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Original Article

OPTIMIZATION OF TENOXICAM LOADED NIOSOMES USING QUADRATIC DESIGN

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ABSTRACT

Objective: The objective of the present study was to obtain an optimized formula of Tenoxicam (TNX) niosomes using Quadratic Design.

Methods: TNX niosomes were prepared by Organic Solvent Injection method and all vehicles were evaluated for their entrapment efficiency (EE%), and Particle Size(nm).

Results: EE% was found to be between 77.88 and 89.98**.** Percentage entrapment efficiency was significantly affected by the applied processing variables such as the concentration of span 60 as well as cholesterol. The mean vesicle size of drug loaded niosomes of the different batches ranged between 79-190 nm. Vesicle size of drug loaded niosomal batches was found to decrease as the concentration of span increases. The effects of all the tested independent variables have P-values<0.05.

Conclusion: Quadratic design succeeded in the optimization of the formulation ingredients on EE% and particle size of Tenoxicam niosomes. Finally the optimization process provides a formula having the optimum level of factors.

Keywords: Quadratic Design, Entrapment efficiency (EE%), Niosomes, Optimization, Tenoxicam (TNX).

INTRODUCTION

Niosomes or non-ionic surfactants vesicles are microscopic lamellar structures formed by the admixture of a non-ionic surfactant, cholesterol and Stabilizer with subsequent hydration in aqueous media [1].

TNX is a nonsteroidal anti-inflammatory drug (NSAID) that exhibits anti-inflammatory, analgesic, especially for rheumatoid arthritis and antipyretic activities. The mechanism of action of TNX, like that of other NSAIDS, is not completely understood but may be related to prostaglandin synthetase inhibition [2]. Optimization may be considered for the search of a result that is satisfactory and at the same time the best possible within a limited field of search. Thus, the type and components of a formulation may be selected, according to previous experience [3]. Some strategies are frequently used to achieve optimization such as full factorial, Box-Behnken, central-composite, Plackett-Burman designs, etc [4].

MATERIALS AND METHODS

Materials

Commercial grade Tenoxicam (TX) was a gift sample obtained from Ramdev chemicals, Mumbai. Span-60, Cholesterol, Sodium deoxycholate (SDC) purchased from sd Fine Chemicals, India. Albino Wistar rats were obtained from Bharat Serum and Vaccines Pvt. Ltd. (Mumbai, India).

Equipment

An electric balance (SARTORIUS AG, Germany), Shimadzu UV spectrophotometer (2401/PC Japan), Buchi rotavapor (R-3000, Switzerland), Digital Sonifier (Branson, Danbury, USA), Dissolution apparatus (Erweka GmbH, Germany), Shaker water bath (Julabo SW-20 C, Germany), pH meter, (JENWAY England), Centrifuge (Biofuge, primo Heraeus, Germany), and JEOL Transmission Electron Microscope (JTEM model 1010, Japan), were used.

Formulation of niosomes

Niosomes were prepared by an organic injection method using two variables include: HLB surfactant, sodium deoxycholate. The quadratic design was established to prepare 9 different formulae of TNX niosomes. The surfactants, lipid and drug were first dissolved in a suitable organic solvent. The prepared organic phase was then added drop wise into the aqueous phase. Surfactant: cholesterol ratio of 1:1 along with tenoxicam and Sodium deoxycholate were dissolved in chloroform: methanol in the ratio of 2:1. Thus, the dissolved organic solution containing drug were injected drop wise through 24 gauge needle into preheated PBS PH 7.4, which was magnetically stirred (Mechanical stirrer, Remi, Mumbai) and maintained at 65 °C. Stirring was continued until all chloroform & methanol evaporated to get drug loaded niosome. Vaporization of chloroform & methanol leads to the formation of single layered vesicles [5]. These were further size reduced by ultrasound cavitation using probe sonicator (Oscar, Japan) to form small unilamellar vesicles.

Entrapment efficiency of niosomes (EE %)

The unentrapped drug was separated from the niosomal dispersions by centrifugation at 15,000 rpm for 45 min. The supernatant was separated, diluted to 100 ml with PBS pH 7.4, filtered using a membrane filter (0.45µm pore size), and measured using a spectrophotometer at 354 nm. EE% was calculated by the following equation [5].

$$EE\ \% = [(Ct - Cr \backslash Ct)] \times 100\%$$

Ct is the concentration of total TNX.

Cr is the concentration of free TNX

Particle size

The vesicle size and distribution were determined by dynamic light scattering method Malvern zeta sizer (Malvern Instruments Ltd.). Measurements were carried out at an angle of 90 ° at 25 °C.

Optimization of formulation

Optimization by means of experimental design may be helpful in shortening the experimenting time. The design of experiments is a structured, organized method used to determine the relationship between the factors affecting a process and the output of that process.

Most experiments consist of an investigation into the relationship between two types of variables. The independent variables, or the factors, are those that are set by or under the control of the

experimenter. The dependent variables, or the responses, are those that are the outcomes of the experiment. Thus, the values of the dependent variables are controlled by the values of the independent variables. One important role of such experimental designs is to establish the relative importance of two or more factors and also to indicate whether or not interaction occurs between these factors, thereby affecting the magnitude of the response. Having established those factors and interactions that determine the response, the same experiments can be used for a predictive purpose, namely, estimating the response at combinations of factors that have not been studied experimentally, and this is the role of response-surface methodology. The surface can be visualized by using contour plots or three-dimensional diagrams. The prediction is carried out by deriving a mathematical model relating the response to the factors. This model is usually empirical and is based on responses to experiments that have been carried out as part of the experimental design.

Response surface methodology permits a deeper understanding of a process or product and has many important applications. The two most important of these are in optimization and in establishing the robustness of that process or product. Hence used for further studies [Amstrong 2006].

Experimental designing

Here, a commercially available software program was used (Design Expert, Version 9, Stat-Ease Inc, and Minneapolis, MN). The experimental design chosen was Response Surface, 2-factors, 3-level factorial; 9 formulations were formulated. Run order was kept in the randomize mode to protect against the effects of time-related variables and also to satisfy the statistical requirement of independent variables. Analysis of variance (ANOVA) and all statistical analyses was also performed using the same software. Calculation of the effects was performed; half-normal plots, response surface plots were plotted. Also ANOVA was used to treat the data, and for proper model selection. The F value was checked to see whether it is within the desired limits. The F value was calculated by comparing the treatment variance with the error variance. Out of these experimental batches, optimised batch was selected for tenoxicam loaded niosome. The factors considered were:

(1) The amount of surfactant span 60.

(2) Amount of stabilizer

Factor A-Surfactant concentration: Surfactant concentration was varied to study the effect of surfactant concentration on particle size and stability as well as its interaction with the lipids. Levels of factor A are shown in table 1. Factor B-span 60 concentration: sodium deoxycholate concentration was varied to achieve maximum entrapment of the drug and to adjust the drug loading and particle size. Levels of factor B are shown in table 2.

Response

Average particle size and Drug entrapment efficiency, the formula of TNX loaded niosomes was based on 32 factorial designs where each of the two factors was considered at three levels. Thus, as shown in table 3, total 9 batches were prepared.

Table 1: Levels of factor A-surfactant span 60 concentration

Level of factor (A)	Coded value	Concentration(mg)
Low	-1	16
Medium	0	18
High	1	20

Table 2: Levels of Factor B-sodium deoxy cholate concentration

Level of factor (B)	Coded value	Concentration(mg)
Low	-1	0.8
Medium	0	1.2
High	1	1.6

Table 3: Design matrix for Experimentation

Batches ingredients	TRS1	TRS 2	TRS 3	TRS 4	TRS 5	TRS 6	TRS 7	TRS 8	TRS 9
Drug (mg)	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Surfactant(mg)	18	18	20	20	16	18	16	20	16
Sodium deoxycholate	0.8	1.2	1.2	1.6	1.6	1.6	0.8	0.8	1.2
Cholesterol (mg)	20	20	20	20	20	20	20	20	20
PBS (as hydrating medium) in ml	20	20	20	20	20	20	20	20	20

Experimental design

The technique of 3^2 factorial designs with 2 factors at 3 different levels affecting the particle size and entrapment efficiency was considered. All experiments were carried out in random order to nullify the effects of extraneous or nuisance variables. The results of the experimental design were analyzed using Design Expert software that provided considerable useful information and reaffirmed the utility of statistical design for conducting experiments. The selected independent variables like amount of span 60 & amount of sodium deoxycholate significantly influenced the particle size and drug entrapment efficiency that is very much evident from the results in table 5.3 which represents the various combinations of independent variables with its resultant affect on the dependent variable.

RESULTS AND DISCUSSION

Design summary

Study type	Response Surface
Runs	9
Initial design	3^2 Level Factorial
Blocks	No Blocks
Design model	Quadratic

Table 4: Factors selected for experimentation

Factor	Name	Units	Type	Low Actual	High Actual	Low coded	High coded
A	Amount of span 60	Mg	Numeric	-1.00	1.00	-1.00	1.00
B	Amount of sodium deoxycholate	Mg	Numeric	-1.00	1.00	-1.00	1.00

Table 5: Response selected for experimentation

Response	Name	Units	Obs	Analysis	Min	Max	Mean	Std. Dev	Model
Y1	Particle size	nm	9	Poly-nominal	79.46	190	134.73	1.2	Quadratic
Y2	Drug entrapment efficiency	%	9	Poly-nominal	77.88	89.98	83.93	2.4	Quadratic

Table 6: Design matrix and responses

Std. [Batch No.]	Run	Block	Factor A amount of span 60 [mg]	Factor B amount of sodium deoxycholate [mg]	Response 1 Avg. Particle Size [nm]	Response 2 entrapment efficiency [%]
F1	1	Block 1	18	0.8	79.46	88.8
F2	2	Block 1	18	1.2	140.8	89.21
F3	3	Block 1	20	1.2	130.4	84.32
F4	4	Block 1	20	1.6	171	85.93
F5	5	Block 1	16	1.6	190	77.88
F6	6	Block 1	18	1.6	188.3	89.98
F7	7	Block 1	16	0.8	95.02	78.49
F8	8	Block 1	20	0.8	97.18	80.11
F9	9	Block 1	16	1.2	119.1	79.84

Model analysis for particle size, Response 1 Particle size, ANOVA For response surface quadratic model

Table 7: Analysis of variance table (Partial sum of squares–Type III)

Source	Sum of squares	Df	Mean square	F Value	P-Value Prob>F	
Model	13066.43	5	2613.29	15.22	0.0242	**Significant**
A-Span60	5.12	1	5.12	0.030	0.8739	
B-Sodium deoxycholate	12847.33	1	12847.33	74.84	0.0032	
AB	111.94	1	111.94	0.65	0.4785	
A^2	11.55	1	11.55	0.067	0.8121	
B^2	90.50	1	90.50	0.53	0.5203	
Residual	514.96	3	171.65			
Cor Total	13581.39	8				

The model F-value of 15.22 implies the model is significant. there is only a 2.42 % chance that an F-value this large could occur due to noise., Values of "prob>f" less than 0.0500 indicate model terms are significant., If there are many insignificant model terms (Not counting those required to support hieraracy)., Model reduction may improve your model.

Table 8: Parameters of selected quadratic model

Std. dev.	13.10	R-SQUARE	0.9621
Mean	134.58	ADJ R-SQUARE	0.8989
C. V%	9.73	PRED R-SQUARE	0.6029
PRESS	5393.00	ADEQ PRECISIO	9.640

The "Pred R-Squared"of 0.6029 is not as close to the "Adj R-Squared"of 0.8989 as one might normally expect i. e the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model &/or data. Things to consider are a model reduction, response transformation, outlines, etc. All empirical models should be tested by doing confirmation runs, "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 9.640 indicates an adequate signal. This model can be used to navigate the design space.

Table 9: Shows VIF and 95%CI

Factor	Coefficient estimate	DF	Standard error	95%CI low	95%CI high	VIF
Intercept	131.70	1	9.77	100.62	162.78	
A-span 60	-0.92	1	5.35	-17.95	16.10	1
B-sodium deoxycholate	46.27	1	5.35	29.25	63.30	1
AB	-5.29	1	6.55	-26.14	15.56	1
A^2	-2.40	1	9.26	-31.89	27.08	1
B^2	6.73	1	9.26	-22.76	36.21	1

Final equation in terms of coded factors

Particle size =+131.70

= -0.92 *A

= +46.27*B

= -5.29*AB

= -2.40 *A^2

= +6.73 *B^2

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factors. By default, the high levels of the factors are coded as+1 & the low levels of the factors are coded as-1. The coded equation is useful for

identifying the relative impact of the factors by comparing the factors coefficients.

Final equation in terms of actual factors

Particle size = -275.76778

= +29.10333* Span 60

= +133.80833* Sodium deoxycholate

= -6.61250* span 60 * sodium deoxycholate

= -0.60083* span 60^2

= +42.04167* Sodium deoxycholate^2

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factors. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

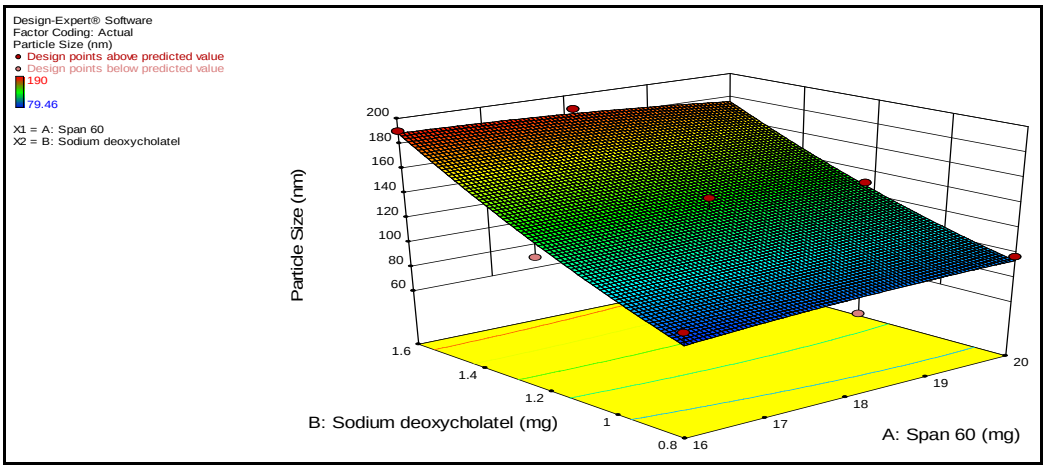

Fig. 1: Response Surface Plot for particle size

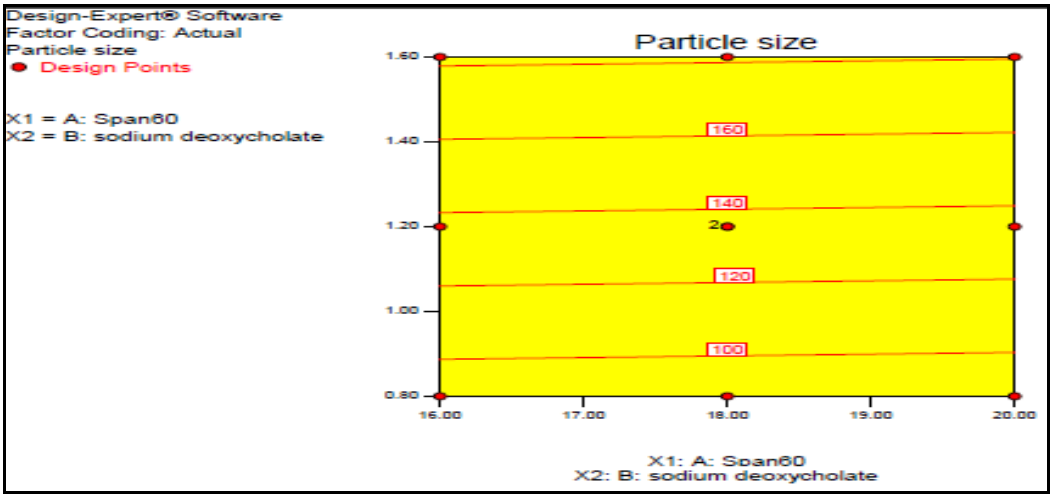

Fig. 2: Contour plot for particle size

Table 10: Analysis of variance table (partial sum of squares-Type III)

Source	Sum of squares	DF	Mean of squares	F value	P-value PROB>F
Model	186.14	4	46.54	50.98	0.0011
A-span 60	33.37	1	33.37	36.56	0.0038
B-sodium deoxy Cholate	6.8	1	6.81	7.46	0.0524
6.81 7.46 0.0524 Cholate					
AB	10.34	1	10.34	10.32	0.0282
A^2	135.63	1	135.63	148.69	0.0003
Residual	3.65	4	0.91	148.59	0.0003
Cor total	189. 79	8			

The model f-value of 50.98 implies the model is significant. There is only a 0.11% chance that an F–value this large could occur due to noise, Values of "prob>F"less than 0.0500 indicate model terms are significant. In this case, A, AB, A^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant if there are many insignificant model terms (not counting those required to support hierarchy). The model reduction may improve your model. The "pred R-square " of 0.86.48 is in reasonable agreement with the "adj r–squared" of 0.9615 i. e the difference is less than 0.2., "Adeq Precision " measures the signal to noise ratio. a ratio greater than 4 is desirable. Your ratio of 17.133 indicates an adequate signal. This model can be used to navigate the design space.

Table 11: Parameters of selected quadratic model

Std. Dev.	**0.96**	**R-Squared**	**0.9808**
Mean	83.84	Adjust R-Square	0.9615
C. V	1.14	Pred R Square	0.8648
Press	25.65	adeq precision	17.133

Table 12: **Shows VIF and 95%CI**

Factor Estimate	**coefficient**	**df error**	**Standard low**	**95% CI low**	**95% CI high**	**VIF**
Intercept	89. 33	1	0.55	87.80	90.86	
A-span60	2.36	1	0.39	1.28	3.44	1
B-sodium deoxycholate	1.07	**1**	0.39	-0.018	2.15	1
AB	1.61	**1**	0.48	0.28	2.93	**1**
A^2	-8.23	**1**	0.68	-10.11	-6.36	**1**

Model analysis for **entrapment efficiency**

Response 2 **Entrapment efficiency**

ANOVAs for response surface reduced quadratic model

Final equation in terms of coded factors

Entrapment efficiency = 89.33+2.36*A+1.07*B+1.61*AB-8.23*A^2

Final equation in terms of actual factors

Entrapment efficiency =-558.722+72.88292* span 60-33.50625 *sodium deoxy cholate+2.00937*span 60 *sodium deoxycholate-2.05875 span 60^2

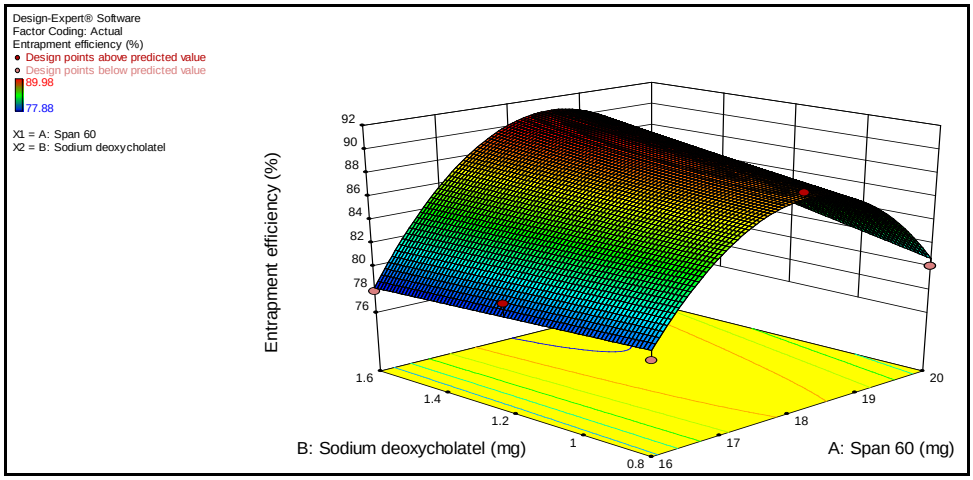

Fig. 3: Shows response surface plot for drug entrapment efficiency

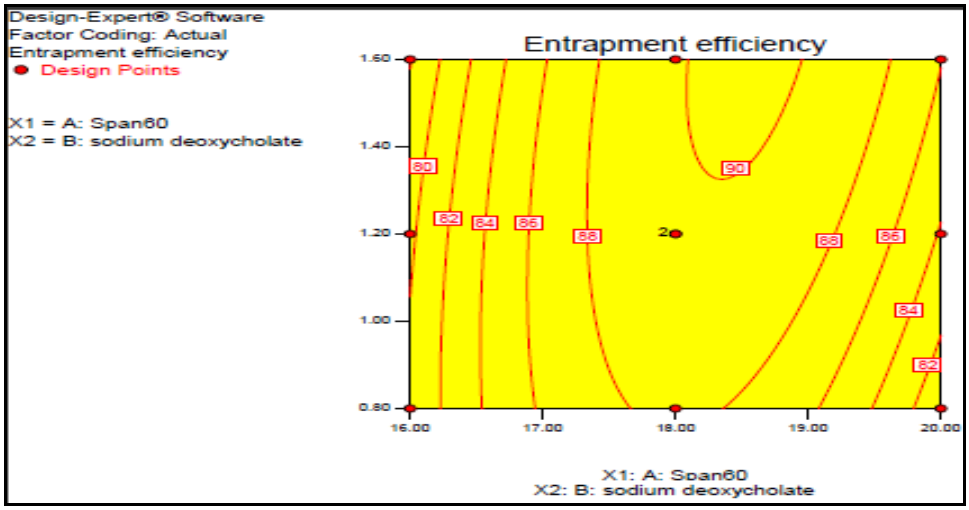

Fig. 4: Shows contour plot for drug entrapment efficiency

Effect of amount of edge activators on niosomes

Response plots [fig. 1 and 3] indicate that an optimum amount of 18 mg i.e. 2% of total niosomal suspension generates particles of optimum size. Increasing or decreasing this amount causes changes in particle size. The entrapment efficiency is also highest when the amount is 2% of total surfactant concentration.

The initial increment in drug entrapment in the presence of low concentrations of EAs may be credited to the growth in vesicle size owing to the incorporation of more amount of drug. The decreased entrapment efficiency beyond a certain optimum concentration of EA can be ascribed to the pore formation/dissolution of cholesterol and surfactant bilayers in EA [Simões *et al.*; 2005]. This may be due to the fact that at a certain concentration, surfactant molecule gets associated with the cholesterol bilayer, resulting in better partitioning of the drug. So above a 2% concentration of the surfactant, molecules may start forming micelles in a bilayer resulting in pore formation in vesicle membranes and complete conversion of vesicle membranes into mixed micelles. These mixed micelles were reported to have a lower drug carrying capacity and poor skin permeation due to their structural features [Gupta *et al.*; 2012]. A particle size in the range of 120-160 nm was desired, as these Niosomes would be further loaded with Tenoxicam. Too small particle size would provide the lesser volume of the aqueous compartment to entrap the Tenoxicam. Also particles greater than 200 nm are readily engulfed and taken up by the lymphatic system leading to lower bioavailability of the drug. Moreover, niosomes prepared with low EA concentrations exhibited a smaller size. This reduction of the particle size diameter of elastic SDC niosomes may be attributed to the increased flexibility and reduced surface tension of these vesicles. It could be known from above data that EA concentration had an influence on not only the drug loading and encapsulation efficiency of niosomes but also their structure and formation. Micelles were formed with excessive concentration of salt ions of sodium deoxycholate.

Role of surfactant in drug entrapment

The effect of different concentrations of Span 60 on percent entrapment is depicted in Fig.2. An increase in Span 60 beyond a ratio of cholesterol/surfactant of 1:4.5 results in spherical vesicles along with aggregates. This shows that surfactant beyond a certain concentration with a low amount of cholesterol will not form stable vesicles with good entrapment (fig. 1a). Our results are similar to the results reported by Ahmed *et al.* which indicate that the lower the HLB of the surfactant, the higher will be the entrapment efficiency. The entrapment efficiency of Span 60 was higher in F6 (89.98) than F2(89.21). The higher entrapment may be due to the solid nature, hydrophobicity, and high phase transition temperature of the surfactant. Our results were similar to those reported in the literature for carboxyfluorescein niosomes. These results are similar to those reported for non-sonicated sorbitan monoester niosomes loaded with doxorubicin confirming the hypothesis that entrapment efficiency may be correlated with the hydrophobicity of the alkyl chain of the sorbitan esters.

The mean vesicle size of drug loaded niosomes of the different batches according to the factorial design ranged between 79-190 nm. The polydispersity index (PdI) was in the range of 0.207–0.341 for drug-loaded niosomes which indicate a narrow vesicle size distribution. The mean vesicle size and PdI of all the nine batches of factorial design is shown in table 2. It was observed that the relative amount of span 60 and cholesterol was found to play an important role in the determining of vesicles size. Vesicle size of drug loaded niosomal batches was found to decrease as the concentration of span increases. Hydrophobic Tenoxicam intercalates into the lipid bilayer leading to appreciable cohesion among a polar portion of the membrane, causing a reduction in the vesicle size. The typical profile of particle size distribution in the prepared niosomes was showed in fig. 1.

A good correlation was observed for both variables $X1$ (Span 60) and $X2$ (Cholesterol) in vesicle size of drug-loaded niosomes ($r2$ = 0.9986). To understand the effect of lipid concentration on vesicle size of the drug loaded niosomes observed coefficient values for the drug-loaded niosomes was fitted in Eq. (1) to generate Eq. (2).

Particle size =-275.76778

= +29.10333*Span60

= +133.80833*Sodium deoxycholate

= -6.61250*span 60 *sodium deoxycholate

= -0.60083*span 60^2

= +42.04167*Sodium deoxycholate^2

CONCLUSION

Quadratic Design succeeded in the optimization of the formulation ingredients on EE% and in particle size of TNX niosomes. The model F-value of 15.22 implies the model is significant for particle size and equation of Particle size =+131.70-0.92 *A+46.27*B-5.29*AB-2.40 *A2+6.73 *B2. The model f-value of 50.98 implies the model is significant for entrapment efficiency and equation for entrapment efficiency is Entrapment efficiency = 89.33+2.36*A+ 1.07*B+ 1.61*AB-8.23*A2 and these observed values of the optimized formula were close to the predicted values.

CONFLICT OF INTERESTS

Declare none

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