Journal of Chromatography A, 1320 (2013) 80–85

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

High performance liquid chromatography with evaporative light scattering detection for the characterisation of a vesicular delivery system during stability studies

Manal M. Alsaadi, K. Christine Carter, Alexander B. Mullen*

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK

ARTICLE INFO

Article history:
Received 9 July 2013
Received in revised form 8 October 2013
Accepted 18 October 2013
Available online 24 October 2013

Keywords:
Non-ionic surfactant
Vesicles
Lipid analysis
Normal phase HPLC
Evaporative light scattering detection

ABSTRACT

A normal phase high performance liquid chromatography method with evaporative light scattering detection was developed for the simultaneous quantification of the lipid constituents of a non-ionic surfactant vesicle (NIV) delivery system consisting of tetra-ethylene glycol mono n-hexadecyl ether, cholesterol and dicetyl phosphate. An accelerated stability study performed at 25 °C/60% relative humidity (RH) and 40 °C/75% RH indicated that the NIV were chemically stable. Similar results were observed when stored at 4 °C for 469 days. This chromatographic method developed is a sensitive, robust and high throughput analytical technique that offers the potential for rapid quantification of lipids in liposomal and vesicular systems. The results of the chromatographic studies were supported by parallel size and zeta potential measurements.

1. Introduction

Vesicular delivery systems are designed to provide a carrier vehicle that can retain entrapped drug and deliver the payload at its intended site to enhance therapeutic outcomes [1]. These types of systems, exemplified by liposomes have proven successful in clinical applications for the delivery of drugs such as doxorubicin, daunorubicin [2,3], amphotericin B [2], cytarabine and morphine [4]. This has encouraged further investigations into other types of drug delivery systems by the parenteral, oral and pulmonary routes. Such systems include non-ionic surfactant vesicles (NIV), or niosomes, which are structurally and functionally similar to liposomes. However, NIV are considered less expensive alternatives which require less stringent storage and handling requirements, and offer greater stability and comparable toxicity to liposomes [5].

However, the successful clinical or commercial development of a vesicular system requires demonstration of physical and chemical stability [6]. Edwards and Baeumner [7] summarised characterisation studies performed on vesicular delivery systems. They concluded that entrapment efficiency, lipid composition and concentration, vesicle diameter and size distribution, lamellarity and surface charge should be determined. Analysis of lipid content in vesicular delivery systems is critical in evaluating the chemical stability of a system as changes in lipid chemistry can have profound effects on physical stability, drug entrapment, biological safety and efficacy of a formulation [8]. The major degradation pathways for most lipids involve hydrolysis or oxidation [9].

Lipid analysis by chromatographic methods are superior to chemical and enzymatic assays as they are simple to perform and can simultaneously separate and quantify a number of lipids [7]. Among the various chromatographic methods available, high performance liquid chromatography (HPLC) is considered as the preferred method [10]. Thin layer chromatography lacks resolution, reproducibility and sensitivity whilst gas chromatography is unsuitable for analysing non-volatile lipids [11]. Mass spectroscopy, although powerful in investigating the molecular diversity within a given lipid class, is unsuitable for simple quantification of lipid content based on class alone [12] and is too expensive for routine analysis [13].

Most lipids lack chromophores and those that do exhibit absorption properties typically absorb in the ultraviolet range of 200–210 nm, thereby limiting solvent choice to minimise spectral interference [14]. Refractive index detectors are sensitive to slight changes in ambient temperature, flow rate and pressure, thereby leading to a variable detector response. Furthermore, refractive index detectors are sensitive to changes in composition of the mobile phase which prevents chromatographic separation employing gradient elution [10]. In contrast, evaporative light scattering detection (ELSD) has been reported as the most suitable detector for HPLC studies in comparison with ultraviolet and

* Corresponding author. Tel.: +44 141 548 4409; fax: +44 141 552 2562.
E-mail address: a.mullen@strath.ac.uk (A.B. Mullen).

0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.10.054

refractive index detectors [15]. ELSD is compatible with most solvents and chromatographic techniques employing gradient elution, it is sensitive and is capable of detecting any compound as long as it is less volatile than the mobile phase [16]. ELSD is based on the passage of eluent into the detector where it is transformed into droplets by a nebuliser. As these droplets pass through the heated drift tube the volatile mobile phase evaporates leaving solute particles. When the resulting particles pass through a laser beam, light scattering occurs which is proportional to mass, and this is converted into an electronic signal [17]. HPLC-ELSD studies are simple, feasible, economical and sensitive with normal phase (NP) HPLC-ELSD regarded the as the analytical method of choice [18].

Various HPLC-ELSD methods have been reported for the determination of lipid content for food-related products and lipidomic applications [10,13,19–22]. Although HPLC-ELSD studies have been used in lipid analysis of liposomal formulations [15,23–25], very few researchers have included lipid content as part of their routine characterisation studies [26].

In the present study, a method to analyse the lipid content of an NIV formulation by NP-HPLC-ELSD was identified and its application demonstrated in a stability study to characterise the NIV formulation. The NIV formulation has been previously used as a carrier for sodium stibogluconate [27,28], amphotericin B [29,30] and recombinant protein *Leishmania donovani* gamma glutamyl cysteine synthetase vaccine [31].

2. Materials and methods

2.1. Materials

Tetra-ethylene glycol mono n-hexadecyl ether (surfactant VIII) was obtained from Nikko Chemicals Co., Ltd. through Jan Dekker Ltd. (Fareham, UK). Cholesterol was obtained from Croda Chemicals Ltd. (Goole, UK). Dicetyl phosphate (DCP), triethylamine, prednisolone and HPLC grade chloroform stabilised with ethanol were obtained from Sigma–Aldrich Inc. (Dorset, UK). HPLC grade propan-2-ol, isohexane, ethyl acetate, methanol, acetonitrile and glacial acetic acid were obtained from Fisher Scientific (Loughborough, UK). Sterile water for irrigation was obtained from Baxter Healthcare Ltd. (Northampton, UK).

2.2. HPLC instrumentation and chromatographic conditions

The HPLC system used in the studies consisted of a Gynkotek® HPLC pump series P580 and autosampler model GINA 50 (Macclesfield, UK) operated by a Chromeleon® software version 6.30 SP3 Build 594, Dionex (Camberley, UK). Separation was carried out on an YMC-PVA Silica column (100 mm × 3.0 mm i.d. and 5 μm particle size) from Hichrom Ltd. (Reading, UK) attached to a guard column (10 mm × 3.0 mm i.d. and 5 μm particle size) containing the same stationary phase from Hichrom Ltd. (Reading, UK). An Alltech ELSD model 500 (Carnforth, UK) was used as the HPLC detector and was operated at 80 °C and a gas flow rate of 2.90 standard litre per minute, supplied by a compressor operated between 60 and 80 pound per square inch gauge.

The chromatographic separation method was developed by evaluating the use of a gradient system which utilised an increasingly polar solvent system to elute analytes of different polarity from the normal phase HPLC column. During method development the impact of typical separation parameters such as solvent flow rate, rate of solvent gradient change were evaluated and optimised with respect to the internal standard and three analytes of interest.

Optimised lipid separation was achieved using a ternary gradient elution at a flow rate of 1 ml/min with column regeneration achieved by a 5 min elution with solvent A prior to the next injection (Table 1).

Table 1
The gradient elution sequence used in the NP-HPLC-ELSD method for lipid analysis using 100% isohexane (A), 100% ethyl acetate (B) and a mixture of 60% propan-2-ol, 30% acetonitrile, 10% methanol, 142 μl/100 ml glacial acetic acid and 378 μl/100 ml triethylamine (C).

Time (min)	Solvent channel		
	A	B	C
0	80	20	0
2	72	25	3
3	64	30	6
4	56	35	9
5	48	40	12
6	35	45	20
7	35	45	20
8	35	45	20
9	72	25	3
10	80	20	0
15	80	20	0

2.3. Preparation of standard calibration

Stock solutions (1 mg/ml in chloroform) of cholesterol, surfactant VIII and DCP were mixed and diluted with chloroform to prepare standard concentrations in the range from 0.025 to 0.25 mg/ml. An internal standard of 0.8 mg prednisolone (400 μl of 2 mg/ml prednisolone in methanol) was added to each 10 ml of lipid standard and efficient mixing was performed to ensure complete miscibility. The internal standard was to the same volume of chloroform as a blank. From each standard, 100 μl was removed and centrifugally evaporated to dryness at 35 °C under normal atmospheric pressure using SpeedVac® evaporator model SPD121P from Savant Instruments Inc. (NY, USA). Each sample was reconstituted in 100 μl chloroform and 20 μl was injected onto the column. Calibration curves for each lipid ingredient were established separately by plotting the area under the curve (AUC) ratio against the standard lipid concentration. The AUC ratio was calculated by dividing the AUC of the lipid of interest by the AUC of the internal standard. Correlation coefficients, $R^2 > 0.99$, were generally obtained.

2.4. Validation of the HPLC method

The HPLC method was validated for linearity, range, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). Linearity was established by injecting standard solutions in triplicates in the concentration range of 0–0.25 mg/ml. The retention time precision for each ingredient from all injections was determined. For precision and accuracy three concentrations levels were prepared: low (0.05 mg/ml), medium (0.10 mg/ml) and high (0.20 mg/ml). For intra-day precision two sets of each concentration were injected in triplicates on the same day. For inter-day precision a third set of each concentration was injected in triplicates on a different day. For accuracy, samples from each concentration level were injected in triplicates and % recovery and precision of recovery were calculated. LOD and LOQ were determined based on a signal to noise ratio of 3:1 and 10:1, respectively, in six replicates.

2.5. Preparation of NIV

Empty NIV were prepared under aseptic conditions in a laminar cabinet using sterilised apparatus. Lipids (30 mmol) consisting of surfactant VIII, cholesterol and DCP were weighed in a 3:3:1 molar ratio to prepare 200 ml of NIV suspension. Weighed lipids were aseptically transferred into a sterile glass bottle inside a laminar cabinet and the bottle was sealed. Lipids were melted in an oil bath for 5 min at 130 °C then cooled down to 70 °C. The bottle was then transferred into a 70 °C water bath located inside the