated injections of increasing volumes from the respective lipid stock solution, or by manual dilution followed by injection of a constant volume. Since the lipid calibration curves of both DOTAP and DOPC did not have a linear response, curves were fitted by a second order polynomial regression. Calibration curves prepared by manual dilution, which had constant injection volumes, were comparable to calibration curves prepared from one stock by automated injection of varying volumes (Fig. 2, table 3). In this study all calibrations curves of both peptides and lipids were therefore prepared by automated injection, which circumvents manual preparation of dilution series.

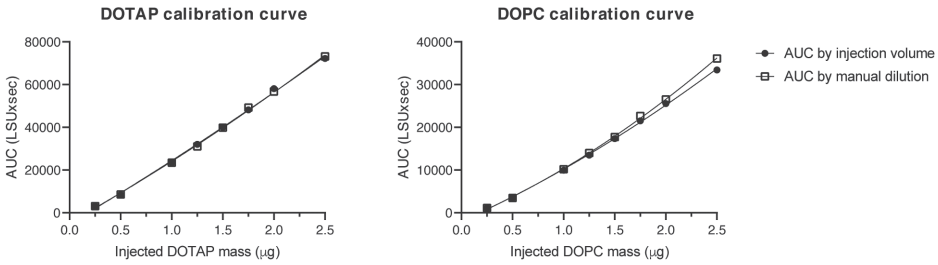

Figure 2. Calibration curves of DOTAP (left) and DOPC (right) prepared by automated injections of different volumes of one stock solution (black circles) or by manual dilutions of the stock solution followed by injections of a constant volume (open squares). Data was fitted by second order polynomial regression.

Table 3. Correlation coefficient of DOTAP and DOPC calibration curves prepared by manual and automated dilution. Data were fitted by making use of second order polynomial regression (Eq. 1).

Calibration curve mode	DOTAP	DOPC
Manual dilution series, constant injection volume	$R^2 = 0.9988$ $\gamma = 1818\chi^2 + 26804\chi - 4578$	$R^2 = 0.9995$ $\gamma = 2058\chi^2 + 10067\chi - 1816$
One stock solution, varying injection volume	$R^2 = 0.9983$ $\gamma = 1191\chi^2 + 28376\chi - 5175$	$R^2 = 0.9993$ $\gamma = 1387\chi^2 + 10823\chi - 2003$

Detection of both peptide A and B was performed by UV detection (λ = 214 nm) and corresponding calibration curves were linear (Fig. 3).

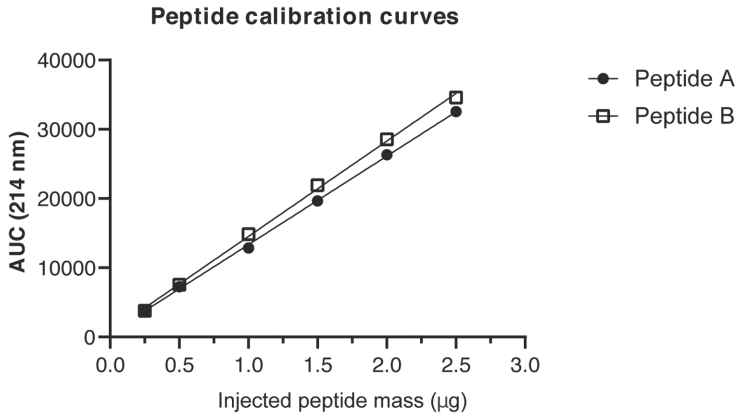

Figure 3. Calibration curves of peptide A (black circles) and peptide B (black squares) prepared by automated injection. Data was fitted by linear regression.

3.3 Precision of lipid and peptide quantification

The precision of RP-UPLC-ELSD based quantification of DOTAP and DOPC was determined by performing six repeated injections of three different lipid quantities (0.5, 1.30 and 2.00 μg), within the range of the calibration curves (Fig. 4). The relative standard deviation (RSD) of integrated AUC for both lipids was dependent on the injected mass and was 1.30 – 3.38 % for DOTAP and 0.97 – 2.33% for DOPC (table 4). The precision of the RP-UPLC-UV quantification for peptide A and B was determined similarly by the injection of three different peptide quantities (0.5, 1.30 and 2.50 μg) and the RSD for the AUC of all samples was smaller than 1% (figure 4, table 4). For the more hydrophilic peptide A the average RSD was 0.41% and for the more hydrophobic peptide B the average RSD 0.22% (table 5).

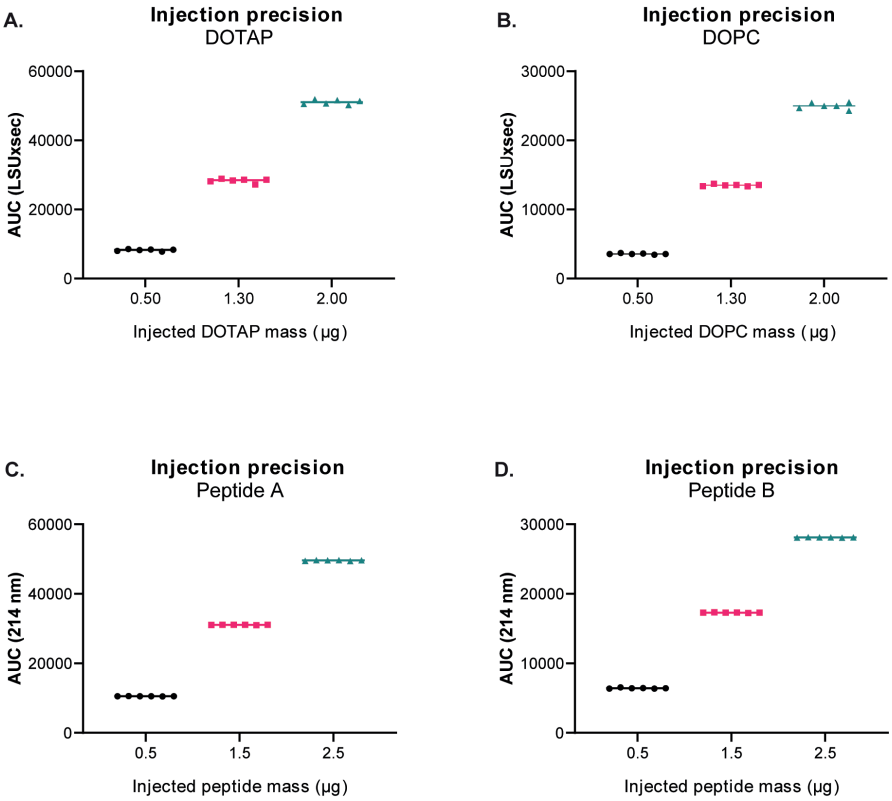

Figure 4. Precision of (A) DOTAP and (B) DOPC (right) quantification and (C) quantification of peptide A and (D) peptide B. Three different quantities of each lipid or peptide were injected 6 times on the same day. The average response and spread in detector signal was calculated (table 4).

Table 4. Precision ELS and UV detectors.

Injected mass (μg)	RSD (%)	
	DOTAP	**DOPC**
0.50	3.38	2.33
1.30	2.06	0.97
2.00	1.30	1.85
	Peptide A	**Peptide B**
0.50	0.97	0.35
1.50	0.16	0.11
2.50	0.08	0.20

Next, the intraday variability (variance within the same day) and interday variability (variance in-between multiple days) were determined for the calculated lipid and peptide content in reference samples. Reference samples for both lipids and peptides, covering the range of the calibration curves, were prepared and the analyte content was determined in triplicate on five different days. The RSD of the intraday variability was below 2.2% for both lipids and the interday variability had a RSD < 4% (table 5). For both peptide A and B the inter- and intraday variability of peptide quantification via RP-UPLC-UV were determined according to the same procedure. The interday variability based on the calculated masses was lower than 3%, the intraday variability was lower than 1% (table 5).

Table 5. Precision of lipid and peptide quantification for DOTAP and DOPC by RP-UPLC-ELSD and for peptide A and B by RP-UPLC-UV. In total, 5 experiments in which all masses were injected in triplicate. RSD of the intraday variability based on triplicate injections within the same day, RSD of interday variability determined on triplicate injections in-between 5 different days (n=15).

Injected mass (μg)	RSD Intraday variability (%)	RSD Interday variability (%)
	DOTAP	
0.4	1.28	3.37
1.0	0.76	1.80
1.6	0.75	2.04
	DOPC	
0.4	2.24	3.94
1.0	1.53	2.50
1.6	1.41	1.96
	Peptide A	
0.75	0.36	3.1
2.25	0.46	3.0
	Peptide B	
0.75	0.95	1.05
2.25	0.20	3.37

3.4 Sensitivity of the RP-UPLC-UV & RP-UPLC-ELSD methods

Both the LOD & LOQ were determined with single calibration samples containing only DOTAP or DOPC using the ELS detector and the same was done for the peptides A and B by using the UV detector (table 6). The LOQ for both lipids were 6- to 12-fold lower than the lowest mass injected for the calibration curve (250 ng). For both peptide A and B the LOQ was 31- to 35-fold lower than the lowest mass injected for the calibration curve (250 ng).

Table 6. LOD and LOQ values for both the lipids and peptides. For both DOTAP and DOPC the average with standard deviation is displayed n=3) and for both peptides average with standard deviation (n=3).

Compound	LOD (ng)	LOQ (ng)
DOTAP	6.2 ± 0.7	20.5 ± 2.2
DOPC	12.1 ± 1.3	40.3 ± 4.3
Peptide A	2.6 ± 0.03	7.99 ± 0.09
Peptide B	2.4 ± 0.06	7.15 ± 0.18

3.5 Accuracy of lipid and peptide quantification

The accuracy displays the difference between the measured value and the expected value. On five different days the lipid and peptide content of reference samples, covering the range of the calibration curve, was determined. Based on the calibration curves and reference samples, the average measured values for both lipids were found to be within the 95% - 105% range of the theoretical mass with a RSD lower than 4% (table 7). For both peptide A and peptide B the average quantified masses were found to be within the 95% - 105% range of the theoretical mass with a RSD lower than 3.5% (table 7).

Table 7. Accuracy of lipid quantification for DOTAP and DOPC. In total, 5 experiments in which all masses were injected in triplicate. Average recovery has been calculated based on all injections (n=15).

Theoretical mass (µg)	Average quantified mass ± SD (µg)	Average accuracy (%)
	DOTAP	
0.4	0.39 ± 0.01	97.2 ± 4.0
1.0	1.01 ± 0.02	100.6 ± 2.5
1.6	1.67 ± 0.03	104.5 ± 2.0
	DOPC	
0.4	0.40 ± 0.02	96.4 ± 3.4
1.0	1.01 ± 0.03	101.1 ± 1.8
1.6	1.67 ± 0.03	104.3 ± 2.0
	Peptide A	
0.75	0.74 ± 0.02	98.0 ± 2.9
2.25	2.19 ± 0.06	97.1 ± 2.8
	Peptide B	
0.75	0.74 ± 0.01	98.4 ± 1.4
2.25	2.21 ± 0.07	98.2 ± 3.1

3.6 Influence of peptide and lipid matrices on method performance

3.6.1. Lipid standards spiked with peptide

The influence of peptide on the quantification of DOTAP and DOPC was determined by the spiking of the lipid standards with increasing amounts of peptide A or peptide B dissolved in $CHCl_3$:MeOH:MQ water (60:36:4, v/v). This solvent was used because dissolution of peptides in 0.04% NH_4OH, previously used as peptide solvent, was found to induce DOTAP degradation (data not shown) (1). Four different lipid : peptide weight ratios were chosen based on average lipid and peptide recoveries in our earlier studies with peptide-loaded cationic liposomes (1, 2, 4, 5). The peptides did not have a detectable influence on lipid quantification at all tested ratios and time points (0, 3 and 6 hours) post sample preparation (Fig 5.).

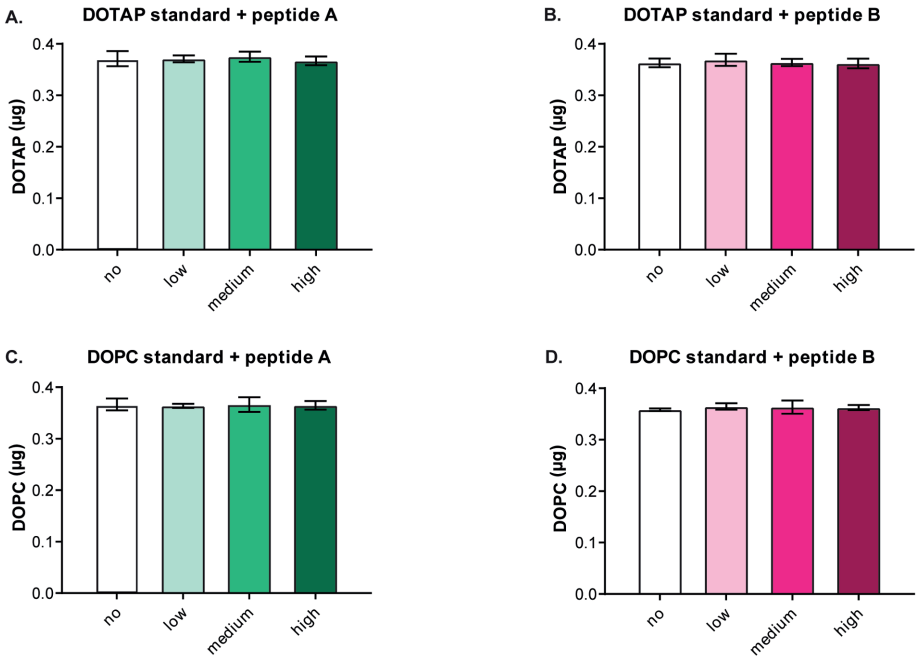

Figure 5. Spiking of DOTAP (A & B) and DOPC (C & D) standards with increasing amounts of peptide A (A & C) or peptide B (B & C). A total of four different weight-based lipid : peptide ratios were used: no (only lipid), low (20 : 0.5), medium (20 : 1.0) and high (20:1.5) in which total lipid concentration was 40 μg/ml and 20 μl per sample was injected. Of each sample 20 μl was injected at 0, 3 and 6 hours post sample preparation. Data shown as mean ± SD of two independent experiments (n=6).

3.6.2. Empty liposomes spiked with peptide

Next, empty liposomes were spiked with increasing amounts of peptide A or peptide B. Again no differences in DOTAP and DOPC quantification were observed for all tested

lipid : peptide ratios (Fig. 4). Sample measurements were performed at 0, 3 and 6 hours post sample preparation, however, no noticeable differences between the time points were observed with respect to the detected amount of DOTAP or DOPC (Fig 6.).

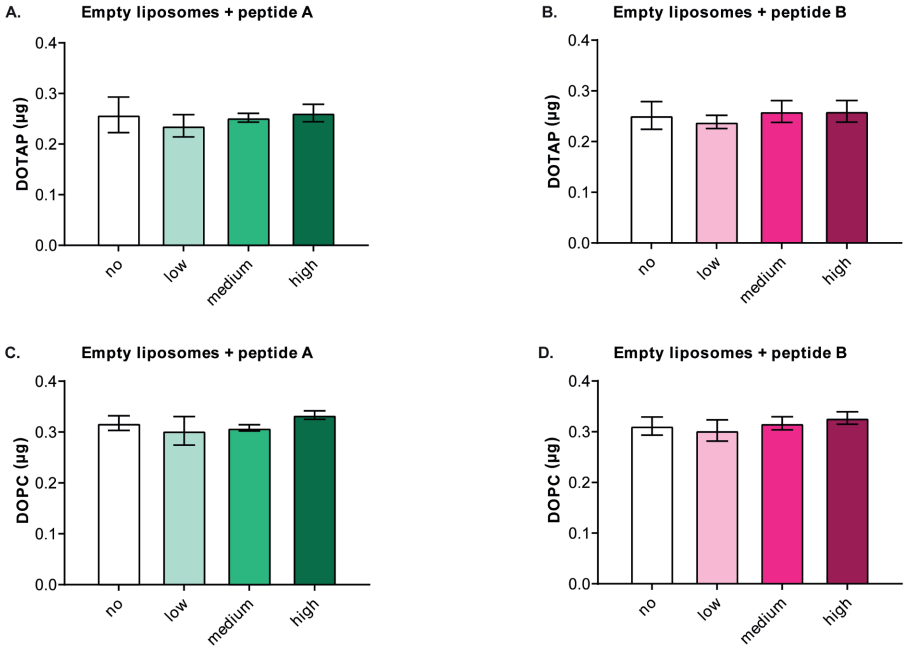

Figure 6. Empty liposomes spiked with peptide A (A & C) or peptide B (B & D). Prior to the measurement, the liposome peptide mixture was diluted in MeOH:MQ (1:1, v/v) and DOTAP (A & B) and DOPC (C & D) were quantified in these mixtures to determine a potential matrix effect. A total of four different weight based liposomes : peptide ratios were used: no (only liposomes), low (20 : 0.5), medium (20 : 1.0) and high (20:1.5) in which total lipid concentration was 65 *µ*g/ml and 10 *µ*l per sample was injected. All ratios were measured at 0, 3 and 6 hours post sample preparation Data shown as mean ± SD of three independent experiments (n=9).

3.6.3. Peptide spiked with empty liposomes

The influence of empty liposomes on peptide quantification was determined by spiking of peptide stock solutions with empty liposomes in various peptide : lipid ratios. No differences were detectable in the quantification of peptide A and peptide B when mixed with empty liposomes in all ratios and at all time points (Fig. 7). Peptides were dissolved in 0.04% NH_4OH prior to dilution, since only the peptide was quantified in these experiments and did not show any degradation within the studied conditions.

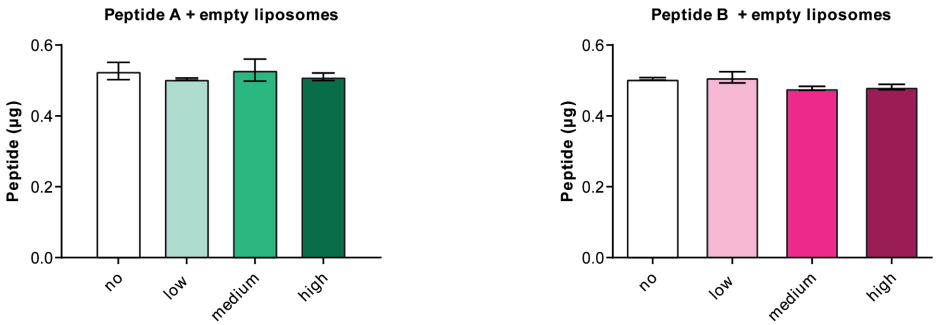

Figure 7. Peptide A (left) and B (right) spiked with empty liposomes. A total of four different weight based peptide : liposomes ratios were used: no (only peptides), low (1 : 10), medium (1 : 20) and high (1 : 30). Prior to the measurement, the liposome peptide mixture was diluted 100 times in 1:1 (v/v) MeOH:MQ water and peptide sample concentration was 50 μg/m and 10 μl per sample was injected. Peptides were quantified in these mixtures to determine a potential matrix effect of the empty liposomes on peptide quantification. Data shown as mean ± SD (n=9).

3.7. Stability of DOTAP and DOPC upon storage

The RP-UPLC-ELS detection was used to study DOTAP and DOPC stability during prolonged storage of peptide loaded liposomes at 4 °C and 40 °C. The storage of liposomes during 28 days at 4 °C did not alter the DOTAP content, however, the DOPC content decreased 10%. The degradation rate could be fitted with first-order degradation kinetics ($R^2 = 0.46$), however, correlation was not strong due to limited degradation (Fig. 8)**.** Storage of the liposomes at 40 °C resulted in a 20% decrease of both the DOTAP and the DOPC content in empty liposomes and degradation followed first-order degradation kinetics ($R^2 = 0.96$ for DOTAP and $R^2 = 0.82$ for DOPC) (Fig. 8). As a reference dried lipid standards were reconstituted in 1:1 (v/v) MeOH:MQ water prior to the measurement.

The RP-UPLC gradient was able to separate degradation products from the DOTAP (elution at 8 minutes) and DOPC (elution at 8.25 minutes) peaks. Upon storage at 40 °C a clear increase of degradation product peaks 6.0 and 7.25 minutes were observed in the ELSD chromatograms (Fig. 8). LC-MS analysis of empty liposomes showed that these peaks contained degradation products. The peak at 6.0 minutes contained the lyso-form of DOTAP and the peak at 7.25 lysoPC. In the peptide loaded liposomes used in these experiments no peptide degradation was observed during storage for 28 days at 4 °C and 40 °C (data not shown).

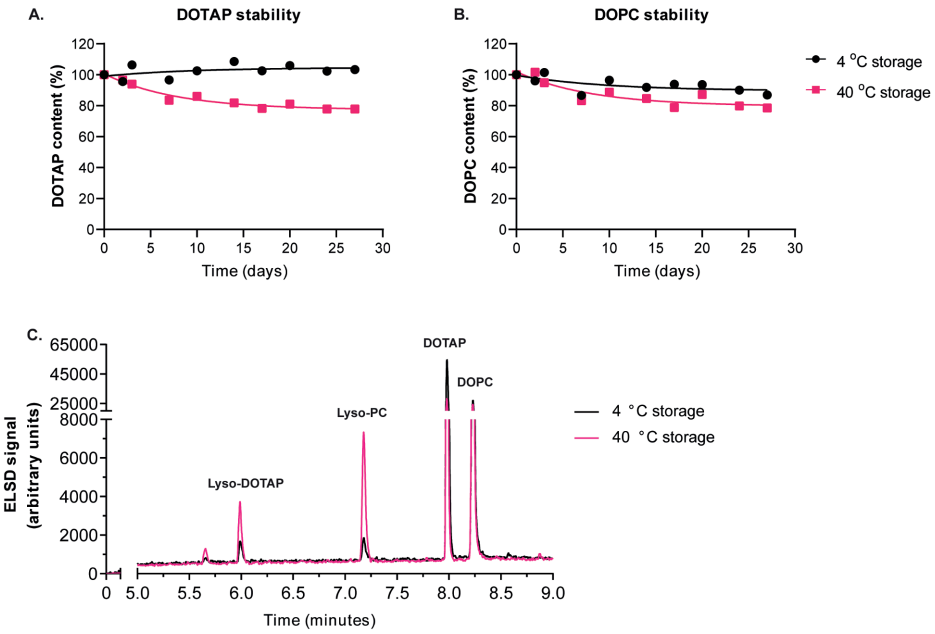

Figure 8. Lipid stability during liposome storage. Peptide-loaded liposomes were stored at 4 °C (black) and 40 °C (pink). Throughout storage both DOTAP (A) and DOPC (B) were quantified (top). For both DOTAP (A) and DOPC (B) data are fitted with first order degradation kinetics as indicated by the trendlines. For both lipids the degradation products could be separated by the RP-UPLC method and were detected by ELSD (bottom). For both DOTAP and DOPC data are fitted with first order degradation kinetics as indicated by trendlines.

4. DISCUSSION

The described RP-UPLC separation method followed by UV detection for peptide detection, and ELS detection for lipid quantification allowed rapid and accurate determination of both peptide and lipid content in antigenic peptide-loaded DOTAP:DOPC liposomes. In order to facilitate cGMP production of liposomal peptide based cancer vaccines, a validated lipid and peptide quantification method is a prerequisite. In this work the method was evaluated according to the ICH guideline *Validation of analytical procedures: Text and Methodology* for two physicochemical different peptides in combination with DOTAP and DOPC. The evaluated lipids and peptides did not mutually effect each other's detection and subsequent quantification. The resolution between both peptides and DOTAP and DOPC was high and the RP-UPLC separation method can be used for different peptides and lipids. Peptide response in UV detection is much higher in comparison to lipid quantification by the ELS detection, therefore peptides were quantified by UV detection in this work. The lipids DOTAP and DOPC do not have UV absorption. Therefore, a second in-line detector (ELSD) is necessary to measure and quantify the lipids by particle formation after evaporation of the mobile phase. The lipid particle size distribution after evaporation of the mobile phase is affected by lipid

concentration. Additionally, it has been reported that solvent composition can influence the ELSD response (22, 26, 27). Small differences in mobile phase composition during gradient elution therefore contribute to a slightly lower precision of lipid quantification by ELSD compared to peptide quantification by UV.

Several reports describe chromatography mediated lipid separation followed by ELSD to quantify lipid content in liposomes. Methods for UPLC measurements of multiple lipids have been reported before and are now combined with the method described here. This indicates the feasibility of RP-UPLC-ELSD methods during product characterization (11, 12, 16). However, these reports described the analysis of empty liposomes. In the current work a chromatograpic method was developed to separate both lipids and peptide in cationic liposomes during one gradient run. Since peptides have different physicochemical properties than lipids, different detectors needed to be coupled to the UPLC system. By diluting the peptide-containing liposomes no lipid extraction was necessary anymore, enabling direct injection of this solution into the UPLC system to quantify the peptide and lipid content. This is especially important for quality control of personalized cancer vaccines, since every patient will have a different set of physichochemically different peptides, for which conventional liquid-liquid extractions can be problematic.

During this study two different peptide solvents were used for sample preprocessing: 0.04% NH_4OH and $CHCl_3$:MeOH:MQ (60:36:4, w/w). For the preparation of fresh peptide calibration samples, 0.04% NH_4OH is a versatile solvent that is able to dissolve a wide range of physicochemically different peptides (1, 28). However, during lipid quantification of the peptide-loaded liposomes upon storage, the presence of 0.04% NH_4OH resulted in DOTAP degradation even though the NH_4OH containing samples were diluted in MeOH : MQ during sample preparation. The strong base, NH_4OH, most likely mediates hydrolysis of DOTAP resulting in free fatty acids and the lyso-form of DOTAP. However, more in depth degradation studies including LC-MS analysis are required to further identify degradation pathways. In the stability studies both peptides A and B were dissolved in $CHCl_3$:MeOH:MQ (60:36:4, w/w) to prevent DOTAP degradation by 0.04% NH_4OH, the peptide solvent, during liposomes preparation. Further sample preparation for the RP-UPLC-ESLD based lipid quantification was performed in MeOH : MQ according to the described method.

Degradation was observed during storage for DOTAP as well as DOPC. The degradation peaks were detectable by the ELSD and the lyso-forms of both lipids were identified by LC-MS. Since both DOTAP and DOPC contain ester bonds in-between the oleic acids and the polar headgroups both lipids are vulnerable for hydrolysis, especially when they are stored in an aqueous buffer. Several other studies reported the lyso-forms of DOTAP and DOPC and resulting free fatty acids in forced degradation experiments (16, 29). In our previous study the physicochemical properties of peptide loaded DOTAP:DOPC liposomes were studied up to 8 weeks of storage at 4 °C. The Z-average, polydispersity

and zeta-potential did not notably change, however, the presence of lyso-forms could result in a destabilization of the lipid bilayer which could increase leakage of hydrophilic peptide (1). More detailed analysis of the degradation peaks by mass spectroscopy can provide more insight into the mechanism of degradation and implications for further formulation development.

5. CONCLUSION

In this work we describe an extraction free RP-UPLC-UV-ELSD method for the quantification of both peptide and lipids in DOTAP:DOPC liposomes loaded with peptide. The accuracy and precision of the method were determined and no matrix effects were observed between the lipids and two different peptides. Two lipid degradation products were separated and identified from the DOTAP and DOPC peaks and offer the possibility to monitor lipid degradation during storage of liposomal formulations. The described quantification method allows reliable quantification of both lipids and peptide in cationic liposomal cancer vaccine.

Acknowledgements
We would like to thank Martin Giera and Rico Derks from the Centre for Proteomics and Metabolomics (CPM) in the LUMC for the LC-MS experiments in which the lipid degradation products were identified. We would like to thank Rob Valentijn from the department of Clinical Pharmacy and Toxicology from the LUMC for the conceptual input regarding GMP production of liposomes.

Funding
JH was funded by the Molecule to Patient program of the Translational Drug Discovery and Development (TDDD) profiling area of University Leiden, The Netherlands. KvdM is the recipient of a H2020-MSCA-Intra European Fellowship-2018 (Grant Number 832455-Need2immune).

Conflict of interest
The authors declare to have no conflict of interest.

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