International Journal of Pharmaceutics 371 (2009) 197–203

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Development of nobiliside A loaded liposomal formulation using response surface methodology

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ARTICLE INFO

Article history:
Received 12 September 2008
Received in revised form 17 December 2008
Accepted 21 December 2008
Available online 31 December 2008

Keywords:
Nobiliside
Liposome
Response surface methodology
Hemolysis
Encapsulation efficiency
Acute toxicity

ABSTRACT

To reduce the hemolysis and toxicity of nobiliside A (Nob), liposomes were used as a carrier in this study. Response surface methodology (RSM) based on central composite rotatable design (CCRD) was applied for formulation optimization. Phosphatidyl choline (PC) proportion, cholesterol (CH) proportion, and lipids/drug ratio were selected as the independent variables while the encapsulation efficiency (EE) and hemolytic rate (HR) of the liposomes as the dependent variables. The results indicated CH proportion and lipids/drug ratio were the major contributing variables for EE and PC/CH ratio was the major contributing variables for HR. The optimum formulation of Nob liposomes, in which PC proportion of 2% (w/v), CH proportion of 0.9% (w/v), and lipids/drug ratio (w/w) of 40, had higher EE (>95%) and lower HR (<1% at the concentration of 80 $\mu g\,mL^{-1}$) with spherical shape and uniform sizes. The intravenous LD_{50} increased to 9.5 $mg\,kg^{-1}$ compared to 4.1 $mg\,kg^{-1}$ of Nob solution. In conclusion, the liposome was a safety and effective carrier for intravenous Nob.

1. Introduction

Holothuria nobilis is widely distributed in sea areas in China, Indonesia, Japan, and Australia (Liao, 1997; Zou et al., 2004). Triterpene glycosides are the predominant secondary metabolites of Holothuria nobilis and exhibit wide spectra of biological activity, such as antifungal, cytotoxic, and cytostatic effects (Wu et al., 2007). Nobiliside A (Nob) is a new triterpenoid saponin isolated from Holothuria nobilis (Fig. 1). It has potent inhibition effect on P-388 mouse lymphoma, A-549 human lung cancer, and other tumor cell strains. Moreover, it is a dual-acting anticancer agent with both cytotoxic and angiogenesis inhibiting effect (Wu et al., 2007; Yi et al., 2006). Unfortunately, in our previous study (data not published), the bioavailability of Nob is low after oral administration due to the hydrolysis and enzymolysis of Nob in the artificial simulation gastric juices. For intravenous injection, Nob is highly toxic because it causes hemolysis of blood cells (Hu et al., 1996). Therefore, choosing a good carrier for reducing its hemolysis and its toxicity is of great importance for its clinical use.

Liposomes are bilayer vesicles built up by amphiphilic phospholipids and other materials, such as cholesterol. Hydrophilic compounds can be trapped within the liposome interior, while lipophilic or amphiphilic compounds normally are incorporated into the liposome membrane (Gløgård et al., 2002). They have been extensively investigated as a carrier of antitumor drugs to enhance the bioavailability and to reduce the adverse effect of antitumor drugs. It has been reported that encapsulating antitumor drugs in liposomes enable drug target to tumor tissues and prevents damage to the normal surrounding tissues (Kshirsagar et al., 2005). Moreover, some publications have demonstrated that liposomes can decrease hemolysis effect of some saponins (Garcon and Friede, 2005; Yuldasheva et al., 2005). Based on these results, the optimal liposomal formulation of Nob possibly reduces the hemolysis effect and toxicity of drug.

The conventional method used for optimization is the "change-one-factor-at-a-time" method in which a single factor or one independent variable is varied while fixing all others at a specific level. This may lead to unreliable results and less accurate conclusions (Oh et al., 1995). Response surface methodology (RSM) is an effective statistical technique for optimizing multifactor experiments (Hamsaveni et al., 2001; Chiang et al., 2003; Zhang et al., 2007). It can be used to depict the relationship between the response and the independent variables (Vicente et al., 1998), and it takes interaction effects of the variables into consideration. In addition, RSM is less laborious and time-consuming than other approaches owing to the decrease of the number of experimental trials (Liyana-Pathirana and Shahidi, 2005; Lee et al., 2006).

The objective of this study was to optimize the formulation of Nob liposomes using RSM and to explore its application for

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doi:10.1016/j.ijpharm.2008.12.031

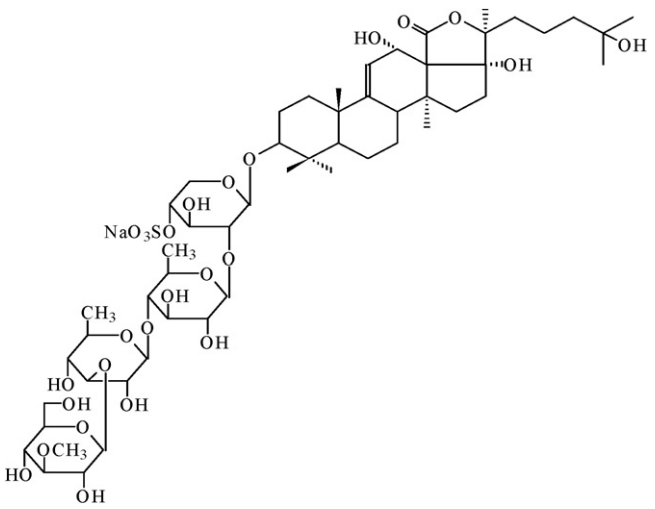

Fig. 1. Chemical structure of Nob.

intravenous administration of Nob. In the present study, Nob liposomes were prepared with three factor central composite rotatable design (CCRD) using a thin-film ultrasonication method. Phosphatidyl choline (PC) proportion (w/v), cholesterol (CH) proportion (w/v), and lipids/drug ratio (w/w) were selected as independent variables while encapsulation efficiency (EE) and hemolytic rate (HR) as dependent variables. The optimal experiment condition was verified and the Nob liposomes with optimized formulation were characterized by particle size, ζ-potential, and transmission electron microscopy (TEM) image. The acute toxicity of optimal Nob liposomes was conducted in mice.

2. Materials and methods

2.1. Materials and animals

Nobiliside A was provided by Research Center for Marine Drugs, College of Pharmacy, Second Military Medical University (Shanghai, China). PC was purchased from Tai-wei-yao-ye Ltd. (Shanghai, China). CH was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Sephadex® G50 was purchased from Amersham Bioscience (A.B. Sweden). All other chemicals were of analytical or HPLC grade. All aqueous solutions were prepared with distilled water.

ICR species mice (6 weeks old, body weight 18–22 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (China). Animal experiments were performed according to the Guiding Principles for the Care and Use of Experiment Animals in Second Military Medical University.

2.2. Preparation of liposomes

Liposomes were prepared by thin-film ultrasonication technique. Briefly, required proportion of PC and CH were co-dissolved in chloroform and evaporated to form a thin lipid film under reduced pressure. The thin film was hydrated with 20.0 mL of aqueous Nob solution, the concentration of Nob solution was determined according to the lipids/drug ratio. The resulting mixture was sonicated for 30 min and further processed by probe sonication for 1 min cycles (3 s working and 3 s rest) at 200 W (Ningbo Xinzhi Bio-tech Co. Ltd., China). The resulting liposomes' suspension was extruded through sterile Millipore Express (PES, Millipore, USA) with 0.22 μm pore size.

2.3. Encapsulation efficiency determination (EE)

2.3.1. Chromatographic conditions

An HPLC method with evaporative light scattering detector (ELSD) was developed for the determination of Nob. The HPLC system comprised an isocratic pump (1100 series), autosampler (1100 series), a degasser (1100 series) (Agilent, CA, USA), and ELSD (2000ES, Alltech, USA). A SB-C_{18} column (4.6 mm × 150 mm, particle size 5 μm, Agilent, USA) and an Agilent SB-C_{18} Guard Pak (4.6 mm × 12.5 mm, 5 μm) were used as analytical and guard column, respectively. A binary mobile phase, consisting of 0.5% acetic acid (solvent A) and methanol (solvent B), was used at a flow rate of 1.0 mL min^{-1}. The analytes were eluted using the following program: 0–5 min, linear gradient 72–80% B; 5–12 min, linear gradient 80–98% B; 12–25 min, linear gradient 98–100% B. The column temperature was kept at 30 °C. ELSD conditions were optimized in order to achieve maximum sensitivity: the purified compressed air as the nebulizing gas was at a flow rate of 2.0 L min^{-1} and temperature of the nebulizer was at 80 °C.

2.3.2. Encapsulation efficiency (EE)

EE was determined by minicolumn centrifugation method as described previously (Fry et al., 1978) with minor modifications. In brief, Sephadex® G50 solution (10%, w/v) was prepared in water and was kept aside for 48 h for complete swelling. To prepare minicolumn, Whatman filter pad was inserted in 1 mL syringe and swollen Sephadex was added carefully to it to avoid air entrapment in the column. Excessive amount of water was removed by spinning the column at 2000 rpm for 3 min using centrifuge (800B, Shanghai Anting scientific instrument Co., Ltd., China). Nob liposomes suspension (100 μL) were slowly added on prepared column and centrifuged at 500 rpm for 3 min, and then the same procedure was repeated by adding 100 μL of water. The remaining free drug bound to the gel, while liposomes passed through the gel and were collected from the first and second stage of centrifugation. The eluted liposomes obtained were ruptured using a mixture of methanol and isopropyl alcohol (7:3, v/v) and percent encapsulation was calculated from total amount of Nob present in 100 μL of liposomes by HPLC-ELSD using Eq. (1) (Essa et al., 2002; Padamwar and Pokharkar, 2006). The free drug was analyzed by HPLC-ELSD.

$$\text{Encapsulation efficiency (EE)} = \frac{Q_e}{Q_t} \times 100 \quad (1)$$

where Q_e is the amount of encapsulated Nob and Q_t is the amount of Nob in 100 μL of liposomes suspension.

2.4. Hemolytic assays

Hemolytic activity was determined by incubating a 2% (v/v) suspension of rabbit blood cells (RBC, 2.5 mL) with serial dilutions of each selected samples (2.5 mL). RBC were rinsed several times in aqueous NaCl solution (0.9%, w/v) by centrifugation for 3 min at 2000 rpm until supernatants were colorless, and diluted with aqueous NaCl solution (1:50, v/v). Samples were incubated for 3 h at 37 °C, and then followed for 5 min at 0 °C to stop hemolysis. Incubations with aqueous NaCl solution (0.9%, w/v) and water were taken as blank and 100% hemolysis, respectively. After centrifugation, 0.2 mL supernatant was imbibed and diluted to 5.0 mL with ethanol. The absorbance at 415 nm was determined with UV–visible spectrophotometer (Agilent 8453 spectrophotometer, USA) (Yu et al., 2007). The lipids in ethanol had negligible OD value at 415 nm, and therefore would not interfere with the hemolytic result. The blank supernatant absorbance was almost negligible, indicating the lack of spontaneous hemolysis during centrifugation (Belokoneva et al., 2004). The relative OD compared to that of the suspension treated with water defined HR.