QTRAP® LC/MS/MS Workflow for Simultaneous Characterization and Quantitation of PEGylated Liposomal Drug Formulation

Characterization of precursor lipids and its PEGylated derivatives allows the determination of liposome compositions using QTRAP® LC/MS/MS System

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ABSTRACT

Liposomes are used to encapsulate pharmacological active compounds for drug delivery. In order to improve the circulation time of liposomes in the blood stream, lipids can be conjugated with low molecular weight PEG. It is important to accurately determine a liposomes molecular composition, because it may be composed of different lipids to modulate its properties. Although amount and ratio of the starting materials for the synthesis of liposomes are known, the lipid composition of the product may not reflect input quantities. We describe the qualitative and quantitative analysis of liposomes composed of PEG-2000-conjugated and unconjugated phospholipids by LC-MS/MS analysis using a QTRAP® 5500, and the data analysis using PeakView® and MultiQuant™ software. The method allows the determination of liposome compositions.

INTRODUCTION

Liposomes are used to encapsulate pharmacological active compounds for drug delivery systems. In order to improve the circulation time of liposomes in the blood stream, lipids in the liposomes can be conjugated with low molecular weight poly-ethylenglycol (PEG). The physicochemical and biological properties may depend on a number of parameters, including acyl chain length of the lipids, the lipid class, and size and branching of the attached PEG molecules or the stoichiometric ratios of the different lipids. Because liposomes may be composed of a variety of different lipids to modulate their properties, it is important to accurately determine their molecular composition.

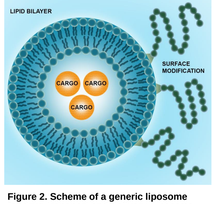

Figure 2. Scheme of a generic liposome for drug transport and delivery. Liposome properties are determined by lipid composition and surface modifications e.g. PEGylation (or attached targeting peptides).

Liposomes are usually produced by a biofilm process, in which a defined amount of various lipids are mixed in an organic solvent. The solvent is then removed *in vacuo* using a rotary evaporator. As a result a lipid film is formed on the glass walls of the vessel. Liposomes are then created by adding an aqueous buffer followed by sonification or other shearing procedures to generate a more or less homogenous liposome population.
Although amount and ratio of the staring materials is known, the lipid composition of the final liposomes may not necessarily reflect the input quantities. Thus, in order to confirm the composition of the liposomes, a method is required which can quantitate the various chemically diverse components of the liposomes.
Here we describe the qualitative and quantitative analysis of liposomes composed of PEG-2000-conjugated and unconjugated phospholipids by LC-MS/MS analysis using linear ion trap 5500, and the data analysis using the PeakView® Bio-Tool Kit and MultiQuant™ software. The method allows the exact determination of the liposome composition.

MATERIALS AND METHODS

Synthesis of Liposomes

Liposome were generated according to the method as described by Avanti Polar Lipids, Inc (see homepage [1]). Two liposome variants were produced. For the DOPC/DOPG liposomes the two educts were provided with an initial ratio of 4:1 (m/m). For the DSPE-PEG-OMe/DSPE-PEG-Mal/Cholesterol/DOPC liposomes the educts were provided with an initial ratio of 0.16:0.66:3:12 (m/m/m/m).

Sample preparation

Phospholipid standards were dissolved in chloroform:methanol (50:50 v/v) resulting in stock solutions with the following concentrations: DOPC 142 mg/ mL, DOPG 109 mg/ mL. PEGylated DSPE-lipid standards were dissolved in CH_2Cl_2:ACN (50:50 v/v) resulting in stock solutions with the following concentrations: DSPE-PEG2000-OMe 33.5 mg/ mL; DSPE-PEG2000-Mal 9.7 mg/ mL).
Liposome formulations DOPC/DOPG and DSPE-PEG2000-OMe/DSPE-PEG2000-Mal/DOPC were obtained as aqueous emulsion. Subsequent 1:100 dilution in pure MeOH yielded stock solutions for quantification.

Chromatography

LC System: Agilent 1200 SL series LC pump
Analytical Column: Waters Acquity BEH C8, 1.7 µm, 2.1 × 100 mm
Oven temperature: 60 ° C
Flow rate: 500 µl/min
Mobile Phase A: H_2O:MeOH, 80:20 v/v, 5 mM NH_4OAc
Mobile Phase B: MeOH:ACN:2-Propanol, 76:19:5 v/v/v, 5 mM NH_4OAc)
High pressure gradient elution was performed by a mixture of 5 mM NH_4OAc in water containing 20 % methanol (eluent A) and 5 mM NH_4OAc in methanol containing 12% acetonitrile and 1% isopropanol (eluent B). The following gradient was applied: 0 min – 25% A; 1 min - 25% A; 2 min – 10% A; 4.5 min – 2% A; 7 min – 2% A; 7.01 min – 25% A; 10 min – 25 % A .This chromatographic method was used for all experiments.

Mass spectrometry

Source Parameter for the QTRAP® 5500 System equipped with a TurboV® Source are given in **Table 1**. LC-MRM quantification of DOPC and DOPG was performed in negative ionization mode using a fatty acid fragment for DOPG (773.4->281.2) and the [M-OAc-Me]$^-$ fragment of DOPG. The respective paramteres are given in **Table 2**. For the quantitation of PEGylated lipids positive ESI was used **Table 3**. Quantitation was performed by preparation of a calibration curve using dilutions of standards

Table 1. Source parameter chosen for the quantification of selected lipids.

Source Parameter	DOPG/ DOPC	DSPE PEG-X
Ionization mode	ESI (-)	ESI (+)
CUR (psi)	35	35
IS (V)	-4500	2500
CAD	Medium	Medium
TEM (°C)	500	400
Gas 1 (psi)	60	70
Gas 2 (psi)	60	60

Table 2. Overview of the chosen MRM transitions for lipid quantification

Analyte	Q1/ Q3 m/z	Dell time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
DOPG	773.4/ 281.2	120	-115	-10	-52	-13
DOPC	844.5/ 770.5	120	-115	-10	-34	-27

Table 3. MRM transitions for the quantification of DSPE-PEG derivatives

Analyte	Q1/ Q3 m/z	Dell time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
DSPE PEG OMe	704.0/724.7	120	50	10	20	21
DSPE PEG Mal	760.1/793.8	120	50	10	22	20

Data processing

Data was acquired with Analyst® 1.6.2 software and was reviewed in PeakView® 2.1 software. Quantitation of measured analytes was performed with MultiQuant™ 3.0 software.

RESULTS

Quantification of DOPG/DOPC Lipids

A calibration curve of authentic standards (10–10,000 pg/mL) was created using MultiQuant™ 3.0 software (**Figure 2**). The detection limit (LOD) for all analytes was below the lowest measured concentration of 10 pg/ mL. Linearity was achieved from 10-10,000 pg/mL with regression coefficients of 0.9995 and 0.9998 for DOPG and DOPC, respectively.

Quantification of PEGylated DSPE Lipids

A calibration curve of authentic standards (10–50,000 pg/mL) was created using MultiQuant™ 3.0 software (**Figure 3**). The detection limit (LOD) for all analytes was below the lowest measured concentration of 10 pg/ mL. Linearity was achieved from 10-50,000 pg/mL with regression coefficients of 0.9996 and 0.9999 for DSPE-OMe and DSPE-Mal, respectively.

Characterization of Liposomes

The stock solution of dispersed liposomes was diluted stepwise to yield signal intensities below 1e6 cps. Peak areas and corresponding lipid concentrations were determined using MultiQuant™ 3.0 software. The liposome composition, that is to say the ratio of lipids used, was specified *via* comparison of the calculated concentrations. The data is summarized in **Table 4** and **5**

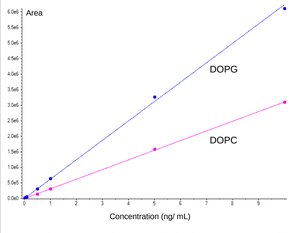

Figure 2. Lipid calibration curve (0.01 – 10 ng/ mL). Linear fitting. Weighting: 1/x. CV (3 injections, 1 ng/ mL):DOPC 1.7%; DOPG 0.6%.

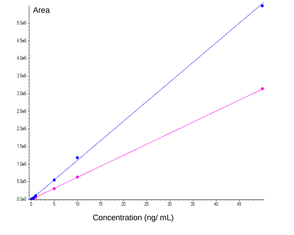

Figure 3. Lipid calibration curve (0.01 – 50 ng/ mL). Linear fitting. Weighting: 1/x. CV (3 injections, 1 ng/ mL):D.-OMe 0.7%; D.-Mal 2.3%.

Table 4. Quantification of DOPC/DOPG liposome components

Analyte	Dilution Factor	Calc. Conc. (µg/ mL)	Ratio
DOPG	5e7	1.04e4	1
DOPC	5e7	4.41e4	4.3

Table 5. Quantification of DSPE-PEG-Mal/OMe and DOPC liposome components

Analyte	Dilution Factor	Calc. Conc. (µg/ mL)	Ratio
DSPE-PEG OMe	1e5	1.140e2	9.6
DSPE-PEG Mal	1e5	1.187e1	1.0
DOPC	1e6	9.396e3	791

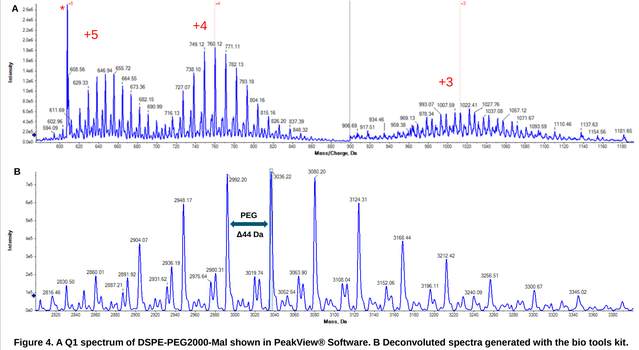

Figure 4. A Q1 spectrum of DSPE-PEG2000-Mal shown in PeakView® Software. B Deconvoluted spectra generated with the bio tools kit. Charge states are given in red scripture. The asterix indicates a major PEG insource fragment.

DISCUSSION

Definition of MRM transitions for unfunctionalized lipids is straightforward. However, it has to be taken in account that certain lipid classes are zwitterionic compounds and some of them are ubiquitous. Accordingly, to determine a sensitive transition it might be necessary to evaluate the lipid's fragmentation for both polarities to facilitate an effective ionization and to keep background or carryover problems at a minimum level.
The analysis of functionalized lipids is more challenging. Conjugation of lipids with PEG modulates the immune response against the liposomes. The resulting constructs have an increased molecular weight which entails absorption of multiple charges.
In addition, unlike peptides, which are explicitly defined biopolymers, PEGs are industrial polymers that suffer from a molecular weight distribution. The PEG chain length heterogeneity in conjunction with multiple charge states complicates method development, because, Q1 or EMS spectra of lipid-PEG-conjugates show a charge distribution superimposed by the polymers molecular weight distribution (see **Figure 4**). Further in depth analysis of the Q1/EMS PEG-lipid spectra reveals a distinct in-source fragmentation. This process is declustering potential-dependent, but the formation of the corresponding fragment ion m/z 607.6 cannot be fully suppressed. Nevertheless, please note that this process can be used to quantify high molecular weight PEG conjugates (e.g. X-PEG 40k).[2] For these compounds the signals are fully overlapped and the choice of a specific signal is impossible. An MRM transition based on an enhanced in-source fragment becomes the only viable option. For lower weight PEG-constructs (up to ~X-PEG 5k) the Q1/ EMS data complexity can be reduced using deconvolution software to factor out the multiple charge effect (see **Figure 4, B**).
To set up an MRM based quantification method for the chosen PEGylated lipids, the charge states were verified using the enhanced resolution QTRAP® scan, that allows for an precise (resolution 9200 for m/z 922) assessment of relevant isotopic distributions. Subsequently, we investigated the fragmentation behavior of the [M+4H]$^{+4}$ species.
As expected the MS/MS spectra show a strong signal at m/z 607.6, but also signals with higher m/z ratios than the actual parent molecule e.g. m/z 724.7 for DSPE-PEG-OMe. Setting specific collision energies both fragment classes are formed with similar efficiency. However, the higher mass fragments featured an unrivalled selectivity with extremely low noise levels (≤10 cps) facilitating an improved LOQ. This effect counteracted the sensitivity loss due the the extensive PEG signal spread and allowed an efficient quantification of PEGylated lipids.

CONCLUSION

- Liposomes are effective drug carriers. Their properties and organ targeting is modulated by the lipid composition and the surface modification.
- We demonstrated efficient workflows for the quantification of lipids and PEGylated derivatives in liposomes.
- Measurement of a non-PEGylated liposome sample yielded component ratios well within the range of expectations (**Table 4**), while analysis of PEGylated liposomes revealed significant differences between expected and measured component ratios (**Table 5**).
- Comparison with the used educt ratios will allow for an enhanced procedure for liposome synthesis.
- The intrinsic concentration differences in surface modified liposomes (c.p. DOPC 9 mg/ mL vs. DSPE PEG-Mal 1.3 µg/ mL) are challenging. Hence, it will be very interesting to transfer this method to the 6500 QTRAP® system featuring a detector with increased dynamic range (detector saturation at 1e8 cps).

REFERENCES

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2 Jiachang Gong, et al. Quantitative Analysis of Polyethylene Glycol (PEG) and PEGylated Proteins in Animal Tissues by LC-MS/MS Coupled with In-Source CID. *Anal. Chem.* June 25, 2014, dx.doi.org/10.1021/ac501507g

Conflict of interest: Pol Harvengt is an employee of the GSK group of companies. Sebastian Fabritz and Cyrus Papan are employees of SCIEX, manufacturer of mass spectrometers. **This work was supported by GlaxoSmithKline Biologicals SA.**