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Research paper

NIR transmission spectroscopy for rapid determination of lipid and lyoprotector content in liposomal vaccine adjuvant system CAF01

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ABSTRACT

It is of crucial importance to determine the concentration of the different components in the formulation accurately, during production. In this respect, near-infrared (NIR) spectroscopy represents an intriguing alternative that offers rapid, non-invasive and non-destructive sample analysis. This method, combined with multivariate data analysis was successfully applied to quantify the total concentration of lipids in the liposomal CAF01 adjuvant, composed of the cationic surfactant dimethyldioctadecylammonium bromide (DDA) and the immunomodulator α,α′-trehalose 6,6′-dibehenate (TDB). The near-infrared (NIR) detection method was compared to a validated high-performance liquid chromatography (HPLC) method and a differential scanning calorimetry (DSC) analysis, and a blinded study with three different sample concentrations was performed, showing that there was no significant difference in the accuracy of the three methods. However, the NIR and DSC methods were more precise than the HPLC method. Also, with the NIR method it was possible to differentiate between various concentrations of trehalose added as cryo-/lyoprotector. These studies therefore suggest that NIR can be used for real-time process control analysis in the production of CAF01 liposomes.

1. Introduction

CAF01 is a novel adjuvant system composed of the cationic surfactant dimethyldioctadecyl ammonium bromide (DDA) and the immunomodulating glycolipid α,α′-trehalose 6,6′-dibehenate (TDB). Upon dispersion in aqueous solution DDA self-assembles into liposome structures, which are cationic due to the quaternary ammonium head-group of DDA. Incorporation of TDB into the liposomes not only enhances and modulates the immune response towards co-injected antigens in a Th1 direction, but also stabilizes the liposomes. This stabilization is probably due to enhanced hydration of the liposome membrane and sterical hindrance, avoiding reduced charge repulsion and aggregation [1,2]. The addition of trehalose to the formulation has enabled freeze-drying of the formulation, which can be readily redispersed in water [3]. Thereby, one of the big hurdles in developing vaccines intended for the developing world can be overcome, making the formulations less dependent on functioning cold-chains for the distribution of the vaccines.

When bringing liposomes like CAF01 into clinic, it is important to address issues such as how to perform product quality control (QC) during the different stages of production. This consideration is important, especially in light of the recent process analytical technology (PAT) initiative and the guidance documents (Q8–Q10) from the International Conference on Harmonization (ICH) [4] which have been implemented by both European Medicines Agency (EMEA) [5] and the US Food and Drug Administration (FDA) [6]. These guidelines encourage pharmaceutical production sites to implement real-time quality control methods into the manufacturing process. Implementation of a PAT system roughly encompasses (1) identification of critical points in the manufacturing process through risk assessment, (2) identification of suitable analytical techniques and (3) subsequent installation of related sampling devices in the production (e.g. in the form of probes) designed to perform timely measurements of the bulk product and/or manufacturing environment. If unwanted changes are observed during production, measures can be taken, e.g. by adjusting process parameters, to ensure that end product quality is maintained.

Several methods for quantification of lipid content in liposomes have previously been reported. These include chemical techniques

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(e.g. the Bartlett assay [7]), high-performance liquid chromatography (HPLC) [8–10] and enzyme-based assays [11–13]. An alternative method is high sensitivity differential scanning calorimetry (hsDSC) usually used to investigate the nature of phase- and/or structural transitions in lipid membranes and proteins.

All of the above mentioned methods are generally used off-line (i.e. manual sampling with transport to remote laboratory) and, in addition, are resource-intensive with respect to sample preparation and analysis time. Furthermore, HPLC requires extraction of the lipid components, which must be validated as complete and non-selective. Due to the apolar nature of the lipids, organic solvents are needed to ensure proper elution, thus requiring specialized equipment and localization. Furthermore, as lipids lack light absorbing moieties, light detectors like UV and fluorescence detectors are usually not efficient for detecting liposomal components. Hence, other spectroscopic methods like evaporating light scattering detector (ELSD) or mass spectroscopy (MS) could be implemented.

The enzyme-based assays are commercially available kits, which only require a spectrophotometer. The main drawback is that they can only detect cholesterol and cholesterol esters, choline-containing phospholipids or non-esterified fatty acids [13]. This excludes the quantification of a long range of lipids including DDA and TDB. DSC enables accurate determination of the concentration-dependent change in enthalpy (ΔH) of the liposomes when going from a lower temperature gel-phase to a higher temperature fluid-phase. The applicability of this method, however, is highly dependent on the composition of the liposomes, since a well-defined gel–liquid phase transition is a prerequisite. Furthermore, DSC is time consuming due to the low scanning speed. Finally, all the above mentioned methods are destructive and thus not suitable for real-time analysis of CAF01 liposomes and trehalose.

Near-infrared (NIR) spectroscopy presents an intriguing alternative, requiring no sample preparation while offering rapid (seconds rather than minutes), non-invasive and non-destructive sample analysis. Furthermore, NIR spectroscopy allows for measurements directly through transparent sample containers, e.g. glass and plastic. This enables monitoring of large numbers of samples in the production line, without affecting the throughput of the production. For these reasons, the technique has gained wide acceptance within the pharmaceutical industry, either as part of off-line quality control or as a PAT tool [14]. NIR spectroscopy measures overtones and combinations of fundamental vibrations from the mid-IR region: –OH, –NH, –SH and –CH. Anharmonicity of the atomic vibrations gives rise to lower absorption (compared to mid-IR) and, as a result, the bands related to particular functional groups of the analyte in question are often broad and overlapping [15]. Hence, data analysis of more than a single wavelength is often needed to adequately describe the physicochemical features of the sample found in the spectra. For this purpose, the multivariate modeling method principal component analysis (PCA) may be used [16]. PCA reduces the number of original (and highly co-linear) variables into so-called principal components (PCs), best describing the systematic variance found in the spectra. These new variables (i.e. the PCs) contain physicochemical information on the sample. PCA therefore facilitates interpretation of NIR spectroscopy data, while modified versions of the algorithm, e.g. partial least squares (PLS) regression, are often used for building quantitative calibration models [17].

NIR spectroscopy has been used extensively for quantification of fatty acids and proteins in the food industry (e.g. [18–20]). Few efforts, however, have been done to thoroughly scrutinize the use of NIR transmission spectroscopy and multivariate data analysis for detection of lipids in liposomes. The work presented in this paper is therefore a feasibility study, in which the objective was to evaluate the applicability of NIR transmission spectroscopy for the quantification of CAF01 liposome content in liquid formulations. The developed model will be compared to results obtained by established HPLC and DSC methods and the results from the three methods will be evaluated using HPLC as the reference method. Furthermore, the possibility to qualitatively determine trehalose content in liposome formulations by NIR transmission spectroscopy is investigated. It is expected that implementation of NIR methods will provide faster – yet reliable – analyses, compared to existing HPLC and DSC methods. Hopefully, this should also help set the scene for future use of in-line NIR spectroscopy methods within the field of liposomology.

2. Materials and methods

2.1. Materials

Dimethyldioctadecylammonium (DDA) bromide and α,α′-trehalose 6,6′-dibehenate (TDB) were obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the compounds was >99% by HPLC. Methanol (extra pure) and chloroform (extra pure) were purchased from Merck (Darmstadt, Germany). Tris base (99%) was obtained from Sigma–Aldrich (St. Louis, MO). Trehalose dihydrate (≥99%) was obtained from Sigma–Aldrich (Brøndby, Denmark). Purified water of Milli-Q quality was used to prepare all buffers.

2.2. Liposome preparation

The adjuvant was prepared by the thin film method as described previously [1]. For the quantitative analysis, weighed amounts of DDA and TDB were dissolved in chloroform/methanol (9:1 v/v), and the organic solvent was removed using a gentle stream of N_2, forming a thin lipid film on the bottom of the test vial. The lipid film was dried overnight under vacuum to remove trace amounts of the organic solvent. The lipid films were hydrated to their final concentration (Table 1) in 10 mM Tris-buffer (pH 7.4) containing 0–100 mg/ml trehalose (Table 1) by heating for 20 min at 10 °C above the main phase transition temperature of DDA ($T_m \approx 47$ °C) [21].

2.3. High-performance liquid chromatography analysis

Standard curves for DDA and TDB were prepared according to the protocol for the QC-validated method at Statens Serum Institut [22] analyzing 1, 2, 3 and 4 mg DDA and 0.2, 0.4, 0.6 and 0.8 mg TDB. Briefly a Vydac 218TP52 reversed-phase C_{18} column (250 × 2.1 mm, 5 μm particles) was used on a Dionex Summit HPLC with a Shimadzu ELSD-LT evaporative light scattering detector. Twenty-five microliters of sample was injected and eluted in 68/32% (v/v) of eluent A (0.1 M ammonium acetate in methanol) and B (chloroform) for 6 min. This gradient was followed by a 10 min plateau at 10/90% eluents A and B, before going back to the initial eluent mixture for 9 min. All runs were performed at 25 °C at a flow rate of 0.400 ml/min. ELSD detection was done at

Table 1
Overview of the liposome preparations containing DDA, CAF01 and CAF01 + trehalose

Formulation	Content		
	DDA (mg/ml)	TDB (mg/ml)	Trehalose (mg/ml)
DDA	0.50–3.50	–	–
CAF01 (DDA + TDB fixed ratio = 5:1)	0.50–3.50	0.10–0.70	–
CAF01 + trehalose	0.625	0.125	0–100
	1.25	0.25	0–100
	2.50	0.50	0–100

DDA and CAF01 were prepared in concentration intervals of 0.50 mg/ml DDA.