Enhancing the *in vitro* release of total flavonoids extract from *Dracocephalum moldavuca* composite phospholipid liposomes optimized by response surface methodology

Cheng Zeng[1-4], Guipeng Xue[1,2], Xiaoyi Yang[2,3], Chenghui He[2], Wei Huang[4] and Jianguo Xing[2]*

[1]School of Pharmacy, Shihezi University, Shihezi, PR China
[2]Xinjiang Institute of Materia Medica, Urumqi, PR China
[3]Xinjiang Medical University, Urumqi, PR China
[4]Department of Pharmaceutics, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, PR China

Abstract: The present study was undertaken to optimize the preparation conditions of total flavonoids extract from *Dracocephalum Moldavuca* composite phospholipid liposome (TFDMCPL) by response surface methodology (RSM) and to investigate the *in vitro* release (IVR) of TFDMCPL. Method of ethanol injection was adopted to prepare TFDMCPL. The single factor experiments were used for the key experimental factors and their test range. Based on the single factor experiments, with encapsulation efficiency (EE) Size of TFDMCPL and polymey disperse index (PDI) as dependent variable, central composite design was adopted to optimize preparation technology by taking content of phospholipid and content of cholesterol as independent variables, fitting of various mathematical equations were performed using a statisitical software of Design-Expert 8.0.6. Preparation parameters were optimized through response surface plotted by optimum fitting equations, optimized procedure was validated through experimental preparation of TFDMCPL. Optimum preparation technology was as following: phospholipid 505mg and cholesterol 50mg. Under these condition, encapsulation efficiency was 90.2±1.2%, size of TFDMCPL was 115.6±4.3nm, PDI was 0.169±0.015 and Zeta potential was -15.38±0.5. These indicated that TFDMCPL with high entrapping efficiency and small particle size could be prepared by the ethanol injection method. And TFDMCPL were found to enhance the release of drugs more effectively than TFDM based on the *in vitro* model.

Keywords: Total flavonoids extract from Dracocephalum moldavuca, composite phospholipid liposomes, response surface methodology, encapsulation efficiency, *in vitro* release.

INTRODUCTION

Dracocephalum moldavica L. (DM) is a *Labiatae* annual herb, which is used as a traditional Uygur medicine for centuries in the name of Baeiranjiboya (Maimaitiyiming D *et al*. 2014). And to the best of our knowledge, it has few previous studies about its reports. DM possesses important medicinal values against the following diseases: Bronchitis, hypertension, hepatitis, dizziness, biliary tract infections, and other related diseases (Tian *et al*., 2012). DM has a long history as many classical books on Uygur medicines have reported its use (Martínez-Vázquez *et al*., 2012). A portion of minority population in Xinjiang region uses this herb along side tea, and currently marketed drugs, such as Yixin Badiranjibuya Granule, was used to treat coronary heart disease, cold nervous headache and bronchitis etc., It is also used to improve the symptoms of myocardial ischemia (Liang *et al*., 2013). It has been demonstrated that DM possess cardiotonic and antioxidative activities, which are ascribed to the flavonoid C-glycosides present in the plant. And TFDM showed remarkable scavenging effects against hydroxyl and super oxide anion radicals *in vitro*. The current study shows that Tilianin, one of the major bioactive flavone glucuronides present in the radix of DM, is generally used in Uygur medicine and possess obvious protective effects on myocardial I/R injury, which may be related to the improvement of myocardial oxidative stress states (Li *et al*., 2001). Due to the glycosyl group on the ring, TFDM has low hydrophilic, poorly absorbed and low permeability after oral administration. These characteristics strongly influence absorption, cause low oral bioavailability and limit therapeutic efficacy (Jiang *et al*., 2014).

Composite phospholipid liposome (CPL) is a new developed technology in recent years it has been applied to pharmaceuticals to modify their drug release characyeristics, and in order to improve the drug loading for poorly soluble drugs (Zeng *et al*., 2015). CPL is an exceptional liposome for incorporating a high content of hydrophobic substances therein ,comprising: a first phospholipid which is a hydrogenated naturally-occurring phospholipid and a saturated phospholipid having long carbon chains, and which has a phase transition temperature (ranging between 40 and 74^{o}C), a second phospholipid is an unsaturated phospholipid and a

*Corresponding author: e-mail: xjguodd@163.com

Cheng Zeng and Guipeng Xue are co-first authors

saturated phospholipid having short carbon chains, and which has a phase transition temperature (ranging between -30 and 10^{o}C) (Kan *et al*., 2004; Cui *et al*., 2006). Liposome-forming materials effective to form a liposome in which the first phospholipid and the second phospholipid coexist in two immiscible phases and create several discontinuous regions to greatly increase solubility of difficult soluble drugs (Chen *et al*., 2009; Liu *et al*., 2007; Hao *et al*., 2012; Shimizu *et al*., 2010). Some studies had shown that vesicles of lipid such as Soybean lecithin (SPC) displayed different behavior during freezing, drying and rehydration process in comparison with vesicles of more saturated Lecithin Hydrogenated (HSPC) (Alavizadeh *et al*., 2014; Chen *et al*., 2012; Souza *et al*., 2014). In order to overcome the difficult solubility of TFDM and problem of poor oral absorption, we provide a liposome-based drug delivery system that is able to incorporate large amounts of hydrophobic compounds and improve the stability and bioavailability.

Response surface methodology (RSM), a collection of mathematical and statistical technique, was used to develop and optimize the formulation as it provided the empirical models that could describe the effect of variables on the response and select the optimized response region from the response surface described by few well-designed experiments. A central composite factor design (CCD), one of experimental design in RSM, is used to fit regression model equations and operating conditions as variables for pharmaceutical blending problems (El-Malah *et al*., 2006). CCD has been extensively adopted to optimize and predict the nanoparticulate preparation. Those studies explained the apparent merits of CCD may produce a more efficient method to optimize multi-responses for drug delivery system design (Xu *et al*. 2014).

The aim of this study was to enhance the release of drugs utilizing technology of CPL (an *in vitro* method for the promoted release of TFDMCPL with dialysis bag was investigated) and prepare and optimize a TFDMCPL by Central Composite Design and Response Surface Methodology.

MATERIALS AND METHODS

Materials

TFDM was obtained from Xinjiang Garst pharmaceutical company (Xinjiang, China).Soybean phospholipids (SPC, purity 96%) was purchased from German lipoid company (German). Hydrogenated soya phosphatide (HSPC, purity, 98%) was purchased from Advanced Vehicle Technology Pharmaceutical Ltd (Shanghai, China). Cholesterol was purchased from Hui Xing biochemical reagent Co. Ltd (Shanghai, China). Tilianin (purity 98%) was purchased from Xinjiang Pharmaceutical Research Institute. Sephadex G-50 was purchased from Pharmacia Company (Beijing, China). Other chemical reagents were chromatographic or analytical grade.

Preparation of TFDMCPL

TFDMCPL were prepared by ethanol injection method. Briefly, ethanol contained total flavonoids of DM (0.2mg/ mL), SPC' HSPC and Cholesterol were dissolved with the solution of ethanol. The mixture was completely homogenized through using the ultrasound, then was injected into the Phosphate Buffered Saline (PBS, pH=7.4) and was stirred for 45 min by magnetic stirrer. Finally, the resulting mixture was sonicate for 5 min by probe sonication for 1 min cycles (1 s working and 2 s rest) at 400W (Ningbo Xinzhi Bio-tech Co. Ltd., China). The resulting CPL suspension was extruded through sterile Millipore Express (PES, Millipore, USA) with 0.22μm pore size.

Size of CPL measurements

The mean diameters and polymey disperse index (PDI) of the vesicles were determined by photon correlation spectroscopy (Zetasizer S90, Malvern Instruments, Malvern, UK) 12h after their preparation. Measurements were performed at a scattering angle of 90^{o} and a temperature of 25^{o}C.

Zeta potential measurements

The zeta potential measurements were performed on a Zetasizer S90 (Malvern Instruments, Malvern, UK) with the optical modulator operating at 1000 Hz using a capillary cell. To ensure valid measurements, the instrument was calibrated through measurements of the Malvern Zeta Potential Transfer Standard (-50±5 mV). Samples of the CPL were prepared by diluting the CPL suspension with an appropriate volume of water to achieve the proper count rate for the samples. All of the zeta potential measurements were performed at 25^{o}C.

Chromatographic conditions

A High Performance Liquid Chromatography (HPLC) method with UV-VIS detector was developed for the determination of total flavonoids of DM. The HPLC system consisted of an SCL-10Avp system controller, an LC-10Avp pump, a DGU-14A degasser and a CTO-10Avp column oven (Shimadzu, Kyoto, Japan).A chromatographic C18 column- SPD-10Avp (4.6mm × 250mm, 5μm,) was used as analytical column. A binary mobile phase, consisting of 0.5% methanol (solvent A) and acetonitrile (solvent B) (80:20,v/v) , was used at a flow rate of 1.0mL min^{-1} and with an injection volume of 10μL. The column temperature was kept at 35^{0}C. The detection wavelength was set at 324nm.

Entrapment efficiency (EE)

EE was determined by via gel-filtration method with SephadexG-50 column. In brief, SephadexG-50 solution (10%, w/v) was prepared in water and was kept aside for 48 h for complete swelling. Cotton was inserted in column (length: 27 cm, inner diameter:1 cm) and swollen Sephadex was added carefully to it to avoid air entrapment in the column. Sephadec was balanced by

PBS (pH=7.4). CPL of total flavonoids of DM (3mL) were slowly added on prepared column, flow rate of elution was set at 1mL min^{-1} and volume of elution was 10 times before HPLC analysis.

$EE(\%) = M_{in}/M_{total} \times 100\%$

Where M_{in} is the drug amount entrapped, M_{total} is the total drug amount used in the preparation.

Transmission electron microscopy (TEM)

The CPL of TFDM were observed by transmission electron microscopy (TEM, H-7000; Hitachi, Tokyo, Japan) at 80 kV. A dispersion of sample was dropped into a copper grid coated with film and left for 1 min then the liquid of remained was removed by a filter paper. And a negative staining of 2% phosphotungstic acid solution (w/w, pH 7.1) was dropped on the deposit during 1 min. Finally, the remained of phosphotungstic solution was wiped by a filter paper and dried samples were observed.

Single factor experiment

Single factor experiments were carried out to study the effect of PH (PBS), drug/lipid (mass ratio) and cholesterol/phospholipids (mass ratio) on the size of liposome of total flavonoids from *Dracocephalum moldevica* - CPL. During the optimization of experimental factors, one factor was changed while the other factors were kept constant in each experiment. All the experiments were repeated three times.

Experimental design

After selecting for the most significant factors influencing the physicochemical properties of the the CPL of total flavonoids of DM. Experiments were performed using a two-factor, five-lever CCD created by Design-Expert software (Zhou HW *et al*.2011). According to the result of single factor tests, two selected independent variables (amount of phospholipids (X_1) and amount of cholesterol (X_2) were taken as the main investigating factors at five different levels coded as -1.414, -1, 0, 1, and +1.414 and EE (Y_1), size of liposome(Y_2), and PDI(Y_3) were selected as dependent variables. The central point was replicated was replicated five times to find the system error (Yang *et al*., 2006), and the CCD experiments (table 2) were carried out in a randomized order. The data were statistically analyzed by ANOVA. The p values of <0.01 were considered to be statistically significant. Response variables viz. EE, size of liposome and PDI were determined spectrophotometrically (table.1).

Experimental data obtained were fitted into a second order polynomial model. The generalized second order polynomial order equation used was:

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + a_{22}X_2^2 + a_{12}X_1X_2$$

Where Y_i(i=1-3) is predicted response for EE, size of liposome and PDI. The a_0 is the fitted response at the centre point; a_1 and a_2 are linear terms; a_{12} is the interaction effect, a_{11} and a_{22} are squared effects. X_1 and X_2 are the independent variables.

In vitro release of TFDM- CPL

The studies of in-vitro release were performed by the dialysis bag method. First of all, the dialysis bag (molecular weight cut off 8000-14,000) was soaked in distilled water for 12 h before use. Then, 10 mL of TFDM or a sample of TFDM- CPL (0.1mg/mL) was placed in the dialysis bag and the receptor compartment was filled with 100 mL of phosphate buffer (pH 7.4) at 37^{o}C with gentle agitation (30 rpm). 1.0mL of the dissolution medium was withdrawn from the receptor compartment at intervals of 1, 2, 3, 4, 6, 8, 10, 12, 24 h and replaced with the same volume of fresh dialysis medium. Finally, the concentration of TFDM was determined by HPLC. All analyses were performed in triplicate to allow proper statistical analysis.

RESULTS

Physicochemical characterization of Drug-Loaded CPL

The addition of the TFDM added gradually the mean particle size of preparation (respectively, 86.4 and 116.2 nm without and with TFDM). And the augment of the mean particle size could be illustrated by the entrapment of the TFDM in the vesicles bilayers.

Single factor experimental analysis

Effect of pH (PBS) on the Size of CPL

The solubility of TFDM is seriously depending on the pH of PBS. Due to this, it is important to select a suitable pH to ensure the size of liposome of CPL. The size of liposome of TFDMCPL increased with the decrease of pH. Thus the pH about 7.4 was the best choice in the present work.

Effect of ratio of drug/lipid(mass ratio,1:6,1:25,1:45) on the size of CPL

The drug/lipid (mass ratio) is an important factor influencing the size of liposome. The size of liposome firstly increased with the decreasing drug/lipid (mass ratio) and then increased with the further increase of drug/lipid(mass ratio). Therefore, the drug/lipid(mass ratio) of 1:25 was chosen for the TFDMCPL.

Effect of cholesterol/ phospholipids (mass ratio,1:30,1:9,1:15) on the Size of CPL

The size of liposome not only depends on the drug/lipid (mass ratio), but also relies on the cholesterol/ phospholipids (mass ratio). The size of liposome increase over the cholesterol/ phospholipids (mass ratio) range of 1:1.5 to 1:30. When the cholesterol/ phospholipids (mass ratio) was higher than 1:9, the size of liposome kept on increasing. As a redult of this, 1 :9 was an appropriate cholesterol/ phospholipids (mass ratio) for the TFDMCPL.

Table 1: Table of factors and levels

Independent variables	Levels				
	-1.414	-1	0	1	1.414
X_1	130	239.83	505	770.17	880
X_2	20	28.79	50	71.21	80
Dependent Variables				constraints	
Y_1 = Entrepment Efficiency (EE)				Maximize	
Y_2 = Particile size (nm)				Maximize	
Y_3 = Ploydispersity index (PDI)				Maximize	

Table 2: Design and results of central composite experiment

NO.	X_1	X_2	The entrapment efficiency (%)	Size of CPL (nm)	PDI
1	770.17	28.79	81.14	196.3	0.269
2	505	20	89.87	129.7	0.216
3	770.17	71.21	75.24	175.1	0.289
4	505	80	85.37	135.9	0.236
5	505	50	92.08	109.6	0.172
6	239.83	71.21	83.64	173.4	0.266
7	130	50	77.87	220.6	0.291
8	505	50	92.13	112.6	0.167
9	880	50	69.64	248.4	0.321
10	239.83	28.79	83.15	161.5	0.241
11	505	50	92.02	113.8	0.164
12	505	50	91.56	110.4	0.171
13	505	50	91.85	115.3	0.169

Table 3: Binomial coefficient of the regression model test of significance (Average size of liposome)

Sources of variance	SS	df	MS	F	P
model	30539.01	5	6107.80	837.67	<0.0001
X_1	718.49	1	718.49	98.54	<0.0001
X_2	114.23	1	114.23	15.67	0.0055
X_1X_2	273.90	1	273.90	37.56	0.0005
X_1^2	29430.88	1	29430.88	4036.36	<0.0001
X_2^2	556.46	1	556.46	76.32	<0.0001

Table 4: Binomial coefficient of the regression model test of significance (PDI)

Sources of variance	SS	df	MS	F	P
model	37433.64	5	7486.73	613.38	<0.0001
X_1	1091.06	1	1091.06	89.39	<0.0001
X_2	671.32	1	671.32	55.00	<0.0001
X_1X_2	6.25	1	6.25	0.51	0.4974
X_1^2	32892.39	1	32892.39	2694.83	<0.0001
X_2^2	5755.00	1	5755.00	471.50	<0.0001

Table 5: Binomial coefficient of the regression model test of significance (The entrapment efficiency)

Sources of variance	SS	df	MS	F	P
model	612.36	5	122.47	1091.32	<0.0001
X_1	47.67	1	47.67	424.73	<0.0001
X_2	17.36	1	17.36	154.67	<0.0001
X_1X_2	10.18	1	10.18	90.67	<0.0001
X_1^2	528.54	1	528.54	4709.58	<0.0001
X_2^2	34.95	1	39.95	311.44	<0.0001

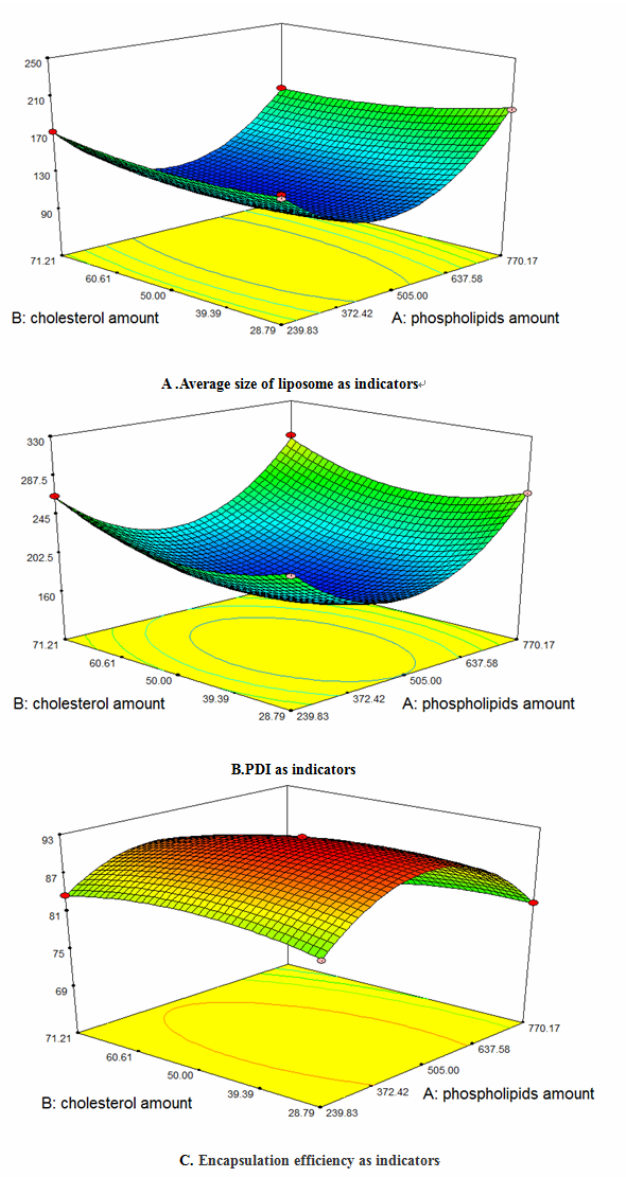

Fig. 1: Response surface model (RSM) showing the influence of the independent variables phospholipids amount and cholesterol amount on average size of liposome(A), PDI(B) and entrapment efficiency(C)

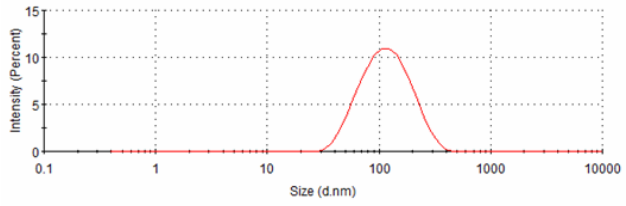

Fig. 2: The particle size of total flavonoid from dracococephalum moldevica- composite phospholipid liposomes

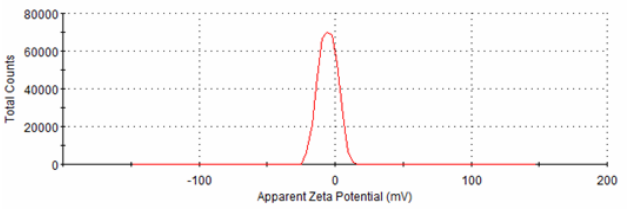

Fig. 3: The zeta particle of total flavonoid from dracococephalum moldevica- composite phospholipid liposomes

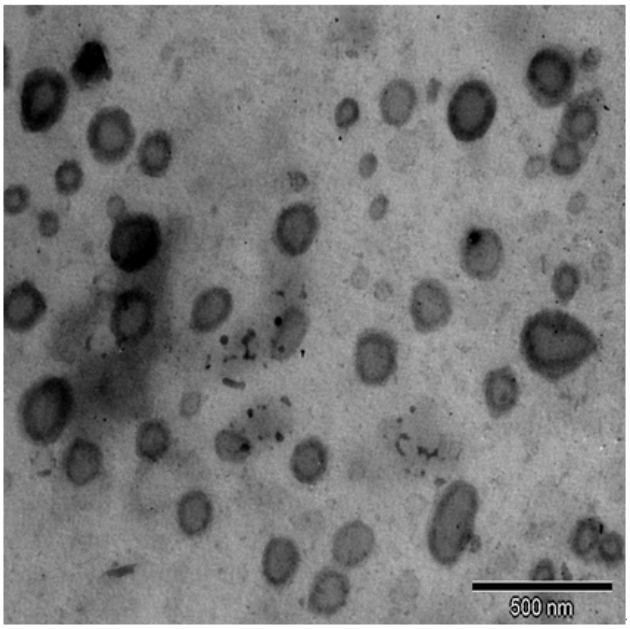

Fig. 4: TEM image of the surface morphology of composite phospholipid liposomes

Optimisation and fitting of the model

The three-dimensional response surface plots for EE, size of CPL and PDI were presented in fig.1 (A-C), respectively. The date were analyzed using Design-Expert software. This software could respectively fit the date to linear, interaction effect between two factors, second-order regression models and even higher order models, and exactly locate the response optimum. The model was judged by multiple correlation coefficient (R^2) and confidence interval (P) of the adjusted model. RSM was applied to determine the effect of phospholipids amount and cholesterol amount on EE, size of CPL and PDI of CPL of DM (table.2). For the good fit of a model, the R^2 value should be 0.80. In the present study, R^2 values for the three responses were higher than 0.80 which implied the adequacy of the applied regression model (Xiong Y *et al*.2009 ; Fan YP *et al*.2014). The lack of fit for all fitted models was found to be not significant ($p>0.05$). Therefore, it can be assumed that the selected model can be used for the simulation and optimisation of variables for the preparation of CPL of TFDM.

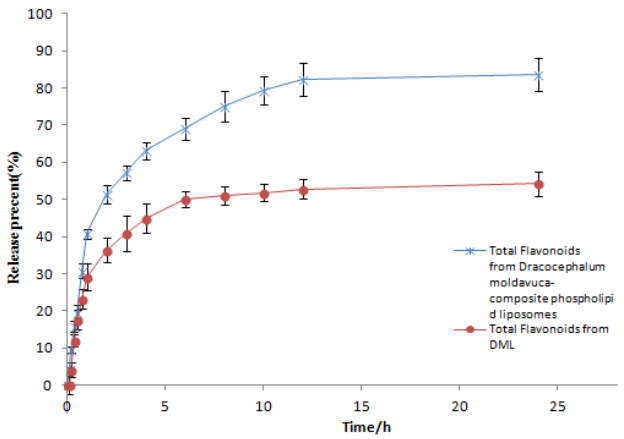

Fig. 5: The release of TFDM and TFDMCPL (n=3)

Effect of phospholipids amount and cholesterol amount on size of CPL

In response plot for variables on size of liposome (fig.1A and table.3), it was seen that a large amount of phospholipids had a detrimental effect on size of liposome. However, like EE, increase in cholesterol amount showed a gradual rise and then slight drop in size of liposome value. A positive interaction effect between the phospholipids amount and cholesterol amount was observed. The regression equation for the response size of liposome is given below :

$Y = +103.5 + 9.75X_1 - 2.82X_2 - 7.72X_1X_2 + 65.22X_1^2 + 7.87X_2^2$

Effect of phospholipids amount and cholesterol amount on PDI

The selected variable conditions had positive effect (fig.1B and table.4) on PDI. With decrease in phospholipids amount and cholesterol amount, decreased PDI was observed. In this case also, interaction effect was found to be positive on PDI. The regression equation for the response PDI is given below :

$Y=+168.60+11.68X_1+9.16X_2-1.25X_1X_2+68.76X_1^2+28.76X_2^2$

Effect of phospholipids amount and cholesterol amount on EE

From the response plot (fig. 1C and table.5), it was observed that initial rise in phospholipids amount to 505mg resulted in increased EE value but beyond 505mg slight drop occurred. However, cholesterol amount had imparted greater posititve effect compared to phospholipids amount. The interaction effect between the two variables showed a positive effect on the response. The regression equation obtained for EE is given below :

$Y = +91.93 - 2.76X_1-1.47X_2-1.6X_1X_2-9.06X_1^2-2.13X_2^2$

Verification of the predictive model

The suitability of the model for the response was determined under the optimum conditions of phospholipids amount(505mg) and cholesterol amount(50mg). As shown in fig.2 and fig.3, the liposome particle size was 115.6±4nm, PDI was 0.169±0.005, indicating a relatively narrow particle size and Zeta potential was -15.38±0.5,which showed that CPL obtained have sufficient charge to inhibit aggregation of vesicles. The experiment value for the responses were found to quite comparable and in agreement with that of the predicted value (table 6).

Surface morphology study by transmission electron microscope (TEM)

CPL prepared with ethanol injection method were observed by Transmission Electron Microscopy (TEM) and TEM micrographs are given in fig. 4. As could be seen, CPL were spherical with multilayered membrane structure specific to multilamellar vesicles. Their size estimated from TEM pictures ranged from 50 to 110 nm. These values were in the range of size evaluated by DLS.

In vitro release of TFDM- CPL

The amount of drug released from CPL was drafted as a function of time. The cumulative amount of TFDM in the receptor over 24 h was charted after administration of TFDM-CPL as shown in fig. 5.

DISCUSSION

When compared to drug-free preparation, the suspension of TFDM-CPL had an upper zeta potential. In addition, the zeta potential of suspension of drug-free CPL was -22.7 mV and the zeta potential of suspension of drug-loaded CPL was -15.4 mV. Measurements of Zeta potential can explained about the surface properties of the carrier which can be useful to determine the mold of the connection and interaction between the active substance and the vesicle (whether the drug is encapsulated in the body or simply adsorbed on the surface).

In this study, the negative surface charge was further covered in the presence of the drug, suggesting that at least a part of the connection was surface-adsorption and the rest was incorporated within the lipidic matrix. These data of zeta potential can predict that preparations have a very good stability (a negative zeta potential higher than 15 mV was sufficient to prevent carrier coalescence).

According to the single factor study, the following conditions could be used for the following experiment: a pH (PBS) of 7.4, drug/lipid(mass ratio, 1:25), cholesterol/ phospholipids (mass ratio, 1:9).

It was obvious that the drug release from CPL lifted significantly. When comparing the results which were consistent with the general summary that composite phospholipid liposomal entrapment of drugs promotes their release.

Table 6: Comparing the predicted values and actual values

Index	Predicted value	Actual value	Deviation%
Size/nm	103.6	105.2	1.54
PDI	0.166	0.169	1.81
EE/%	91.93	90.23	1.85

Note :bias (%) = (predicted values - experimental values) / predicted values × 100%

CONCLUSION

TFDM (a poorly soluble drug) were successfully incorporated into CPL by the method of ethanol injection. Based on the single factor experiment, RSM was used to further optimize the TFDMCPL condition including amount of phospholipids and cholesterol amount. The optimal condition were as the following : phospholipids amount of 505mg and cholesterol amount of 50mg resulted in TFDMCPL with high EE, small size, well suited PDI and the final CPL was able to potentially promote releasing of TFDM.

ACKNOWLEDGMENTS

This research work was supported by the National Natural Science Foundation of China (81260681), Autonomous Region High Technology Research and Development Program of China (201517109), Project of Xinjiang Medical University Graduate Innovation and Entrepreneurship (CXCY066) and project of Autonomous region science and technology (201591145).

REFERENCES

Alavizadeh SH, Badiee A, Golmohammadzadeh S and Jaafari MR (2014). The influence phospholipid on the physicochemical properties and anti-tumor efficacy of liposomes encapsulating cisplatin in mice bearing C26 colon carcinoma. Int J Pharm., 473(1-2):326-333.

Chen J, Su X, Cai BC, Wang W and Qi Y (2009). Determination of Content and Encapsulation Efficiency of Liposomes Containing Total Alkaloids from Seed of Strychnos nux-vomica L. Traditional Chinese Drug Research and Clinical pharmacology., 3(20):249-252.

Chen J, Sun X, Yu Z, Gao J and Liang W (2012). Influence of lipid components on gene delivery by polycation liposomes: Transfection efficiency, intracellular kinetics and *in vivo* tumor inhibition. Int J Pharm., 422(1-2):510-515.

Cui J, Li C, Deng Y, Wang Y and Wang W (2006). Freeze-drying of liposomes using tertiary butyl alcohol/water cosolvent systems. *Int. J. Pharm.*, **312**(1-2): 131-136.

El-Malah Y, Nazzal S and Khanfar NM (2006). D-optimal mixture design: Optimization of ternary matrix blends for controlled zero-order drug release from oral dosage forms. *Drug Dev. Ind. Pharm.*, **32**(10): 1207-1218.

Fan YP, Song XP, Gao YY, Chen Y, Ma L Zhang WM, Hou WF, Guo C and Tong DW (2014). Preparation and optimization of ophiopogon polysaccharide liposome and its activity on Kupffer cells. *Int. J. Pharm.*, **477**(1-2): 421-430.

Hao J, Wang F, Wang X, Zhang D, Bi Y, Gao Y, Zhao X and Zhang Q (2012). Development and optimization of baicalin-loaded solid lipid nanoparticles prepared by coacervation method using central composite design. *Eur. J. Pharm. Sci.*, **47**(2): 497-505.

Jiang J, Yuan X, Wang T, Chen H, Zhao H, Yan X, Wang Z, Sun X and Zhen Q (2014). Antioxidative and cardioprotective effects of total flavonoids extracted from Dracocephalum moldavical L. against acute ischemia/reperfusion-induced myocardial injury in isolated rat heart. *Cardiovasc. Toxicol.*, **14**(1): 74-82.

Kan P, Wang AJ, Chen WK and Tsao CW (2004). Liposome for incorporating large amounts of hydrophobic substances. U.S. Patent 20,040,126,886, A1, July 1.

Li JB and Ding Y (2001). Studies on chemical constituents from Dracocephalum moldavica L. *Zhong guo Zhong Yao Za Zhi.*, **26**(10): 697-698.

Liang Y, Xu XW and Zhao ZY (2013). Protection effect of Xiangqinglan Dripping Pill on acute myocardium ischemia in rats induced by coronary artery ligation. *Drug & Clinic.*, **28**(3): 312-316.

Liu ZB and Song QG (2007). Application of star point design in optimization the formulation of non viral gene delivery vector:the procationic liposomes. WCJ.PS,. **22**(1): 496-498.

Maimaitiyiming D, Hu G, Aikemu A, Hui SW and Zhang X (2014). The treatment of Uygur medicine *Dracocephalum moldavica L* on chronic mountain sickness rat model. *Pharmacoqn. Mag.*, **10**(40): 477-482.

Martínez-Vázquez M, Estrada-Reyes R, Martínez-Laurrabaquio A, López-Rubalcava C and Heinze G (2012). Neuropharmacological study of Dracocephalum moldavica L. (Lamiaceae) in mice: Sedative effect and chemical analysis of an aqueous extract. *J. Ethnopharmacol.*, **141**(3): 908-917.

Shimizu K, Osada M, Takemoto K, Yamamoto Y, Asai T and Oku N (2010). Temperature-dependent transfer of amphotericin B from liposomal membrane of AmBisome to fungal cell membrane. *J. Control Release.*, **141**(2):208-215.

Souza AC, Grabe-Guimarães A, Souza J, Botacim WE, Almeida TM, Frézard FJ and Silva-Barcellos NM (2014). Development and characterization of multilamellar liposomes containing pyridostigmine. *Pharm Dev Technol.,* **19**(4): 454-459.

Tian Y, Shang J, He T, Cai M and Abdelkader D (2012). Study on material basis of Dracocephalum moldavica for protecting cardiomyocyte against hypoxia/ reoxygenation injury by traditional Chinese medicine serum chemical and pharmacological methods. *Zhong guo Zhong Yao Za. Zhi.,* **37**(5): 620-624.

Zeng C, Huang W, He CH and Xing JG (2015). Research progress of composite phospholipids liposomes. *J. Int. Pharm. Res.,* **42**(1): 8-12.

Xiong Y, Guo D, Wang L, Zheng X, Zhang Y and Chen J (2009). Development of nobiliside A loaded liposomal formulation using response surface methodology. *Int. J. Pharm*., **371**(1-2): 197-203.

Xu H, Paxton J, Lim J, Li Y and Wu Z (2014). Development of a gradient high performance liquid chromatography assay for simultaneous analysis of hydrophilic gemcitabine and lipophilic curcumin using a central composite design and its application in liposome development. *J. Pharm. Biomed. Anal.,* **98**(9): 371-378.

Yang T, Huan SS and Cheng XM (2006). Optimization of andrographolide solid lipid Nanoparticles by Central Composite Design and Response Surface Methodology. *Zhongguo. Zhong. Yao. Za. Zhi.,* **31**(8): 650-653.

Zhou HW，Zhou M and Hou DY (2011). Optimization of ginsenoside Rg1 liposome preparation by Central Composite Design and Response Surface Methodology. *Zhong. Yi. Yao Za. zhi.,* **4**(2): 38-41.