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Development of nobiliside A loaded liposomal formulation using response surface methodology

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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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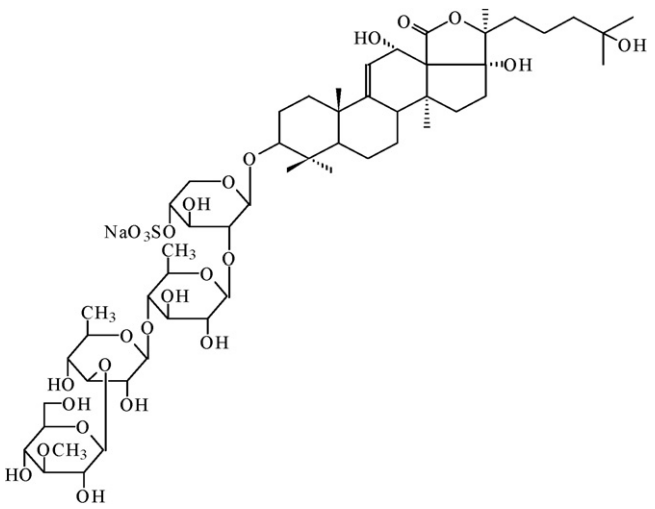

Fig. 1. Chemical structure of Nob.

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.

Table 1
Levels of factors used in CCRD.

Factors	Code	Range and levels				
		−1.682	−1	0	+1	+1.682
PC% (w/v)	x_1	1.00	1.41	2.00	2.59	3.00
CH% (w/v)	x_2	0.20	0.36	0.60	0.84	1.00
Lipids/drug ratio	x_3	55	49	40	31	25

2.5. Experimental design and optimization

RSM as a generic means for optimization was applied to optimize the formulation of Nob liposomes. The optimization was designed based on a three-factor central composite rotatable design (CCRD) with a total of 20 experimental runs that involved 8 factorial points, 6 axial points and 6 replicates at the center points (Montgomery, 1997; Moyo et al., 2003). Based on the preliminary experiments and our previous studies, three formulation parameters, PC proportion (x_1), CH proportion (x_2), and the lipids/drug ratio (x_3), were identified as key factors responsible for EE and HR. In view of the feasibility of liposome preparation and drug loading ratio for clinical dose, the ranges of the three factors were determined as follows: PC proportion (1–3%, w/v), CH proportion (0.2–1%, w/v), and lipids/drug ratio (25–55, w/w) (Table 1). The experimental runs for CCRD were shown in Table 2. Each experimental run was performed in duplicate except at the central point (15–20 runs) of the design. The response could be related to the selected variables by a second-order polynomial model. In this study, a second-order polynomial Eq. (2) was used to generate response surfaces.

$$\hat{Y}_i = \beta_0 + \sum_i \beta_i x_i + \sum_i \beta_{ii} x_i^2 + \sum_{i \neq j} \beta_{ij} x_i x_j \quad (2)$$

where $\hat{Y}_i$ represents the predicted responses, x_i and x_j are the coded values of independent variables, β_0 is the intercept coefficient, β_i are the linear coefficients, β_{ii} are the squared coefficients, and β_{ij} are the interaction coefficients (Zhang et al., 2007).

Prior to modeling, each independent variable was divided by its maximum value to treat the dimension uniformly. The least square technique was used to calculate the coefficients of variables in the models. The statistical significances were judged by Student's *t*-test at a probability of 0.01. The significance of the estimated effects was tested by analysis of variance. The accuracy of the statistical model used was described by the determination coefficient R^2. R^2 was the fraction of the data explained by the model, and value close to 1 indicated a good model. All calculation programs were coded in MATLAB 7.0 (The Mathworks Inc., Natick, MA, USA).

Table 2
Scheme of CCRD with the results of responses on three independent factors.

No.	Levels of independent factors			Responses	
	PC% (w/v)	CH% (w/v)	Lipids/drug ratio	EE (%)	HR (%)
1	1.41	0.36	49	91.3	3.6
2	2.60	0.36	49	90.2	102.6
3	1.41	0.84	49	93.0	0.0
4	2.60	0.84	49	96.2	0.8
5	1.41	0.36	31	87.3	3.4
6	2.60	0.36	31	83.6	106.1
7	1.41	0.84	31	98.8	0.6
8	2.60	0.84	31	92.8	5.7
9	1	0.6	40	101	13.0
10	3	0.6	40	84.4	74.4
11	2	0.2	40	74.6	102.6
12	2	1	40	98.5	4.3
13	2	0.6	55	81.0	2.4
14	2	0.6	25	81.9	17.4
15	2	0.6	40	92.0	4.9
16	2	0.6	40	93.1	8.5
17	2	0.6	40	93.0	10.3
18	2	0.6	40	94.2	9.9
19	2	0.6	40	97.4	8.9
20	2	0.6	40	93.2	9.7

2.6. Verification of the results

Optimal conditions and the maximum predicted acquired EE as well as the minimum predicted HR were obtained using the second-order polynomial model of RSM. The practical acquired EE and HR were achieved under the optimal conditions. The experimental and predicted acquired results were compared in order to confirm the validity of the model.

2.7. Characteristic of Nob liposomes of optimized formulation

2.7.1. Particle size distribution and ζ-potential measurement

Average particle size and size distribution of liposomes was determined using Zetasizer Nano S (Malvern Instruments, UK) based on photon correlation spectroscopy. ζ-Potential was measured using a Zetasizer Nano Z (Malvern, UK). Each experiment was repeated three times and all the measurements were conducted at 25 °C and at an angle of 90°.

2.7.2. Transmission electron microscopy (TEM)

Liposome suspension was placed onto a carbon-coated copper grid to form a thin liquid film, and then the excess solution was removed with a filter paper. After thorough air-drying, the films were then observed on a TEM (JEOL-2010, JEOL Ltd.).

2.8. Acute toxicity studies in mice

The median lethal dose (LD_{50}) values for Nob solution and liposomes were determined in ICR mice. Ten mice (five males and five females) were used for each dose group, between which an equal dose ratio of 1.18 was designed. The dosing range for the Nob solution and liposomes was from 6.0 to 3.1 mg kg^{-1} and from 14.5 to 7.54 mg kg^{-1}, respectively. The normal saline as control was injected via the tail vein. The injection volume of 20 mL kg^{-1} was administered to a mouse. Mice were observed shortly after dosing over the next 4 h, at least three times daily thereafter for 14 days. Dominant signs of toxicity and mortality were recorded. The animals died and the survivors killed after 14 days were examined for pathological changes. The LD_{50} values and their confidence limits were calculated according to the Bliss software.

2.9. Statistical analysis

Data were analyzed using MATLAB 7.0 software (The Mathworks Inc., Natick, MA, USA) to yield regression equations and regression coefficients. For analysis of variance and Student's two-tailed *t*-test, differences between test and control groups were judged to be statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Formulation conditions optimization

3.1.1. Fitting the model

The combined effects of PC proportion, CH proportion, and the lipids/drug ratio on EE and HR were presented in Table 2. From the results, two second-order polynomial Eqs. (3) and (4) representing EE and HR can be expressed as a function of the three operating parameters of Nob liposomes formulation, and their relationships were obtained as follows:

The model equation for EE:

$$EE = -0.1208x_1 + 0.2133x_2 + 2.3680x_3 - 1.5968x_3^2 \quad (3)$$

The model equation for HR:

$$HR = 3.1348x_1^2 + 1.9908x_2^2 - 5.1163x_1x_2 \quad (4)$$

The P-values were lower than 0.01 for both the fitted models, which indicated that the regression equations were statistically significant. Positive and negative sign in front of the terms indicates synergistic and antagonistic effect, respectively. The quality of the model developed was evaluated based on the correlation coefficient value. Fig. 2 showed the predicted values versus the experimental values for EE and HR. The R values for Eqs. (3) and (4) were 0.8043 and 0.9821, respectively. Both the R values obtained were higher than 0.8 (close to unity), indicating that there was a good agreement between the experimental and the predicted values from the models, especially for HR model, and that the models developed were successful in achieving the correlation between the experimental factors. The R^2 values for Eqs. (3) and (4) were 0.6469 and 0.9645, respectively, which indicated that 64.7% and 96.5% of the total variation in the EE and HR, respectively, were attributed to the experimental variables studied. The standard deviations were 0.0019 and 0.0005, respectively, for Eqs. (3) and (4). The closer the R^2 value to unity and the lower the standard deviation, the better the model. This indicated that the predicted value for HR would be more accurate and closer to its actual value, compared to EE.

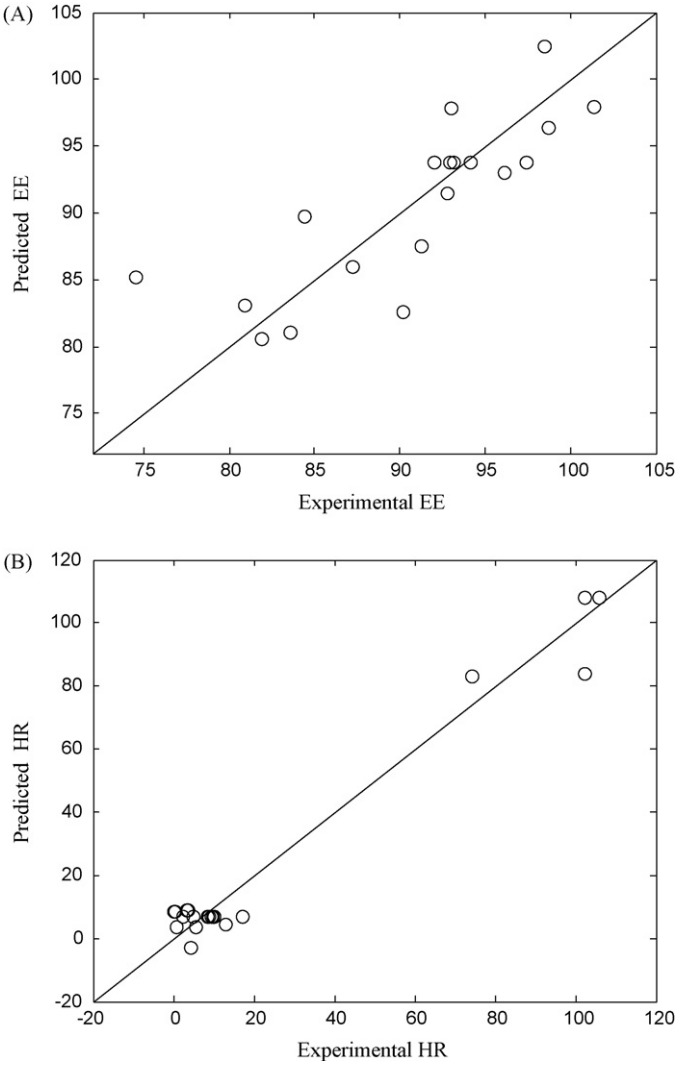

Fig. 2. Predicted values vs. experimental values of EE (A) and HR (B).

3.1.2. Analysis of response surface

The contour plots as response surfaces of PC proportion, CH proportion, and lipids/drug ratio were obtained by keeping one of the variables constant. The contour plots were shown in Fig. 3.

Fig. 3A showed the contour plots for the response of PC proportion and CH proportion to the acquired EE at fixed lipids/drug ratio of 40. The contours indicated that EE would be enhanced by the increment of CH proportion but not the increase of PC proportion. As shown in Eq. (3), EE was negatively related to PC proportion but positively related to CH proportion. The maximum acquired EE could be obtained at PC proportion of 1–2.5% and CH proportion of 0.7–1%.

Fig. 3B showed the contour plots for the response of CH proportion and lipids/drug ratio to the acquired EE at the PC proportion of 2% (central point of CCRD). The contours showed that EE was increased with the increase of CH proportion. The higher CH proportion could be favorable to entrap the drug into the liposomes. At the fixed CH proportion, high lipids/drug ratio could increase the EE. However, when the lipids/drug ratio exceeded 40, the EE began to decrease. In other words, it was not advisable to use higher lipids/drug ratio in order to enhance EE. As shown in Eq. (3), EE was found to be a function of the linear and quadratic effects of lipids/drug ratio. The linear effect was positive and the quadratic effect was negative. The maximum EE (100%) could be obtained when CH proportion ranged from 0.9 to 1 with fixed lipids/drug ratio of 40.

Fig. 3C showed the contour plots for the response of PC proportion and CH proportion to the HR at fixed lipids/drug ratio of 40. The contours indicated that HR significantly related to the PC/CH ratio (w/w). When PC/CH ratio ranged from 2 to 3, no hemolysis was observed. When PC/CH ratio was more than 3 or less than 2, HR tended to increase. Therefore, the optimum PC/CH ratio for HR ranged from 2 to 3. The interaction between PC proportion and CH proportion could also be deduced from the coefficients in Eq. (4). The value of b_{12} (−5.1163) was found to be maximal, indicating that the interaction effect of PC proportion and CH proportion might be a major contributing variable for HR of Nob liposomes.

Nob is an amphipathic compound. Its hydrophobic aglycone backbone could probably incorporate into the membrane bilayer of liposomes, while its glycoside chain could interact with the polar group (Francis et al., 2002). So Nob maybe has strong interaction with both PC and CH, leading to high EE. But it must have a stronger interaction with CH, when fixing the lipids/drug ratio, EE would increase with the increasing of CH proportion while not the increasing of PC proportion.

Monte Carlo simulation studies of the effect of CH on PC large unilamellar vesicles (LUVs) showed that it caused a reduction in the density of the head group at the interfacial region of the bilayer and an increase in the package of the phospholipids tails in the middle of the bilayer (Jedlovsky and Mezei, 2003). And also Monte Carlo simulations suggested that the lipid head group and the interface bilayer region would be more hydrated in CH rich membranes than in membranes without CH (Yau et al., 1998). Perhaps, the more hydrated and polar interface of the liposomes containing CH may facilitate the superficial binding of Nob, thus resulting in the enhancement of the EE.

The factors influencing HR seemed more complicated. CH could decrease hemolysis of saponin at the ratio of CH to saponin above 1:1 (mol/mol) (Garcon and Friede, 2005). However, in this study,

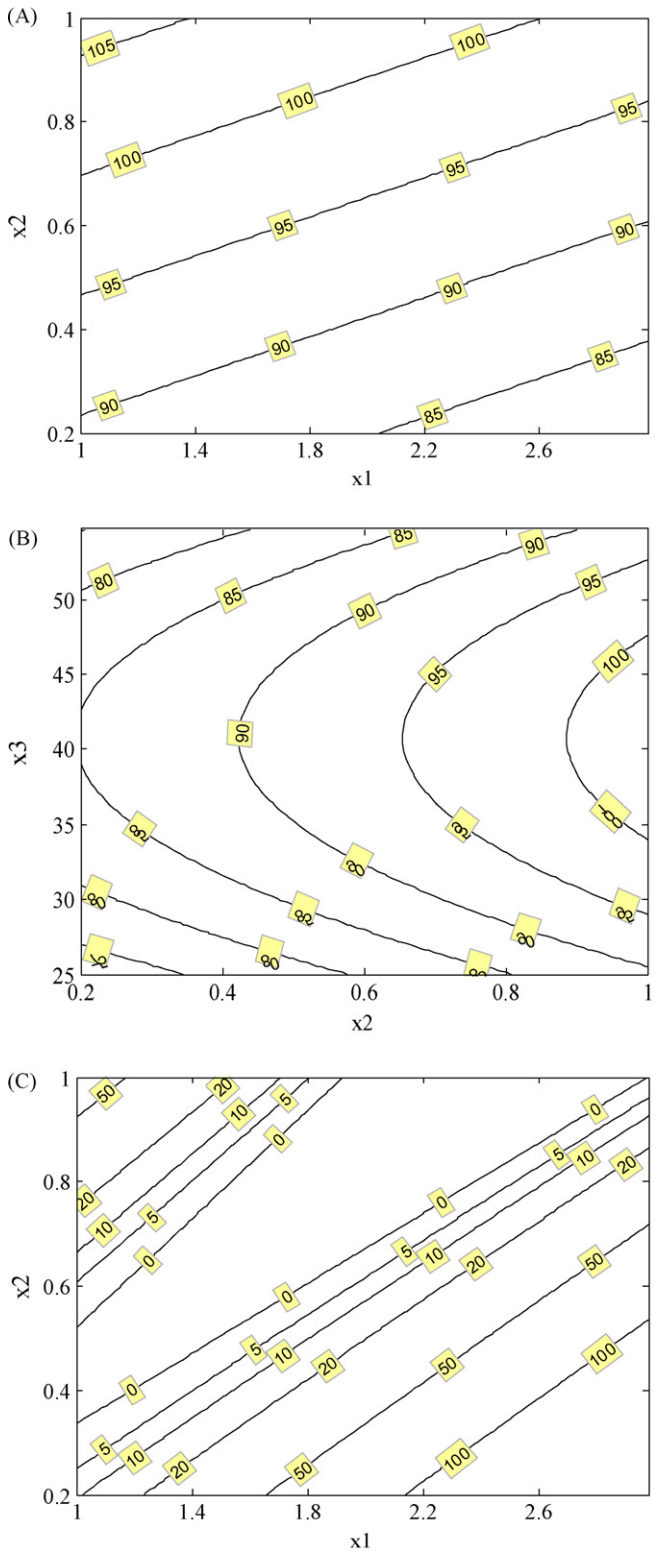

Fig. 3. Contour plots showing the effects of experimental factors on EE and HR. (A) PC and CH proportion on EE when lipids/drug ratio was 40; (B) CH proportion and lipids/drug ratio on EE when PC proportion was set at the central level of CCRD (2%, w/v); (C) PC proportion and CH proportion on HR.

molar ratio of CH to Nob was above 1:1 in all formulations, HR was quite different. The single factor experiment carried out previously demonstrated that HR was positively and negatively correlated with CH/drug ratio and PC/CH ratio, respectively. At fixed amount of CH and drug, PC/CH ratio and lipids/drug ratio increased with the increase in PC proportion while HR tended to increase. As deduced from the theory reported previously (Jedlovsky and Mezei, 2003; Yau et al., 1998), it was expected that the hydrophobic moiety of Nob would more favorably incorporate into PC liposomes than into liposomes containing CH, while the hydrophilic glycoside of Nob would more favorably incorporate into liposomes containing CH. The hemolytic effect of Nob was mainly due to its glycoside. The interaction between the head group of PC increased with the increase in PC/CH ratio, which would be unfavorable for the incorporation of the glycoside. Then the glycoside might be exposed out the bilayer, thus leading to hemolysis. In addition, there were also reports that the thickness and rigidity of PC membrane were significantly affected by CH (Gent and Prestegard, 1974; Bloom et al., 1991; Raffy and Teissie, 1999; Matsuoka and Murata, 2002). Nezil and Bloom (1992) reported that CH (33 mol%) increased the thickness of l-palmitoyl-2-oleoyl-sn-glycero-3 phosphocholine (POPC) bilayer by about 4 A°. The density of the head group and the thickness of the bilayer might account for the hemolytic effect of Nob liposomes.

3.1.3. Optimization of the formulation of Nob liposomes

After the effects of PC proportion, CH proportion, and the lipids/drug ratio on the formulation of Nob liposome were investigated, the optimum ranges for each independent variable were found to generate Nob liposomes with high EE and low HR by overlapping contour plot. The contour plots for the response of PC and CH proportion to the acquired EE and HR were shown in Fig. 4. The optimum formulation conditions were as follows: PC/CH ratio of 2%/0.9% (w/w) and the lipids/drug ratio of 40 (w/w). The models showed that the maximum acquired EE and the minimum HR were 100% and 0%, respectively.

3.2. Verification of the optimal formulation

The suitability of the model equation for predicting the optimum response values was tested using the recommended optimum conditions. The set of optimum conditions, determined using the RSM optimization approach, were tested experimentally according to the model equation. Three batches of liposomes were prepared according to the optimized formulation (Batch numbers 20080218, 20080222, and 20080225, respectively). Then EE and HR of each batch were determined. The mean experimental EE (97.1%) and HR (0.31%) at the concentration of 80 $\mu g\,mL^{-1}$ were close to the predicted results.

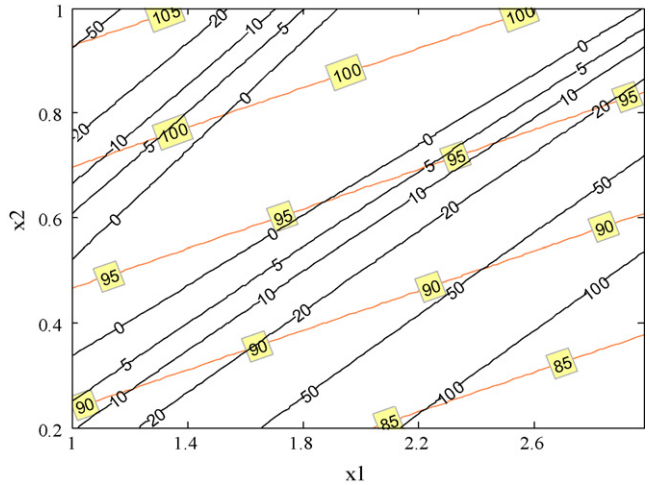

Fig. 4. Contour plot showing PC and CH proportion on EE and HR when lipids/drug ratio was 40.

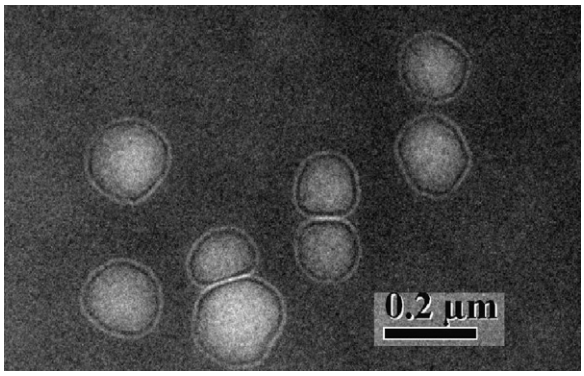

Fig. 5. Transmission electron micrograph of Nobiliside A liposomes without staining.

3.3. Characteristic of Nob liposome with optimized formulation

Nob liposomes (Batch number 20080218) of optimized formulation had been used for determination of particle size distribution, ζ-potential and hemolytic effect.

3.3.1. Particle size and ζ-potential measurements

The particle size of Nob liposomes was 109.3 ± 0.54 nm, indicating a relatively narrow particle size. Values of ζ-potential for Nob loaded liposomes was -19.5 ± 0.67 mV, which showed that liposomes obtained have sufficient charge to inhibit aggregation of vesicles. The ζ-potential of Nob liposomes was negative, because the PC included some ingredients with negative charge such as phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylglycerol (PG).

3.3.2. Transmission electron microscopy (TEM)

The TEM of Nob liposomes was shown in Fig. 5. The liposomes were small homogenous vesicles with bilayer lipid membrane.

3.3.3. Hemolytic effect

The hemolytic effect of Nob solution and liposomes were shown in Table 3. Nob solution had potent hemolytic effect. When the concentration of Nob solution was in the range of 0.4–16.0 $\mu g\,mL^{-1}$, the HR of Nob solution was in a dose-dependent manner with the increase in its concentration. However, the correlation between HR and drug concentration was not linear or logarithmic. When the concentration of Nob solution exceeded 16.0 $\mu g\,mL^{-1}$, the HR reached 100%. As incorporated into liposomes with optimized formulation, the hemolytic effect of Nob was hardly observed, even at higher concentration of 80 $\mu g\,mL^{-1}$. The hemolytic process caused by Nob completed within 1 min irrespective of the concentration of Nob solution, and thereafter the HR was not further increased with incubation time (data not shown). These results demonstrated that the rupture of erythrocytes might be of damage type, but not of channel type (Knopik-Skrocka and Bielawski, 2002). And hemolysis was not the consequence of a colloid-osmotic process (Katsu et al., 1988).

Table 3
In vitro hemolysis rate of Nob solution and Nob liposome ($n = 3$, average ± SD).

[a]Nob concentration ($\mu g\,mL^{-1}$)	Hemolysis rate of Nob solution (%)	Hemolysis rate of Nob liposome (%)
0.4	6.31 ± 2.1	−0.04 ± 0.02*
0.8	23.3 ± 3.8	−0.03 ± 0.02*
2.0	55.2 ± 3.2	−0.02 ± 0.02*
4.0	87.5 ± 3.7	−0.05 ± 0.03*
8.0	90.8 ± 2.0	0.02 ± 0.01*
16.0	102.1 ± 5.5	−0.03 ± 0.02*
24.0	100.3 ± 6.1	−0.04 ± 0.02*
32.0	104.5 ± 4.9	0.01 ± 0.02*
40.0	101.9 ± 7.2	0.25 ± 0.05*
80.0	103.4 ± 5.3	0.31 ± 0.09*

[a] Nob concentration = Nob A amount/volume of NaCl solution and RBC suspension.
* $P < 0.01$, vs. Nob solution group by two-tailed t-test.

3.4. Acute toxicity studies

The toxic signs for intravenous Nob solution were as follows: (1) serious hemolysis; after Nob solution was injected into mouse via tail vein, the mouse tail turned black within 1 h and necrosis occurred at the injection site during several days at the dose higher than 2 $mg\,kg^{-1}$. Necropsy showed that hyperaemia of the mesentery could be noted in all mice died, especially in those died within 5 min after intravenous administration. (2) Cardiac toxicity; at the dose of 6 $mg\,kg^{-1}$, tachycardia and myocardial tetanus could be observed. While for Nob liposomes (Batch number 20080222) at all doses, no toxic signs were observed except slight hepatosplenomegaly. This might be due to the macrophage uptake of liposomes in liver and spleen. The LD_{50} values for intravenous Nob liposomes and Nob solution in mice were 9.5 $mg\,kg^{-1}$ (8.8–10.2 $mg\,kg^{-1}$) and 4.1 $mg\,kg^{-1}$ (3.7–4.4 $mg\,kg^{-1}$), respectively, indicating that the acute toxicity of Nob liposomes was lower. This was related to the low HR of Nob liposomes.

4. Conclusions

Preparation of liposomes using RSM was found to be well suited and sound approach to obtain optimal liposomal formulation of Nob. Variables such as PC/CH ratio and the lipids/drug ratio had a profound effect on the EE and HR. PC/CH ratio of 2%/0.9% and the lipids/drug ratio of 40 resulted in Nob liposomes with high EE and low HR. Compared to the Nob solution, the acute toxicity of Nob liposomes was lower. In conclusion, the liposome was a suitable carrier for Nob to decrease the hemolytic effect and acute toxicity, which made it possible for clinical use.

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