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A four parameter optimization and troubleshooting of a RPLC – charged aerosol detection stability indicating method for determination of S-lysophosphatidylcholines in a phospholipid formulation

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ABSTRACT

A four parameter optimization of a stability indicating method for non-chromophoric degradation products of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1-stearoyl-*sn*-glycero-3-phosphocholine and 2-stearoyl-*sn*-glycero-3-phosphocholine was achieved using a reverse phase liquid chromatography-charged aerosol detection (RPLC-CAD) technique. Using the hydrophobic subtraction model of selectivity, a core-shell, polar embedded RPLC column was selected followed by gradient-temperature optimization, resulting in ideal relative peak placements for a robust, stability indicating separation. The CAD instrument parameters, power function value (PFV) and evaporator temperature were optimized for lysophosphatidylcholines to give UV absorbance detector-like linearity performance within a defined concentration range. The two lysophosphatidylcholines gave the same response factor in the selected conditions. System specific power function values needed to be set for the two RPLC-CAD instruments used. A custom flow-divert profile, sending only a portion of the column effluent to the detector, was necessary to mitigate detector response drifting effects. The importance of the PFV optimization for each instrument of identical build and how to overcome recovery issues brought on by the matrix effects from the lipid–RP stationary phase interaction is reported.

1. Introduction

Phospholipids are surface-active, amphiphilic molecules having a glycerol backbone, esterified with 2 fatty acids and a phosphate assigned to position 3. The nomenclature of a phospholipid is 1,2-diacyl-*sn*-glycero-3-phosphate, where *sn* stands for stereospecific numbering of the three positions of glycerol. If the phosphate group is further esterified with choline (like in membrane phospholipids), the resulting triester is a phosphatidylcholine.

Phospholipids are used in pharmaceutical technology as wetting agents, emulsifiers, and builder or components of mesophases like liposomes, micelles, mixed micelles, cubosomes, etc. These functional properties are used in many formulation types, like suspensions, various types of emulsions, mixed micelles, solid dispersions, drug–phospholipid complexes, etc. Due to their physiological role, phospholipids possess a very low toxicity profile and can be used for any route of administration [1].

The use of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) for pulmonary delivery has been reported [2]. Phospholipids such as DSPC may undergo hydrolysis [3] of the stearate ester at the *sn*-1 or *sn*-2 position of the glycerophosphocholine backbone to yield 2-stearoyl-*sn*-glycero-3-phosphocholine (1-S-Lyso-PC; 1-SLPC) or 1-stearoyl-*sn*-glycero-3-phosphocholine (2-S-Lyso-PC; 2-SLPC), respectively (Scheme 1). Lyso is a prefix applied to various phospholipids to indicate the removal of one of the two fatty acids, in our case stearate (S) at the position indicated by the prefix number (e.g., 1-S, 2-S). Each S-Lyso-PC isomer may also undergo intra-molecular acyl migration to yield the other isomer, with 1-S-Lyso-PC being the predominant isomer [4]. Lysophosphatidylcholine (LPC) is believed to play an important role in atherosclerosis and inflammatory diseases [5,6]. Lysophospholipids (LPLs) are bioactive lipids and have been shown to bind to G-protein-coupled receptors (GPCRs) in lymphocytes and a number of tissues, including the aorta, and can trigger signal transduction cascades involved in the initiation and development of atherosclerosis [5]. LPC is a lipid component of oxidized LDL cholesterol and has an important role in the antimigratory activity of the lipoprotein with a near complete inhibition at 20 mM for 1-palmitoyl-lyso-PC [6]. There-

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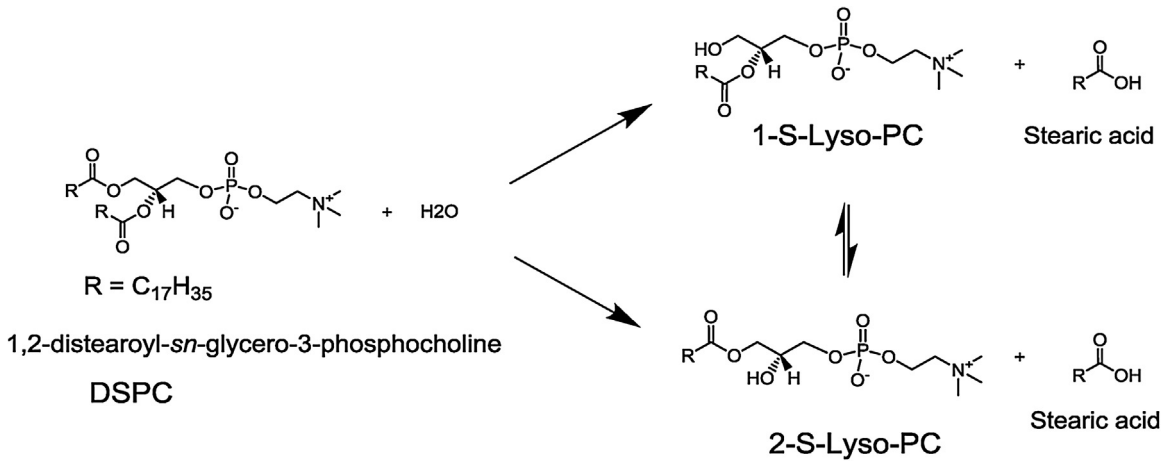

Scheme 1. Hydrolysis of DSPC and acyl migration of S-Lyso-PC.

fore, the levels of stearoyl-LPC (S-Lyso-PC) need to be controlled and monitored in formulations containing DSPC.

DSPC and all of its related degradation products lack a UV chromophore amenable to conventional UV absorbance detection. Therefore, other types of detection methods are needed. Non-chromophoric detectors make possible the application of chromatographic methods for analysis of these type of compounds [7]. Direct analysis of LPC and related phospholipids *via* chromatographic methods coupled with non-chromphoric detectors such as evaporative light scattering detection (ELSD) [1,8,9,10] refractive index (RI) [11], mass spectrometry (MS) [12,13], and the first generation Corona charged aerosol detector (Corona CAD) have been reported [14,15]. Three isomeric lysophosphatidylcholines (1-palmitoyl-*sn*-glycero-3-phosphorylcholine, 2-palmitoyl-*sn*-glycero-3-phosphorylcholine, and 3-palmitoyl-*sn*-glycero-2-phosphorylcholine) were studied by ^{31}P NMR spectroscopy [17]. Using this detection technique it was reported that at high pH, the ratio between *sn*-1 and *sn*-2 isomers was 90:10 and the second order rate constant for the acyl migration was $4 \times 10^{-4}\ M^{-1}\ s^{-1}$. At pH 4 and 4 °C, the intra-molecular acyl migration was completely eliminated [13].

For a quality control (QC) method, the ELSD and CAD techniques are of interest. However, it is debatable which mode of separation is better. Hydrophilic interaction chromatography (HILIC) has become increasingly popular for the separation of phospholipids [14]. A major problem using reversed-phase liquid chromatography (RPLC) is the high hydrophobicity of the stationary phase, leading to irreversible adsorption of the phospholipids and altering the nature of the stationary phase. This does not mean that RPLC cannot be used for this type of separation. As we report here, under certain RPLC conditions, the analysis of phospholipids in a drug product matrix is feasible, with the advantage that the method can also be used for the quantitation of the degradation products from the active pharmaceutical ingredient (API). Other stationary phases such as perfluorinated phases have been reported to achieve good separation of the analytes [9,14]. The CAD response repeatability was 0.14, 0.95 and 1.68% RSD for 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-hydroxy-2-palmitoyl-*sn*-glycero-3-phosphocholine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), respectively [14]. The CAD parameters optimization for an aminoglycoside analytical method has been reported [16], but phospholipids analysis by CAD brings unique challenges.

This report describes the RPLC method development for the determination of 1-S-Lyso-PC and 2-S-Lyso-PC in DSPC placebo and related formulations using the current generation charged aerosol detector (Thermo Scientific Dionex Corona Veo RS). Column selection, HPLC conditions, detector parameters optimization, troubleshooting and validation results are presented. We also report the importance of the power function values (PFV) optimization for each instrument (of identical build) and how to overcome recovery issues brought on by the matrix effects from the lipid–RP stationary phase interaction.

2. Materials and methods

2.1. Materials

Placebo containing DSPC and calcium chloride (2:1 mol ratio) and API formulations were in-house material (Novartis Pharmaceuticals Corporation). 1-Stearoyl-*sn*-glycero-3-phosphocholine reference standard was obtained from Corden Pharma (Liestal, Switzerland) or from Sigma Aldrich (St. Louis, MO, USA). 2-Stearoyl-*sn*-glycero-3-phosphocholine reference material was obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). HPLC grade Trifluoroacetic acid (TFA) was from J.T. Baker (Center Valley, PA, USA). LC/MS grade methanol was from EMD (Billerica, MA, USA). HPLC grade (or equivalent) water was dispensed from an EMD Milli-Q® water purification system (Billerica, MA, USA) or obtained from Honeywell Inc. (Muskegon, MI, USA). All other chemicals and reagents were ACS reagent grade.

2.2. Instrumentation

The Agilent 1260 HPLC systems (Agilent Technologies, Santa Clara, CA, USA) comprised of a quaternary pump, online degasser, auto-injector, and thermostated column compartment. The column outlet was connected to the divert valve on the Thermo Scientific Dionex Corona Veo RS Charged Aerosol Detector (Thermo Fisher Scientific, Sunnyvale, CA), which allowed the column effluent to flow either to the CAD detector or to waste. The system dwell volume was measured to be 1.15 mL. Chromeleon 6.80 software (Thermo Fisher Scientific, Waltham, MA) was used for data acquisition and analysis.

2.3. HPLC method

The final method used mobile phases consisting of: (A) 0.1% (v/v) TFA in water and (B) 0.1% (v/v) TFA in methanol. The mobile phase gradient program was as follows: 0–8 min, 60–95% B; 8–10 min, 95% B; 10.1–15 min, 60% B. The HPLC column was the Agilent Poroshell Bonus-RP, 2.7 μm, 4.6 × 100 mm. The column tempera-