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# OPTIMIZING PRONIOSOMES FOR CONTROLLED RELEASE OF KETOPROFEN USING BOX-BEHNKEN EXPERIMENTAL DESIGN

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## ABSTRACT

The present study deals with the investigation of the effect of formulation variable on ketoprofen (KP) proniosomes prepared by spray method. A three factor, three level Box-Behnken design (DOE) with response surface methodology (RSM) was run to evaluate the main and interaction effect of several independent formulation variables that included cholesterol concentration % ($X_1$), total lipid concentration µmole ($X_2$), and total amount of drug mg ($X_3$). The dependent variable included entrapment efficiency EE% ($Y_1$) and % drug released at 6 hrs ($Y_2$). A desirability function was used to maximize EE% and minimize the release percent to attain a controlled release formula. The transformed values of the independent variables and the dependent variables were subjected to multiple regressions to establish a full-model second-order polynomial equation. Contour plots were constructed to show the effects of $X_1$, $X_2$ and $X_3$ on the $Y_1$ and $Y_2$. The computer optimization process and contour plots predicted the levels of independent variables $X_1$, $X_2$, and $X_3$ (30, 2000, and 75 respectively), for maximized response of EE% (82.77%) and controlled release of drug (40.65%). 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 ketoprofen proniosomes. This study proved that Box-Behnken design could efficiently be applied for modeling of ketoprofen proniosomes.

**INTRODUCTION:** Ketoprofen (KP) is a poorly water-soluble non-steroidal anti-inflammatory, antipyretic and analgesic drug, frequently used for the treatment of rheumatoid arthritis, osteoarthritis [1], anklylosing spondylitis, a variety of other acute and chronic musculoskeletal disorders and mild to moderate pain [2]. Ketoprofen is a potent non-steroidal anti-inflammatory drug that inhibits prostaglandin synthetase cyclooxygenase. Its oral administration is associated with a high risk of adverse effects such as irritation, ulceration of the gastrointestinal tract, oedema, dizziness, and peptic ulceration when taken orally for a prolonged period [3]. 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 [4]. 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.

Non-ionic surfactant vesicles (Niosomes) are unilamellar or multilamellar vesicles that are made up of nonionic surfactants. Niosomes can entrap hydrophilic drugs and other bioactives upon encapsulation or hydrophobic material by partitioning of these molecules into hydrophobic domains. Moreover, niosomes possess great stability, cost-effectiveness, and simple methodology for the routine and large-scale production without the use of hazardous solvents. In recent years, niosomes have been extensively studied for their potential to serve as carriers for delivery of drugs, antigens, hormones and other bioactive agents. Niosomes are biodegradable, biocompatible, nontoxic and capable of encapsulating large quantities of material in relatively smaller volume of vesicles [5].

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, but they are associated with problems related to physical stability, such as fusion, aggregation, sedimentation, and leakage on storage [6]. The proniosome approach 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 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.

In the present study, the spray method was used for the preparation and optimization of ketoprofen proniosomes. Many others formulation variables, such as cholesterol concentration %, total lipid concentration and amount of drug, also affect the characteristics of proniosome-derived niosomes. Traditional experiments require more effort, time, and materials when a complex formulation needs to be developed. Various experimental designs [6] 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 [7] was used to optimize proniosomes containing ketoprofen and sorbitol as a carrier. The independent variables selected were cholesterol concentration ($X_1$), total lipid concentration ($X_2$), and total amount of drug ($X_3$) to evaluate their separate and combined effects on entrapment efficiency ($Y_1$) and % drug released at 6 hrs ($Y_2$).

**MATERIALS AND METHODS:** Ketoprofen (KP) was a gift sample kindly supplied by Amriya Pharmaceutical Industries, Alexandria, Egypt. Sorbitan monostearate (Span 60), cholesterol (Chol), were purchased from Sigma Chemical Co., St. Louis, MO, USA. Diethyl ether was purchased from s.d. Fine Chem. Ltd. (India). Sorbitol was purchased from El-Gomhorea Chemical Company, Cairo, Egypt. Chloroform and all other chemicals were obtained from El-Nasr Pharmaceutical Chemical Co., Cairo, Egypt. All ingredients were used as received.

**Preparation of Proniosomes:** The proniosomes were prepared according to the method developed by Hu and Rhode [8] with some modifications. The lipid mixture and KP were dissolved in 10ml chloroform-diethyl ether (1:1 v/v). The prepared solution was sub sequentially sprayed onto the surface of sorbitol powder in 100ml round bottom flask so that sorbitol: surfactant ratio was 10:1 [9]. During the spraying period, the rate of application was controlled at 2ml/min so that the powder bed of sorbitol didn't become overly wet such that slurry would form. The evaporator was then evacuated and the rotating flask was lowered into water bath maintained at 65-70$^{o}$C.

The flask was rotated in the water bath under vacuum for 15-20 min or until sorbitol powder appeared to be dried, then another aliquot of solution was introduced. This process was repeated until all the solution was applied. After addition of the final aliquot, evaporation was continued for about 20-30 min until the powder was completely dry producing free flowing product [10]. The loaded powder was further dried in the desiccator under vacuum at room temperature overnight. This dry preparation referred as proniosomal powder was stored in a tightly closed container and was used for the preparation of proniosome-derived niosomes and for further evaluation and further study on powder properties.

Proniosomes-derived niosomal dispersions were obtained by hydrating the proniosomal powder with 10 ml phosphate buffer solution (PBS) pH 7.4 at 80$^{o}$C using vortex mixer for 2min [11]. The resulting niosomal dispersion was used for the determination of the entrapment efficiency, morphological study and in-vitro release studies.

**Microscopic Examination:** The morphology of hydrated niosomes prepared from proniosomes was determined using optical microscope (Zeiss, Me 63 C, West Germany) with varied magnification powers. The prepared sample was spread on a glass slide and examined under microscope for niosomal vesicles formation [12]**.** Photomicrographs were taken for niosomes using Samsung digital camera.

**Determination of KP Entrapment Efficiency in Niosomes:** The KP-entrapped niosomes was separated from the un-entrapped free drug by the dialysis method as discussed by [13]. 1 ml of the prepared niosomal dispersion formed from proniosomes, was placed into a glass tube to which a cellophane membrane was attached to one side, the un-entrapped free KP was exhaustively dialyzed for one hour each time against 100ml of PBS (pH 7.4). The dialysis of free KP was completed after about five changes of buffer solution when no KP was detected in the solution [14]. The drug content was determined spectrophotometrically at 260 nm using PBS (pH 7.4) as a blank. The entrapment efficiency was defined as the percentage ratio of the entrapped drug concentration to the total drug concentration and calculated according to the following equation**.** Amount of entrapped drug was obtained by subtracting amount of free drug from the total drug incorporated.

$$EE\% = \frac{\text{Total drug concentration} - \text{Free drug concentration}}{\text{Total drug concentration}} \times 100$$

***In-vitro*** **release of KP from Niosomes:** The *in-vitro* release of KP from niosomes was determined by a simple dialysis method. One milliliter of the dialyzed vesicle dispersion or KP solution was placed into a glass tube to which a cellophane membrane was attached to one side, the tube was suspended in 250 ml beaker containing 100 ml PBS (pH 7.4). The solution was maintained at 37$^{o}$C$\pm$0.5$^{o}$C and stirred at 100 rpm in a thermostatically controlled water bath shaker. At different time intervals for 48 hrs, 4 ml samples were withdrawn from the receptor compartment, and replaced with an equal volume of fresh buffer solution (pH 7.4) at the same temperature (37$^{o}$C$\pm$0.5$^{o}$C) to keep the volume of the solution constant during the experiment. The samples were analyzed spectrophotometrically at 260 nm against PBS (pH 7.4) as a blank. Drug solution of the same concentration as in niosomal dispersion was also studied [10]**.** The percentage of the drug release was plotted as a function of time**.**

**Box-Behnken Experimental Design:** The traditional approach to developing a formulation is to change one 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 [6]**.** The use of experimental design allows for testing a large number of factors simultaneously and precludes the use of a huge number of independent runs when the traditional step-by-step approach is used. Systematic optimization procedures are carried out by selecting an objective function, finding the most important or contributing factors and investigating the relationship between responses and factors by the so-called response surface methodology [15]. The objective functions for the present study was selected as maximizing the % encapsulation efficiency while controlling the % drug release.

Hence, a Box-Behnken statistical design with 3 factors, 3 levels, and 15 runs was selected to statistically optimize the formulation parameters and evaluate the main effects, interaction effects and quadratic effects of the formulation ingredients on the % encapsulation efficiency of proniosomes and % drug released [7]**.** A 3-factor, 3-level design was used to explore the quadratic response surfaces and for constructing second order polynomial models thus helping in optimizing a process using a small number of experimental runs [16]**.** The Box-Behnken design was specifically selected since it requires fewer runs than a central composite design, in cases of three or four variables**.** 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**.

**TABLE 1: VARIABLES AND THEIR LEVELS IN BOX-BEHNKEN DESIGN**

| Independent variables | Levels | | |
|---|---|---|---|
| | Low (-1) | Medium (0) | High (1) |
| $X_1$= Cholesterol concentration (%). | 10 | 20 | 30 |
| $X_2$= Total lipid concentration (µmole). | 250 | 1125 | 2000 |
| $X_3$= Total drug concentration (mg). | 25 | 75 | 125 |
| **Dependant variables** | | **Constraints** | |
| $Y_1$= entrapment efficiency % | | Maximize | |
| $Y_2$= % drug released after 6 hours. | | Minimize | |

The polynomial equation generated by this experimental design (DOE PRO XL) is as follows:

$$Y_i = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_1 X_2 + b_5 X_1 X_3 + b_6 X_2 X_3 + b_7 X_1^2 + b_8 X_2^2 + b_9 X_3^2 \quad \text{.....(1)}$$

Where; $Y_i$ is the dependent variable; $b_0$ is the intercept; $b_1$ to $b_9$ are the regression coefficients computed from the observed experimental values of Y from experimental runs; and $X_1$, $X_2$ and $X_3$ are the independent variables that were selected from the preliminary experiments. The terms $X_1X_2$ and $X_i^2$ (i = 1, 2 or 3) represent the interaction and quadratic terms, respectively**.** Independent variables studied were the cholesterol concentration % ($X_1$), total lipid concentration (µmole) ($X_2$) and total amount of drug (mg) ($X_3$). The dependent variables were the entrapment efficiency % ($Y_1$), and % drug release ($Y_2$). The concentration range of independent variables under study is shown in table (1) along with their low, medium and high levels, which were selected based on the results from preliminary experiments.

**Optimum Formula:** After developing the polynomial equations for the responses EE% and % drug released after 6 hrs with the independent variables, the formulation was optimized for the responses EE% and % drug released at 6 hrs. 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 EE% and controlled release of drug.

**RESULTS AND DISCUSSION:** Proniosomes-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 1**).

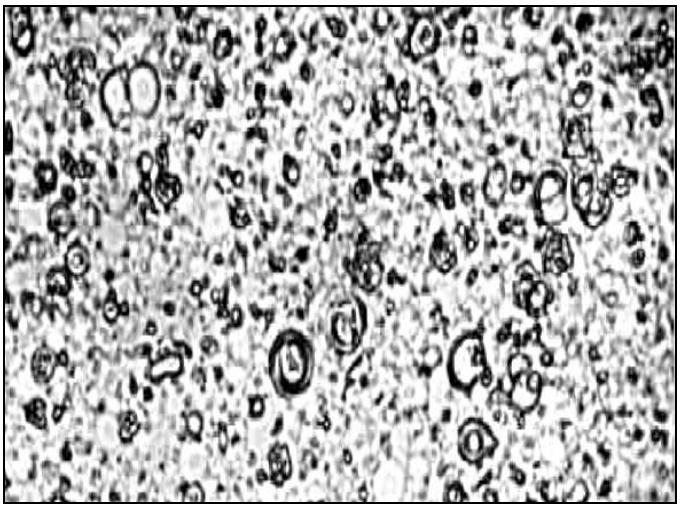

**FIG. 1: OPTICAL PHOTOMICROGRAPH OF PRONIOSOMES-DERIVED NIOSOMES**

**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 within the experimental design yielded niosomes on hydration, and these were evaluated for the entrapment efficiency (EE %) and % drug released at 6 hrs. 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**. Batches 4, 8, and 12 had the highest EE% (> 70%). **Tables 3, 4** show the observed and predicted values with residuals and percent error of responses for all the batches. **Figures 2-4** indicate the *in-vitro* release of KP from niosomes prepared by hydration of proniosomal powders.

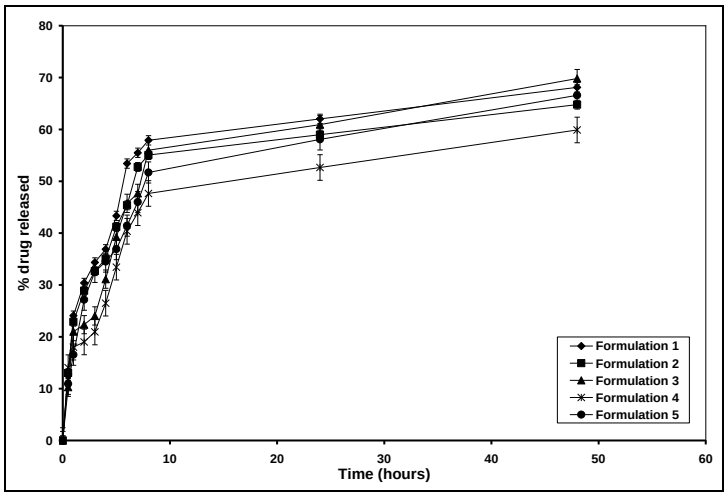


**FIG. 2: *IN-VITRO* DRUG RELEASE OF KETOPROFEN FROM NIOSOMES PREPARED BY HYDRATION OF PRONIOSOMES POWDER FOR BATCHES 1-5**

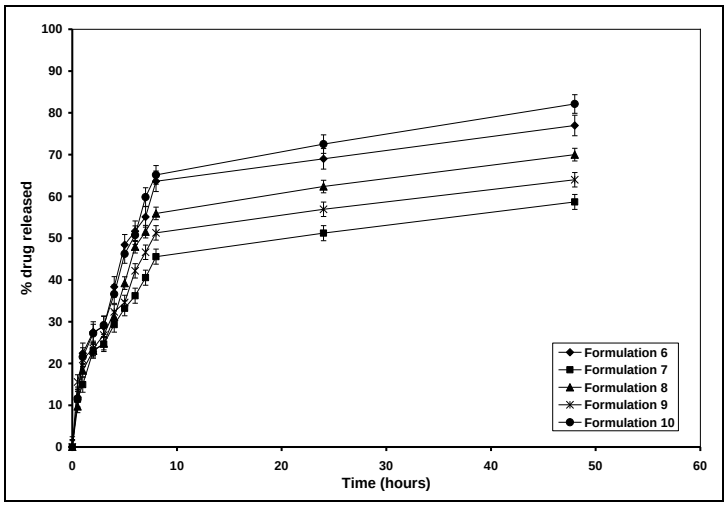


**FIG. 3: *IN-VITRO* DRUG RELEASE OF KETOPROFEN FROM NIOSOMES PREPARED BY HYDRATION OF PRONIOSOMES POWDER FOR BATCHES 6-10.**

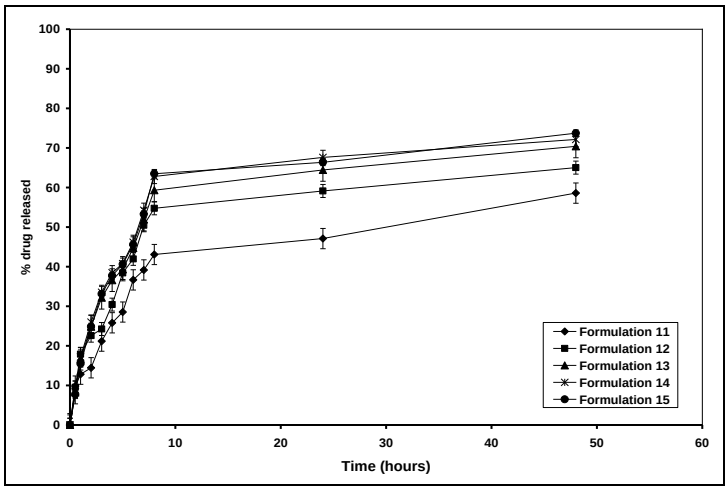


**FIG. 4: *IN-VITRO* DRUG RELEASE OF KETOPROFEN FROM NIOSOMES PREPARED BY HYDRATION OF PRONIOSOMES POWDER FOR BATCHES 11-15**

**TABLE 2: OBSERVED RESPONSES IN BOX-BEHNKEN EXPERIMENTAL DESIGN FOR KETOPROFEN PRONIOSOMES**

| Batch No. | Independent Variables | | | Dependent Variables | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $Y_1$ (EE% ± SD) | $Y_2$ (%release at 6 hrs ± SD) |
| 1 | 10 | 250 | 75 | 46.79±2.15 | 53.41±1.37 |
| 2 | 10 | 2000 | 75 | 65.62±1.27 | 45.35±1.77 |
| 3 | 30 | 250 | 75 | 48.29±1.74 | 45.73±1.93 |
| 4 | 30 | 2000 | 75 | 85.11±1.38 | 40.34±1.84 |
| 5 | 10 | 1125 | 25 | 48.98±1.89 | 41.42±1.47 |
| 6 | 10 | 1125 | 125 | 58.77±1.61 | 51.62±1.43 |
| 7 | 30 | 1125 | 25 | 55.58±1.20 | 36.22±1.77 |
| 8 | 30 | 1125 | 125 | 72.53±1.87 | 47.95±1.00 |
| 9 | 20 | 250 | 25 | 39.59±1.69 | 42.19±1.64 |
| 10 | 20 | 250 | 125 | 61.58±1.42 | 50.72±1.55 |
| 11 | 20 | 2000 | 25 | 64.69±1.92 | 36.24±1.19 |
| 12 | 20 | 2000 | 125 | 74.08±1.56 | 41.92±1.99 |
| 13 | 20 | 1125 | 75 | 58.56±1.86 | 44.69±1.17 |
| 14 | 20 | 1125 | 75 | 54.60±1.46 | 46.18±1.37 |
| 15 | 20 | 1125 | 75 | 55.66±1.52 | 45.58±0.85 |

The EE% (dependent variable) obtained at various levels of the 3 independent variables ($X_1$, $X_2$, and $X_3$) was subjected to multiple regressions to fit the response with the experimental data [17] and to yield a second-order polynomial equation (full model):

$$EE\% = 56.27 + 5.17\ X_1 + 11.66\ X_2 + 7.27\ X_3 + 4.49\ X_1 X_2 + 1.79\ X_1 X_3 - 3.15\ X_2 X_3 + 2.08\ X_1^2 + 3.10\ X_2^2 + 0.61\ X_3^2 \quad (2)$$

The value of the correlation coefficient ($r^2$) of Equation 2 was found to be 0.9735, indicating good fit. The analysis of variance for the three variables (cholesterol concentration (%), total lipid concentration (µmole), and total drug concentration (mg)) indicated that the responses could be well described by the polynomial model with a relatively high coefficient of determination. The statistical analysis of the full model in **table 5** shows that the independent variables had a significant effect on the responses.

The EE% values measured for the different batches showed wide variation (i.e., values ranged from a minimum of 39.59 to a maximum of 85.11). The results clearly indicate that the EE% 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 one 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 EE% changes when 2 variables are simultaneously changed.

The positive coefficients for all 3 independent variables indicate a favorable effect on the EE% (synergistic effect), while the negative coefficients for the interactions between 2 variables ($X_2X_3$) indicate an unfavorable effect on the EE% (antagonistic effect). The standardized effect of the independent variables and their interaction on the dependent variable was investigated by preparing a Pareto chart (**figure 5**), which depicts the main effect of the independent variables and interactions with their relative significance on the EE%. The length of each bar in the chart indicates the standardized effect of that factor on the response. The small coefficients for these terms in equation 2 indicate that these terms contribute the least in prediction of EE%.

Hence, these terms are omitted from the full model to obtain a reduced second-order polynomial equation (equation 3) by multiple regression of the EE% and the significant terms ($P < 0.05$) of equation 2: $EE\% = 56.27 + 5.17\ X_1 + 11.66\ X_2 + 7.27\ X_3 + 4.49\ X_1\ X_2$…… (3)

The theoretical (predicted) values and observed values were in reasonably good agreement as shown from **table 3**. The significance of the ratio of mean square variation due to regression and residual error was tested using analysis of variance (ANOVA). In ANOVA, the prob > F parameter is the observed significance probability (P-value) of obtaining greater F-value by chance alone if the specified model fit no better than the overall response mean. Observed significance probability of 0.05 or less are often considered evidence of a regression effect.

A prob > F of 0.002 for $Y_1$ and 0.0025 for $Y_2$ indicated a significant effect of the independent factors on the responses $Y_1$ and $Y_2$. This implies that the main effect of the cholesterol concentration %, total lipid concentration and the amount of drug added is significant. 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 [6]**.** In this study, the model was checked for lack of fit for the response EE%. For lack of fit P value was obtained 0.2329 for EE%, and hence the current model provided a satisfactory fit to the data ($P > 0.05$) and had no lack of fit.

**TABLE 3: OBSERVED AND PREDICTED VALUES WITH RESIDUALS OF THE RESPONSE $Y_1$**

| Batch No. | Experimental (observed) value of EE% | Theoretical (predicted) value of EE% | Residuals | %Error |
|---|---|---|---|---|
| 1 | 46.79 | 49.13 | -2.24 | 5.00 |
| 2 | 65.62 | 63.44 | 2.18 | 3.32 |
| 3 | 48.29 | 50.47 | -2.18 | 4.51 |
| 4 | 85.11 | 82.78 | 2.24 | 2.74 |
| 5 | 48.98 | 48.32 | 0.66 | 1.35 |
| 6 | 58.77 | 59.27 | -0.50 | 0.85 |
| 7 | 55.58 | 55.08 | 0.50 | 0.90 |
| 8 | 72.53 | 73.19 | -0.66 | 0.91 |
| 9 | 39.59 | 37.91 | 1.68 | 4.24 |
| 10 | 61.58 | 58.74 | 2.84 | 4.61 |
| 11 | 64.69 | 67.53 | -2.84 | 4.39 |
| 12 | 74.08 | 75.76 | -1.68 | 2.27 |
| 13 | 58.56 | 56.27 | 2.29 | 3.91 |
| 14 | 54.60 | 56.27 | -1.67 | 3.06 |
| 15 | 55.66 | 56.27 | -0.61 | 1.10 |

The relationship between the dependent and independent variables was further elucidated by constructing the surface plots. The effects of $X_1$ and $X_3$ with their interaction on EE% at a fixed level of $X_2$ (medium level) are shown in **figure 6**. The plot was found to be linear up to 64% EE, but below this value, the plot was found to be nonlinear indicating a non linear relation ship between $X_1$ and $X_3$. It was determined from the contour plot that a higher value of EE% (> 64%) could be obtained with an $X_1$ level range from 20 to 30%, and an $X_3$ level range from 78 to 125 mg. It is evident from the contour that the high level of both $X_1$ and $X_3$ favors EE% of proniosome-derived niosomes. This observation is in agreement with the observation of [18] who reported that the cholesterol increased the entrapment efficiency. The positive effect of $X_3$ on EE% could be due to the saturation of the media with drug that forces the drug to be encapsulated into niosomes [19].

**TABLE 4: OBSERVED AND PREDICTED VALUES WITH RESIDUALS OF THE RESPONSE $Y_2$**

| Batch No. | Experimental value of %release at 6 hrs | Predicted value of %release at 6 hrs | Residuals | %Error |
|---|---|---|---|---|
| 1 | 53.41 | 53.09 | 0.32 | 0.60 |
| 2 | 45.35 | 44.71 | 0.64 | 1.41 |
| 3 | 45.73 | 46.37 | -0.64 | 1.40 |
| 4 | 40.34 | 40.66 | -.32 | 0.79 |
| 5 | 41.42 | 42.86 | -1.44 | 3.48 |
| 6 | 51.62 | 51.13 | 0.49 | 0.95 |
| 7 | 36.22 | 36.71 | -0.49 | 1.35 |
| 8 | 47.95 | 46.51 | 1.44 | 3.00 |
| 9 | 42.19 | 41.06 | 1.13 | 2.68 |
| 10 | 50.72 | 51.52 | -0.80 | 1.58 |
| 11 | 36.24 | 35.43 | 0.80 | 2.24 |
| 12 | 41.92 | 43.05 | -1.13 | 2.70 |
| 13 | 44.69 | 45.48 | -0.79 | 1.77 |
| 14 | 46.18 | 45.48 | 0.69 | 1.52 |
| 15 | 45.58 | 45.48 | 0.09 | 0.22 |

**TABLE 5: RESULTS OF** ANOVA **TEST FOR EE % AND % DRUG RELEASED AT 6 HRS OF PRONIOSOMES-DERIVED NIOSOMES**

| Regression | Df | SS | MS | F value | P value |
|---|---|---|---|---|---|
| EE% | 9 | 1904.6 | 211.6 | 20.39 | 0.0020 |
| % drug released at 6 hrs | 9 | 351.6 | 39.1 | 18.41 | 0.0025 |

ANOVA indicates analysis of variance; EE % indicates entrapment efficiency percentage of drug; Df, degrees of freedom; SS, sum of squares; MS, mean of squares; F, Fischer's ratio.

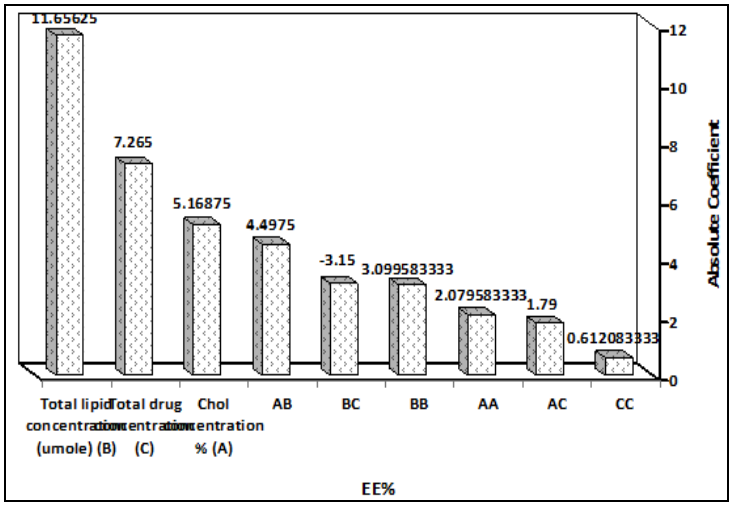


**FIG. 5: Y-HAT PARETO CHART SHOWING THE STANDARDIZED EFFECT OF INDEPENDENT VARIABLES AND THEIR INTERACTION ON THE PERCENTAGE DRUG ENTRAPMENT OF PRONIOSOME-DERIVED NIOSOMES**

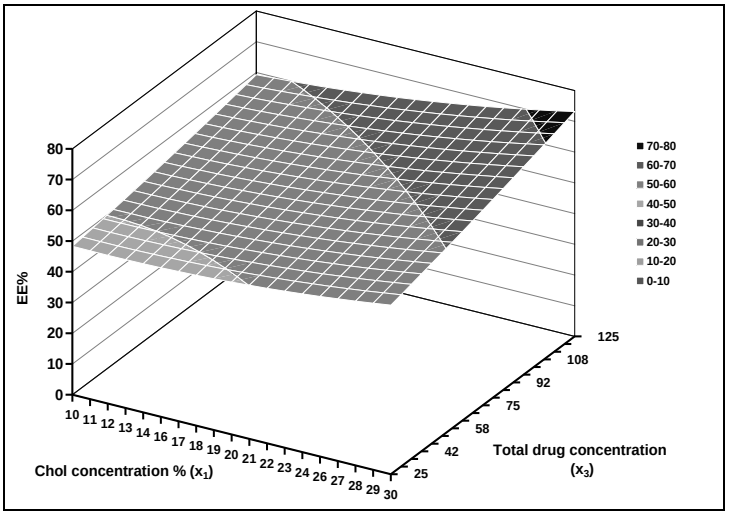


**FIG. 6: Y-HAT SURFACE PLOT SHOWING THE EFFECT OF CHOLESTEROL CONCENTRATION % ($X_1$) AND THE TOTAL AMOUNT OF DRUG ADDED ($X_3$) ON THE PERCENTAGE DRUG ENTRAPMENT OF PRONIOSOME- DERIVED NIOSOMES AT CONSTANT $X_2$= 0**

**Figure 7** show the surface plot drawn at a 0 level of $X_3$. The EE% values up to 70% were found to be linear between $X_1$ and $X_2$. The high value of EE% can be obtained for a combination of the 2 independent variables, at the $X_1$ level in the range of 19 to 30 %, and the $X_2$ level in the range of 1465 to 2000 µmole. But below this value, EE% < 70%, the plot was found to be nonlinear indicating a non linear relation ship between $X_1$ and $X_2$.

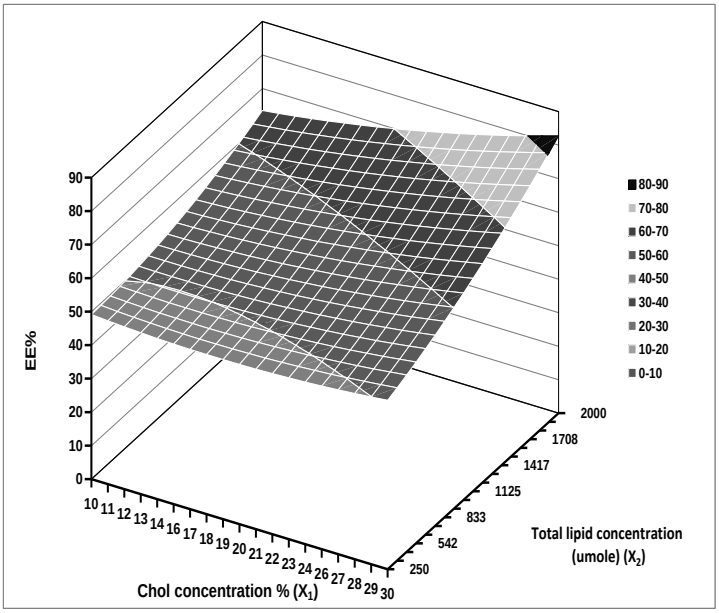


**FIG. 7: Y-HAT SURFACE PLOT SHOWING THE EFFECT OF CHOLESTEROL CONCENTRATION % ($X_1$) AND THE TOTAL LIPID CONCENTRATION ADDED ($X_2$) ON THE PERCENTAGE DRUG ENTRAPMENT OF PRONIOSOME-DERIVED NIOSOMES AT CONSTANT $X_3$= 0**

Similarly, **figure 8** show the surface plot plotted at a 0 level of $X_1$. The plot corresponding to EE% up to 72% is linear, but below this value of EE%, plots were found to be nonlinear in relationship to $X_2$ and $X_3$, and a high value of EE% (> 70%) can be obtained with an $X_2$ level range of 1750 to 2000 µmole and an $X_3$ level range of 88 to 125 mg.

The percentage of drug released after 6 hrs from niosomal batches was found to be in the range of 36.22% to 53.41%. A polynomial equation was also developed for % drug released at 6 hrs:

% drug released after 6 hrs = 45.48 - 2.69 $X_1$- 3.53 $X_2$+ 4.52 $X_3$ + 0.67 $X_1$ $X_2$ + 0.38 $X_1$ $X_3$ - 0.71 $X_2$ $X_3$ + 1.13 $X_1^2$- 0.41$X_2^2$ – 2.31 $X_3^2$……(4)

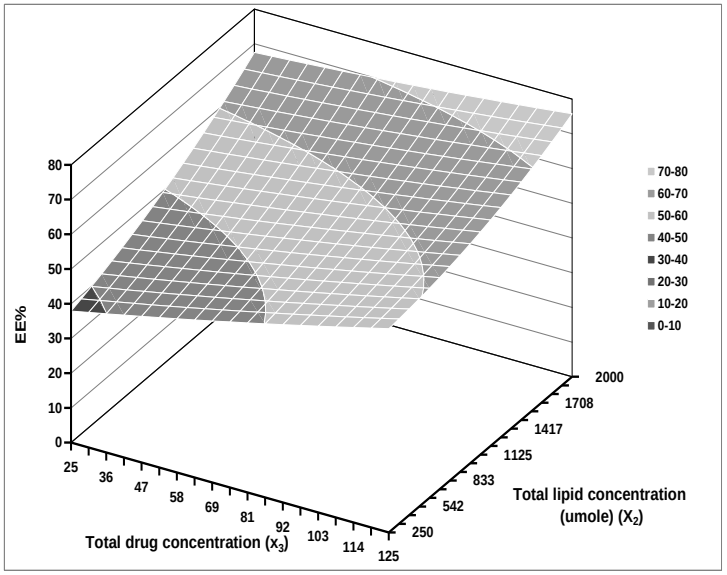


**FIG. 8: Y-HAT SURFACE PLOT SHOWING THE EFFECT OF THE TOTAL LIPID CONCENTRATION ADDED ($X_2$) AND AMOUNT OF DRUG ADDED ($X_3$ ) ON THE PERCENTAGE DRUG ENTRAPMENT OF PRONIOSOME-DERIVED NIOSOMES AT CONSTANT $X_1$= 0**

The value of the correlation coefficient ($r^2$) of equation (4) was found to be 0.9707, indicating good fit. Among the independent variables selected and their interactions, $X_1$, $X_2$, $X_3$, $X_3^2$ were found to be significant ($P < 0.05$), indicating a major contributing effect of $X_1$, $X_2$, $X_3$, $X_3^2$ on % drug released at 6 hrs.

Values of the % drug released after 6 hrs measured for the different batches showed wide variation (i.e., values ranged from a minimum of 36.24% to a maximum of 53.41%). The results clearly indicate that the values of % drug released after 6 hrs value is strongly affected by the variables selected for the study. This is also reflected in the wide range of values for coefficients of the terms of eq. (4). The main effects of $X_1$, $X_2$, and $X_3$ represent the average result of changing one 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 % drug released after 6 hrs changes when the two variables are simultaneously changed. The negative coefficients for the two independent variables, $X_1$ and $X_2$ and interactions between 2 variables $X_2X_3$, $X_2^2$, and $X_3^2$ indicate an unfavorable effect on % drug released after 6 hrs, while the positive coefficients for $X_3$ (total drug concentration (mg)) and the interactions between two variables $X_1X_2$ , $X_1X_3$ , and $X_1^2$ indicate a favorable effect on % drug released after 6 hrs.

The significance level of coefficients $b_4$, $b_5$, $b_6$, $b_7$, and $b_8$ was found to be more than 0.05 ($p>0.05$), hence it was omitted from the full model to generate the reduced model. Coefficients $b_1$, $b_2$, $b_3$, and $b_{33}$ were found to be significant at $p < 0.05$; hence they were retained in the reduced model to obtain a reduced second-order polynomial equation [eq. (5)] by multiple regression of % drug released after 6 hrs and the significant terms ($p < 0.05$) of Eq. (4): % drug released after 6 hrs = $45.48 - 2.69\ X_1 - 3.53\ X_2 + 4.52\ X_3 - 2.31\ X_3^2$.(5)

This implies that the main effect of the cholesterol concentration %, total lipid concentration and the amount of drug added is significant, as it is evident from their high coefficients. In this study, the model was checked for lack of fit for the response $Y_2$. For lack of fit P value was obtained to be 0.1546 for $Y_2$, and hence the current model provided a satisfactory fit to the data ($P > 0.05$) and had no lack of fit, **figure 9**.

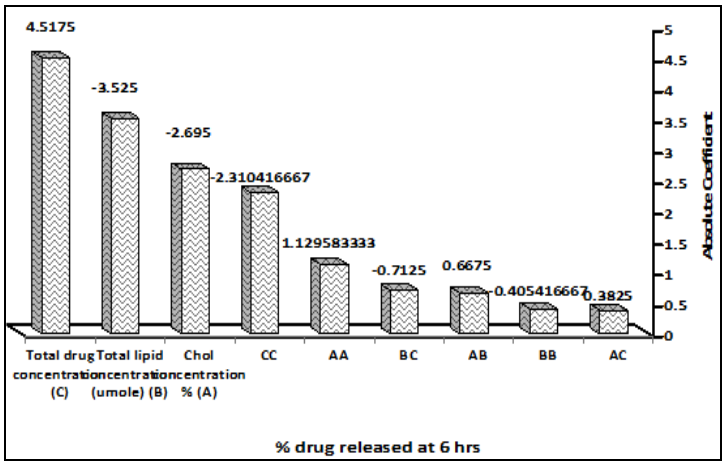


**FIG. 9: Y-HAT PARETO CHART SHOWING THE STANDARDIZED EFFECT OF INDEPENDENT VARIABLES AND THEIR INTERACTION ON THE %DRUG RELEASE AFTER 6 HRS FROM PRONIOSOME-DERIVED NIOSOMES**

The relationship between the dependent and independent variables was further elucidated by constructing surface plots. The effects of $X_1$ and $X_3$ with their interactions on$Y_2$ at a fixed level of $X_2$ (medium level) are shown in **figure 10**. The plots were found to be nonlinear, indicating a nonlinear relationship between $X_1$ and $X_3$. It was determined that a lower value of $Y_2$ could be obtained with an $X_1$ level ranging from 30 to 11.5% and an $X_3$ level ranging from 25 to 85 mg. It is evident from the contour that the high level of $X_1$ and low level of $X_3$ favors % drug released after 6 hrs. This observation is in accordance with the observation of Vora *et al.,* [20] who reported that the increased concentration of drug led to higher % of drug release.

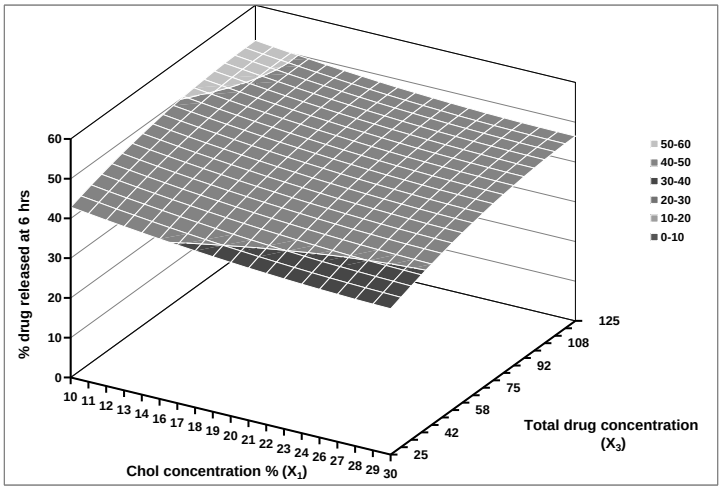


**FIG. 10: Y-HAT SURFACE PLOT SHOWING THE EFFECT OF CHOLESTEROL CONCENTRATION % ($X_1$)AND THE TOTAL AMOUNT OF DRUG ADDED ($X_3$) ON THE %DRUG RELEASE AFTER 6 HRS FROM PRONIOSOME- DERIVED NIOSOMES AT CONSTANT $X_2$= 0**

**Figure 11** show the surface drawn at a 0 level of $X_3$. The plots were found to be linear up to 42% drug released after 6 hrs, but below this value, the plots were found to be nonlinear indicating a nonlinear relationship between $X_1$ and $X_2$. It was determined that a low value of $Y_2$ could be obtained for a combination of the two independent variables, the $X_1$ level in the range of 18 to 30%, and the $X_2$ level in the range of 1660 to 2000 µmole. It is evident from the plots that the high level of both $X_1$ and $X_2$ favors the % drug released after 6 hrs from proniosomes- derived niosomes.

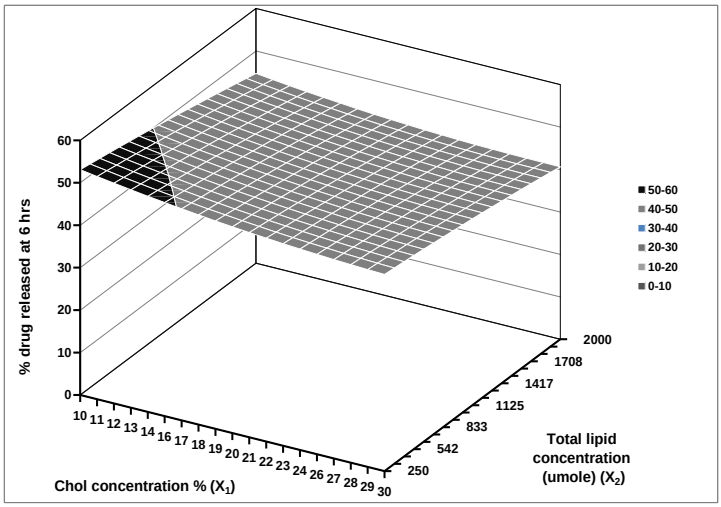


**FIG. 11: Y-HAT SURFACE PLOT SHOWING THE EFFECT OF CHOLESTEROL CONCENTRATION % ($X_1$) AND THE TOTAL LIPID**

**CONCENTRATION ADDED ($X_2$) ON THE %DRUG RELEASE AFTER 6 HRS FROM PRONIOSOME- DERIVED NIOSOMES AT CONSTANT $X_3$= 0**

The effects of $X_2$ and $X_3$ with their interaction on % drug released after 6 hrs at a fixed level of X1 (medium level) are shown in **figure 12**. The plots were found to be nonlinear, indicating a nonlinear relationship between $X_2$ and $X_3$. It was determined from the surface plot that a lower value of % drug released after 6 hrs could be obtained with an $X_2$ level ranging from 250 to 2000 µmole and an $X_3$ level ranging from 25 to 81 mg. It is evident from the contour that the high level of $X_2$ and medium level of $X_3$ favor % drug released after 6 hrs.

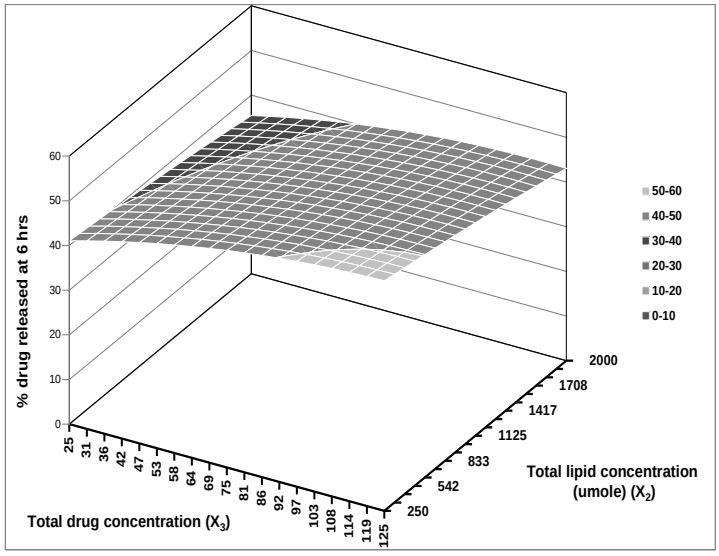


**FIG. 12: Y-HAT SURFACE PLOT SHOWING THE EFFECT OF THE TOTAL LIPID CONCENTRATION ADDED ($X_2$) AND AMOUNT OF DRUG ADDED ($X_3$) ON THE %DRUG RELEASE AFTER 6 HRS FROM PRONIOSOME- DERIVED NIOSOMES AT CONSTANT $X_1$= 0**

**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. The optimum formulation is one that gives a high value of EE% and a controlled drug release with a high total amount of drug entrapped and a low amount of carrier in the resultant niosomes. It is evident from the polynomial equation and plots that increasing the amount of cholesterol increases the EE% and decreases the % drug released after 6 hr.

Cholesterol is known to abolish the gel-to-liquid phase transition of niosomes, and the resulting niosomes are known to be less leaky. So, cholesterol is able to effectively prevent leakage of drug from niosomes [21]. Hence, the high level was selected as optimum for the cholesterol concentration % ($X_1$). It is clear that, the total lipid concentration increases the EE% within niosomes and decreases the % drug released after 6 hr from niosomes. Hence, the high level was selected as optimum for the total lipid concentration % ($X_2$). Using a computer optimization process and the contour plots for $X_3$, we selected the medium level of 75 mg of drug, which gives the theoretical value of 82.77%, 40.65% for EE% and % drug released after 6 hr, respectively.

Hence, 30% level for the cholesterol concentration ($X_1$), 2000 µmole of total lipid concentration ($X_2$), and 75 mg 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 EE% and % drug released at 6 hrs were found to be 81.25%, 41.87%, 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 demonstrated the use of a Box-Behnken design in optimization of proniosome batches. The derived polynomial equations and contour plots aided in predicting the values of selected independent variables for the preparation of optimum proniosome batches with desired properties.

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