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Sirolimus Nano Liposomes

Optimization of sirolimus nano liposome prepared by modified ethanol injection method using response surface methodology

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ABSTRACT

The aim of the present study was to apply experimental design methodology in the development and optimization of sirolimus liposomes employing a modified ethanol injection method. Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (Chol) as well as dioleoylphosphoethanolamine (DOPE) were utilized in the preparation of liposomes. Particle size (PS), encapsulation efficiency percent (EE%) and cumulative percent of drug release in 24 h (D_{24h}) were investigated. Analysis of drug was performed using a validated RP-HPLC method. The response surface methodology, utilizing the polynomial equations, graphical plots and response optimization were used to evaluate the effects of each formulation variables on the responses and prediction of the optimal formulation for sirolimus liposomes as well. DPPC/Chol and DOPE/DPPC molar ratios were selected as independent variables and correspondingly, results of PS, EE% and D_{24h} were considered as responses of the experimental design. Results ranges for PS, EE% and D_{24h} were 89-200 nm, 63.3-97.4 % and 13.18-22.86 %, respectively. The effects of DPPC/Chol and DOPE/DPPC molar ratios on D_{24h} were significant, but in the case of PS and EE%, only the DPPC/Chol molar ratio had a significant effect ($P< 0.05$). Therefore based on the obtained results, the ethanol injection method could be a rapid, simple and inexpensive method for the preparation of liposomes.

KEY WORDS

- Ethanol injection
- Factorial design
- Liposome
- Response surface methodology
- Sirolimus

ZUSAMMENFASSUNG

Sirolimus-Nanoliposomen/Optimierung von durch eine modifizierte Ethanolinjektions-Methode hergestellten Sirolimus-Nanoliposomen unter Verwendung einer Reponse Surface-Methode

Ziel der vorliegenden Studie war die Anwendung von Experimentdesign in der Entwicklung und Optimierung von Sirolimus-Liposomen unter Verwendung einer modifizierten Ethanolinjektions-Methode. Dipalmitoylphosphatidylcholin (DPPC), Cholesterol (Chol) und Dioleoylphosphoethanolamin (DOPE) wurden zur Herstellung von Liposomen verwendet. Untersucht wurden Partikelgröße (PS), prozentuale Verkapselungseffizienz (EE%) und kumulative prozentuale Wirkstofffreisetzung in 24 h (D_{24h}). Die Wirkstoffanalyse erfolgte mit einer validierten RP-HPLC-Methode. Die Wirkungen der Formulierungsvariablen auf Ansprechverhalten und Vorhersage der optimalen Zubereitung für Sirolimus-Liposomen wurden mit Hilfe der Reponse Surface-Methode bewertet unter Verwendung polynomer Gleichungen, graphischer Plots und Responsoptimierung. Die molaren Verhältnisse DPPC/Chol und DOPE/DPPC wurden als unabhängige Variablen ausgewählt. Deshalb wurden die Ergebnisse von PS, EE% und D_{24h} als Reaktion des Experimentdesigns betrachtet. Die Ergebnisse für PS, EE% und D_{24h} betrugen 89 – 200 nm, 63.3 – 97.4 % und 13.18 – 22.86 %. Die Wirkungen der molaren Verhältnisse von DPPC/Chol und DOPE/DPPC auf D_{24h} waren signifikant, aber im Fall von PS und EE% zeigte nur das Molverhältnis DPPC/Chol signifikante Wirkung ($P< 0,05$). Die Ergebnisse deuten darauf hin, dass die Ethanolinjektions-Methode eine schnelle, einfache und kostengünstige Methode zur Herstellung von Liposomen sein könnte.

1. Introduction

Sirolimus (Rapamycin, SRL, CAS 53123-88-9) is a macrolide lactone produced by *Streptomyces hydroscopicus*. It is an immunosuppressive agent used for the prophylaxis of renal allograft rejection. SRL was first isolated from a soil sample from Rapa Nui, an island in the south Pacific. SRL unlike tacrolimus, cyclosporine, mycophenolate and other immunosuppressants has a unique mechanism of action involving the suppression of T-lymphocyte proliferation through inhibition of rapamycin protein kinase complex target.

Since 1999 SRL has been approved by the FDA and now it is available as tablet, suspension and stent [1–6]. Liposomes are vesicles consisting of phospholipid bilayers which enclose aqueous compartments and are utilized as delivery systems for both water and lipid soluble drugs such as peptides, proteins, RNA and DNA [7–9]. Liposomes are biodegradable and biocompatible structures, and their composition and properties can be finely modulated to improve their interaction and penetration through cell membranes. Liposomes are usually prepared by amphipathic phospholipids, and in most cases cholesterol is included to increase the stability of phospholipid bilayers and decrease the leakage of liposomes. The interactions between the liposomal vesicles and cells can occur via one or more processes, such as stable physical adsorption, endocytosis, lipid exchange and fusion. Fusogenic liposomes can be prepared by incorporating special lipids, which are capable of undergoing a bilayer-to-hexagonal II transition, such as dioleoylphosphatidylethanol-

amine (DOPE). They make vesicles more fluid and capable to promote the destabilization of biological membranes and hence they can deliver their contents into the cytoplasm by fusing with cellular membrane [10–12]. Liposomes can be made by several different methods, using different lipid compositions which both greatly influence the characteristics of liposomes including drug entrapment, drug release rate and particle size of liposomes. The most commonly used methods for liposomes formation are the dry-film method, reverse phase evaporation method, supercritical fluid method and ethanol injection method [13–18].

Reducing the number or cost of experiments is beneficial and important for the development as well as optimization of pharmaceutical formulations. Statistical experimental design, also called design of experiments (DoE), is a well-established concept in experiment planning. Factorial design is an efficient tool to obtain an appropriate mathematical model with minimum experiments for optimization of formulation. Studies based on factorial design allow all factors to be varied simultaneously, thus enabling the evaluation of each variable's effect and also the interrelationship between them [19–21]. Response surface methodology (RSM) is a rapid technique used to empirically derive a functional relationship between experimental responses and a set of input variables. It reduces the number of necessary experimental runs and allows for determination of the optimum level of experimental factors required to achieve desired responses. Reduction in the number of experiments by optimizing formulation during development of a drug delivery process results in significant decrease in production costs [22, 23]. In the present study, the conventional and fusogenic liposomes prepared by modified ethanol injection method were investigated in regard of particle size (PS), encapsulation efficiency percent (EE %) and drug release rate. The liposomes were composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol) and DOPE. A 3^2 full factorial design and central composite design (CCD) from RSM approach were used to evaluate the influence of different lipid content on the characteristics and *in vitro* drug release of SRL liposomes.

2. Material and methods

2.1 Material

Sirolimus was obtained from Poli Company (Lazio, Italy). DPPC and DOPE were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was provided by Merck (Darmstadt, Germany). HPLC grade solvents and analytical grade chemicals were obtained from Merck (Darmstadt, Germany).

2.2 Methods

2.2.1 Liposome preparation

The ethanol injection method was used for liposome formation. After dissolving different proportions of lipids and SRL in ethanol (in concentration of 30 µmol lipid/mL and 500 µg/mL SRL, respectively), 10 mL of ethanolic solution were slowly (during 5 min) injected into 90 mL of distilled water under homogenizer mixing at 10,000 rpm of homogenizer speed (Heidolph, Germany).

2.2.2 Statistical design

A 3^2 full factorial design was applied to examine the individual and combined effects of two formulation variables, each at three levels, and the nine proposed SRL liposome formulations were prepared as well (Table 1). Experimental factors and factor levels were determined based on preliminary studies. The molar ratios of DPPC/Chol (X1) and DOPE/DPPC (X2) were taken as independent variables. The encapsulation efficiency percent, particle size and cumulative release percent in 24 h were considered as dependent variables or responses. The order of the experiments was randomized to avoid any bias. All other parameters (temperature, homogenizer speed, amount of ethanol, lipid concentration, etc.) were kept constant to minimize fluctuations.

For better understanding and visualization, different graphic plots such as response surface plot, counter plot and overlaid plot using Minitab 17 software from the RSM approach were constructed. In the overlaid plot, the unshaded region represents the ranges of variables levels which resulted in the desired responses. Following these preliminary screening experiments, optimization of formulation was carried out and suggested formulation based on optimization plot was prepared. Assessing the validity of 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.

2.2.3 Particle size measurement

The liposome size was measured using a Shimadzu SALD series (Kyoto, Japan) laser diffraction particle size analyzer. All measurements were performed in triplicate.

2.2.4 Encapsulation efficiency determination

Liposome suspension is a mixture of drug-encapsulated liposomes and unencapsulated drug. Methods for determination of the amount of encapsulated drug within liposomes typically include the destruction of the lipid bilayer and subsequent quantification of the released drug. In the present study, the liposome encapsulation efficiency was determined using the dynamic dialysis method at a temperature less than the glass transition temperature (Tg) to separate unencapsulated drug and subsequently liposome disruption by methanol. Encapsulation efficiency percent was calculated using following equation [24, 25]:

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

where C_{total} is the total drug concentration which was added and C_{free} is the unentrapped drug concentration.

2.2.5 *In vitro* drug release study

The drug release study was conducted immediately after separation of free SRL from liposomes. One ml of liposomal suspension was placed in the dialyzer and 250 mL of distilled water was used as dissolution medium (37±2 °C). Samples (2 mL) were collected at predetermined time intervals, replaced with equal volumes of fresh medium and analyzed with a previously developed RP-HPLC method at λ=278 nm [26]. Drug concentration was calculated using a standard calibration curve (r^2=0.998) and expressed as cumulative percent of drug release. The release study was performed in triplicate.

3. Results

The results for particle size, EE% and D_{24h} of the nine suggested batches by the 3^2 full factorial designs are shown in Table 1.

■ Tab. 1 ■

The sizes of the liposomes were in the range of 89 to 200 nm. Considering the preparation of liposomes without using the extrusion method, it could be a great advantage for this method. The range of EE% of the pre-

pared liposomes was 63.3-97.4 %, which was predictable due to the high lipophilic property of SRL. Drug release profiles for nine formulations are illustrated in Fig. 1. All formulations released less than 22.86 % of SRL during 24 h.

■ Abb. 1 ■

Response surface regression analysis using data in uncoded units was employed to determine the optimum conditions and interaction effects of DPPC/Chol and DOPE/DPPC on the particle size, EE% and D_{24h}. The estimated regression coefficients were obtained as well.

Obtained equations for particle size, EE% and D_{24h} were as follows:

$Y_{PS} = 212.429 - 21.558\ X_1 - 12.683\ X_2 - 0.687\ X_1X_1 + 19.6\ X_2X_2 + 3.25\ X_1X_2$

$Y_{EE\,\%} = 103.91 - 7.292X_1 - 2.45X_2 - 0.164X_1X_1 - 0.733\ X_2X_2 + 0.65\ X_1X_2$

$Y_{D24h} = 20.802 - 2.847X_1 + 4.875\ X_2 + 0.277X_1X_1 - 0.795\ X_1X_2$

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_1 has maximum effect on particle size and EE% and accordingly, X_2 has maximum effect on D_{24h}. Besides X_1 and X_2 have negative effects on particle size as well as EE%; however, X_2 has positive effect on D_{24h}.

The obtained equations for each three responses were used to calculate the results of prepared formulations. The mean percentage error (MPE) 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:

$$MPE = \frac{calculated - observed}{observed} \times 100$$

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

■ Tab. 2 ■

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

The effects of DPPC/Chol and DOPE/DPPC molar ratios on particle size, EE% and D_{24h} are illustrated via surface plot and counter plots (Fig. 2, 3 and 4, respectively). Fig. 2 shows that although increasing DPPC/Chol ratio decreased particle size from 200 to 89 nm, increasing in DOPE/DPPC ratio had not significant effect on particle size ($P > 0.05$).

In Fig. 3 it is revealed that changing the DPPC/Chol ratio from 1 to 5 caused a decrease in EE% value from 97.4 to 63.3 %. DOPE/DPPC value similar to particle size had no significant effect on EE% ($P > 0.05$).

Correspondingly, the results indicated that decreasing the DPPC/Chol value and increasing the DOPE/DPPC value increased the D_{24h} value from 13.18 to 22.86 % ($P=0.019$ and $P=0.013$, respectively) (Fig. 4).

To investigate the effect of variables on the SRL liposome formulation, desired ranges of each response were input to Minitab software and an overlaid contour plot

was obtained (Fig. 5). White area shows the ranges of independent variables to produce liposome with particle size, EE% and D_{24h} in desired ranges of 120-160 nm, 80-90 % and 15-20 %, respectively.

An optimization plot for desired responses was designed. The proposed levels of variables to form the optimum formulation were as follows: DPPC/Chol: 2.3 and DOPE/DPPC: 0.6 (Fig. 6). These levels were in the white area of the overlaid plot. This proposed formulation was prepared and observed response values were compared with calculated values employing above mentioned equations. MPE for particle size, EE% and D_{24h} were 12.48, 6.54 and 8.3, respectively.

■ Abb. 2-6 ■

4. Discussion

Several methods of liposome preparation and various lipid compositions could greatly influence the characteristics of liposomes including particle size, drug entrapment and drug release rate. The ethanol injection method is one of the simplest methods for liposome preparation. The force of injection of ethanolic solution and stirring rate of aqueous solution usually lead to complete mixing. Ethanol is diluted almost immediately in medium and phospholipid molecules are dispersed uniformly throughout the medium. Photon correlation spectroscopy of liposomes showed homogeneous nanoliposomes in the size range of 89 to 200 nm without need to extrusion. High mean EE% (79.78±13.65 %) in the present study and previous studies including our previous research (72.6±10.67 %) as well as investigations by Haeri et al. (86.5±6.3), Wang etal. (82.11±2.13 %) and Zhao et al. (90.02±2.25 %) came to the conclusion that high EE% is due to the high lipophilicity and high solubility of SRL in phospholipids (Table 1) [14, 18, 27-30]. Recent researches have also shown that incorporation of cholesterol into liposome formulation increases the rigidity of the bilayer and reduces the drug permeability as well. Consequently the fluidity of liposome bilayers is considered an important factor influencing the drug release rate. High drug entrapment, even in formulations with low cholesterol level, indicates that this method is useful for preparation of cholesterol-free liposomes. Drug release study revealed that the release of SRL from liposomes was very slow and incomplete after 24 h, which is in accordance with previous studies including our previously published paper, where liposomes prepared by thin film hydration method only could release 17.63 % of loaded SRL. Accordingly liposomes prepared by Haeri et al. and Rouf et al. could not release more than 20 % of incorporated drug in 24 h, respectively [14, 24, 27]. Nevertheless, referring to our previous study [24] ■**ok?,** comparing to liposomes prepared by thin film hydration method, the drug release rate was increased significantly ($P< 0.05$). Overall the low rate of SRL from the liposomal formulation is due to the high hydrophobicity of SRL and lipophilic property of the liposomal bilayer. The main shortcoming of this technique is the limitation of solubility of some lipids and drugs in ethanol as well as the volume of ethanol that can be injected into the aqueous medium (maximum 10 % v/

v). Due to these limitations, prepared the liposome suspension is usually relatively diluted. Generally, this method due to the rapidity of process is suitable for thermo sensitive drugs and phospholipids. Furthermore, as it is a simple, inexpensive and rapid method it is suitable for large scale production [31–36]. As a matter of fact, considering the many raw materials and variables in the formulation process whose control and optimization is a complicated process and depends on previous experience and knowledge of the formulator, systematic design of experiments and optimization techniques were utilized successfully to model the relationships between variables and responses. [22, 23, 31, 37, 38]. In this study by using statistical methods, the optimum conditions to achieve desired responses in formulation were achieved and calculated MPE for train and test set were in acceptable ranges (train set < 10 % and test set < 20 %).

5. Conclusion

Numerous published reports indicated that optimization of the ethanol injection method to achieve desired encapsulation efficiency percent, particle size and appropriate release rates requires much experimental work by using traditional trial and error technique. Factorial design approach and response surface methodology were found to be efficient methods to determine the effect of variables and their interaction effects on the responses as well as formulation optimizing with a minimum number of experiments. Factorial design indicated that the evaluated responses were dependent on the DPPC/Chol and DOPE/DPPC molar ratio. Optimization technique was successfully used for preparing of liposomes with desired characteristics. In conclusion, ethanol injection is a very easy method for preparing liposomes. Its simplicity and reproducibility make it suitable for producing nanoliposomes on a large scale.

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Conflict of interest

The authors report no conflicts of interest.

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Table 1

Response [particle size (PS), encapsulation efficiency percent (EE %) and released drug over 24 h (D_{24h})] values of 3^2 factorial design.

Formulation code	DPPC	DOPE	Cholesterol	PS (nm)	PDI	EE (%)	±SD	D_{24h} (%)	±SD
F_1	1	0.5	1	187.3	0.36	94.3	3.6	19.55	2.3
F_2	1	1	1	200.3	0.23	94.1	4.2	22.86	1.6
F_3	1	0	1	193.3	0.45	97.4	2.3	18.41	2.9
F_4	3	1.5	1	159.6	0.41	80.6	3.9	17.16	1.3
F_5	5	5	1	109.3	0.39	62.6	5.6	14.45	2.5
F_6	3	0	1	136.6	0.36	80.3	6.1	15.41	2.6
F_7	5	2.5	1	93.6	0.29	65.8	5.1	14.26	3.1
F_8	3	1.5	1	148.6	0.28	79.6	6.3	15.45	3.6
F_9	5	5	1	89.3	0.26	63.3	6.7	13.18	3.2

Table 2

Mean percentage error for PS, EE % and D_{24h}.

Response		PS	EE %	D_{24h}
MPE	Train set	3.56	3.38	2.26
	Test set	4.86	6.69	5.65

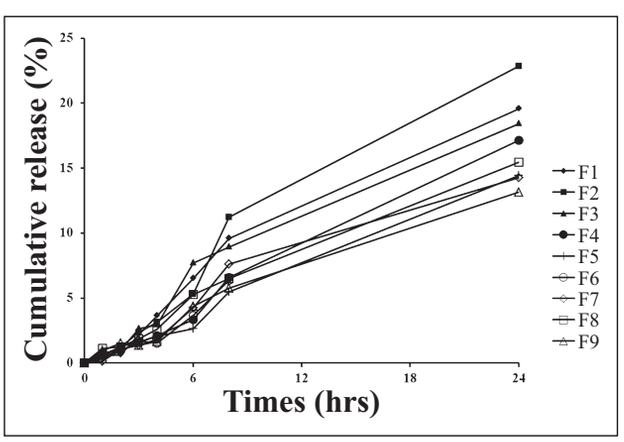

Fig. 1: Drug release profile for nine prepared formulations. Source: all figures by the authors.

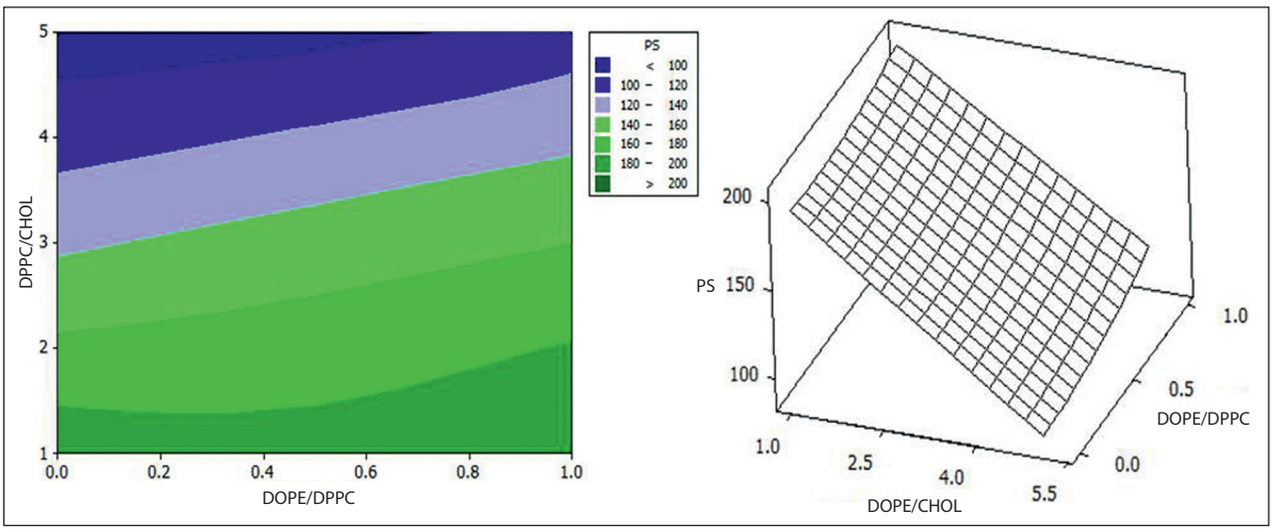

Fig. 2: Response surface plot and counter plot for the effect of the lipid content on particle size (PS).

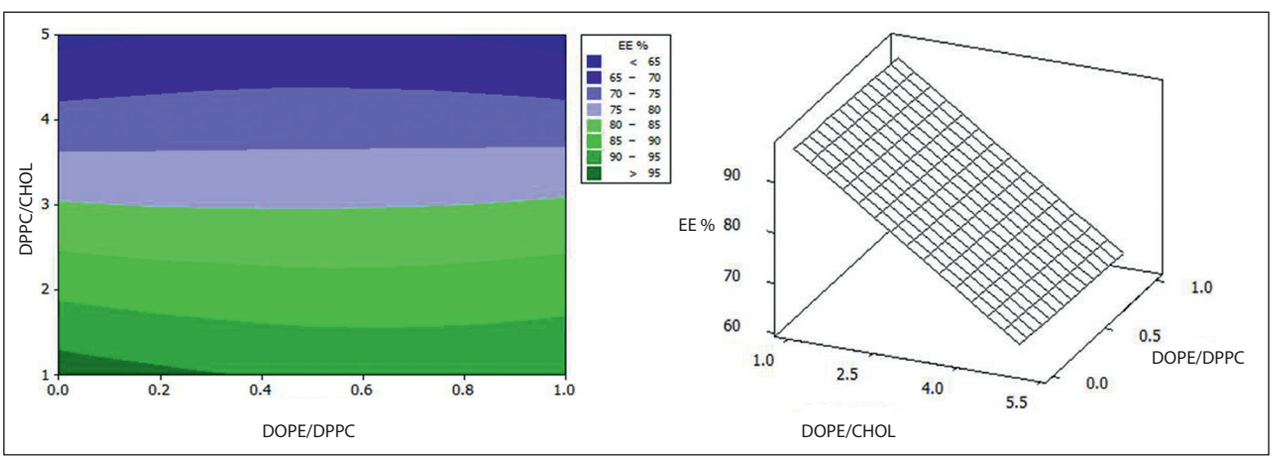

Fig. 3: Response surface plot and counter plot for the effect of the lipid content on encapsulation efficiency percent (EE %).

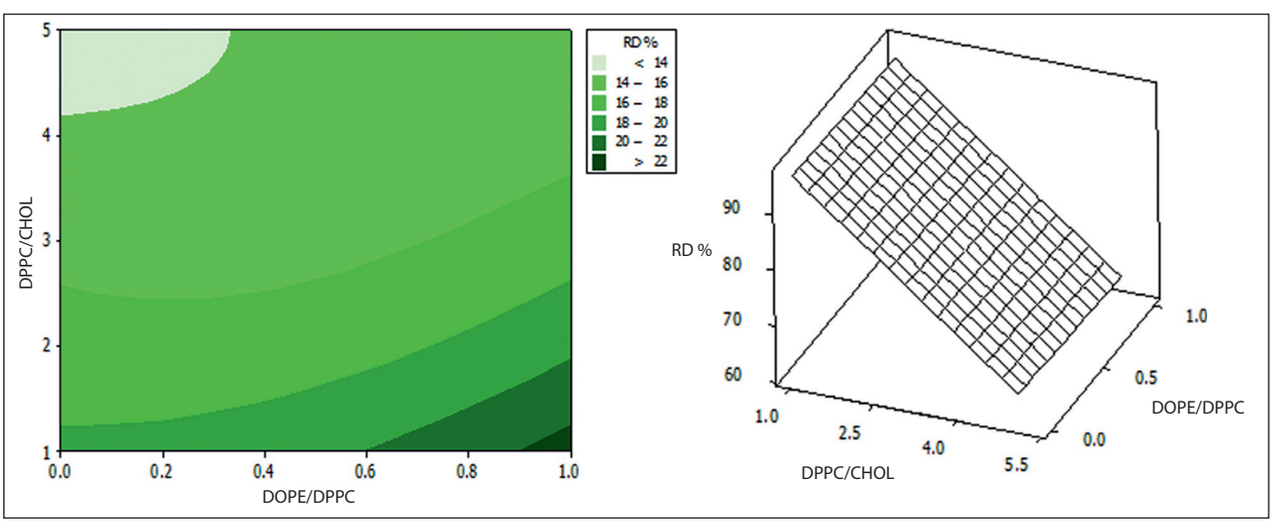

Fig. 4: Response surface plot and counter plot for the effect of the lipid content on drug released (RD %) over 24 h (D_{24h}).

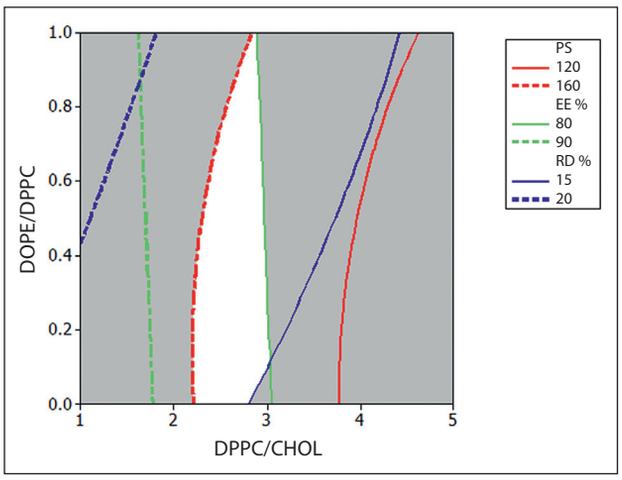

Fig. 5: Overlaid contour plot for desired ranges of particle size (PS), on encapsulation efficiency percent (EE %) and drug released (RD %) over 24 h (D_{24h}).

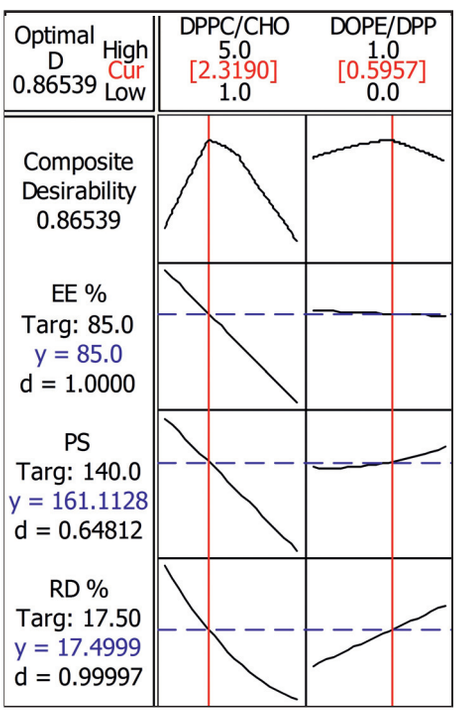

Fig. 6: Optimization plot to produce formulation with desired particle size (PS), on encapsulation efficiency percent (EE %) and drug released (RD %) over 24 h (D_{24h}) values.

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