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A versatile, quantitative analytical method for pharmaceutical relevant lipids in drug delivery systems

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ABSTRACT

Over the past few years, liposomal formulations as drug carrier systems have markedly advanced in pharmaceutical research and development. Therefore, analytical methods to characterize liposome-based formulations are required. One particular issue in liposome analysis is the imbalance of lipid ratios within the vesicle formulations and the detectability of degradation products such as lysophospholipids and fatty acids caused by hydrolysis, especially in low molar ranges. Here, a highly sensitive and selective reversed-phase high-performance liquid chromatography (rp-HPLC) method is described by the combination of an organic solvent/trifluoroacetic acid (TFA) triggered gradient and the application of an evaporative light scattering detector (ELSD). Gain setting adjustments of the ELSD were applied to obtain an optimal detection profile of the analyzed substances. This optimization provides simultaneous separation and quantification of 16 components, including different phosphatidylcholines, phosphatidylglycerols and their degradation products, as well as cholesterol. Parameters such as limit of detection (LOD) and limit of quantification (LOQ) were determined for each of the components and had ranges from 0.25–1.00 mg/mL (LOD) and 0.50–2.50 μg/mL (LOQ), respectively. The intra-day precision for all analytes is less than 3% (RSD) and inter-day precision is about 8%. The applicability of the method was verified by analyzing two different liposome formulations consisting of DSPC:DPPC:DSPG:Chol (35:35:20:10) and DSPC:DPPC:DSPG (38:38:24). For degradation studies, both formulations were stored at 4 °C and at ambient temperature. Additionally, forced degradation experiments were performed to determine hydrolysis mass balances. A total recovery of 96–102% for phospholipid compounds was found. Analytical data revealed that the sensitivity, selectivity, accuracy, and resolution are appropriate for the detection and quantification of phospholipids and their hydrolysis products. These results as well as additional preliminary analyses of other relevant components used in liposomal formulations indicate that the developed method is suitable for the development, characterization, and stability testing of liposomal based biopharmaceuticals.

1. Introduction

Liposomes are spherical vesicles consisting of phospholipid bilayers that enclose an aqueous inner compartment. Due to their characteristic ability to incorporate drug substances in the lipid layer or aqueous compartment, they are used in various pharmaceutical formulations as drug delivery systems [1].

These carrier systems are designed particularly for intended purposes such as chemotherapeutics, antifungals, gene therapeutics, and vaccines [2]. The lipid membrane composition is responsible for stability and release mechanism. Most liposomes consist of neutral lipids as a main component as well as alternating ratios of charged lipids, modified lipids, or adjuvants. Phosphatidylcholines (PC), belong to the neutral lipid class, and phosphatidylglycerols (PG), belong to the anionic lipid class, are widely applied in drug delivery formulations. There is also growing interest in the field of cationic lipids such as DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), which are applied for the encapsulation of drugs such as nucleic acid and peptides [3].

According to Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines, the chemical and physical stability of liposome formulations must be demonstrated. Both agencies recommend the implementation of stability tests, which include methods for the quantification of critical degradation products [4,5]. The primary chemical degradation process of

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phospholipids is the hydrolysis of ester bonds resulting in corresponding lysoforms and fatty acids. It has been shown that hydrolysis processes occur in aqueous media as a function of pH, temperature, concentration, and ionic buffer strength [6]. Lysophospholipids affect the membrane permeability of the liposomes significantly and the incorporation of the resultant fatty acids in the membranes can promote fusion of the liposomes, leading to the leakage of the active pharmaceutical ingredient (API) [7]. Therefore, the degradation products affect the stability of lipid membranes and may alter size, structure and other physicochemical properties such as the phase transition temperature [8].

With the increasing number of approved drug carriers on the market and in clinical studies, ever more sensitive and selective analytical methods are required to ensure the safety and quality of those pharmaceuticals. High-performance liquid chromatography (HPLC), commonly used for this approach, can be equipped with various detectors such as mass spectrometers (MS) [9], ultraviolet detectors (UV) [10], diode array detectors (DAD) [11], fluorescence detectors (FLD) [12], refractive index detectors (RID) [13], charged aerosol detectors (CAD) [14], and evaporative light scattering detectors (ELSD) [15]. For the simultaneous detection of phospholipids and degradation products, ELSD and CAD are favored due to their high sensitivity and compatibility with various solvents used in HPLC. Moreover, neither complex lipid extraction nor derivatization is required. At a fundamental level, both CAD and ELSD share some similarities, in that mobile phase exiting the column is first nebulized and then vaporized to form analyte particles. While, both CAD and ELSD are non-linear, the mechanism by which these techniques measure analyte mass differ [16,17]. For this novel method, an rp-HPLC equipped with an ELSD was chosen for several reasons. First, this equipment is well established in the quality control of liposome suspensions [18–20]. Second, solvents commonly used in rp-HPLC lipid analysis (methanol, isopropanol, and acetonitrile) cause higher levels of background noise with CAD [17]. The challenge for the analysis of degradation profiles comes from the presence of additional analytes in the sample and their unbalanced concentration. Therefore, higher noise may interfere with the results. Furthermore, ELSD detectors provide the opportunity to adapt the gain of signal during analysis. The sensitivity of the detector can be adapted to individual sample characteristics such as unbalanced lipid molar ratios or low amounts of degradation products [19].

Both reversed-phase [15] and normal-phase [20] gradients are well studied in the simultaneous separation of lipids in drug delivery products. Advantages of reversed-phase systems include compatibility with the ELSD detector, simplicity, inexpensive solvents, and the requirement of only a binary pump. Trifluoroacetic acid (TFA), the most common additive in reversed-phase HPLC, is utilized in lipid analysis to elute charged lipid classes. Increasing the TFA concentration in the mobile phase achieves an enhanced separation profile for neutral and anionic lipids [19].

The majority of applied analytical methods are designed for specific lipid-based drug carrier systems [11,14,18,20]. In the present study, we introduce a versatile, quantitative rp-HPLC–ELSD method including a TFA gradient in order to analyze complex liposome formulations of various phosphatidylcholines, phosphatidylglycerols, the respective degradation products, as well as cholesterol, a common membrane stabilizing molecule [21]. Based on promising results when testing standard mixtures, we selected two liposomal carrier models used for cisplatin administration, a widely used chemotherapeutical agent [22]. These liposome formulations consisted of DSPC:DPPC:DSPG:Chol (35:35:20:10) and DSPC:DPPC:DSPG (38:38:24). Thus, the reliable applicability of the developed method for the quantitative lipid analysis as well as simultaneous sensitive determination of hydrolysis products could be demonstrated. These selected model membrane systems have different cholesterol molar ratios, which might affect the shelf life of the formulation [23]. In combination with the phospholipid composition and long storage conditions at proper pH and temperature, the formation of lipid lysoforms and fatty acids can differ. Forced degradation experiments were conducted to indicate that the presented method is suitable to analyze lipid components and degradation products with appropriate selectivity and sensitivity expressed as mass balance.

2. Materials and methods

2.1. Materials

The neutral lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and the anionic lipids 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol), sodium salt (DMPG), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol), sodium salt (DPPG), 1,2-distearoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol), and sodium salt (DSPG) were purchased from Lipoid (Ludwigshafen, Germany). The corresponding lysolipids 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 14:0), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 16:0), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC 18:0), 1-myristoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-*rac*-glycerol), sodium salt (LPG 14:0), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phospho-(1′-*rac*-glycerol), sodium salt (LPG 16:0), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), and sodium salt (LPG 18:0) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The fatty acids myristic acid (FA 14:0), palmitic acid (FA 16:0), and stearic acid (FA 18:0) were purchased from Sigma–Aldrich Inc. (Dorset, UK) and cholesterol (Chol) from Solvay, (Weesp, The Netherlands). HPLC analysis was performed with gradient grade isopropanol (IPA), methanol (MeOH) and trifluoroacetic acid (TFA). All solvents including chloroform and 0.1 M hydrochloric acid were purchased from Merck (Darmstadt, Germany).

For the liposome preparations, phosphate buffered saline (PBS) obtained from PAA (Pasching, Austria), was used.

2.2. HPLC instrumentation and chromatographic conditions

All analyses were performed using an Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with a binary pump, an online membrane degasser, a temperature controlled autosampler, and a column oven operated by an Agilent software, version B.04.01SP1[647]. For separation, a Luna C18 column, 5 μm, 100 Å (Phenomenex LTP, Aschaffenburg, Germany) was used. Detection was performed with a low temperature evaporative light scattering detector (ELSD), Sedex-85 (Sedere, Alfortville, France).

Separation of the lipids was carried out at a constant flow rate of 1 mL/min at 30 °C using a binary gradient (Table 1). Solvent A consisted of 95% methanol, 5% water (v/v) and solvent B consisted of 100% isopropanol, 0.045% trifluoroacetic acid (v/v). Samples were brought to at 20 °C in the autosampler and 100 μL aliquots were injected. For detection, the ELSD filter setting was adjusted to 10 s. The nebulizer gas pressure (compressed air) was set to 3.5 bar at 30 °C. Sensitivity of the detector was adjusted by selecting the appropriate gain setting according to the requirements of the measured standards and samples, as the detector offers the opportunity to switch the gain within a single run. Gain setting evaluation is displayed in the corresponding chapters.