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Research Paper

A simple and rapid measurement method of encapsulation efficiency of doxorubicin loaded liposomes by direct injection of the liposomal suspension to liquid chromatography

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ABSTRACT

A simple and rapid chromatographic measurement method for determining doxorubicin (DOX) encapsulation efficiency (EE) into PEGylated liposomes using nanoparticle exclusion chromatography (nPEC) was developed. In this work, Doxil® and two PEGylated liposomes spiked with DOX were employed as model liposomes, and unencapsulated DOX was measured by high performance liquid chromatography with diode-array detector using an *N*-vinylpyrrolidone modified nPEC column without any sample pretreatment. Only 5 μL of an intact liposomal suspension and 3 min analysis time were required for the determination of the quantity of unencapsulated DOX. The method was validated in terms of linearity, accuracy, precision, and recovery in the range from 0.00–1.0 mg/mL (corresponding to 100–50% EE). Applicability of the method was confirmed using the measurement of time-dependent DOX loading% into the liposomes with the remote loading method using an ammonium sulfate gradient. Furthermore, it was found that the peak area of DOX-loaded liposomes in the chromatogram was proportional to DOX EE%. As this simple and rapid analytical method can measure the EE precisely, it is expected that this method will be applicable to the in-process control of liposome preparation manufacturing and the quality control of the liposome drug products.

1. Introduction

Nanoparticle formulations, such as liposomes, show efficacy and toxicity based on their pharmacokinetics, distribution, and drug release characteristics after administration. In the development of nanomedicines, evaluation of drug encapsulation, drug release, and particle size is essential to confirm the quality and performance of the formulations (2013; 2015; 2016). The drug encapsulation would be one of the critical quality attributes that would affect its *in vivo* pharmacokinetic and pharmacodynamic properties. Encapsulation efficiency (EE) of a drug should be determined with appropriate analytical method.

Today, EE of a drug is determined using various pretreatment methods, *i.e.*, dialysis, centrifugation, ultrafiltration, size-exclusion chromatography, solid-phase extraction, and centrifugation ultrafiltration prior to analysis (Edwards and Baeumner, 2006; Gómez-Hens and Fernández-Romero, 2006), which involve difficult and time-consuming procedures. In some cases, they provide different results and have limitations for the sample concentration (Ran et al., 2016). These difficulties are primarily attributed to the drug release from nanoparticles and/or adsorption of unencapsulated drug such as doxorubicin (DOX) onto devices (Chao et al., 2017; Yuan et al., 2016) during the procedure. These difficulties in EE% measurement necessitate the development of a reliable evaluation methodology that is superior to previous versions. In addition, to our knowledge, optimal EE measurement methods have not been established for in-process control of nanoparticle formulation manufacturing. Hence, rapid EE measurement method applicable to the in-process control is strongly required.

In the present study, to develop a novel EE measurement method suitable for in-process control of liposome preparation manufacturing and quality control of liposome drug products, we have investigated a rapid, accurate, precise, and practical EE measurement method for DOX-loaded PEGylated liposomes by using monolith column chromatography given the name nanoparticle exclusion chromatography (nPEC) (Itoh et al., 2017) with diode-array detection (DAD). Applicability of the method was confirmed by comparison of EE% with a centrifugation method which is one of the EE measurement methods for

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DOX-loaded liposomes (Fritze et al., 2006; Haran et al., 1993; Mayer et al., 1990; Ohnishi et al., 2013).

2. Materials and methods

2.1. Materials

Doxil® (Barenholz, 2012) was purchased from Janssen Pharmaceutical K.K., (Tokyo, Japan). DOX hydrochloride was purchased from Sicor (Santhia, Italy). High performance liquid chromatography (HPLC) grade methanol, ammonium sulfate, sodium sulfate, disodium hydrogen phosphate, acetic acid, sodium acetate and diammonium hydrogen citrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrogenated soy phosphatidylcholine (HSPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Nippon Fine Chemical (Osaka, Japan). *N*-(carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (MPEG2000-DSPE) was purchased from Corden Pharma Switzerland LLC (Liestal, Switzerland). Saline was purchased from Otsuka Pharmaceutical (Tokyo, Japan). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Monolithic column preparation

Monolithic silica with a through pore size of 2 μm, a mesopore size of 11 nm, and a surface area of 200 m^2/g was utilized as a starting material. The monolithic silica was chemically modified with *N*-vinylpyrrolidone (VP), and the nPEC column was prepared in a dimension of 3.0 mm I.D. × 20 mM, as previously reported (Itoh et al., 2017).

2.3. nPEC

A Shimadzu HPLC system composed of a LC-10ADvp pump, SIL-10Avp autosampler, CTO-10ACvp column oven and SPD-M10A diode-array detector. The autosampler temperature was set at 5 °C. The injection needle was washed with 50% methanol, and the column temperature was set at 30 °C. The mobile phase consisted of 20 mM acetate buffer (pH 4.6)-methanol (95:5, v/v) containing 50 mM sodium sulfate. The mobile phase flow rate was 1.0 mL/min. The VP-modified monolithic silica column [GL Sciences MonoSelect Diol for Liposome (prototype)] was employed as the nPEC column. An intact liposome suspension (5 μL) was injected directly to the system, and the DAD wavelength was set from 200–400 nm.

2.4. Standard solution preparation

DOX was dissolved in 10 mM potassium dihydrogen phosphate at 10 mg/mL with gentle warming (stock solution). Standard solutions (DOX concentration: 0.02, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/mL) were prepared in saline with 10 mM L-histidine (pH 7.4).

2.5. Preparation of liposomes

The formulation of the liposome membrane was HSPC: cholesterol: MPEG2000-DSPE, at 56.5:37.5:6.0 (mol). Two liposome batches were prepared using the hydration technique; one was prepared for DOX encapsulation and one for the blank liposomes used in method validation. In preparation of the DOX liposomes, DOX was remote-loaded using an ammonium sulfate gradient method. Briefly, dried lipids were hydrated with 250 mM ammonium sulfate or 300 mM diammonium citrate, and extruded at 65 °C to make the particle size of the liposomes approximately 80 nm. After extrusion, the external solution was exchanged to saline with 10 mM L-histidine (pH 7.4) by centrifugation. Blank liposomes with varying particle sizes were prepared in saline with 10 mM L-histidine (pH 7.4) and extruded in the same manner.

Particle size of the liposomes was measured using dynamic light scattering for 100-fold diluted samples (water, 20 °C) on a Malvern Zetasizer Nano ZS (Worcestershire, UK). The DOX concentration and lipid concentration were determined by HPLC.

2.6. HPLC assay of lipids in prepared liposomes

The HPLC system was composed of two LC-20AD pumps (Shimadzu), SIL-20A autosampler (Shimadzu), CTO-20AC column oven (Shimadzu), SPD-M20A (Shimadzu), and Corona Veo RS charged aerosol detector (ThermoFisher Scientific, NYSE: TMO, US). Inertsustain C18 (3.0 mm I.D. × 10 mM, 3 μm) was utilized as the HPLC column (GL science, Tokyo, Japan), and the column temperature was set at 60 °C. The mobile phase flow rate was 500 μL/mL, and the sample injection volume was 5 μL. Mobile phases was 10 mM ammonium formate-isopropanol (35:65, v/v).

2.7. Performance evaluation for nPEC

The method performance was evaluated in terms of linearity, accuracy, precision, and recovery for unencapsulated DOX in the liposomal suspension.

Linearity of DOX in the standard solution and DOX spiked blank liposomes was assessed in a range of 0.00–2.0 mg/mL. The accuracy and precision were evaluated at 0.02, 0.1, 0.2, 0.5, and 1.0 mg/mL (corresponding to 99, 95, 90, 75, and 50% EE in liposomes). Precision was determined by the coefficient of variation (CV, %), and accuracy (%) was determined using the following equation.

$$\text{Accuracy (\%)} = \frac{\text{Spiked concentration} - \text{found concentration}}{\text{Spiked concentration}} \times 100$$

DOX recovery rates (%) from the DOX-spiked liposomes were determined at 0.02, 0.1, 0.2, 0.5, and 1.0 mg/mL (n = 3). The recovery rate corresponds to the accuracy defined in the ICH guideline on validation of analytical procedures (ICH, 2005).

2.8. Comparison of the nPEC method and centrifugation method

Next, 10 mg/mL DOX in 10 mM potassium dihydrogen phosphate, liposomes prepared for DOX encapsulation (36 mM), and saline with 10 mM L-histidine (pH 7.4) were mixed (2:5:3, v/v/v). This suspension was agitated for 40 min at 50 °C (2 mL, n = 3), and DOX was remotely loaded into preformed PEGylated liposomes.

During the loading procedure using the ammonium sulfate gradient method, a 200 μL aliquot of the liposome suspension was placed in polypropylene tubes at the time points of 0, 10, 20, 30, and 40 min and immediately placed in ice water for analysis by the nPEC method (n = 3). Another 200 μL aliquot of the sample was diluted to 1.0 mL with a mixture of saline and 10 mM L-histidine (pH 7.4). A 900 μL aliquot of the DOX liposomes was placed in a polycarbonate tube (S/N S300535A, Hitachi Koki, Tokyo, Japan) and centrifuged 600,000 × *g* for 30 min at 4 °C using a Hitachi himac CS120FX ultracentrifuge for the centrifugation method (n = 3). The diluted liposomes and obtained supernatants were analyzed using HPLC for the determination of total DOX and unencapsulated DOX, respectively. The HPLC analysis was conducted as follows. The HPLC system was composed of two LC-20AD pumps, SIL-20A autosampler, CTO-20AC column oven, SPD-M20A. Inertsustain C18 (3.0 mm I.D. × 10 mM, 3 μm) was utilized as the HPLC column, and the column temperature was set at 60 °C. The mobile phase flow rate was 500 μL/mL, and the sample injection volume was 5 μL. Mobile phases was 10 mM ammonium formate-acetonitrile-isopropanol (70:18:12, v/v).

For the nPEC method, intact liposomes were injected into the HPLC. EE (%) was calculated as follows: