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Original Article

DESIGN AND OPTIMIZATION OF DOXORUBICIN HCL PRONIOSOMES BY-DESIGN OF EXPERIMENT

SRIKANTH[1*], ANAND KUMAR Y.[2], MALLIKARJUNA SETTY C.[3]

[1*]Research scholar at JNTU Hyderabad and V. L. College of Pharmacy, Raichur, India, [2]Department of Pharmaceutics, V. L. College of Pharmacy, Raichur, India, [3]Department of Pharmaceutics, Oxford College of Pharmacy, Bengaluru, India
Email: srikanthyerigeri@gmail.com

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ABSTRACT

Objective: The present research work was designed to formulate and optimize doxorubicin HCl proniosomes by design of experiment (DoE).

Methods: A 4-factor, 3-level Box-Behnken design was used to explain multiple linear regression analysis and contour 3D plot responses. The independent variables selected were tween 20, cholesterol, hydration volume and sonication time; dependent variables percentage entrapment efficiency (PEE), mean vesicle size (MVS). Based on the Box-Behnken design 29 trial runs were studied and optimized for PEE and MVS. Further "Model F-Value" was calculated to confirm the omission of insignificant terms from the full-model equation to derive a multiple linear regression analysis to predict the PEE and MVS of niosomes derived from proniosomes. 3D plots were constructed to show the influence of independent variables on dependent variables.

Results: PEE of doxorubicin HCl proniosomes was found to be in the range of 40.21-87.5%. The polynomial equation for PEE exhibited a good correlation coefficient (0.5524) and the "Model F-Value" of 7.41 implies the model is significant. P-values less than 0.0500 indicate model terms are significant. The MVS of doxorubicin HCl proniosomes was found to be in the range of 325.2 nm to 420.25 nm. The mathematical model generated for MVS (R2) was found to be significant with model F-value of 54.22. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise (P<0.0500) and R^2 value of 0.9004.

Conclusion: The DoE of Box-Behnken design demonstrated the role of the derived equation, 3D plot in predicting the values of dependent variables for the preparation and optimization of doxorubicin HCl proniosomes. The results suggest that doxorubicin HCl proniosomes can act as a promising carrier.

Keywords: Doxorubicin HCl, Proniosomes, Tween 20, cholesterol, Hydration volume, Sonication time, Box-Behnken design

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INTRODUCTION

In new and existing development of formulations pharmacist generally experiments by a series of logical steps, carefully controlling the different variables and changing one at a time until satisfactory results are obtained. This is how the optimization is done in the pharmaceutical company. Optimization is defined as follows: "Choosing the best element from some set of available alternatives". It is the process of finding the best way of using the existing resources while taking in to the account of all the factors that influence decisions in any experiment. The objective of designing quality formulation is achieved by various Optimization techniques like DoE (Design of Experiment) [1].

DoE is a mathematical tool, for systematically planning and conducting scientific studies that change experimental variables together in order to determine their effect on a given response [2-7]. It makes controlled changes to input variables in order to gain maximum amounts of information on cause and effect relationships with a minimum sample size for optimizing the formulation. There are mainly four steps associated with DoE viz., design of the experiment (By using various models); a collection of the data; statistical analysis of the data and conclusions reached and recommendations made as a result of the experiment. In optimization method various types of the model used from preliminary screening of factors to select their level and for finally study of their effect so it's depend upon the formulator to choose a suitable model for study and help in minimizing the experimenting time and expenses.

An experimental design is a statistical design that advises a set of combination of variables. The number and layout of these design points within the experimental region, depends on the number of effects that must be estimated. Depending on the number of factors, their levels, possible interactions and order of the model, various experimental designs are chosen. Each experiment can be represented as a point within the experimental domain, the point being defined by its co-ordinate (the value given to the variables) in the space [8-10].

Doxorubicin HCl is an antineoplastic agent occurred as orange-red, crystalline, hygroscopic powder soluble in water and slightly soluble in methanol. The mechanism of action of doxorubicin HCl is related to its ability to bind to DNA and inhibit nucleic acid synthesis. Cell culture studies have demonstrated rapid cell penetration and perinucleolar chromatin binding, rapid inhibition of mitotic activity and nucleic acid synthesis, mutagenesis and chromosomal aberrations. The oral bioavailability of about 0.5%-1%, excretion is about 4-5% of the administered dose in five days, the half-life is observed phase of 12 min, second phase 3.3h and prolonged third phase about 29.6h [11].

In the present study, the conventional slurry method was used for the preparation of doxorubicin HCl proniosomes and optimized. To check the influence of formulation variables on responses optimization technique was studied. A Box-Behnken design was created using Design Expert 11 (Trial Version 11, Stat-Ease Inc., Minneapolis, MN) to interpret the results.

MATERIALS AND METHODS

Materials

Doxorubicin HCl was obtained as a gift sample from Shilpa antibiotic Pvt Ltd, Raichur. Maltodextrin was procured from Himedia, Hosur, cholesterol, tween 20 and DCP (Dicetyl phosphate) were purchased from Loba chem Pvt Ltd, Mumbai. All the other ingredients and reagents used were of analytical grade.

Methods

Design of experiments (DoE)

Initially, primary experiments (one factor at a time approach) were performed to determine the main factors and the appropriate ranges in which the optima lie. Among all the non-ionic surfactants tween 20 was selected based on results of the preliminary experiments [12].

Further, the effect of four factors (concentrations of surfactant, cholesterol, hydration volume and sonication time), three levels on the (PEE) and MVS were optimized. The independent factors and the dependent variables used in the design are listed in table 1.

Table 1: List of dependent and independent variables in box-behnken design

Independent variables		Levels		
Variables names		Low	Medium	High
1	Conc. of tween 20	40%	60%	90%
2	Conc. of cholesterol	10%	30%	60%
3	Sonication time	5 min	10 min	15 min
4	Hydration volume	5 ml	10 ml	15 ml
	Dependent variables	Goals		
R1	Percentage entrapment efficiency (PEE %)	Maximize		
R2	Mean vesicle size (MVS nm)	Minimize		

Preparation of proniosomes

The proniosomes were prepared by the slurry method.250 µmol stock solution of tween 20, cholesterol and sonication time was prepared in chloroform: methanol (2:1). The accurately measured volumes of tween 20 and cholesterol stock solutions and doxorubicin HCl (50 mg) dissolved in chloroform: methanol (2:1) solutions were added into a 250 ml round bottom flask containing previously 900 mg of maltodextrin powder used as carrier. Additional chloroform: methanol (2:1) solution added form slurry. Further the flask was attached to a rotary flash evaporator rotated at 60 to 70 rpm. The solvent is allowed to evaporate at the temperature of 45±2 °C in a reduced pressure of 600 mm/Hg until the mass in the flask become a dry, free-flowing product. The obtained proniosome powder was further dried overnight in desiccators under vacuum at room temperature. The obtained dry proniosome powders were stored in airtight amber colored vials and kept in a refrigerator for further evaluation [13].

Characterization of proniosomes

The proniosomes were used for the preparation of niosomes and characterization of the surface characteristics by optical microscopy. Proniosomes were transformed to niosomes by hydrating with phosphate buffer saline (PBS) pH 7.4 at 80 °C using vortex mixer for 2 min. The niosomes were sonicated twice for specified time and were characterized for morphology, PEE and MVS.

Particle size and size distribution analysis

For all the batches of doxorubicin HCl proniosomes particle size analysis was carried out using a compound microscope (optical microscope). The freshly prepared hydrated proniosomes were dispersed in double distilled water (DDW) and was used to characterize the particle size. Size measurements were done in triplicate for each sample. Polydispersity Index (PDI) was also determined as a measure of homogenecity.

Entrapment efficiency

Niosome entrapped doxorubicin HCl was estimated by the dialysis method. The calculated amount of prepared niosomes was placed in the dialysis bag (presoaked for 24 h). Free doxorubicin HCl was dialyzed for 30 min each time in 100 ml of phosphate buffer pH 7.4. The dialysis of free doxorubicin HCl always completed after12-15 changes, when no doxorubicin HCl was detectable in the recipient solution. The dialyzed doxorubicin HCl was determined by finding out the concentration of bulk of solution by UV spectrophotometer at 235 nm. The samples from the bulk of solution diluted appropriately before going for absorbance measurement. The free doxorubicin HCl in the bulk of solution gives us the total amount of unentrapped drug. The percentage entrapment efficiency is calculated by using the following formula [14].

$$\% \text{ Entrapment effeciency} = \frac{\text{Amount of drug entrapped}}{\text{Total amount of drug}} \times 100$$

***In vitro* drug release and release kinetics**

The release of doxorubicin HCl from niosomes derived from proniosome was determined using membrane diffusion technique. The niosome suspension prepared from proniosomes equivalent to 50 mg of doxorubicin HCl was taken in a glass tube having a diameter 2.5 cm with an effective length of 8 cm that was previously covered with soaked osmosis cellulose membrane, which acts as a donor compartment. The glass tube was placed in a beaker containing 200 ml of phosphate buffer pH 7.4, which acts as a receptor compartment. The whole assembly was fixed in such a way that the lower end of the tube containing suspension was just touched (1-2 mm deep) the surface of diffusion medium. The temperature of receptor medium maintained at 37±1 °C and the medium was agitated at 100 rpm speed using magnetic stirrer. Aliquots of 5 ml sample were withdrawn periodically and after each withdrawal, same volume of medium was replaced to maintain sink condition. The collected samples were analyzed at 235 nm for doxorubicin HCl using phosphate buffer pH 7.4 as blank. The diffusion studies were carried out in triplicate and the data were interpreted, model fitted by using dissolution software PCP-DISSO V.3.

RESULTS AND DISCUSSION

Preparation of doxorubicin HCl proniosomes

In this study, the doxorubicin HCl proniosomes were formulated, optimized and evaluated for its efficacy in drug delivery to overcome the major issues associated with its oral delivery. Primary experiments conducted using different non-toxic and biocompatible non-ionic surfactants like span together with cholesterol and DCP. The phase transition temperature plays a crucial role in the doxorubicin HCl proniosomes formulation. Based on the results of preliminary experiments, tween 20 was selected as a suitable surfactant for the preparation of doxorubicin HCl proniosomes.

Optimization of formulation variables

Through primary experiments the four factors viz., concentration of tween 20, the concentration of cholesterol, hydration volume and sonication time were identified as the most significant variables influence the PEE (R1) and MVS (R2).

The formulations were further optimized by considering the parameters like maximum entrapment efficiency and smaller particle size. Twenty-nine runs performed for the response surface methodology based on the Box–Behnken design. Based on the DoE, the factor combinations yielded different responses as presented in table 2. These results clearly specify that the dependent variables are strongly dependent on the selected independent variables as they show a wide variation among all the 29 batches to obtain analysis of variance (ANOVA), regression coefficients and regression equation. Mathematical relationships were generated using multiple linear regression analysis for the mentioned variables as shown in table 3. These equations represent the quantitative effect of concentration of tween 20, concentration of cholesterol, hydration volume and sonication time and their interaction on PEE (R1) and MVS (R2).

Table 2: Box-behnken DoE and observed responses

Run	Factor 1	Factor 2	Factor 3	Factor4	Response 1	Response 2
	Surfactant	Cholesterol	Hydration volume	Sonication time	PEE	MVS
	%	%	ml	min	%	nm
1	-1	1	1	1	40.21	400.1
2	-1	-1	-1	-1	43.21	325.2
3	0	0	0	0	45.21	351.21
4	1	1	1	1	61.1	402.12
5	-1	-1	1	1	45.25	329.21
6	1	-1	1	-1	63.25	332.21
7	1	1	-1	-1	65.75	406.21
8	0	0	0	0	47.52	355.4
9	-1	-1	1	-1	47.25	335.75
10	1	0	0	0	68.45	360.12
11	0	0	0	0	48.95	362.75
12	-1	0	0	0	48.75	368.12
13	1	-1	-1	1	70.12	339.25
14	1	-1	1	1	72.15	341.21
15	0	0	0	1	50.21	370.1
16	-1	1	-1	1	50.75	408.95
17	0	0	0	-1	52.85	372.12
18	1	1	-1	1	73.76	410.85
19	0	0	1	0	54.95	376.12
20	1	-1	-1	-1	75.95	346.75
21	0	1	0	0	87.5	412.35
22	-1	-1	-1	1	52.21	348.21
23	0	0	0	0	59.35	380.12
24	0	0	0	0	62.25	385.21
25	-1	1	1	-1	53.75	415.95
26	-1	1	-1	-1	55.75	418.85
27	0	-1	0	0	65.32	351.21
28	0	0	-1	0	65.85	391.21
29	1	1	1	-1	79.11	420.25

Each formulation containing doxorubicin HCl 50 mg

Table 3: Regression equation for the responses-PEE (R1) and MVS (R2)

Response	Regression equation
R1	57.9706+9.78944A-0.340556B-3.99556C-0.79056D+0.084375AB+0.053125AC+0.41625AD-0.545625BC-0.723125BD-0.171875CD+0.443932A^2+5.05393B^2-1.17107C^2-3.51607D^2
R2	373.004+0.479444A+35.9239B-2.36444C-1.29389D.

The values of the coefficients of 1, 2, 3 and 4 are related to the effect of these variables on the responses R1and R2. Coefficients with more than one-factor term and those with higher order terms represent interaction terms and quadratic relationship respectively. A positive sign represents a synergistic effect, while a negative sign indicate antagonistic effect.

PEE of doxorubicin HCl proniosomes was found to be in the range of 40.21–87.5% as shown in table 2. The polynomial equation for PEE exhibited a good correlation coefficient (0.5524) and the model F-value of 7.41 implies the model is significant. There is only a 0.05% chance that a "Model F-Value" this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. The 3D response surfaces plots of the response R1are shown in fig. 1 and 2 to depict the interactive effects of independent variables on response R1, one variable was kept constant while the other three variables varied in a certain range.

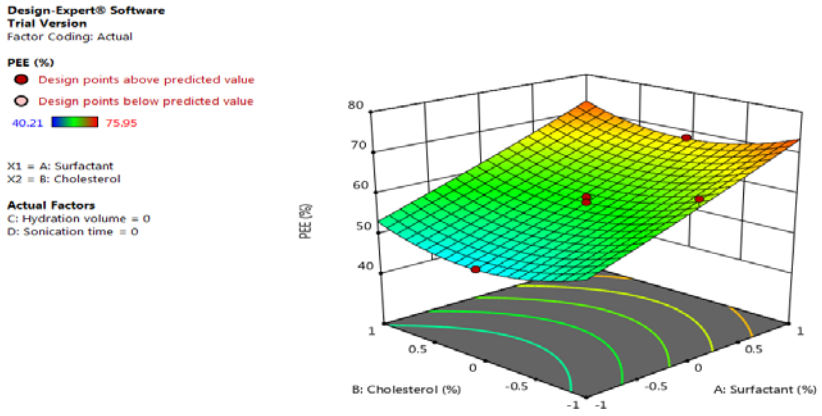

Fig. 1: Response surface plots showing the interactions between concentration of tween 20 and concentration of cholesterol on PEE

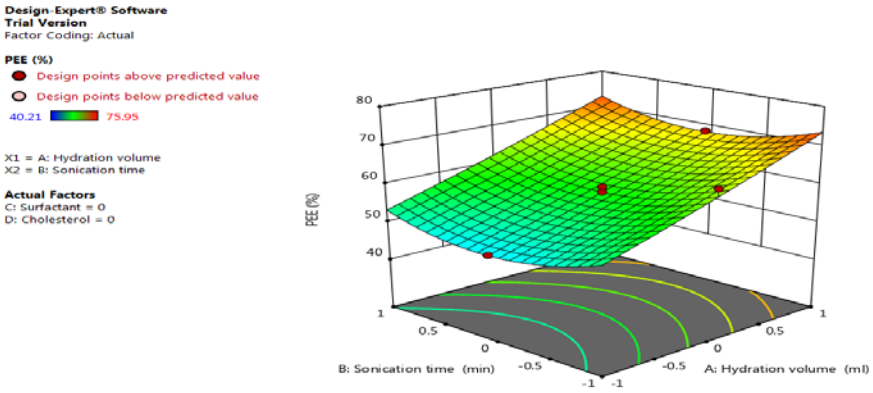

Fig. 2: Response surface plots showing the interactions between sonication time and hydration volume on PEE

The mean vesicle size of doxorubicin HCl proniosomes was found to be in the range of 325.2 nm to 420.25 nm. The mathematical model generated for MVS (R2) was found to be significant with Model F-value of 54.22. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise (P<0.0500) and R^2 value of 0.9004. The model indicated the linear relationship between particle size and independent variables. The independent variables 1, 2, 3 and 4 have linear effects on the particle size. The influence of the main and interactive effects of independent variables on the particle size was further elucidated using the perturbation and 3D response surface plots. The relationship between the dependent and independent variables was further elucidated using 3D response surface plots fig. 3 shows the interactive effect of 1 and 2 on the particle size (R2) at a fixed level of 3 and 4 and fig. 4 shows the interactive effect of 3 and 4 on the particle size (R2) at a fixed level of 1 and 2. At low levels of 2 (concentration of cholesterol), R2 increases from 325.2 nm to 451.21 nm. Similarly, at high levels of 2, R2 increases from 400.1 nm to 420.25 nm.

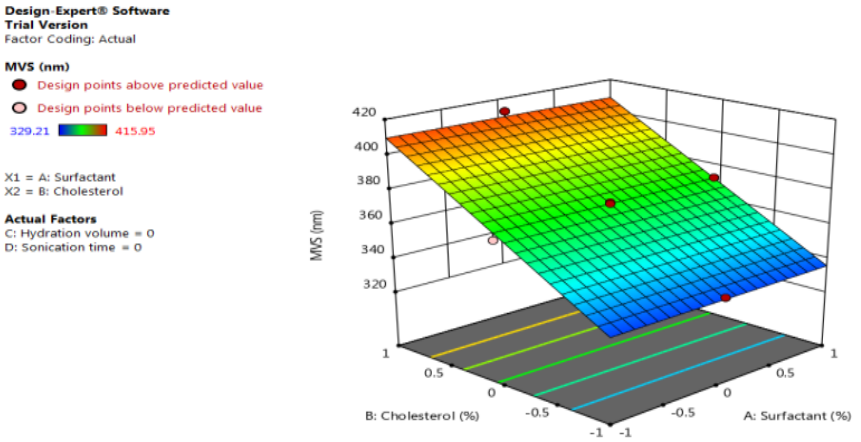

Fig. 3: Response surface plot showing the influence of concentration of tween 20 and concentration of cholesterol on particle size

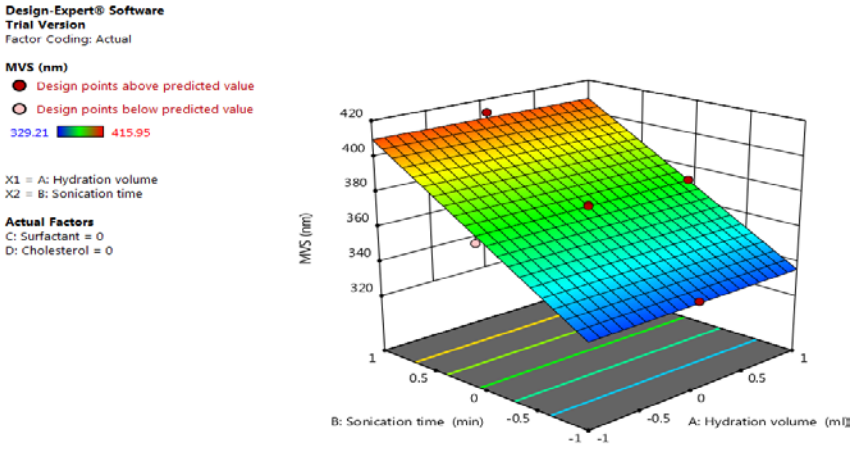

Fig. 4: Response surface plot showing the influence of sonication time and hydration volume on particle size

Optimization and confirmation of DoE

A numerical optimization technique using the desirability approach was employed to prepare maltodextrin based doxorubicin HCl Proniosomes with the desired responses. Constraints like minimizing the MVS in addition to maximizing the PEE were set as goals to locate the optimum settings of independent variables. The optimized levels and predicted values of R1 and R2 are shown in table 4. All the four batches of obtained doxorubicin HCl proniosomes were subjected to further characterization.

Table 4: Optimized values obtained by the constraints apply on R1 and R2

Independent variables	Nominal values	Predicted values		Codes	Observed values	
		PEE % R1	MVS nm R2		PEE % R1	MVS nm R2
Conc. of surfactant(1)	60%	90.11	386.43	FD-1	73.76	412.35
Conc. of cholesterol(2)	30%			FD-2	75.95	415.95
Hydration volume (3)	10 ml			FD-3	87.54	376.96
Sonication time (4)	10 min			FD-4	79.11	418.95

n=3, Drug-50 mg

The mean vesicle size of diluted doxorubicin HCl proniosomes are shown in table 4, it ranges from 376.96±1.25 nm to 418.95±1.20 nm with unimodal particle size distribution, which favors drug delivery of doxorubicin HCl. Since the smaller particle size is advantageous to decrease the irritation, cardiac arrest and improve the penetration of particles into the tissues thereby decreases toxicity. The PDI in all the formulations was low (<0.024), which indicated the homogenecity of the preparation. All the maltodextrin-based doxorubicin HCl proniosomes formulations were negatively charged, which was due to the negative charge present on the DCP (table 5).

Table 5: PEE, PDI and mean vesicle size of optimized formulations

Codes	PEE %±SD	PDI	MVS(nm)±SD	PDI
FD-1	73.76±0.25	0.117	412.35±0.23	0.025
FD-2	75.95±0.35	0.113	415.95±0.52	0.024
FD-3	87.54±0.29	0.098	376.96±0.42	0.027
FD-4	79.11±0.55	0.109	418.95±0.22	0.024

n=3($p<0.05$)

Doxorubicin HCl proniosomes prepared using the optimum ratio of surfactant (tween 20) and cholesterol demonstrated lamellar structures under the compound microscope. Hydration of dry proniosomes with pH 7.4 phosphate buffer solution leads to swelling of bilayers as well as particles due to the interaction of water with polar groups of surfactant. The bilayer tends to form spherical structure randomly giving rise to multilamellar, multivesicular structures. When shaken with aqueous phase, complete hydration takes place leading to the formation of niosomes. Observation under optical microscope revealed that maltodextrin based doxorubicin HCl proniosomes was rapidly converted to niosomes almost completely within minutes.

***In vitro* drug release and release kinetics**

It is clear that the cumulative release of drug from control (pure drug) was faster than from the doxorubicin HCl proniosomes. This was due to the fact that doxorubicin HCl was sufficiently hydrophilic and it partitions in favour of the proniosomes, which resulted in the slower release of doxorubicin HCl proniosomes.

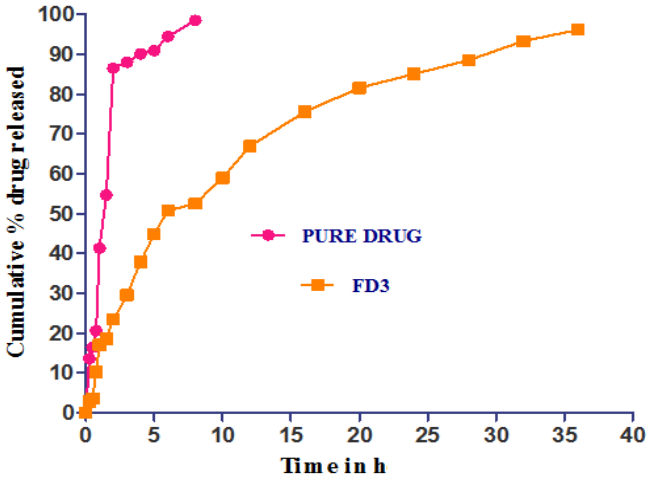

Fig. 5: *In vitro* release of doxorubicin HCl and doxorubicin HCl proniosomes (FD-3)

The *in vitro* drug release from the pure doxorubicin HCl was found to be 94.50±0.16 FD-3 was found to be 40.2±0.24 after 6h and 53.7±0.54 after 9h. The drug release about 28 to 43% after 6h is mainly due to initial bursting of improper niosome and adhered drug particles in formulations and at the start of 24h the release was found to be steady because stable niosomes retains and the release

was extended up to 36h with sustained action. The best fit model was found to be Hix. Crow for pure doxorubicin, best fit model was found to be Ist order for FD-3. The korsemayer peppas exponential n<0.84 indicate the release mechanism was non fickanian (anomalous transport) i.e. drug released by erosion followed by diffusion mechanism.

CONCLUSION

The present study conclusively demonstrates the use of Box-Behnken design in formulation and optimization of maltodextrin based doxorubicin HCl niosome derived from proniosomes formulations to avoid its systemic toxicity. This study indicated that niosome can be optimized to achieve desired properties using tween 20 concentrations, cholesterol concentration, sonication time, and hydration volume. The optimized niosome formulation demonstrated enhanced PEE and optimum MVS.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declare none

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