Iranian Journal of Pharmaceutical Sciences
2018: 14 (2): 45-54
www.ijps.ir

Original Article

Investigation of the Effect of Gammaoryzanol Addition on the Properties of the Nanoliposomes by using Response Surface Methodology: Preparation, Characterization and Optimization

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Abstract

Liposomes are the most important lipid-based nanocarriers which are used for encapsulation of both hydrophilic and hydrophobic active compounds. The aim of the present study was to investigate the effect of addition of Gammaoryzanol (GO) in the lipid bilayer formulation on the liposomes characteristics prepared by a modified ethanol injection method. GO bearing nanoliposomes were prepared with different phosphatidylcholine (PC): GO molar ratios, which were selected as the independent variables. Subsequently, the particle size (PS), encapsulation efficiency percent (EE%), and drug release over 24 h (D_{24h}), which were considered as dependent variables, were analyzed and optimized by employing response surface methodology (RSM). Graphical response surface and contour plots were drown to understand the interaction effects of different variables. Finally, the optimum points for the variables levels were obtained from the optimization plot. The mean PS, EE% and D_{24h} of Celecoxib-loaded nanoliposomes were found as 102.6 ± 9.5 nm, 67.6 ± 11.2 %, and 53.97 ± 9.6 %, respectively. The results indicated that both PC: GO and PC: Drug ratio were the important contributing variables for PS, and EE% of nanoliposomes; however, only PC: GO had effect on D_{24h} ($P<0.05$). The optimum formulation of Celecoxib nanoliposomes, where PC: GO and PC: Drug ratio were 4.6 and 3, have PS = 102 nm, EE% = 74.2 % and D_{24h} = 59.9 %. In conclusion, addition of GO and using ethanol injection method besides RSM techniques presented a simple, rapid, and beneficial approaches for preparation of nanoliposomes with optimum characteristics.

Keywords: Gammaoryzanol, nanoliposomes, Response surface methodology, Ethanol injection method

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Cite this article as: Esmaeli T, Barati M, Hayatdavoudi P, Tila D, Ghanbarzadeh S, Investigation of the Effect of Gammaoryzanol Addition on the Properties of the Nanoliposomes by using Response Surface Methodology: Preparation, Characterization and Optimization, 2018, 14 4): 45-54

1. Introduction

Antioxidant agents are mainly used in pharmaceutical and cosmetic formulations to prevent auto-oxidative deterioration of lipidic raw materials and also was used as free radicals scavenger [1]. Gammaoryzanol (GO) has been reported to possess some health-beneficial properties including plasma lipid pattern improvement ability, total plasma cholesterol reduction capability, and platelet aggregation inhibitory effect. Furthermore, GO has been proposed as a natural antioxidant to improve the stability of food, as a UV-A filter in sunscreen cosmetics and also as antioxidant agent for pharmaceutical purposes [2, 3]. Celecoxib (CLX), a selective Cyclo-oxygenase-2 (COX-2) inhibitor and non-steroidal anti-inflammatory drug is a highly lipophilic (log P = 3.68) and poorly water soluble (3–7 μg/mL) drug. The amide group in GO structure gives a weak acidic property with pKa value of about 11 [4]. Liposomal formulations are used in pharmaceutical field as drug delivery systems due to their biodegradability, biocompatibility and low toxicity due to their capability of entraping both lipophilic and hydrophilic drugs. On the other hand, these formulations have been used in different forms such as oral, parenteral, and topical [5]. The fundamental properties important in liposomal formulations are size, encapsulation efficiency percent (EE %) and drug release rate, which are all dependent on the amount and structure of the membrane ingredients used. Therefore, for efficient encapsulation and optimum release it is requred to figure out the molecular interactions of the liposomal membrane composition [6]. Optimization of liposome composition *in vitro* is the initial step of developing a liposomal formulation for bioactive agents' delivery. However, it is very difficult to study the effect of several variables and interactions between these variables by a conventional technique. Factorial design is an important statistical tool to study the effect of several factors influencing responses by varying them concurrently and running limited number of tests. Response surface methodology (RSM) is a collection of mathematical and statistical methods, which calculates the functional correlation between a number of measured response variables and several explanatory factors to obtain an optimal response by using a series of experiments [7]. The main advantage of RSM is decrease of the experimental runs necessary and it is already widely applied to optimize formulation design in pharmaceutical studies. The desired

objectives can be reached by systematic formulation method in the direct possible time. It reduces the number of experimental runs necessary to establish a mathematical tendency in the experimental design allowing to the determination of the optimum level of experimental factors mandatory for a given results [8]. The literature review showed that there is no research to clarify the effect of existence and concentration of GO on the physical properties of the prepared vesicles. Therefore, in this study we aimed to investigate the effect of different molar ratio of GO in the lipid bilayer of liposomes on the particle size, encapsulation efficiency and drug released over 24 h of CLX, by a systematic statistical survey using RSM.

2. Materials and Methods

2.1. Materials

Soya Lecithin / Soybean Phosphatidylcholine (PC 98%) was supplied from Lipoid Company (Germany). GO was received as a gift from Tsuno Rice Fine Chemicals Company, (Japan). Celecoxib was kindly provided by Zahravi Pharmaceutical Company. (Iran). All of the solvents and chemicals were of the HPLC and analytical grades.

2.2. Preparation of Liposomes

Liposomes were prepared by a modified ethanol injection method as described in our previous paper. In brief, required amount of soya lecithin and GO (Table 1) were dissolved in ethanol containing CLX and slowly injected into double distilled water under high speed (20000 rpm) homogenization (DIAX 900, Heidolph, Germany) for 10 min.

2.3. Characterization of Liposomes

2.3.1. Particle Size Analysis

Particle size distribution of formulations was analyzed using Dynamic Light Scattering (DLS) system and reported as intensity-weighted average (z average) and the polydispersity index (PDI), which quantifies size and distribution width, respectively. Zeta potential of prepared nanoethosomes was also analyzed by the same system (Nano ZS, Malvern, UK). All measurements were performed in triplicate.

2.3.2. The Encapsulation Efficiency

The encapsulation efficiency percent (EE %) of incorporated CLX in the nanoliposomes was calculated by using following equation:

$$EE\% = [(W_{(\text{Total added CLX}} - W_{(\text{Free CLX})} / W_{(\text{Total added CLX})}] \times 100$$

Where, $W_{(\text{Total added CLX})}$ is the amount of initial administered CLX used for the preparation of nanoliposomes, $W_{(\text{free CLX})}$ is the amount of un-loaded CLX, measured in the lower chamber of Millipore Amicon® filter after (100K, Millipore, Billerica MA, USA) centrifugation. One mL of the formulation was diluted with 4 mL of water (to dissolve possible non-entrapped CLX) and then placed in the upper chamber of Amicon® centrifugal filter and centrifuged (Hettich EBA 20, Germany) at 25 °C and 3000 rpm for 10 min. One mL of filtrate in the lower chamber of centrifugal filter was withdrawn and the amount of non-entrapped CLX was

determined by measuring the absorbance of CLX spectrophotometrically (Shimadzu 8400 S, Japan) at 254 nm.

2.3.3. In Vitro Drug Release

After separation of un-entrapped drug, the dialyzer having 1 mL of nanoliposome dispersion was placed in the center of the beaker containing PBS (pH 7.4) at 37 ± 2 ºC. Dissolution sample of 2 mL was withdrawn after 24 h from the beaker and were analyzed spectrophotometrically (Shimadzu 8400 S, Japan) at 254 nm employing the previously prepared standard curve (linear in the range of 2-30 µg/mL, r^2=0.9999).

2.4. Effect of Variables Analysis

In many processes, the relationship between the response and the independent variables is usually unknown. RSM is a collection of useful mathematical and statistical techniques for analyzing the effects of several independent variables. To study the effect of variables on nanoliposomes characteristics, different batches were prepared using 3^2 factorial design. Ratio of PC: GO and lipid: drug (L: D) were selected as two independent variables. The amount of CLX, volume of ethanol, temperature as well as homogenizer speed were kept constant. Particle size, EE%, and amount of released drug over 24 h were selected as dependent variables and three levels were chosen for each factor, which were defined on the basis of the single factor experiment result and possibility of preparing liposome samples at the maximum and minimum levels. Design of factors and levels of all variables were as shown in table 1.

2.5. Response Surface Methodology Approach for Optimization of Factors

For better understanding and visualization, different graphic plots such as response surface plot, counter plot and overlaid plot were constructed using Minitab 15 software from RSM approach. In the overlaid plot, the un-shaded region represents the ranges of variables levels which resulted in the desired responses. Following these preliminary screening experiments, optimization of formulation was also carried out and suggested formulation based on optimization plot was prepared. Validity assessment of the obtained equations and suggested optimized formulation was performed by calculating the mean percent error between calculated and observed values. Correlation coefficients were checked to evaluate the suitability of the model in the significance level of 0.05.

3. Results and Discussion

3.1. Experimental Design and Data Analysis

RSM was employed to investigate the relationship between the influencing factors and characteristics of prepared liposomes by modified ethanol injection method. In all cases, the number of experiments and their factor combinations allowed the measured responses to be fitted by stepwise method to initial lineal polynomial models using coded factors and the final equations are simplified

models that contain only significant parameters ($P<0.05$). All results were evaluated by analysis of the variance (ANOVA).The results for particle size, EE % and D_{24h} of the nine suggested batch by the 3^2 full factorial designs are illustrated in Table 1.

3.2. Effect of Variables on Particle Size, Encapsulation Efficiency and in Vitro Drug Release

As shown in table 1, the size of nanoliposomes was in the range of 89 to 118.7 nm, which by considering the speed and steps of preparation method, without using extrusion and sonication techniques, was found as a great advantage for this method in comparison with other preparation approaches. The range of EE % of prepared liposomes was 51.7 - 84.0 %, which showed that using GO instead of cholesterol in the bilayer of liposomes resulted in high drug encapsulation which is reported for the first time in this study.

Response surface regression analysis using data in uncoded units was employed to determine the optimum conditions and interaction effects of L/GO and L/D on the particle size, EE% and D_{24h}, and the estimated regression coefficients were obtained as well. Obtained equations for particle size, EE% and D_{24h} were as follow:

$$Y_{PS} = 152.325 - 23.990\ X_1 - 26.147\ X_2 + 3.576 X_1 X_1 + 7.128\ X_2 X_2$$

$$Y_{EE\ \%} = 62.496 + 4.1237\ X_1 - 8.606\ X_2$$

$$Y_{D24h} = 49.906 - 3.293\ X_1$$

Negative sign indicates the negative effect of the parameter on the responses, and coefficient values exhibit the magnitude of variables effect on them. Coefficient values show that X_2 has maximum effect on particle size and EE% and accordingly, only X_1 has negative effect on D_{24h}.

Obtained equations for each three responses were used to calculate the results of prepared formulations. The mean percentage error (MPE) percent is the computed average of percentage errors between predicted by a model (using the obtained equations) and actual values of the responses and was obtained using the following equation:

$$\text{MPE\%} = \frac{\left|\text{Calculated} - \text{observed}\right|}{\text{observed}} \times 100$$

Table 1. Particle size (PS), encapsulation efficiency percent (EE%) and released drug over 24 hrs (D_{24h}) values for nine prepared batches. Data are presented as mean ± SD.

FORMULATION CODE	L: GO	L: D	PS (NM)	EE%	D_{24H} (%)
F1	1	1	113.8 ± 8.5	59.9 ± 5.5	47.84 ± 3.1
F2	5	3	105.9 ± 11.7	78.3 ± 4.5	62.49 ± 4.9
F3	1	3	118.7 ± 9.7	51.7 ± 3.9	41.29 ± 3.9
F4	3	3	96.74 ± 7.9	63.9 ± 4.8	51.06 ± 4.0
F5	3	2	89.35 ± 8.6	66.9 ± 3.6	53.39 ± 5.1
F6	5	2	98.48 ± 9.1	79.2 ± 5.0	63.27 ± 4.8
F7	1	2	105.7 ± 12.5	54.5 ± 4.1	43.51 ± 4.0
F8	5	1	101.6 ± 6.9	84.0 ± 6.1	67.04 ± 5.6
F9	3	1	93.09± 7.4	69.9 ± 5.6	55.83 ± 3.7

Table 2. Mean percentage error (MPE) for particle size (PS), encapsulation efficiency percent (EE%) and drug released during 24 hrs (D_{24h}).

RESPONSE		PS	EE%	D_{24H}
MPE	**Train set**	7.41	6.78	8.98
	Test set	9.54	11.45	12.34

To evaluate the validity of these equations, three formulations were prepared and MPE values for each response were calculated and illustrated in table 2.

3.3. Graphical Plots for the Effects of Variables on the Responds

For better visualization of the effect of variables on responses, graphical plots were constructed using RSM approach. Graphical presentation of the data is helpful in learning about both main and interaction effects of the independent variables.

The effects of L:GO and L:D molar ratios on particle size, EE% and D_{24h} are illustrated using surface plot and counter plots (Figure 1, 2 and 3, respectively). Figure 1 shows that increasing L:GO and L:D ratios decreased particle size from 118.7 to 93.09 nm ($P<0.05$).

In figure 2 it is revealed that increasing L:GO ratio from 1 to 5 increased the EE% value and L/D value had negative effect on EE% ($P<0.05$). It is indicated that addition of lower amount of GO resulted in high encapsulation of cargo which by considering the low cost of GO is a valuable results.

Correspondingly, results indicated that decreasing L/GO value decreased D_{24h} value ($P<0.05$) and changing L/D ratio had no significant effect on D_{24h} (Figure 3). It was showed that high amount of GO in the bilayer decreased the release rate of encapsulated drug and could be modified the release of drug from liposomes.

To investigate the overall effects of variables on nanoliposomal formulation,

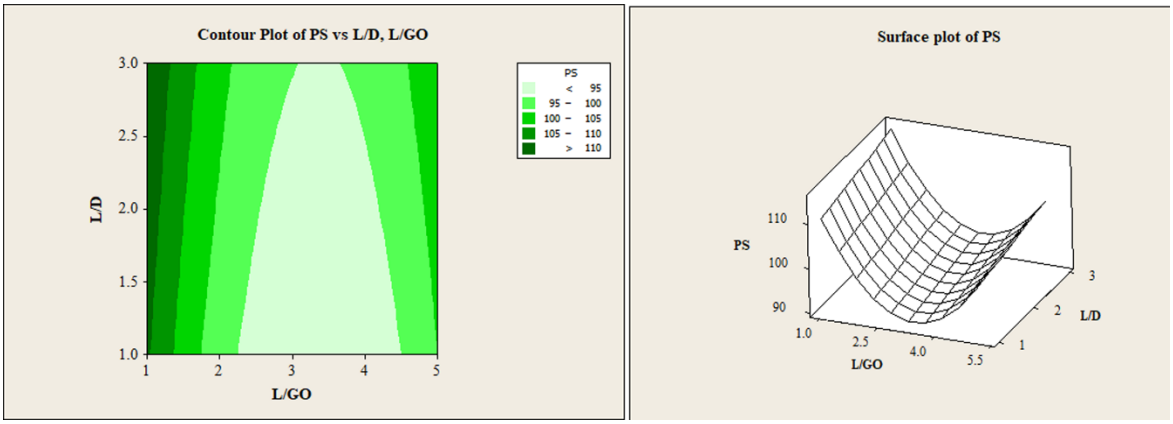

Figure 1. Response surface plot and counter plot for the effect of the lipid/Gammaoryzanol (L/GO) and lipid/Drug (L/D) on particle size.

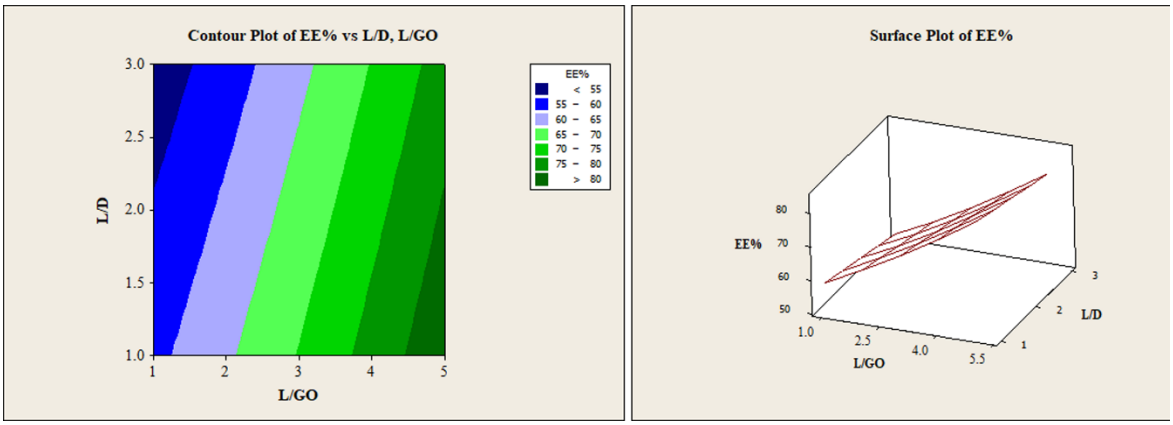

Figure 2. Response surface plot and counter plot for the effect of the lipid/Gammaoryzanol (L/GO) and lipid/Drug (L/D) on encapsulation efficiency percent of liposomes

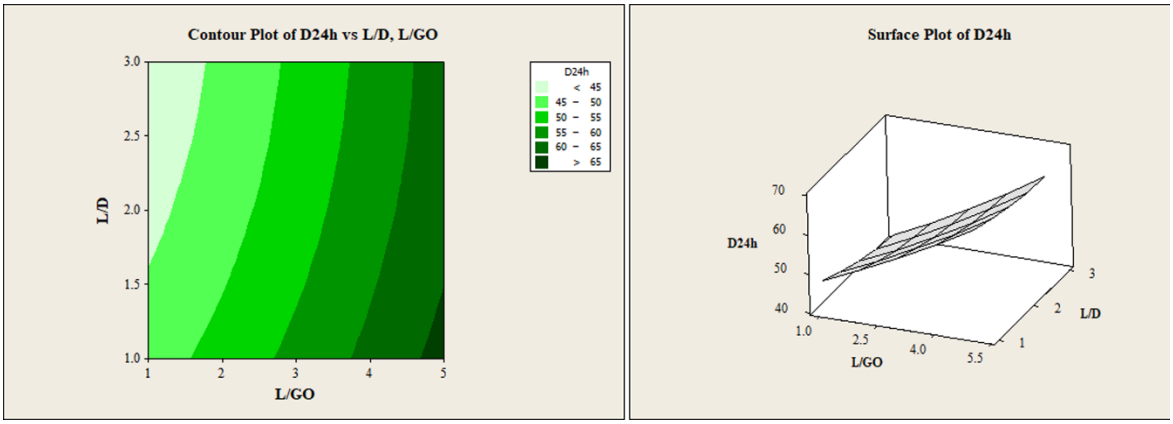

Figure 3. Response surface plot and counter plot for the effect of the lipid/Gammaoryzanol (L/GO) and lipid/Drug (L/D) on drug released during 24 hrs (D_{24h}).

desired ranges of each response were input to Minitab software and overlaid contour plot was obtained (Figure 4). White area shows the ranges of independent variables to produce liposome with particle size, EE%, and D_{24h} values in desired ranges of 90-110 nm, 70-80% and 50-60%, respectively.

3.4. Optimization Plot

Optimization plot to obtain desired responses was designed and the proposed levels of variables to form optimum formulation were found as follow: L/GO= 4.6 and L/D= 3 (Figure 5), which were in the white area of the overlaid plot (Figure 4). This proposed formulation was prepared and observed responses (particle size, EE% and D_{24h}) values were compared with calculated values and the MPE% for particle size, EE% and D_{24h} were found 9.54%, 11.45% and 12.34%, respectively, which were in acceptable range (train set <10% and test set <20%).

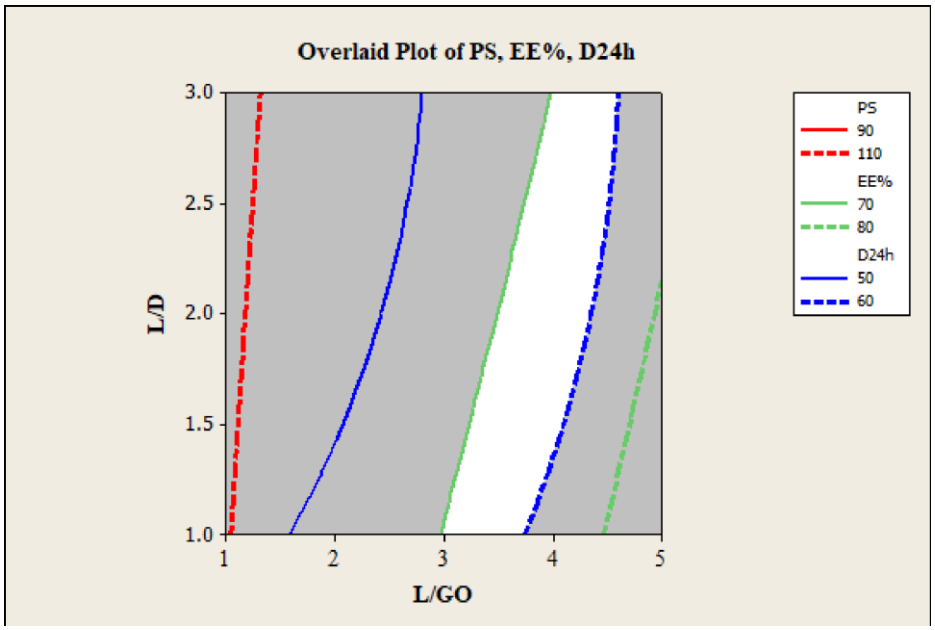

Figure 4. Overlaid contour plot for desired ranges of particle size (PS), encapsulation efficiency percent (EE %) and drug released over 24 hrs (D_{24h}).

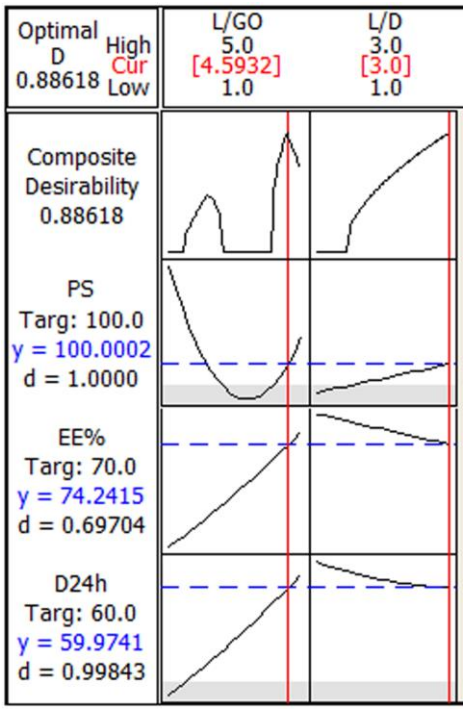

Figure 5. Optimization plot to produce formulation with desired particle size (PS), encapsulation efficiency percent (EE%) and drug released during 24 hrs (D_{24h}).

4. Conclusion

RSM is a rapid technique which widely used to empirically derive a functional relationship between experimental responses and a set of input variables. Statistical techniques allow researchers to evaluate more than one independent variable at a time and are useful and powerful tool for the estimation of the effects of the factors on the responses. This study demonstrates the use of factorial design and RSM for the preparation of CLX-loaded nanoliposomes with desired characteristics in the case of particle size, encapsulation efficiency and released drug over 24 h. Prepared nanoliposomes characteristics were found to be influenced by lipid/GO and lipid/drug ratio of bilayer structure. Experimental results demonstrate that the observed responses were in close agreement with the predicted values and this demonstrates the reliability of the optimization procedure in prediction of PS, EE % and D_{24h} in CLX-loaded nanoliposomes preparation. Preparation of nanoliposomes using RSM was found to be well suited and provides a prediction model for the response of the variables range and the optimum conditions in order to achieve the highest performance. Furthermore, ethanol injection method was shown as a very easy method for preparing liposomes and its simplicity and reproducibility make it suitable for producing vesicles in large industrial scale.

Acknowledgments

We thank the Research Deputy of Zanjan University of Medical Sciences (ZUMS) for financial support of these projects (Grants No. A-12-1077-3 and A12-1077-5).

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