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Original Article

Preparation, Characterization, Optimization, and Stability Studies of Aceclofenac Proniosomes

Ajay Solanki[a*], Jolly Parikh[a] and Rajesh Parikh[b]

[a]*Department of Pharmaceutics and Pharmaceutical Technology, A. R. College of Pharmacy and G. H. Patel Institute of Pharmacy, P.O. Box 19, Vallabh Vidyanagar-388 120 Gujarat, India.*

Abstract

The aim of this investigation was to prepare, characterize and optimize the aceclofenac proniosomes using central composite design and carry out stability studies. Three independent variables selected were molar ratio of drug to lipid (X_1), surfactant loading (X_2) and volume of hydration (X_3). Based on central composite design, 16 batches of proniosomes were prepared by slurry method and evaluated for the percentage drug entrapment (PDE) and mean volume diameter (MVD). The PDE and MVD (dependent variables) and the transformed values of independent variables were subjected to multiple regressions to establish a second order polynomial equation. Contour plots were constructed to further elucidate the relationship between the independent and dependent variables. The conformity of the polynomial equations was checked by preparing three checkpoint batches. From the computer optimization process and contour plots, predicted levels of independent variables X_1, X_2, and X_3 (-0.77, -0.8 and 0 respectively), for an optimum response of PDE with constraints on MVD were determined. The optimized batch was subjected to stability studies. The polynomial equations and contour plots developed using central composite design allowed us to prepare proniosomes with optimum responses. Proniosomes stored refrigerated and at room temperature, were both found to be stable.

Keywords: Proniosomes; Niosomes; Central composite design; Aceclofenac, Optimization.

Introduction

Many drugs, those currently available in the market and those under development, have poor aqueous solubilities that result in variable bioavailabilities. This problem can be overcome by entrapping the drug into niosomes. Niosomes are non-ionic surfactant vesicles that can entrap a solute in a manner analogous to liposomes. They are osmotically active, and are stable on their own, while also increasing the stability of the entrapped drugs (1, 2). Handling and storage of surfactants require no special conditions. Niosomes possess an infrastructure consisting of hydrophilic and hydrophobic moieties together, and as a result, can accommodate drug molecules with a wide range of solubilities (3). Although niosomes as drug carriers have shown advantages such as being cheap and chemically stable, they are associated with problems related to physical stability such as fusion, aggregation, sedimentation and leakage on storage. All methods traditionally used for preparation of niosomes are time consuming and many involve specialized equipments. Most of these methods

* Corresponding author:
E-mail: ajay_solanki76@yahoo.com

allow only for a predetermined lot size so material is often wasted if smaller quantities are required for particular dose application (4).

The proniosome approach minimizes these problems as it is a dry and free flowing product which is more stable during sterilization and storage. Ease of transfer, distribution, measuring and storage make it a versatile delivery system. Proniosomes are water-soluble carrier particles coated with surfactant, which can be measured out as needed and hydrated to form niosomes immediately before use on brief agitation in hot aqueous media (4-6).

In the present study the slurry method was used for the preparation and optimization study of aceclofenac, as this method is simple and easy to scale up. Aceclofenac is a poorly water soluble, non-steroidal anti-inflammatory drug which acts specifically on inflammatory sites and thereby decreases the inflammation. It is highly effective as an anti-inflammatory drug for various inflammatory conditions like rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (7).

Apart from surfactant loading, other formulation variables like molar ratio of drug to lipid and volume of hydration at the time of reconstitution also affect the characteristics of proniosome-derived niosomes. The proniosomes are thus needed to be optimized for the desired response. Many statistical experimental designs have been recognized as useful techniques to optimize the formulation and process variables (8). Different types of experimental design include 3-level factorial design (9), D-optimal design (10), and Central composite design (11). Central composite design requires fewer runs in a 3 factor experimental design and hence was selected for the present study.

The aim of the present study was to prepare, characterize and optimize the aceclofenac proniosomes for percentage drug entrapment (PDE) with constraints on the mean volume diameter (MVD) using central composite design and to carry out stability studies on them. The independent variables selected for the present study are molar ratio of drug to lipid (X_1), surfactant loading (X_2) and volume of hydration (X_3). The dependent variables included are PDE and MVD of proniosome-derived niosomes.

Experimental

Materials

Aceclofenac was a gift from Alembic ltd. (Vadodara, India). Span 60 and cholesterol were purchased from S.D. Fine Chemicals (Mumbai, India). Dialysis tube (DM-70; Capacity: 2.41ml/cm, width: 29.31 mm, Avg. diameter 17.5 mm and molecular weight cut off: 12000 to 14000) was purchased from Hi-Media Laboratories (Mumbai, India). Chloroform, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride were procured from National Chemicals. (Vadodara, India). All chemicals used in the study were of analytical grade and used without further purification.

Method

Central composite experimental design

Traditionally pharmaceutical formulations are developed by changing one variable at a time. By this method it is difficult to develop an optimized formulation, as it does not give an idea about the interactions among the variables. Hence, a central composite experimental design with 3 factors, 3 levels and 16 runs was selected for the optimization study. This design consists of 8 full factorial design points, 6 axial points, and 2 center points.

Independent variables with their levels and the dependent variables selected are listed in Table 1. The second order polynomial equation generated from this experimental design using Microsoft Excel is described as:

$$Y_i=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2 \quad \text{..... (1)}$$

Where Yi is the dependent variable while 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 variables (12) levels of which were selected from the preliminary experiments.

Preparation of proniosomes

Proniosomes were prepared by the slurry method (4). For the ease of preparation 250 mmol stock solutions of span 60 and cholesterol in chloroform were prepared. All the batches were

Table 1. Variables and their levels in central composite design.

Independent variables	Levels		
	Low	Medium	High
X_1 = Molar ratio of drug: lipid	1:40	1:30	1:20
X_2 = Surfactant loading	1.5%*	3%	4.5%
X_3 = Volume of hydration	3 ml	5 ml	7 ml
Transformed values	-1	0	1
Dependent variables			
Y_1 = Percentage drug entrapment			
Y_2 = Mean vesicle diameter			

* 1.5% corresponds to 1.5 mmol per g of carrier.

prepared according to the experimental design given in Table 2. The required volume of span 60 and cholesterol stock solution per g of carrier, and the drug dissolved in chloroform were added to a 100 ml round bottom flask containing the maltodextrin as a carrier. Additional chloroform was added to form a slurry in in stances of lower surfactant loading. The flask was attached to a rotary flash evaporator (EIE-R, India.) to evaporate chloroform at at the speed of 60-70 rpm, temperature of 45±2°C and under vacuum (600 mmHg) until the mass in the flask resulted in a dry free flowing product. These proniosomes were used for preparation of niosomes and characterization of the surface characteristics by scanning electron microscopy.

Proniosomes were transformed to niosomes by hydrating with phosphate buffer saline (PBS) with a pH of 7.4 at 80°C using vortex mixer for 2 min. The niosomes were sonicated twice for 30 s using a 250-W probe-type sonicator (MAGNA-PAK-250, Libra Ultrasonic, India). Niosomes were prepared in such a manner that total surfactant concentration remained at 10 mmol in all the batches. Niosomes were characterized for morphology, PDE and vesicle size in terms of MVD.

Scanning electron microscopy

Proniosomes were sprinkled on to the double-

Table 2. Central composite experimental design with measured responses of aceclofenac proniosomes.

Batch code	X_1	X_2	X_3	PDE ± SD*	MVD(μm)
BN1	-1	-1	-1	71.5±1.86	4.17
BN2	-1	-1	1	74.34±1.64	3.46
BN3	-1	1	-1	70.53±2.17	4.64
BN4	-1	1	1	73.3±2.37	4.22
BN5	1	-1	-1	35.6±1.98	4.3
BN6	1	-1	1	63.7±1.73	4.74
BN7	1	1	-1	56.76±2.32	5.28
BN8	1	1	1	61.12±4.36	5.85
BN9	-1.68	0	0	74.86±4.39	3.97
BN10	1.68	0	0	57.43±2.78	4.86
BN11	0	-1.68	0	68.72±4.43	4.12
BN12	0	1.68	0	66.48±2.82	5.51
BN13	0	0	-1.68	63.38±4.63	5.56
BN14	0	0	1.68	70.8±4.3	4.89
BN15	0	0	0	68.76±2.14	5.77
BN16	0	0	0	66.1±1.33	6.84

*n=3

sided tape that was affixed on aluminum stubs. The aluminum stub was placed in the vacuum chamber of a scanning electron microscope (XL 30 ESEM with EDAX, Philips, 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, Netherlands).

Optical microscopy

The hydrated niosome dispersions prepared from proniosomes were observed using optical microscopy. After suitable dilution, the noisome dispersions on glass slide and viewed by a microscope (Medilux-207R (II), Kyowa-Getner, India) with a magnification of 1200X.

Percentage drug entrapment (PDE)

The entrapped aceclofenac within niosomes was determined after removing the unentrapped drug by dialysis (13). The dialysis was carried out by taking niosomal dispersion in dialysis tube (donor compartment), which was dipped in a beaker containing 400 ml of PBS with a pH of 7.4 (receptor compartment). The beaker was placed on a magnetic stirrer run for 4 h with a speed of 80-120 rpm. Then, the solution inside the receptor compartment was studied for unentrapped aceclofenac at 275 nm using an UV spectrophotometer (UV 1601, Shimadzu, Japan). The PDE in the niosomes was calculated from the ratio of the difference of the total amount of drug added and the amount of unentrapped drug detected, to the total amount of drug added.

Measurement of vesicle size

The vesicle dispersions were diluted about 100 times in the same buffer used for their preparation. Vesicle size was measured on a particle size analyzer (Laser diffraction particle size analyzer, Sympatec, Germany). The apparatus consists 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 multielement detector and a small volume sample holding cell (Su cell). The sample was stirred using a stirrer before determining the vesicle size.

Stability studies

To determine the stability of proniosomes,the optimized batch was stored in airtight sealed vials at 2-8°C temperature and room temperature (R. T.). Surface characteristics and percentage drug retained in proniosomes and proniosome-derived niosomes were selected as parameters for evaluation of the stability, since instability of the formulation would reflect in drug leakage and a decrease in the percentage drug retained. The proniosomes were sampled at regular intervals of time (0, 1, 2, and 3 months), observed for color change and surface characteristics, and tested for the percentage drug retained after being hydrated to form niosomes. The percentage drug retained was determined from the ratio of the entrapment to the initial entrapment of the drug.

Results and Discussion

Morphology of dry proniosomes and proniosome-derived niosomes

Proniosomes were prepared by the slurry method using maltodextrin as a carrier. Scanning electron microscopy (SEM) of uncoated maltodextrin powder (Figure 1a) shows the highly porous surface, which would provide more surface area to be coated with surfactant mixture. Proniosomes were made with different proportions of drug and surfactant coating. Figure 1b, c and d are SEM images of different proniosome batches made at different surfactant loading. Surface of the proniosomes batches PA2 and PA15, made at 1.5X and 3X respectively, was observed as being smooth and uniform while that of batch PA8, made at 4.5X surfactant loading was seen rough, thick and uneven. Morphology of proniosome-derived niosomes were studied under optical microscope. Niosomes prepared from proniosomes were spherical in shape (Figure 2).

Optimization study of proniosomes

An optimization using central composite design for 3 factors, 3 levels offers an advantage of fewer experimental runs (16 runs) as compared with that of 3 factors, 3 levels full factorial design, which requires 27 runs. The experimental runs and the observed responses for the 16 batches are given in Table 2. The different levels of

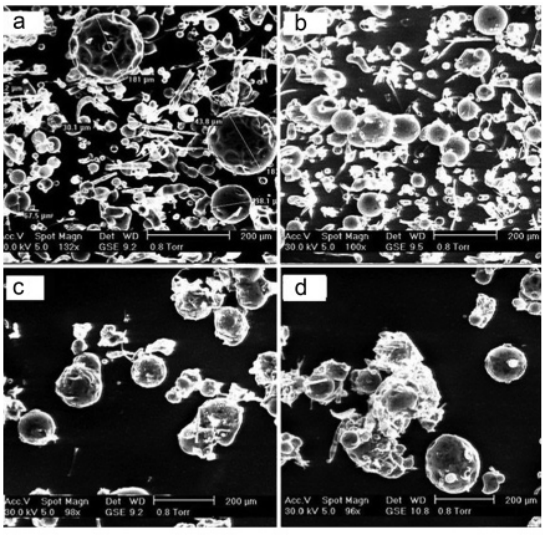

Figure 1. Scanning electron micrographs of proniosomes prepared: (a) with pure maltodextrin, (b) at 1.5X surfactant loading, (c) at 3X surfactant loading, and (d) at 4.5X surfactant loading.

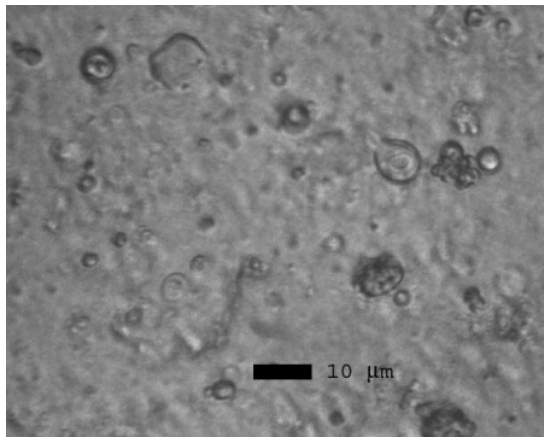

Figure 2. Optical photomicrograph of proniosome-derived niosomes (Batch PA2).

independent variable combinations resulted in different PDE and MVD values. The PDE values observed were in the range of 56.76% in batch PA7 (minimum) to 74.86% in batch PA9 (maximum). This indicates selected three independent variables have a profound effect on the PDE within proniosome-derived niosomes. The second order polynomial equation relating the response PDE and the independent variables was:

$$PDE=67.53-5.79X_1-0.73X_2+1.59X_3-0.34X_1X_2+0.29X_1X_3+0.09X_2X_3-0.69X_1^2-0.17X_2^2+0.001X_3^2 \quad \text{.....(2)}$$

The values of the coefficients X_1-X_3 are related to the effect of these variables on the PDE. Coefficients of more than one terms represents interaction and show how the response changes when two factors are simultaneously changed. Coefficients of higher order terms represent quadratic relationship and are included to investigate nonlinearity. The polynomial equation can be used to draw conclusions after considering the magnitude of each coefficient and the mathematical sign it carries (i.e., positive or negative). The high value (0.98) of correlation coefficient (R^2) for Equation 2 indicates a good fit. Proniosomal batches PA1, PA2, PA3, PA4, PA9 and PA14 exhibited high PDE value, i.e.more than 70% (Table 2). A negative sign of coefficient for molar ratio of drug: lipid (X_1) and surfactant loading (X_2) represents antagonistic effect of these variables. In this study at different levels of X_1, lipid was kept constant and the amount of drug was increased for each level to give a different molar ratio. So at a low level of X_1 high PDE value might be due to more availability of lipophilic ambience for the drug entrapment. A positive sign of the coefficient for volume of hydration (X_3) represents a favourable effect. This may be due to efficient hydration that takes place at a high level of X_3 during transformation of proniosomes to niosomes, resulting in a high PDE within niosomes. The significance of the different formulation variables and their interactions was compared using analysis of variance (ANOVA) at a significance level of $p<0.05$. From the *P* value for PDF analysis given in Table 3, it can be concluded that the molar ratio of drug: lipid and volume of hydration have significant effects on the PDE of aceclofenac proniosome-derived niosomes and no interaction term has a significant effect on the PDE.

Vesicle size (MVD) of the niosome batches was measured by low angle laser light scattering technique and was found to be in the range of 3.46 µm to 8.4 µm. A polynomial equation was developed for MVD, described as:

Table 3. Analysis of variance for PDE of aceclofenac proniosomes*.

Source	SS	DF	MS	F	P
X_1	438.38	1	438.38	256.00	0.000004
X_2	7.17	1	7.17	4.01	0.09
X_3	34.43	1	34.43	19.23	0.0046
X_1X_2	0.92	1	0.92	0.51	0.50
X_1X_3	0.69	1	0.69	0.39	0.56
X_2X_3	0.06	1	0.06	0.03	0.86
X_1^2	4.39	1	4.39	2.45	0.17
X_2^2	0.28	1	0.28	0.15	0.71
X_3^2	0.00	1	0.00	0.00	1.00
Lack of Fit	7.21	5	1.44	0.43	0.83
Pure Error	3.34	1	3.34		
Total SS	517.76	15			

* SS indicates sum of squares, DF, degree of freedom; MS, mean of squares; F, Fischer's ratio.

$$MVD=6.32+0.38X_1+0.41X_2-0.09X_3+0.11X_1X_2+0.27X_1X_3+0.05X_2X_3-0.7X_1^2-0.56X_2^2-0.41X_3^2 \quad (3)$$

The value of the correlation coefficient (R^2) of Equation 3 was found to be 0.95, indicating a good fit. A positive sign of the coefficients for the molar ratio of drug: lipid and surfactant loading indicates favorable effects on MVD. Positive effects of X_1 could be attributed to hydrophobic interaction between drug and surfactant. Favourable effect of X_2 may be due to efficient hydration of the uniform and thin film of surfactant at low surfactant loading compared to the film obtained at a high surfactant loading.

Negative sign of the coefficient for the volume of hydration (X_3) indicates a negative effect. As shown in Table 4, among the independent variables selected the terms X_1 and X_2 were found to be significant ($P<0.05$) in predicting the MVD. It is also evident from Table 4 that the quadratic effects of all the independent variables i.e. X_1^2, X_2^2 and X_3^2 have significant effects on MVD.

As the central composite design includes two center points, we can estimate the pure error of the experiments and enable the model's to be checked for lack of fit. For the experimentally obtained data, the test for lack of fit did not yield statistical significance ($P>0.05$), and the results indicated that the models for PDE and MVD were satisfactory (Table 3 and 4).

Contour plots

Presentation of the data as graphs can help to show the relationship between the independent and dependent variables. First contour plot was constructed at medium level of X_2, as this term is not significant in predicting the PDE value (Table 3). The effects of X_1 and X_3 with their interaction on PDE and MVD at a fixed level of X_2 (medium level) are shown in Figure 3. The plots for PDE were found to be linear which indicates a linear relationship between X_1 and X_3. It was determined from the contour plot that high values of PDE ($\geq 70\%$) could be obtained with different combinations of an X_1 value below -0.73 level and X_3 values in the entire range from -1 level to 1 level. It is evident from the contour that the low level of X_1 favurs high PDE value of proniosome-derived niosomes. Lipid was present in high proportion at low level of X_1, which can accommodate more drug, as where at a high level of X_1 (as the drug is present in a higher amount compared to a low level) saturation of lipid domains with reference to drug provides limited PDE value (14). Furthermore, Figure 3 also indicates low values of MVD can be obtained with low level of X_1 and high level of X_3. Coefficient value for the term X_3 in equation 2 ($b_3=1.59$) indicates positive effect

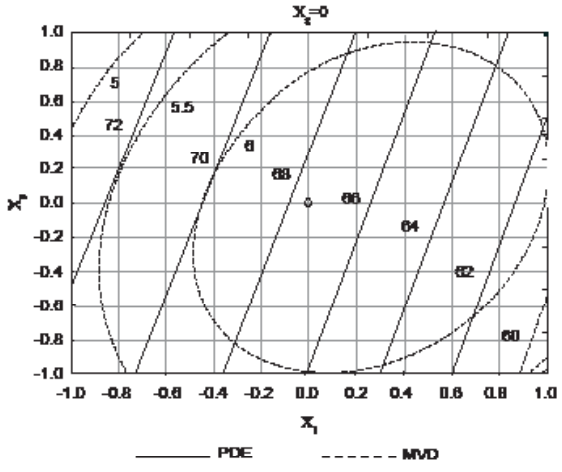

Figure 3. Combined contour plot of PDE and MVD at medium level of X_2.

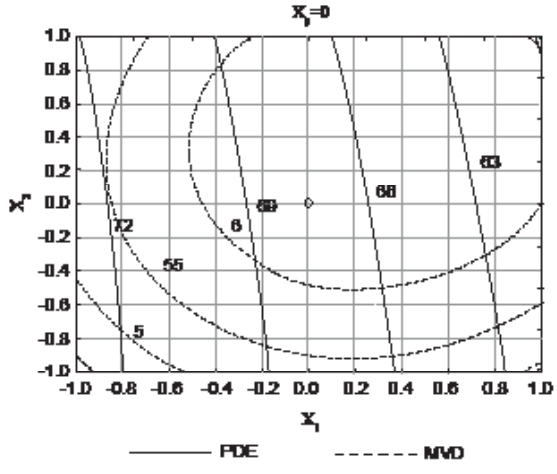

Figure 4. Combined contour plot of PDE and MVD at medium level of X_3.

on the PDE of proniosome-derived niosomes but at a high level of X_3 dilution of the niosomal dispersion takes place. Hence, another contour plot was constructed at medium level of the X_3.

Figure 4 is a contour plot drawn at 0 level of X_3, showing the effect of X_1 and X_2 on MVD and PDE of proniosome-derived niosomes. The contours for all the values of MVD were found to be nonlinear. It was evident from Figure 4 that low value of MVD could be obtained with low level of both X_1 and X_2 and that high values of PDE (≥72%) can be obtained for different combinations of the two independent variables, X_1 in the range of less than -0.8 level and X_2 in the entire range of -1 level to 1 level.

Checkpoint analysis

Three checkpoint batches were prepared for different combinations of independent variables and evaluated for PDE and MVD. The results shown in Table 5 indicate that the measured PDE and MVD values were as expected from the theoretical values computed from the polynomial equations and contour plots. When

Table 4. Analysis of variance for MVD of aceclofenac proniosomes*.

Source	SS	DF	MS	F	P
X_1	1.96	1	1.96	13.19	0.011
X_2	2.34	1	2.34	15.75	0.007
X_3	0.12	1	0.12	0.81	0.404
X_1X_2	0.09	1	0.09	0.62	0.461
X_1X_3	0.57	1	0.57	3.85	0.098
X_2X_3	0.02	1	0.02	0.15	0.714
X_1^2	4.58	1	4.58	30.77	0.001
X_2^2	2.92	1	2.92	19.65	0.004
X_3^2	1.58	1	1.58	10.62	0.017
Lack of Fit	0.32	5	0.06	0.11	0.97
Pure Error	0.57	1	0.57		
Total SS	11.40				

*SS, indicates sum of squares; DF, degree of freedom; MS, mean of squares; F, Fischer's ratio.

Table 5. Checkpoint batches of aceclofenac proniosomes with their predicted and measured responses.

Source	SS	DF	MS	F	P
X_1	1.96	1	1.96	13.19	0.011
X_2	2.34	1	2.34	15.75	0.007
X_3	0.12	1	0.12	0.83	0.404
X_1X_2	0.09	1	0.09	0.62	0.461
X_1X_3	0.57	1	0.57	3.85	0.098
X_2X_3	0.02	1	0.02	0.15	0.714
X_1^2	4.58	1	4.58	30.77	0.001
X_2^2	2.92	1	2.92	19.65	0.004
X_3^2	1.58	1	1.58	10.62	0.017
Lack of Fit	0.32	5	0.06	0.11	0.97
Pure Error	0.57	1	0.57		
Total SS	11.40				

*SS indicates sum of squares; DF, degree of freedom; MS, mean of squares; F, Fischer's ratio.

compared with the predicted PDE and MVD using student t-test the differences were found to be insignificant (P> 0.05). Thus, we can conclude that the obtained mathematical equations and contour plots are valid for predicting the value of PDE and MVD.

Optimum formula

After studying the effects of the independent variables on the responses, the levels of these variables that give the optimum responses were determined. Volume of hydration is a critical factor for preparation of niosomes from proniosomes as inadequate volume of hydration results in improper hydration of the film. Although, high values of PDE could be obtained with the entire range of volume of hydration (X_3), and it affects the final concentration of the lipid in niosomal dispersions. Hence, medium level of X_3 was selected as an optimum for the aceclofenac proniosomes.

The optimum formulation is one that gives a high value of PDE (≥70%) and is constrained to a low MVD (≤ 5 µm) as well as having a high total amount of drug entrapped and low amount of carrier present in the resultant niosomes. Using a computer optimization process and the contour plots shown in Figure 4, the levels selected for both X_1 and X_2 were -0.77 and -0.8 respectively, which gives the theoretical value of 71.84% and 4.99 µm for PDE and MVD, respectively.

Decreasing the level of X_2 from the optimum level resulted in a significant increase in the amount of carrier but an insignificant increase in the PDE value. However, an increase in the level of X_1 above the selected level led to an increase in the PDE value but as well an increase in the vesicle size above the desired value. Hence, -0.77 level of molar ratio of drug: lipid (X_1), -0.8 level of surfactant loading (X_2) and 0 level of volume of hydration (X_3) were selected as optimum. For a confirmation, a fresh formulation (Batch PAO) was prepared at the

Table 6. Stability studies-percentage drug retained in aceclofenac proniosome-derived niosomes (Batch PAO).

Batch code	X_1	X_2	X_3	PDE		MVD	
				Measured*	Predicted	Measured	Predicted
C1	-0.5	0	0.5	68.73 ± 1.64	70.58	3.86	3.74
C2	-0.5	-0.5	0	68.42 ± 1.38	70.45	3.7	3.64
C3	-1	-0.5	0	74.28 ± 2.16	72.83	4.48	4.64

*n=3

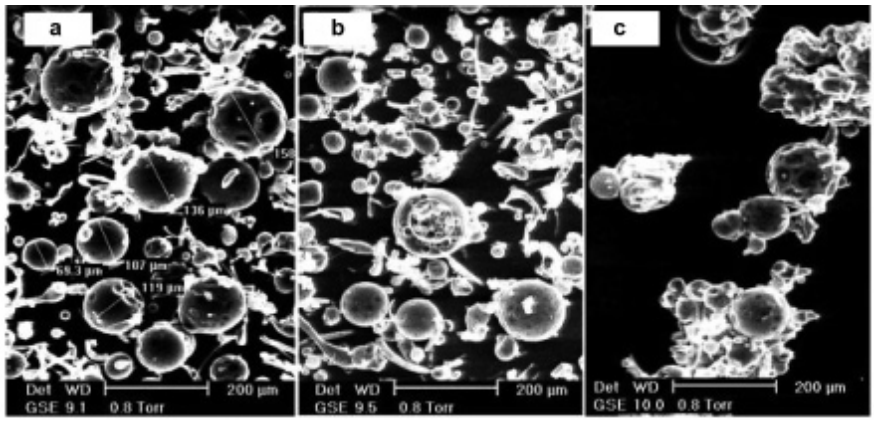

Figure 5. Stability studies - Scanning electron micrograph of aceclofenac proniosomes (a) at zero time (b) after 3 months at 2 - 8 °C and (c) after 3 months at R. T.

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 70.28% and 5.12 µm respectively, which were in close agreement with the theoretical values.

Stability studies

It was observed that there was no change in color of the proniosomes up to 3 months of storage. Figure 5 shows SEM images of the PAO batch at initial time and after 3 months at both storage conditions. It is evident from Figure 5a, b and c that surface characteristics of the proniosomes did not alter. The results of the percentage of drug retained are depicted in Table 6. Proniosomes of batch PAO were found to be stable and showed no significant difference in percentage of drug retained at both storage temperatures.

Conclusion

The slurry method was found to be simple and suitable for laboratory scale preparation of aceclofenac proniosomes. The statistical approach for optimization of formulation is a useful tool, when several variables are to be studied simultaneously. The polynomial equations and contour plots developed by using central composite design allowed us to prepare proniosomes with optimum characteristics. Proniosomes, stored at refrigerated and room temperature were both found to be stable.

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