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Formulation and Optimization of Piroxicam Proniosomes by 3-Factor, 3-Level Box-Behnken Design

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ABSTRACT

The aim of this study was to investigate the combined influence of 3 independent variables in the preparation of piroxicam proniosomes by the slurry method. A 3-factor, 3-level Box-Behnken design was used to derive a second-order polynomial equation and construct contour plots to predict responses. The independent variables selected were molar ratio of Span 60:cholesterol (X_1), surfactant loading (X_2), and amount of drug (X_3). Fifteen batches were prepared by the slurry method and evaluated for percentage drug entrapment (PDE) and vesicle size. The transformed values of the independent variables and the PDE (dependent variable) were subjected to multiple regression to establish a full-model second-order polynomial equation. F was calculated to confirm the omission of insignificant terms from the full-model equation to derive a reduced-model polynomial equation to predict the PDE of proniosome-derived niosomes. Contour plots were constructed to show the effects of X_1, X_2 and X_3 on the PDE. A model was validated for accurate prediction of the PDE by performing checkpoint analysis. The computer optimization process and contour plots predicted the levels of independent variables X_1, X_2, and X_3 (0, -0.158 and –0.158 respectively), for maximized response of PDE with constraints on vesicle size. The Box-Behnken design demonstrated the role of the derived equation and contour plots in predicting the values of dependent variables for the preparation and optimization of piroxicam proniosomes.

KEYWORDS: Proniosomes, niosomes, Box-Behnken design, optimization.

INTRODUCTION

Piroxicam is a poorly water soluble, potent nonsteroidal anti-inflammatory drug used for the treatment of rheumatoid arthritis or osteoarthritis. Although piroxicam has a strong therapeutic effect, it is associated with several side effects such as gastrointestinal irritation, edema, dizziness, and peptic ulceration when taken orally for a prolonged period. One of the major obstacles in designing the formulation of novel drugs is their limited aqueous solubility. This problem can be overcome by entrapping the drug in a vesicular structure. Encapsulation of a drug in vesicular structures like liposomes and niosomes can be expected to prolong the existence of the drug in the systemic circulation, enhance penetration into target tissue, and reduce toxicity, if selective uptake can be achieved.[1]

Niosomes are unilamellar or multilamellar vesicles that are made up of nonionic surfactant and can entrap amphiphilic and hydrophobic solutes.[2,3] Stability is a prime concern in the development of any formulation. Niosomes have shown advantages as drug carriers, such as being cheap and chemically stable alternatives to liposomes,[4] but they are associated with problems related to physical stability, such as fusion, aggregation, sedimentation, and leakage on storage. The proniosome approach[5-7] minimizes these problems by using dry, free-flowing product, which is more stable during sterilization and storage. Ease of transfer, distribution, measuring, and storage make proniosomes a versatile delivery system. Proniosomes are water-soluble carrier particles that are coated with surfactant and can be hydrated to form a niosomal dispersion immediately before use on brief agitation in hot aqueous media. The resulting niosomes are very similar to conventional niosomes and more uniform in size.[5] Reported methods for preparation of proniosomes are the spraying of surfactant on water-soluble carrier particles[5] and the slurry method.[6,7]

In the present study the slurry method was used for the preparation and optimization of piroxicam proniosomes, as this method is simple and easy to scale up. Rhodes et al[5-7] studied the effect of a wide range of surfactant loading on encapsulation of an amphiphilic drug in proniosome-derived niosomes. Many others formulation variables, such as surfactant-to-cholesterol ratio and amount of drug, also affect the characteristics of proniosome-derived niosomes. The proniosomes are thus of interest from a technical viewpoint and allow a wider scope to be used to study the influence of various

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formulation variables; proniosomes need to be optimized for desired response.

Traditional experiments require more effort, time, and materials when a complex formulation needs to be developed. Various experimental designs[8-10] are useful in developing a formulation requiring less experimentation and providing estimates of the relative significance of different variables. In the work reported here, a Box-Behnken design[11] was used to optimize proniosomes containing piroxicam and maltodextrin as a carrier. Independent variables selected were molar ratio of Span 60:cholesterol (X_1), surfactant loading (X_2), and amount of drug (X_3) to evaluate their separate and combined effects on percentage drug entrapment (PDE) and vesicle size expressed as the mean volume diameter (MVD).

Materials and Methods

Span 60 and cholesterol were purchased from S.D. Fine Chemicals (Mumbai, India). Chloroform, disodium hydrogen phosphate, potassium dihydrogen phosphate, and sodium chloride were procured from National Chemicals (Vadodara, India). Piroxicam was received as a gift sample from Elysium Pharmaceuticals (Vadodara). A dialysis tube (DM-70; capacity 2.41 mL/cm, width 29.31 mm, average diameter 17.5 mm, and molecular weight cutoff 12 000 to 14 000) was purchased from Himedia Laboratories (Mumbai). All chemicals used in the study were of analytical grade and used without further purification.

Box-Behnken Experimental Design

The traditional approach to developing a formulation is to change 1 variable at a time. By this method it is difficult to develop an optimized formulation, as the method reveals nothing about the interactions among the variables. Hence, a Box-Behnken statistical design with 3 factors, 3 levels, and 15 runs was selected for the optimization study. The experimental design consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube. The independent and dependent variables are listed in Table 1. The polynomial equation generated by this experimental design (using Statistica Release 6, Statsoft Inc) is as follows:

$$Y_i = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 \quad (1)$$

where Y_i is the dependent variable; b_0 is the intercept; b_1 to b_{33} are the regression coefficients; and X_1, X_2 and X_3 are the independent variable that was selected from the preliminary experiments.

Table 1. Variables and Their Levels in Box-Behnken Design

	Levels		
Independent Variables	Low	Medium	High
X_1 = molar ratio of Span 60:cholesterol	3:2	1:1	2:3
X_2 = surfactant loading	1X*	3X	5X
X_3 = amount of drug	2.5 mg	5 mg	7.5 mg
Transformed values	–1	0	1
Dependent variables			
Y_1 = percentage drug entrapment			
Y_2 = vesicle size			

* 1X corresponds to 1 mmol per gram of carrier.

Preparation of Proniosomes

Proniosomes were prepared by the slurry method.[6,7] For ease of preparation, a 250-mmol stock solution of Span 60 and cholesterol was prepared in chloroform. All the batches were prepared according to the experimental design in Table 2. The required volume of Span 60 and cholesterol stock solution per gram of carrier and drug dissolved in chloroform was added to a 100-mL round-bottom flask containing the maltodextrin carrier. Additional chloroform was added to form a slurry in the case of lower surfactant loading. The flask was attached to a rotary flash evaporator (EIE-R, Ahmedabad, India) to evaporate chloroform at 60 to 70 rpm, a temperature of 45°C ± 2°C, and a reduced pressure of 600 mm Hg until the mass in the flask had become a dry, free-flowing product. These proniosomes were stored in a tightly closed container until further evaluation.

Scanning Electron Microscopy

The surface characteristics of the proniosome batches were studied by scanning electron microscopy (SEM). Double-sided carbon tape was affixed on aluminum stubs. The powder sample of proniosomes was sprinkled onto the tape. The aluminum stubs were placed in the vacuum chamber of a scanning electron microscope (XL 30 ESEM with EDAX, Philips, Eindhoven, The Netherlands). The samples were observed for morphological characterization using a gaseous secondary electron detector (working pressure: 0.8 torr, acceleration voltage: 30.00 kV) XL 30, Philips (Eindhoven, The Netherlands). The particles were observed for surface characteristics.

Preparation of Niosomes From Proniosomes

Proniosomes were transformed to niosomes by hydrating with phosphate-buffered saline (PBS) pH 7.4 at 80°C using a vortex mixer for 2 minutes. The niosomes were sonicated twice for 30 seconds using a 250-W probe-type sonicator

Table 2. Box-Behnken Experimental Design With Measured Responses*

Batch No	X_1	X_2	X_3	Y_1† (PDE ± SD)	Y_2 (μm)
1	0	–1	–1	85.72 ± 1.23	3.48
2	0	–1	1	74.92 ± 0.95	4.10
3	0	1	–1	80.42 ± 1.76	6.85
4	0	1	1	70.12 ± 1.18	7.22
5	–1	0	–1	83.64 ± 0.80	5.34
6	–1	0	1	75.36 ± 2.34	4.88
7	1	0	–1	68.37 ± 1.52	6.19
8	1	0	1	69.58 ± 3.47	5.64
9	–1	–1	0	81.79 ± 2.12	2.98
10	–1	1	0	71.15 ± 0.68	8.40
11	1	–1	0	66.26 ± 0.83	3.73
12	1	1	0	68.76 ± 1.38	7.52
13	0	0	0	78.60 ± 1.07	4.53
14	0	0	0	77.44 ± 1.62	4.27
15	0	0	0	81.88 ± 1.90	5.36

* PDE indicates percentage drug entrapment.
† n = 3.

(MAGNA-PAK-250, Libra Ultrasonic, Kolkata, India). Niosomes were prepared in such a manner that total surfactant concentration remained at 10 mmol in all the batches.

Microscopy

The niosomes were mounted on glass slides and viewed under a microscope (Medilux-207R II, Kyowa-Getner, Ambala, India) with a magnification of 1200X for morphological observation after suitable dilution.

Vesicle Size Determination

The vesicle sizes of niosomes were determined by using a particle size analyzer (laser diffraction particle size analyzer, Sympatec, Clausthal-Zellerfeld, Germany). The apparatus consisted of a He-Ne laser beam of 632.8 nm focused with a minimum power of 5 mW using a Fourier lens (R-5) to a point at the center of a multielement detector and a small-volume sample holding cell (Su cell) The sample was stirred before determining the particle size as MVD.

PDE

The PDE of piroxicam niosomes was calculated after determining the amount of unentrapped drug by dialysis.[12] The dialysis was performed by adding the niosomal dispersion to a dialysis tube (donor compartment) and then dipping the tube into a beaker containing 400 mL of PBS pH 7.4 (receptor compartment) on a magnetic stirrer, rotated at a speed of 80 to 120 rpm for 4 hours. After 4 hours, the solution in the receptor compartment was estimated for unentrapped drug at 253.5 nm by using a UV spectrophotometer (UV 1601, Shimadzu, Kyoto, Japan). The PDE of the niosomes was calculated from the following ratio: (difference between the total amount of drug added and amount of unentrapped drug):total amount of drug added.

Checkpoint Analysis

A checkpoint analysis was performed to confirm the role of the derived polynomial equation and contour plots in predicting the responses. Values of independent variables were taken at 3 points, 1 from each contour plot, and the theoretical values of PDE were calculated by substituting the values in the polynomial equation. Proniosomes were prepared experimentally at 3 checkpoints, transformed to niosomes, and evaluated for the responses.

Optimum Formula

After developing the polynomial equations for the responses PDE and MVD with the independent variables, the formulation was optimized for the response PDE. Optimization was performed to find out the level of independent variables (X_1, X_2, and X_3) that would yield a maximum value of PDE with constraints on MVD.

Results and Discussion

Maltodextrin was used as a carrier for the preparation of proniosomes. Figure 1 shows SEM images of pure maltodextrin particles and proniosomes of batch 5 (at medium-level surfactant loading) and batch 10 (at high-level surfactant loading). Figure 1 shows the porous surface of the pure maltodextrin particles, which makes them effective carriers and provides more surface area for the coating of the surfactant mixture. SEM images of proniosomes (batch 5 and batch 10) show the coating of the surfactant mixture on the carrier particles. Comparison of the various proniosome images revealed that the surface of the carrier particles at the medium level of surfactant loading appeared to be more uniform and thinner than the rough and uneven coating at high surfactant loading. Some particles in the images are broken, which might be due to handling and processing. Proniosome-derived niosomes were observed under a microscope to examine their morphology. Multilamellar niosomes with an aqueous core were observed to be mostly spherical, with a few being slightly elongated (Figure 2).

Data Analysis

A Box-Behnken experimental design with 3 independent variables at 3 different levels was used to study the effects on dependent variables. All the batches of proniosomes

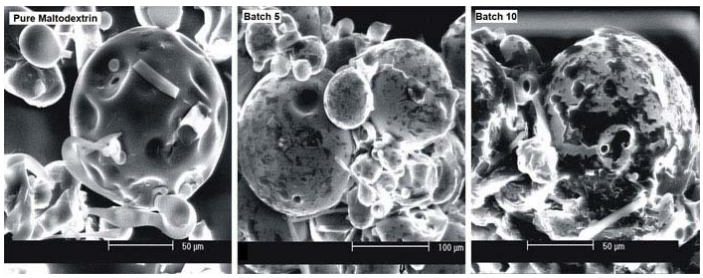

Figure 1. Scanning electron micrographs of various batches of proniosomes.

within the experimental design yielded niosomes on hydration, and these were evaluated for PDE and vesicle size. A Box-Behnken experimental design has the advantage of requiring fewer experiments (15 batches) than would a full factorial design (27 batches). Transformed values of all the batches along with their results are shown in Table 2. Formulations 1, 3, 5, 9, and 15 had the highest PDE (>80%). Table 3 shows the observed and predicted values with residuals and percent error of responses for all the batches. The PDE (dependent variable) obtained at various levels of the 3 independent variables (X_1, X_2, and X_3) was subjected to multiple regression to yield a second-order polynomial equation (full model):

$$\begin{aligned} PDE &= 79.31 - 4.87X_1 - 2.28X_2 - 3.52X_3 \\ &\quad + 3.29X_1X_2 + 2.37X_1X_3 + 0.13X_2X_3 \\ &\quad - 5.44X_1^2 - 1.88X_2^2 + 0.37X_3^2 \end{aligned} \qquad (2)$$

The value of the correlation coefficient (r^2) of Equation 2 was found to be 0.933, indicating good fit. The PDE values measured for the different batches showed wide variation

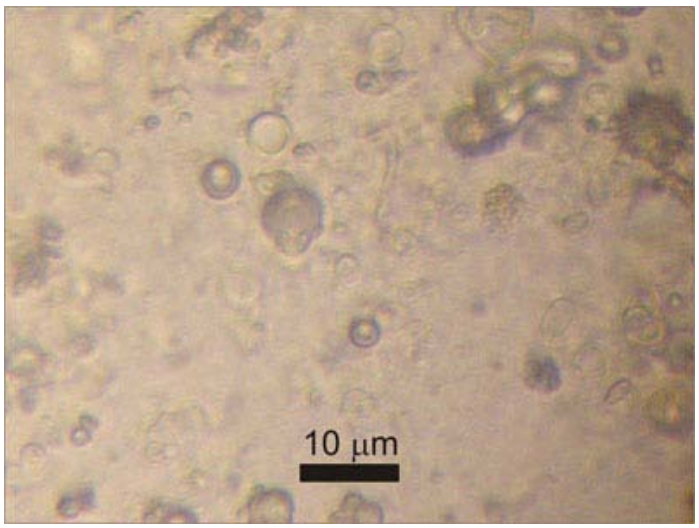

Figure 2. Optical photomicrograph of proniosome-derived niosomes (batch 5).

Table 3. Observed and Predicted Values With Residuals of the Response Y_1*

Batch No	Observed PDE	Predicted PDE	Residuals	% Error
1	85.72	83.72	2.00	2.48
2	74.92	76.43	–1.51	2.18
3	80.42	78.91	1.51	1.72
4	70.12	72.12	–2.00	2.67
5	83.64	85.00	–1.36	1.21
6	75.36	73.22	2.14	0.30
7	68.37	70.52	–2.14	6.61
8	69.58	68.22	1.36	5.37
9	81.79	82.43	–0.64	3.24
10	71.15	71.30	–0.15	4.82
11	66.26	66.11	0.15	4.74
12	68.76	68.12	0.64	5.70
13	78.60	79.31	–0.71	0.90
14	77.44	79.31	–1.87	2.41
15	81.88	79.31	2.57	3.14

* PDE indicates percentage drug entrapment.

(ie, values ranged from a minimum of 68.58 to a maximum of 85.72). The results clearly indicate that the PDE value is strongly affected by the variables selected for the study. This is also reflected by the wide range of values for coefficients of the terms of Equation 2. The main effects of X_1, X_2, and X_3 represent the average result of changing 1 variable at a time from its low level to its high level. The interaction terms (X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2) show how the PDE changes when 2 variables are simultaneously changed. The negative coefficients for all 3 independent variables indicate an unfavorable effect on the PDE, while the positive coefficients for the interactions between 2 variables (X_1X_2, X_1X_3, and X_2X_3) indicate a favorable effect on the PDE. Among the 3 independent variables, the lowest coefficient value is for X_2 ($b_2 = -2.28$ and $P > .05$), indicating that this variable is insignificant in prediction of PDE. The standardized effect of the independent variables and their interaction on the dependent variable was investigated by preparing a Pareto chart (Figure 3), which depicts the main effect of the independent variables and interactions with their relative significance on the PDE. The length of each bar in the chart indicates the standardized effect of that factor on the response. The fact that the bar for X_2, X_1X_2, X_1X_3, X_2X_3, X_2^2, and X_3^2 remains inside the reference line in Figure 3, and the small coefficients for these terms in Equation 2, indicate that these terms contribute the least in prediction of PDE. Hence, these terms are omitted from the full model to obtain a reduced second-order polynomial equation (Equation 3) by multiple regression of the PDE and the significant terms ($P < .05$) of Equation 2:

$$PDE = 78.44 - 4.87X_1 - 3.52X_3 - 5.33X_1^2 \qquad (3)$$

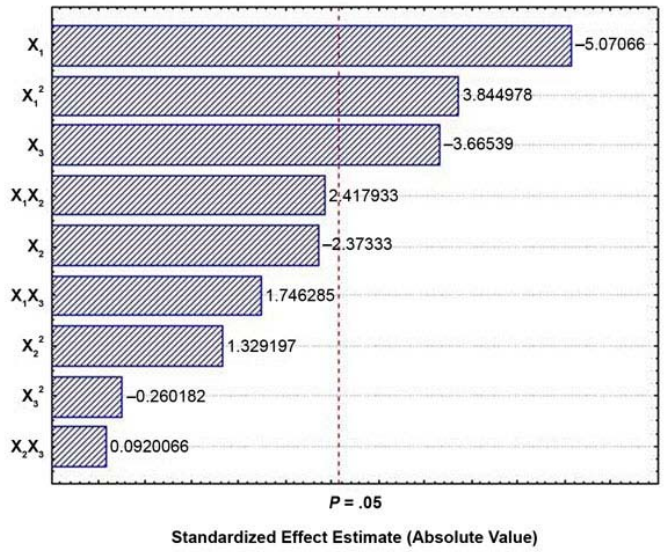

Figure 3. Pareto chart showing the standardized effect of independent variables and their interaction on the percentage drug entrapment of proniosome-derived niosomes.

To confirm the omission of nonsignificant terms, an F statistic was calculated after applying analysis of variance for the full model and the reduced model; the results are recorded in Table 4. The F calculated value (2.74) is less than the tabled value of F (4.95) at a 0.05 confidence interval, $v_1 = 6$ and $v_2 = 5$. Hence it is concluded that the omitted terms do not significantly contribute to predicting the PDE. This implies that the main effect of the molar ratio of Span 60: cholesterol and the amount of drug added is significant, as is evident from their high coefficients and the fact that the bars corresponding to variables X_1, X_3, and X_1^2 extend beyond the reference line in Figure 3. Vesicle size (MVD) of the niosome batches, measured by using a low-angle laser light scattering technique, was found to be in the range of 2.98 μm to 8.4 μm. A polynomial equation was also developed for MVD:

$$\begin{aligned} MVD = {} & 4.72 + 0.19X_1 + 1.96X_2 - 0.003X_3 \\ & - 0.41X_1X_2 - 0.02X_1X_3 - 0.06X_2X_3 \\ & + 0.52X_1^2 + 0.42X_2^2 + 0.27X_3^2 \end{aligned} \quad (4)$$

The value of the correlation coefficient (r^2) of Equation 4 was found to be 0.932, indicating good fit. Among the independent variables selected and their interactions, only X_2 was found to be significant ($P < .05$), indicating a major contributing effect of X_2 on MVD. A positive value of the coefficient for X_2 (surfactant loading) indicates a favorable effect on MVD. Vesicles obtained at low surfactant loading are smaller than are those obtained at high surfactant loading. The smaller size may result from efficient hydration of a uniform and thin film of surfactant mixture at low surfactant loading, compared with higher surfactant loading. These results are in agreement with the results reported by Rhodes et al.[7]

The 3 replicated center points in the Box-Behnken experimental design made it possible to assess the pure error of the experiments and enabled the model's lack of fit to be checked. In this study, the model was checked for lack of fit for both the responses PDE and MVD (by using Statistica Release 6). For lack of fit *P* values we obtained 0.398 and 0.369 for PDE and MVD, respectively, and hence the current model provided a satisfactory fit to the data ($P > .05$) and had no lack of fit.

The relationship between the dependent and independent variables was further elucidated by constructing contour plots. The effects of X_1 and X_3 with their interaction on PDE at a fixed level of X_2 (medium level) are shown in Figure 4. The plots were found to be linear up to 74% PDE, but above this value, the plots were found to be nonlinear indicating a nonlinear relationship between X_1 and X_3. It was determined from the contour plot that a higher value of PDE (≥80%) could be obtained with an X_1 level range from –0.1 to 0.44, and an X_3 level range from –0.1 to 0.12. It is evident from the contour that the low level of both X_1 and X_3 favors PDE of proniosome-derived niosomes. This observation is in agreement with the observation of Baillie et al,[3] who reported that the cholesterol decreased the entrapment efficiency. Span 60 is present in a higher proportion at the low level of X_1, which is hydrophobic, resulting in high entrapment because of hydrophobic interaction with the drug. When the coefficient values of 2 key variables, X_1 and X_3, were compared, the value for variable X_1 ($b_1 = -4.87$) was found to be higher, indicating that it contributes the most to predicting the PDE. The negative effects of X_3 on PDE may be attributed to partitioning of the drug in the hydrophobic interior of vesicles until the interior becomes saturated with drug. After that, incorporation of more drug results in an increase in the concentration of free drug and hence a

Table 4. Results of ANOVA of Full and Reduced Models for PDE of Proniosome-Derived Niosomes*

ANOVA	Df	SS	MS	F value	*P* value
Regression					
A	9	516.40	57.38	7.77	0.018
B	3	395.05	131.68	9.15	0.0025
Residuals					
A	5	36.92 (C_1)	7.38 (D_1)		
B	11	158.27 (C_2)	14.39		

* ANOVA indicates analysis of variance; PDE, percentage drug entrapment; A, full model; B, reduced model; Df, degrees of freedom; SS, sum of squares; MS, mean of squares; F, Fischer's ratio. $F_{CAL} = [(C_2 - C_1)/N_{TO}]/D_1 = 2.74$, where N_{TO} is the number of terms omitted (having a *P* value more than .05).

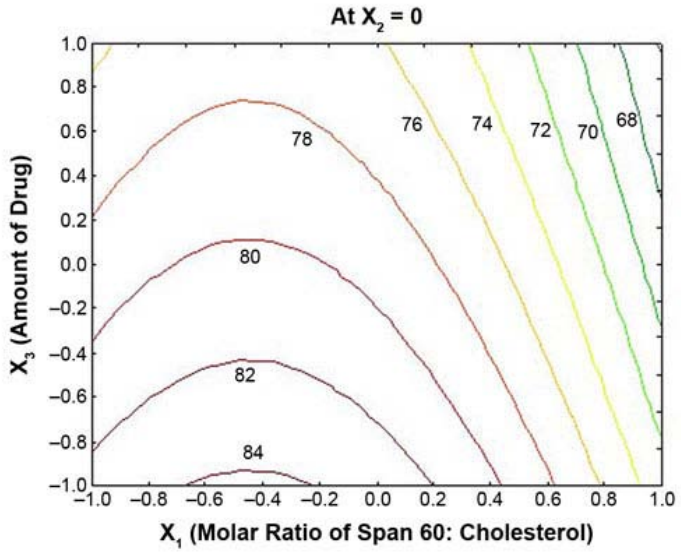

Figure 4. Contour plot showing the effect of molar ratio of Span 60:cholesterol (X_1) and amount of drug added (X_3) on the percentage drug entrapment of proniosome-derived niosomes.

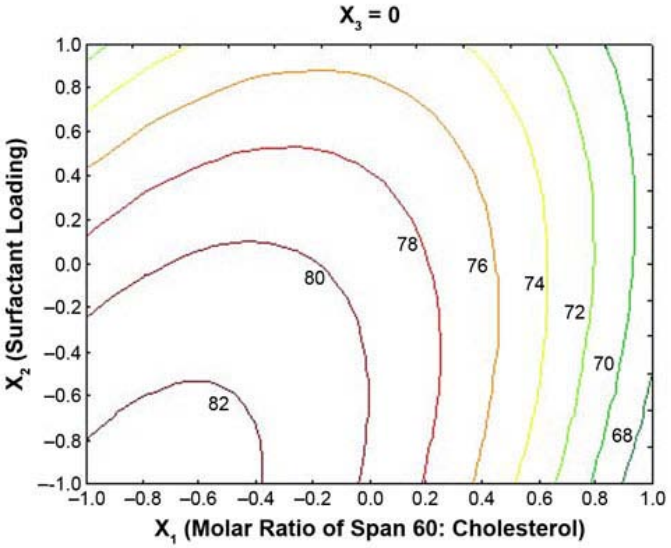

Figure 5. Contour plot showing the effect of molar ratio of Span 60:cholesterol (X_1) and surfactant loading (X_2) on the percentage drug entrapment of proniosome-derived niosomes.

decrease in the PDE within the niosomes. Figure 5 shows the contour plot drawn at a 0 level of X_3. The contours of all the PDE values were found to be curvilinear and indicated that a high value of PDE (≥80%) can be obtained for a combination of the 2 independent variables, the X_1 level in the range of –1 to 0, and the X_2 level in the range of –1 to 0.12. As discussed earlier, the high PDE value may be due to efficient hydration of the thin and uniform coating at a low level of X_2 (surfactant loading). Similarly, Figure 6 shows the contour plot plotted at a 0 level of X_1. The plot corresponding to 74% PDE is linear, but above this value of PDE, plots were found to be nonlinear in relationship to X_2 and X_3, and a high value of PDE (≥80%) can be obtained with an X_2 level range of –1 to 0.81 and an X_3 level range of –1 to 0. From Figures 4, 5, and 6 the following observations can be made. All the contour plots for a high value of PDE were found to be nonlinear. This signifies that there is no direct linear relationship among the selected independent variables. A high value of PDE can be obtained up to a certain level of all 3 independent variables, but above this an increase in the level of independent variables leads to a decrease in the PDE of proniosome-derived niosomes.

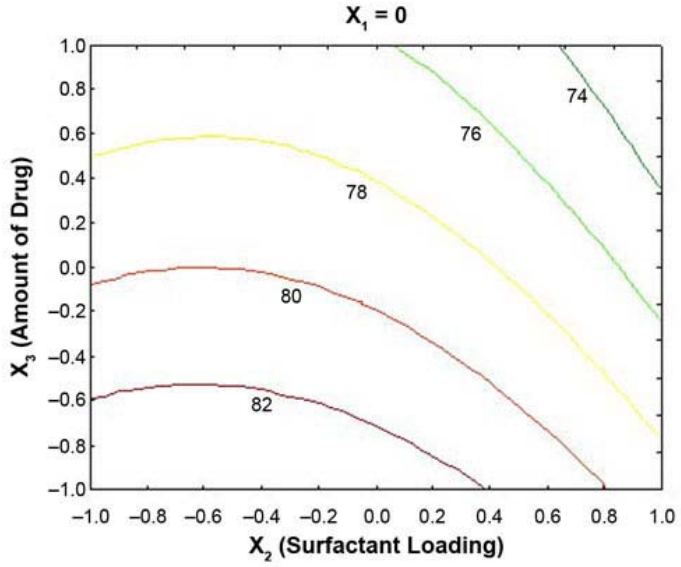

Figure 6. Contour plot showing the effect of surfactant loading (X_2) and amount of drug added (X_3) on the percentage drug entrapment of proniosome-derived niosomes.

Checkpoint Analysis

Three checkpoint batches were prepared and evaluated for PDE, as shown in Table 5. Results indicate that the measured PDE values were as expected. When measured PDE values were compared with predicted PDE values using Student *t* test, the differences were found to be insignificant

Table 5. Checkpoint Batches With Their Predicted and Measured Value of PDE*

				PDE	
Batch Code	X_1	X_2	X_3	Measured†	Predicted
C_1	0	–0.5	0.5	77.38 ± 0.31	78.28
C_2	0.5	0	–0.5	78.81 ± 1.28	76.78
C_3	–0.5	0.5	0	79.56 ± 1.73	77.95

* PDE indicates percentage drug entrapment.

† n = 3.

($P > .05$). Thus, we can conclude that the obtained mathematical equation is valid for predicting the PDE.

Optimum Formula

After studying the effect of the independent variables on the responses, the levels of these variables that give the optimum response were determined. It is evident from the polynomial equation and contour plots (Figure 4 and 5) that cholesterol decreases the PDE within niosomes. Also, cholesterol is known to abolish the gel-to-liquid phase transition of niosomes, and the resulting niosomes are known to be less leaky. Hence, the medium level was selected as optimum for the molar ratio of Span 60:cholesterol (X_1), as up to this level a high value of PDE can be obtained. The optimum formulation is one that gives a high value of PDE (≥80%) and a low MVD (≤5 μm) along with a high total amount of drug entrapped and a low amount of carrier in the resultant niosomes. Using a computer optimization process and the contour plot shown in Figure 6, for both X_2 and X_3 we selected a level of –0.158, which gives the theoretical values of 80.15% and 4.44 μm for PDE and MVD, respectively. Below the selected (optimum) level of X_2, decreases in surfactant loading result in a significant increase in the amount of carrier and an insignificant increase in the PDE. However, a decrease in the level of amount of drug (X_3) below the selected level leads to a decrease in the total amount of entrapped drug. Hence, a 0 level (1:1) for the molar ratio of Span 60:cholesterol (X_1), a –0.158 level of surfactant loading (X_2), and a –0.158 level of amount of drug (X_3) were selected as optimum. For confirmation, a fresh formulation was prepared at the optimum levels of the independent variables, and the resultant proniosomes were transformed to niosomes and evaluated for the responses. The observed values of PDE and MVD were found to be 82.16% and 4.86 μm, respectively, which were in close agreement with the theoretical values.

CONCLUSION

Optimization of a proniosome formulation is a complex process that requires one to consider a large number of variables and their interactions with each other. The present study conclusively demonstrates the use of a Box-Behnken design in optimization of proniosome formulations. The derived polynomial equations and contour plots aid in predicting the values of selected independent variables for preparation of optimum proniosome formulations with desired properties.

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