

Formulation and *in vitro* evaluation of berberine containing liposome optimized by 3² full factorial designs

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ARTICLE INFO

Article history:

Received on: 01/06/2015

Revised on: 10/07/2015

Accepted on: 19/07/2015

Available online: 27/07/2015

Key words:

Berberine; 3² full factorial design; desirability function; lipid based formulation .

ABSTRACT

The present study demonstrates the application of 3² full factorial design for optimization of berberine loaded liposome for oral administration. Thin film hydration method was used to prepare liposome and optimization was done by 3² full factorial designs combined with desirability function. Nine formulations were prepared by using different drug : lipid and soyphosphatidylcholine : cholesterol (SPC:CHOL) ratios and evaluated for entrapment efficiency and vesicle size. The statistical validity of model was done by analysis of variance (ANOVA). Response surface graph and contour plots were used to understand the effect of variables on responses. The optimized formulation with 0.782 desirability value was prepared and evaluated for responses. The results of entrapment efficiency and vesicle size were found to be very close with the predicted values. In addition, an optimized formulation was also characterized for zeta potential, *in vitro* drug release and morphology. The formulation was found to be spherical shape with an average diameter of 0.823 nm and -1.93 mV zeta potential and also shows sustained release pattern. These results support the fact that 3² full factorial designs with desirability function could be effectively used in optimization of berberine loaded liposome.

INTRODUCTION

Berberine (BER) is a quaternary isoquinoline alkaloid obtained from various plants of *Berberis* species. It has been historically used as an anti-diarrheal, anti-protozoal, and anti-microbial agent in Ayurvedic and Chinese medicine. It also possesses multitude of biological effects, including anti-inflammatory, antidiabetic, lipid peroxidation, and neuro-protective activity (Liu *et al.*, 2009; Lee *et al.*, 2010; Wu *et al.*, 2010; Zhou *et al.*, 2010; Zhao *et al.*, 2011). However, quaternary amine cation of BER causes poor water solubility, resulting in low bioavailability. In addition, BER also induce the activity of multidrug efflux transporter P-glycoprotein (P-gp) in the intestine, responsible for active efflux of drug from cells, cause its own ejection resulting in 90% reduction in BER transport (Zhang *et al.*, 2011; Di Pierro *et al.*, 2012; Shan *et al.*, 2013). Moreover, intramuscular and intravenous administration may leads to risk of adverse reactions, such as drug rash and anaphylactic shock.

Oral route is the most easiest and convenient way for administration of drugs. However, some of the drugs have a very low oral bioavailability because of poor aqueous solubility and permeability, multidrug resistance protein (MRP) efflux and metabolic stability (Choi *et al.*, 2004). Recently lipid based formulations are widely used for the oral administration of phytoconstituents. Nevertheless, lipid-based formulation can also be formulated in different dosage form like self-emulsifying systems, multiple emulsions, microemulsions, liposomes, and solid lipid nanoparticle. There are various mechanisms responsible for the absorption enhancement of drug from lipid based formulation for instance, altering the intestinal environment, interacting with enterocyte-based transport, stimulation of lymphatic transport, and active ingredients release modification. Furthermore, degradation of active ingredient in gastrointestinal tract can be protected by phospholipids (Fricker *et al.*, 2010).

Among the lipid based systems, liposome seems to be the most promising system for its ability to enhance the permeability of drug across the enterocyte, to stabilize drugs, and provide the opportunity of controlled release (Charman *et al.*, 1986). Liposomes are spherical-shaped vesicle consisting of one or several phospholipid bilayers separated by aqueous inner compartments and are

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nontoxic, biocompatible and biodegradable. These vesicles have ability to incorporate hydrophobic, hydrophilic and amphiphilic substances. It has also been demonstrated that liposomes can improve solubility, stability and encapsulation efficiency, and drug protection against degradation. Many researchers indicated that bioavailability of orally administered drug with poor solubility and permeability was obviously enhanced after encapsulation with liposomes and changes the *in vivo* distributions of entrapped drugs. (Moutardier *et al.*, 2003; Deshmukh *et al.*, 2008; Jain *et al.*, 2012a; Jain *et al.*, 2012b; Niu *et al.*, 2012; Gradauer *et al.*, 2013). In the present investigation, we prepared a BER loaded liposome using thin film hydration technique, and was optimized using 3^2 full factorial design. They were further characterized for their entrapment efficiency, vesicle size and zeta potential, *in vitro* drug release and morphology.

MATERIALS AND METHODS

Materials

Berberine (BER) was purchased from Yucca Enterprize, Mumbai. Soyphosphatidylcholine (SPC, purity, 98%) was provided as a gift sample from Lipoid GmbH Company (Ludwigshafen, Germany). Cholesterol (CHOL) and all other solvents and reagents used were analytical grade and purchased from S D Fine-Chem Ltd (Mumbai, India).

Preparation of liposome

Thin film hydration method was used to prepare berberine loaded liposome (Szoda, 1981; Law *et al.*, 1998; Fresta *et al.*, 1999). In this method, SPC (Lipoid S 100), CHOL and BER were firstly dissolved in chloroform in different molar ratio (Table 1).

Table 1: 3^2 Factorial designs of independent variables with measured responses.

| Batch | Independent Variables | | Dependent Variables | |
|-------|-----------------------|----------------|---------------------|--------------------|
| | X ₁ | X ₂ | Y ₁ (nm) | Y ₂ (%) |
| BL1 | 1 | 1 | 876 | 82.38 |
| BL2 | -1 | -1 | 982 | 56.08 |
| BL3 | 0 | 1 | 642 | 77.13 |
| BL4 | -1 | 0 | 854 | 67.4 |
| BL5 | 1 | 0 | 1104 | 80.24 |
| BL6 | 1 | -1 | 1105 | 75.76 |
| BL7 | 0 | -1 | 1021 | 69.08 |
| BL8 | 0 | 0 | 995 | 74.51 |
| BL9 | -1 | 1 | 571 | 69.24 |

X₁ = Drug: Lipid (Molar ratio), X₂ = SPC: Cholesterol (% of total lipid)
Y₁ = Vesicle size (nm), Y₂ = Entrapment efficiency (%)

The chloroform was evaporated at 60 °C for 1 h under vacuum at 150 rpm by rotary evaporator (Remi Instruments, Mumbai, India) to form a thin lipid film. The dried thin lipid film was hydrated by adding phosphate buffer saline (PBS) pH 6.8 at 45°C in rotary vacuum evaporator rotated at 100rpm until the dispersion of all the lipids in the aqueous phase. For vesicle size reduction, the dispersion was subjected to bath sonication (Toshniwal Instruments, Ajmer) for 20-30 min at a frequency of about 30±3KHz at 40°C. Thereafter, the mixture was kept for 1 h at room temperature for the formation of vesicle followed by 4°C

for 24h in an inert atmosphere. The formulation was centrifuged for 1h at 15000 rpm in a cold centrifuge (Remi Instruments, Mumbai, India). Then, the supernatant containing the vesicles in each case was separated and taken for further studies in a suspended form.

Experimental design

3^2 factorial designs

The formulations were optimized by 3^2 factorial designs consisting of drug: lipid molar ratio (X₁) and SPC: cholesterol (X₂) as a independent variables while vesicle size (Y₁) and entrapment efficiency (Y₂) as response (Table 1). Nine formulations were prepared and evaluated for response. The obtained data were fitted into Design Expert software (Design Expert 9.0.4, Stat-Ease, Minneapolis, MN). Analysis of variance (ANOVA) was used to validate design.

Response surface plot

Contour plot and (3D) response surface plots were constructed to establish the understanding of relationship of variables and its interaction.

Optimization using desirability function

The formulations were optimized by keeping the X₁ and X₂ within the range used in present work while Y₁ at minimum and Y₂ at maximum using Design-Expert software. On the basis of these assigned goals, software determines the possible formulation composition with maximum desirability value.

Checkpoint analysis

According to desirability value and composition of variables, formulation was prepared and evaluated for response. The predicted and observed response was compared and percentage prediction error was calculated to confirm the validity of design for optimization.

Characterization of Liposome

Morphology of liposome

Shape and lamellarity of vesicle was observed by placing the suspension under optical microscope (Olympus BX 41, USA). Photomicrographs were taken by a camera attached to the optical microscope in 10x100 magnifications.

Vesicle size

The optimized formulation, serially diluted 100-fold with Double distilled water, was used to determine mean vesicle size and polydispersity index (PDI) using Zetasizer HAS 3000 (Malvern instrument Limited, UK).

Zeta potential

Zeta potentials of the optimized formulations was measured by Zetasizer HAS 3000 (Malvern instrument Limited, UK) at 25°C. (Law *et al.*, 1998)

Entrapment efficiency

Liposome suspension was centrifuge at 15000 rpm to separate untrapped drug. Free drug present in supernatant was determined using UV spectrophotometer at 345 nm. EE(%) was calculated by following equation:

$$EE (\%) = [(C_{total} - C_{free}) / C_{total}] \times 100$$

Where, C_{total} = total drug added, C_{free} = untrapped drug

In vitro diffusion study

Membrane diffusion technique was used to determine release of BER from plain drug suspension and formulation. Liposomal suspension (1.5 mL) with known amount of drug was filled in dialysis bag (Mw cut-off = 12000-14000, Hi-media laboratories, Mumbai), previously soaked in distilled water for 24h. The bag was placed in 25mL of phosphate buffer saline (PBS, pH 6.8), continuously stirred by magnetic stirrer, maintained at 37°C. Samples (1 mL) were withdrawn at specified time interval and substituted with fresh PBS (pH 6.8). UV spectrophotometer was used to determine drug from sample at 345 nm.

Stability Study

Berberine loaded liposomes were stored in glass vials and kept at 4-8°C, 25±2°C and 37±2°C for one month. The samples were taken after one month and entrapment efficiency was determined as described earlier.

RESULTS AND DISCUSSION

Experimental design

The three level two factor design is an effective approach for investigating variables at different levels with a limited number of experimental runs (Table 2). The vesicle size and EE of total 9 batches showed a wide variation from 571 to 1105 nm and 56 to 82%, respectively.

Table 2 Variables in 3² Factorial designs for liposome

| Variable | Levels [Coded (Actual)] | | |
|--|-------------------------|------------|------------|
| | Low (-1) | Medium (0) | High (+1) |
| Independent variables | | | |
| X ₁ = Drug: Lipid (Molar ratio) | -1 (1:5) | 0 (1:10) | +1 (1:15) |
| X ₂ = SPC: Cholesterol (% of total lipid) | -1 (70:30) | 0 (60:40) | +1 (50:50) |

Fitting the model to data

Response data of all formulations were fitted to cubic, linear and quadratic model. According to Design Expert software, best-fitted model was linear for response Y₁ and quadratic for response Y₂. All the responses were fitted to model to establish full model (FM) polynomial equation.

$$Y_1 = 964.78 + 113. X_1 - 169.83 X_2 + 45.50 X_1 X_2 - 29.33 X_1^2 - 118.17 X_2^2$$

$$Y_2 = 75.20 + 7.61 X_1 + 4.64 X_2 - 1.64 X_1 X_2 - 1.72 X_1^2 - 2.44 X_2^2$$

Statistical validity of the polynomials was established on the basis of ANOVA provision in the Design Expert @software. Further analysis using ANOVA indicated significant effects of the

independent factors ($p > F$) on response Y₁ and Y₂. F-value for Y₁=53.25 and Y₂=40.88, while resulted R² for Y₁=0.9875 and Y₂=0.9876. Statistical models were generated for each response parameter and tested for significance. Further Adj-R² and Pred-R² values for all responses were in reasonable agreement, indicating that the data were described adequately by the mathematical model. Values of ‘‘p’’ less than 0.05 indicated that model terms were significant except for responses Y₁, two model terms X₁² and X₁X₂ were at $p > 0.05$ (p value: 0.3197, 0.0797, respectively), and for Y₂, model term X₁², X₂² and X₁X₂ were at $p > 0.05$ (p value: 0.1949, 0.1001, 0.1119, respectively) indicated necessary model reduction to improve the model (Table 3).

Table 3 Analysis of Variance of the factorial models for the responses.

| | Source | Sum of squares | df | Mean square | F value | p-value Prob>F |
|---------------------------|--------------|----------------|----|-------------|---------|----------------|
| Vesicle size (nm) | Model | 287600 | 5 | 57520.56 | 47.31 | 0.0047 |
| | A-Drug:Lipid | 76614.00 | 1 | 76614.00 | 63.01 | 0.0042 |
| | B-SPC:CHO | 173100 | 1 | 173100 | 142.34 | 0.0013 |
| | AB | 8281.00 | 1 | 8281.00 | 6.81 | 0.0797 |
| | A2 | 1720.89 | 1 | 1720.89 | 1.42 | 0.3197 |
| | B2 | 27926.72 | 1 | 27926.72 | 22.97 | 0.0173 |
| | Residual | 3647.44 | 3 | 1215.81 | | |
| | Cor Total | 291300 | 8 | | | |
| Entrapment efficiency (%) | Model | 505.08 | 5 | 101.02 | 47.02 | 0.0047 |
| | A-Drug:Lipid | 347.47 | 1 | 347.47 | 161.75 | 0.0010 |
| | B-SPC:CHO | 129.08 | 1 | 129.08 | 60.09 | 0.0045 |
| | AB | 10.69 | 1 | 10.69 | 4.98 | 0.1119 |
| | A2 | 5.94 | 1 | 5.94 | 2.76 | 0.1949 |
| | B2 | 11.89 | 1 | 11.89 | 5.54 | 0.1001 |
| | Residual | 6.44 | 3 | 2.15 | | |
| | Cor Total | 511.53 | 8 | | | |

Response surface (3D) and Contour plot analysis

The obtained results can be observed visually in the response surface (3D) and contour plots (Fig.1, 2). Response surface graph of Y₁ shows that vesicle size of liposome was decreased with decreasing SPC concentration because phospholipids constitute the liposome membrane. With increasing total lipid (SPC:Cholesterol) concentration more drug could be incorporate into liposome. In addition, response surface graph of Y₂ shows that the increase in SPC:Cholesterol ratio significantly increased the drug entrapment efficiency. These results supported by the fact that, movement of fatty acids hydrophobic tails was reduced by incorporation of a bulky molecule of cholesterol in the lipid bilayer of liposome. It leads to permeability reduction of liposome membrane via resistance of phospholipids exchange with apoprotein. These ultimately improve the drug retention in liposome by prevention of drug leakage from lipid bilayer.

Optimization of formulation

The search for the optimized formulation composition was carried out using the desirability function approach with

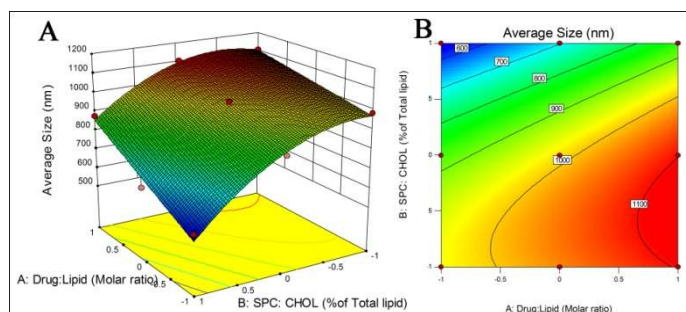


Fig. 1 Response surface (A) and its Contour plot (B) shows effect of X_1 and X_2 on vesicle size.

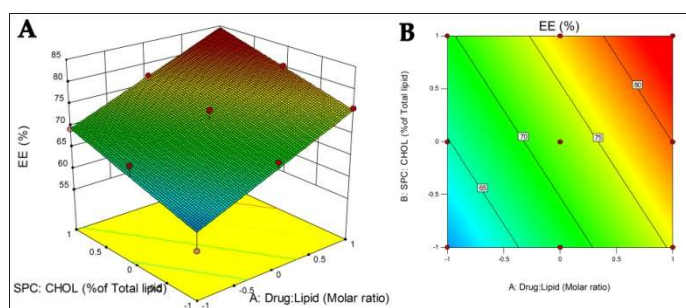


Fig. 2 Response surface (A) and its Contour plot (B) shows effect of X_1 and X_2 on entrapment efficiency.

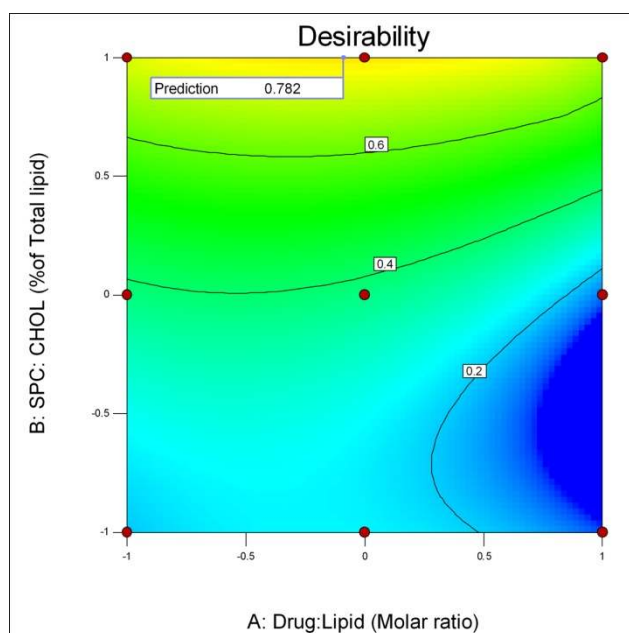


Fig. 3 Contour plot for overall desirability of liposome as a function of X_1 and X_2 .

Design expert software, criterion being one having the maximum desirability value. The optimization process was performed by setting the Y_1 at minimum and Y_2 at maximum while X_1 and X_2 within the range obtained. The optimized formulation was achieved at $X_1=1:9.56$, $X_2=50:50$ with the corresponding desirability (D) value of 0.782 (Fig.3). This factor level combination predicted the responses $Y_1=654$ nm, $Y_2=75.68\%$.

Checkpoint Analysis

The comparisons of predicted and experimental results shows very close agreement, indicating the success of the design combined with a desirability function for the evaluation and optimization of liposome formulations (Table 4).

Table 4. Checkpoint batch with their predicted and observed value of responses

| Batch | Independent Variables | | Vesicle size (Y_1) | | Entrapment efficiency (Y_2) | |
|---------------------------------|-----------------------|---------------|------------------------|-----------|---------------------------------|-----------|
| | X_1 | X_2 | Observed | Predicted | Observed | Predicted |
| BL10 | -0.089 (1:9.56) | +1 (50:50) | 648 | 654 | 77.91 | 75.68 |
| Percentage prediction error (%) | | | -0.92 | | +2.86 | |

Characterization of Optimized Formulation

Vesicle size and shape

Vesicle size determination is essential parameters for application of liposome (Maherani *et al.*, 2012). Several methods are available for preparation of liposome with different size, composed of one or more lipid bilayer. Generally, lipid film hydration is used for preparation of multilamellar vesicles. Sonication was done to produce small unilamellar vesicle. The optimized liposome (BL 10) was spherical in shape and found to be unilamellar to multilamellar (Fig. 4). The average vesicle size was found to be 0.823 nm with 0.354 polydispersity index (Fig. 5).

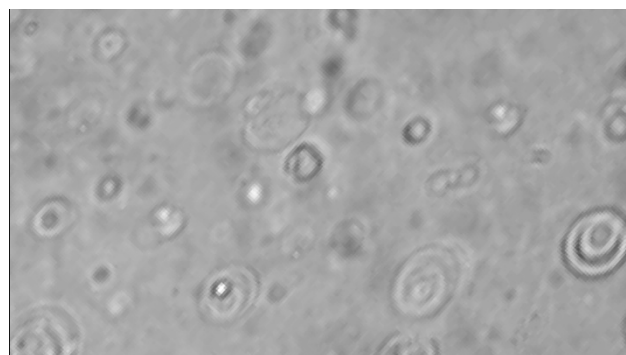


Fig. 4. Microscopy of optimized liposome (BL10).

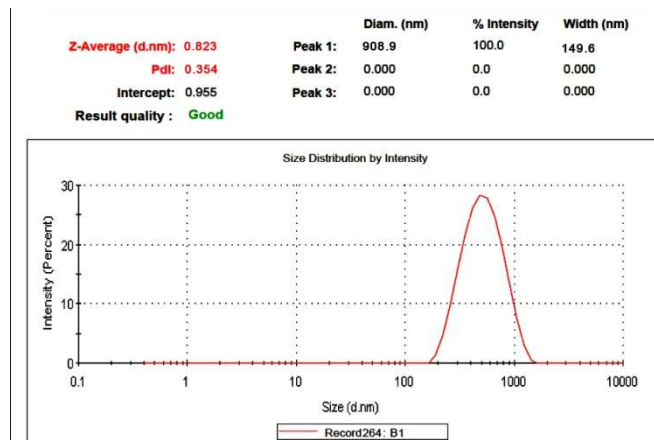


Fig. 5. Particle size of optimized liposome (BL10).

Zeta potential

Zeta potential of liposome ensures stability and entrapment efficiency and also used to predict *in vivo* behavior (Maherani *et al.*, 2012). Entrapment efficiency was increased due to electrostatic attraction between charged molecule and liposomes. Any subsequent modifications of the liposomal surface, such as cholesterol incorporation, also influence zeta potential. The higher values of zeta potential enhance the stability of liposome by increasing the repulsion of vesicle, and thereby preventing aggregation. Liposome prepared by using different lipids acquires different surface charge. Liposome employing phosphatidylserine, stearylamine or dioleoyltrimethylammonium propane and phosphatidylcholine get negative, positive and neutral charge respectively (Brgles *et al.*, 2008). On the contrary, in present study liposome prepared with phosphatidylcholine possess slightly negative charge (-1.93 mV) (Fig. 6). It may be due to the effect of cholesterol on surface charge.

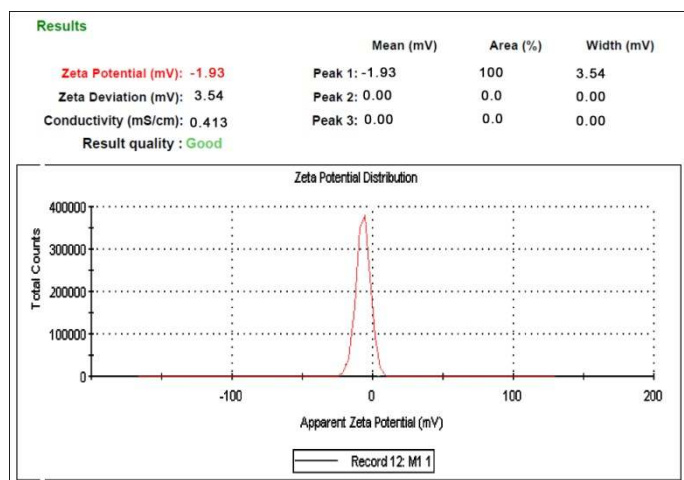


Fig. 6 Zeta potential of optimized liposome (BL10).

Entrapment efficiency

Drug can be incorporated into liposome by several ways depending on various properties like polarity and solubility. It can be adsorbed on surface of membrane, entrapped in lipid bilayer, encapsulated in inner aqueous core, attached between polar head or supported by a hydrophobic tail (Maherani *et al.*, 2011). Method of preparation and composition of lipid can also influence the entrapment efficiency. The present study shows 78.43% entrapment efficiency indicating good electrostatic interaction between bioactive agent and liposomes.

In vitro diffusion study

Release characteristics of BER from liposome was evaluated *in vitro* and compared to that of pure drug. It was observed that the release of BER suspension was completed within 10 h while liposomal formulations shows 70% release within 24 h (Fig. 7). This results supported support by the fact that the layer of drug-encapsulated liposomes attached to the semi-permeable membrane breaks and leaches its contents slowly before

another layer replaces the leached vesicles. Due to this mechanism controlled release of drug in liposomes can be expected over a prolonged period of time.

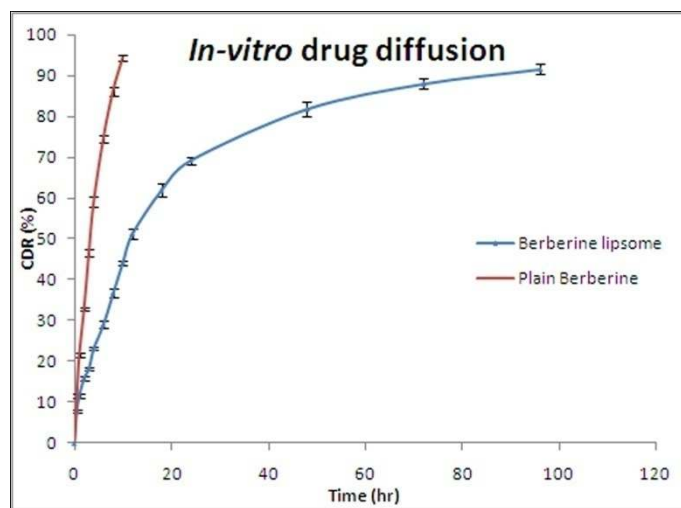


Fig. 7 *In vitro* drug diffusion of berberine loaded liposome and plain drug.

Stability Study

Stability study reveals considerable drug loss (approx. 12%), was marked from formulation storage at high temperature, i.e., $37 \pm 2^\circ\text{C}$. On contrary, formulation stored at $4-8^\circ\text{C}$ and $25 \pm 2^\circ\text{C}$, could retain 93% and 97% of the entrapped drug, respectively. Substantial loss of drug at high temp may be due to the deprivation of phospholipids leads to disturbance in packing of membrane. In addition, high temperature also cause change in gel to liquid transition of lipid bilayer. The results of the study indicate that the development of BER loaded liposome can overcome the limitation of the molecule related to poor oral absorption and can enhance the bioactivity of the BER.

CONCLUSION

In this study, 3^2 full factorial designs were used for predicting the optimum condition for preparation of liposome. The formulations were successfully prepared by thin film hydration method to observe the effect of drug:lipid and soyphosphatidylcholine:cholesterol ratio on vesicle size and entrapment efficiency. Increase in lipid concentration was found to produce liposome with highest entrapment efficiency. On the other hand, decrease in SPC concentration produce smaller vesicle. These effects were fitted into polynomial model to identify the significant effects of independent variables on response and visually observed by contour plot and response surface (3D) plots. The effectiveness of experimental design was confirmed by close agreement of experimental value with estimated value of optimized formulation prepared in accordance with desirability value. Thus, 3^2 full factorial design with desirability function is an effective means to optimize berberine loaded formulations.

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How to cite this article:

Girish Sailor, A K Seth, Ghanshyam Parmar, Sachin Chauhan, Ankur Javia. Formulation and *in vitro* evaluation of berberine containing liposome optimized by 3² full factorial designs. *J App Pharm Sci*, 2015; 5 (07): 023-028.