OPTIMIZATION OF FORMULATION PARAMETERS OF CYTARABINE LIPOSOMES USING 3^3 FACTORIAL DESIGN

N.Subramanian and R.S.R.Murthy
Department of Pharmacy,
Faculty of Technology and Engineering,
M.S.University of Baroda,
Kalabhavan,
Vadodara-390001,
India

Corresponding address:
Prof.R.S.R.Murthy,
Department of Pharmacy,
Faculty of Technology and Engineering,
M.S.University of Baroda,
Kalabhavan,
Vadodara-390001, India
Phone: 091-265-2434187 / 2794051
e.mail: nsubbu2000@rediffmail.com

ABSTRACT

In the present study, 3^3 factorial design was used to investigate the combined influence of three independent variables in the preparation of Cytarabine liposomes by thin film hydration method. The process variables like rotation speed, temperature, vacuum applied and hydration time were kept constant through out the study. The formulation variables, Drug (Cytarabine) / Lipid [Phosphatidyl choline (PC) and Cholesterol (Chol)] molar ratio (X_1), PC / Chol in percent ratio of total lipids (X_2) and the volume of hydration medium (X_3) were selected as the independent variables. Based on the factorial design, twenty seven batches of cytarabine liposomes were prepared. The liposome batches were evaluated for its drug entrapment within the liposomal vesicles. The percent drug entrapment (PDE) was selected as the dependent variable. The transformed values of independent variables were subjected to multiple regression analysis to establish a second order polynomial equation (full model). F-Statistic was applied to establish a reduced polynomial equation (reduced model) by neglecting the nonsignificant ($P > 0.01$) terms from the full model. Based on the coefficient values obtained for independent variables from the regression equation, it was clear that the drug/lipid molar ratio (X_1), which was having the maximum value ($b_1 = 5.36$) was the major contributing variable for PDE within the liposomes. The reduced polynomial equation was used to plot three two-dimensional contour plots at a fixed levels of -1, 0 and 1 of the variable X_3 to obtain various combination values of the two other independent variables (X_1 and X_2) at predetermined PDE. The established equation was validated by preparing three batches three times taking values of independent variables from the contour plots for the prefixed value of PDE. Response surface curves were also plotted to show the effects of X_1, X_2 and X_3 on the PDE within liposomes. Thus the derived equation, response surface plots and contour plots helps in predicting the values of the independent variables for maximum PDE in the preparation of cytarabine liposomes by thin film hydration method.

KEYWORDS

Contour plots, cytarabine liposomes, factorial design, multiple regression, response surface plots.

INTRODUCTION

The toxic side effects associated with the administration of anticancer drugs makes them ideal candidates for site specific delivery. Most small molecule chemotherapeutic agents have a large volume of distribution on intra venous administration which often leads to a narrow therapeutic index due to a high level of toxicity in healthy tissues (Speth et al, 1998). Through encapsulation of drugs in macromolecular carriers, such as a liposome, the volume of distribution is significantly reduced which results in decreased nonspecific toxicities and an increase in the amount of drug that can be effectively delivered to the tumor (Papahadjopoulos et al, 1995; Gabizon et al 1997; Martin et al, 1998). Under optimal conditions, the drug is carried within the liposomal aqueous compartment while in the circulation but leaks at a sufficient rate to become bioavailable on reaching the tumor. The liposome also protects the drug from metabolism and inactivation in the plasma, and due to size limitations in the transport of large molecules or carriers across healthy endothelium, the drug accumulation in healthy tissues is reduced (Mayer et al, 1989; Working et al, 1994). However, discontinuities in the endothelium of the tumor vasculature have been shown to result in an increased extravasation of large carriers and, in combination with an impaired lymphatics, an increased accumulation of liposomal drug in tumor (Yuan et al, 1994; Yuan et al, 1995; Huang et al, 1993l; Hobbs et al, 1998).

Cytarabine is one of the most effective anticancer agents used for various types of tumors (Roberts et al, 1985; Tricot et al, 1984; Winter et al, 1985). The narrow therapeutic index, high volume of distribution and poor tissue specificity requires cytarabine to be delivered as liposomes. Drugs which are freely soluble in water like cytarabine, pose a great challenge to entrap them into the liposomes as they have very low entrapment efficiency (Allen et al, 1992; Zou et al, 1994). The entrapment may vary significantly from batch to batch as the number of formulation variables increases. Hence it is very difficult to optimize the preparation of cytarabine liposomes by the conventional method of optimization as it involves varying one parameter at a time and keeping the others constant and also the conventional optimization method does not allow to study the effect of interaction of various parameters governing the process. Factorial design (Cochran et al, 1992), contour plot and response surface methodology are useful models for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments.

The present investigation is aimed to optimize the process of formulating liposomes containing cytarabine by thin film hydration method. Cytarabine being water soluble, drug loading in the

liposome is highly sensitive towards formulation parameters and so percent drug entrapment (PDE) is taken the response parameters for the study. The process variables like temperature, vacuum applied and hydration time are kept constant while the formulation variables, Drug / Lipid [Phosphatidyl choline (PC) and Cholesterol (Chol)] molar ratio (X_1), PC / Chol in percent ratio of total lipids (X_2) and the volume of hydration medium (X_3) which have been predicted to play a significant role in enhancing the PDE are taken as variable parameters. 3^3 factorial design, contour plots and response surface plots are used to study the main and interaction effects of the variables on the PDE (Fannin et al, 1981; Deshayes, 1980; Matthews et al, 1981).

MATERIALS AND METHODS

Chemicals:

Cytarabine, a gift from Dabur research foundation, Ghaziabad; Egg Phosphatidyl choline, purchased from Sigma chemical Co., St.Louis, M.O.; Cholesterol, purchased from S.D.fine chemicals, Mumbai; DL - α– tocopherol, purchased from E.Merck India limited, Mumbai. All other chemicals and solvents were of analytical reagent grade.

Preparation of Liposomes:

In the present study, Drug / lipid (PC and Chol) in molar ratio, PC / Chol in percent of total lipids and the volume of hydration medium were selected as independent variables, whereas percent drug entrapment (PDE) within the liposomes was selected as dependent variable. The values of these selected variables along with their transformed values are shown in Table 1.

Twenty seven batches of cytarabine liposomes were prepared by thin film hydration method (New R.R.C, 1990) according to the experimental conditions as shown in the table 2. PC , Chol and α– tocopherol (0.5 ml of 0.1 % w/v solution in chloroform) were dissolved in 5 ml of chloroform and methanol (2:1 by volume ratio) in a 250ml round bottom flask. The flask was rotated in the rotary flash evaporator at 100 rpm for 20 minutes in a thermostatically controlled water bath at 37°C under vacuum (600mm of mercury). To the thin dry lipid film formed, drug solution (5mg of drug dissolved in distilled water [hydration medium]) was added and the flask was rotated again at the same speed and temperature as before but without vacuum for 30 minutes for lipid film removal and dispersion. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 30 minutes using a probe sonicator (model – RR-120, Ralsonics, Mumbai) in an ice bath for heat dissipation. The sonicated dispersion was then allowed to stand undisturbed for about 2 hours at room temperature for swelling. Each batch was prepared three times and stored in refrigerator.

Assay of Cytarabine:

Methanolic solutions of Cytarabine (2 to 24 µg) were prepared and the absorbance was measured at 270nm using a Hitachi U-2000 double beam spectrophotometer. An equation was generated by fitting linear regression model to the data obtained in six repetitions yielding the linear regression equation ($Y = 0.0346X + 0.0115$) and the correlation coefficient of 0.9998 (Bolton S, 1997).

Estimation of Entrapped Drug in Liposomes:

Cytarabine entrapped within the liposomes was estimated after removing the un entrapped drug. The un entrapped drug was separated from the liposomes by subjecting the dispersion to centrifugation (New R.R.C, 1990) in a cooling centrifuge (Remi equipments, Mumbai) at 15,000 rpm at a temperature of -4°C for 30 minutes whereupon the pellets of liposomes and the supernatant containing free drug was obtained. The liposome pellets were washed again with distilled water to remove any un entrapped drug by centrifugation. The combined supernatant was analyzed for the drug content after suitable dilution with methanol by measuring absorbance at 274 nm using Hitachi U-2000 double beam spectrophotometer. The PDE in the liposomes was calculated from the difference between the initial drug added and the drug detected in the supernatant. The amount of drug exactly present within the liposomes was also analyzed by dissolving the liposomes in methanol to counter check the PDE and to arrive at a mass balance. The analysis of drug in liposomes was carried out using the empty liposomes dissolved in methanol as blank in order to nullify the interference of the excipients. The mean PDE of all the twenty seven batches is shown in Table 2.

Factorial Design and Optimization:

Traditionally pharmaceutical formulations are developed by changing one variable at a time. The method is time consuming and it is difficult to evolve an ideal formulation using this classical technique since the combined effects of the independent variables are not considered. It is therefore important to understand the complexity of pharmaceutical formulations by using established statistical tools such as factorial design. The number of experiments required for these studies is dependent on the number of independent variables selected. The response is measured for each trial and then either simple linear equation (1), or interactive equation (2) or quadratic (3) model is

$$(Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3) \quad (1)$$

$$(Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3) \quad (2)$$

$$(Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_1{}^2X_{11} + b_2{}^2X_{22} + b_3{}^2X_{33} + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{123}X_1X_2X_3) \quad (3)$$

fitted by carrying out multiple regression analysis and F-statistic to identify statistically significant terms. A prior knowledge and understanding of the process and the process variables under

investigation are necessary for achieving a more realistic model. Based on the results obtained in preliminary experiments, Drug / lipid ratio, PC / Chol ratio and hydration volume were found to be the major variables in determining the PDE. Hence these variables were selected to find the optimized condition for higher PDE using 3^3 factorial design, Response surface plots and Contour plots.

In developing the regression equation, the test factors were coded according to the equation 4.

$$x_i = (X_i - X^X_i) / \Delta X_i \qquad (4)$$

where x_i is the coded value of the i^{th} independent variable, X_i is the natural value of the i^{th} independent variable, X_i^X is the natural value of the i^{th} independent variable at the center point and ΔX_i is the step change value.

$$Y = b_0 + \sum_i b_i X_i + \sum_i \sum_y b_{ij} X_i X_j + \sum b_{ii} X_i^2 \qquad (5)$$

where Y is the measured response, b_0 is the intercept term, b_i, b_{ij} and b_{ii} are, respectively the measures of the variables X_i, X_iX_j and X_i^2. The variable X_iX_j represents the first order interactions between X_i and X_j ($i < j$).

Twenty seven batches of different combinations were prepared by taking values of selective variables X_1, X_2 and X_3 at different levels as shown in Table 1. The prepared batches were evaluated for PDE, a dependent variable and the results are recorded in Table 2. Mathematical modeling was carried out by using equation 6 to obtain a second order polynomial equation (Anthony Armstrong et al, 1996).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_1{}^2X_{11} + b_2{}^2X_{22} + b_3{}^2X_{33} + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{123}X_1X_2X_3 \qquad (6)$$

where Y is the dependent variable (PDE) while b_0 is the intercept, b_i (b_1,b_2 and b_3), b_{ij} (b_{12}, b_{23} and b_{13}) and b_{ijk} (b_{123}) represents the regression coefficient for the second order polynomial and X_i represents the levels of independent formulation variables. A full model (equation 7) was established after putting the values of regression coefficients in equation 6. The predicted values were calculated by using the mathematical model derived from the coefficients of the model as shown in Table 4 and the predicted values along with their observed values are shown in Table 3

which gives information about the percent of error obtained when predicted value was compared with the observed values.

$$Y = 69.141 + 5.366\ X_1 + 2.323\ X_2 - 0.356\ X_3 + 7.745\ X_1^2 - 2.322\ X_2^2 - 7.59\ X_3^2 - 0.253\ X_1X_2 - 3.658\ X_2X_3 - 4.008\ X_1X_3 + 5.863\ X_1X_2X_3 \quad (7)$$

Neglecting nonsignificant ($p<0.01$) terms from the full model established a reduced model (equation 8) which facilitates the optimization technique by plotting contour plots and response surface plots keeping one independent formulation variable constant and varying other two independent formulation variables, to establish the relationship between independent and dependent variables.

$$Y = 67.556 + 5.394\ X_1 + 2.29\ X_2 + 7.71\ X_1^2 - 7.53\ X_3^2 - 3.658\ X_2X_3 - 4.008\ X_1X_3 + 5.863\ X_1X_2X_3 \quad (8)$$

Results of ANOVA of full model and reduced model were carried out and the F-Statistic was applied to check whether the nonsignificant terms can be omitted or not from the full model which is shown in Table 5.

Contour Plots:

Two dimensional contour plots were established using reduced polynomial equation (equation 8). Values of X_1 and X_2 were computed at prefixed values of PDE. Three contour plots were established between X_1 and X_2 at fixed level if -1, 0 and 1 level of X_3 as shown in Figure 1 (A, B and C).

Checkpoint Analysis:

A check point analysis was performed to confirm the utility of established contour plots and reduced polynomial equation in the preparation of cytarabine liposomes. Values of independent variables (X_1 and X_2) were taken from three check points each on contour plots plotted at fixed levels of -1, 0 and 1 of X_3 and the values of PDE were calculated by substituting the values in the reduced polynomial equation. Cytarabine liposomes were prepared experimentally by taking the amounts of the independent variables (X_1 and X_2) on the same check points. Each batch was prepared three times and mean values were determined as shown in table 6. Difference of theoretically computed values of PDE and the mean values of experimentally obtained PDE was compared by using student ‘t’test method.

Response Surface Plots:

Response surface plots (Box et al, 1951; Kenneth et al 1995) as a function of two factors at a time maintaining all other factors at fixed levels are more helpful in understanding both the main and the interaction effects of these two variables. These plots can be easily obtained by calculating from the model the values taken by one factor where the second varies (from -1 to 1 for instance) with constraint of a given Y value. The yield values for different levels of variables can also be predicted from the respective response surface plots depicted in Figure 2 (A, B and C).

RESULTS AND DISCUSSION

By using 3^3 factorial design (Table 1) , twenty seven batches of cytarabine liposomes were prepared by lipid film hydration method varying three independent variables, Drug : lipid (molar ratio) (X_1), PC : Chol (in percent of total lipids) (X_2) and volume of hydration medium (X_3). The percent drug entrapment (PDE) which was taken as dependent variable was determined and the results are recorded (Table 2). A substantial high drug entrapment achieved in liposomes prepared by lipid film hydration method was 83.5% at 1 level of X_1 (1 : 13), 0 level of X_2 (60 : 40) and 0 level of X_3 (2 ml).

The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to yield a second order polynomial equation (full model). 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 to high value. The interactions (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) show how the PDE changes when two or more variables were simultaneously changed. The PDE values for the twenty seven batches showed a wide variation from 43.5 to 83.5 % (Table 2). This is reflected by the wide range of coefficients of the terms of equation 2 representing the individual and combined variables. Small values of the coefficients of the terms X_3, X_2^2 and X_1X_2 in equation 7 are regarded as least contributing in the preparation of cytarabine liposomes by lipid film hydration method. Hence, these terms are neglected from the full model considering non-significance and a reduced polynomial equation (equation 8) obtained following multiple regression of PDE and very significant terms ($p<0.01$) of equation 7.

The significance of each coefficient of the equation 7 was determined by student‘t’ test and p-value, which are listed in table 4. The larger the magnitude of the t value and the smaller the p value, the more significant is the corresponding coefficient (Adinarayana et al, 2002; Akhnazarova et al, 1982). This implies that the quadratic main effects of Drug / Lipid ratio and PC / Chol ratio are significant. The second order main effects of both Drug / Lipid ratio and volume of hydration are significant, as is evident from their p-values. The interaction between X_2X_3, X_1X_3 and $X_1X_2X_3$ are found to be very significant from their p-values (Table 4).

In Table 3, each of the observed values Y_f (O) is compared with the predicted values Y_f (P) from the model. The percent error was calculated to show the correlation between the observed and the predicted values. The results of ANOVA of the second order polynomial equation are given in Table 5. F-Statistic of the results of ANOVA of full and reduced model confirmed omission of

non-significant terms of equation 4. Since the calculated F value (1.086) is less than the tabled F value (3.25) (α = 0.05, V_1 = 3 and V_2 = 16), it was concluded that the neglected terms do not significantly contribute in the prediction of PDE. When the coefficients of the three independent variables in equation 7 were compared, the value for the variable X_1 (b_1 =5.366) was found to be maximum and hence the variable X_1 was considered to be a major contributing variable for PDE of cytarabine liposomes. The fisher F test with a very low probability value ($P_{model} > F$ = 0.000001) demonstrate a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the values of the determination coefficients (R^2 = 0.9178 for full model and 0.9011 for reduced model) indicated that over 90 % of the total variations are explained by the model. The values of adjusted determination coefficients (adj R^2 = 0.8664 for full model and 0.8646 for reduced model) are also very high which indicates a high significance of the model. A higher values of correlation coefficients (R = 0.958 for full model and 0.9493 for reduced model) signifies an excellent correlation between the independent variables (Box et al, 1978). All the above considerations indicate an excellent adequacy of the regression model (Adinarayana et al, 2002; Akhnazarova et al, 1982; Box et al, 1978; Cochran et al, 1992; Yee et al, 1993).

Contour Plots:

Figure 1A shows the contour plot drawn at -1 level of X_3 (1 ml), for a prefixed PDE value of 60 %, 65 %, 70 %, 75 % and 80 %. The plots were found to be linear for 65 %, 70 % and 75 %, but for 60 % and 80 % PDE, the plots were found to be non linear having upward and downward segment for 60 % and a curved segment for 80 % PDE signify nonlinear relationship between X_1 and X_2 variables. It was determined from the contour that maximum PDE (80%) could be obtained with X_1 range at 0.74 level to 1 level and X_2 at 0.58 level to 1 level. It was concluded from the contour that higher amount lipid could be necessary to entrap the drug within the liposomes when 1 ml of hydration medium was used.

Figure 1B shows the contour plot drawn at 0 level of X_3 (2 ml), for a prefixed PDE value of 70 %, 75 %, 80 % and 83 %. The contours of 70 % and 75 % were found to be linear where as the contours were found to be linear only between 0.6 to 1 level of X_1 and -0.52 to 0.52 level of X_2 for 80 % and 0.74 to 1 level of X_1 and -0.4 to 0.4 level of X_2 for 83 % PDE. It was concluded that higher amount of Drug: Lipid (1: 12.22 to 13 molar ratios) and optimum PC: Chol ratio (56-64 % of PC and 36-44 % of Chol of total lipids) was required to achieve a maximum PDE (83 %) when 2 ml of hydration volume used.

Figure 1C shows the contour plot drawn at 1 level of X_3 (3 ml), for a prefixed PDE value of 60 %, 65 %, 70 %, and 75 %. The contour of 65% and 70 % were found to be linear after a certain level of X_2 (from -0.4). The contour of 75 % was found to be linear only between 0.8 to 1 level of X_1 and -0.24 to 0.4 level of X_2. It was concluded from the contour that higher amount lipid and optimum ratio of PC and Chol could be necessary to have maximum PDE (75%) within the liposomes when 3 ml of hydration medium was used. Thus the results of the contour plots revealed the range of Drug to lipid ratio and PC to Chol ratio in the preparation of cytarabine liposomes at all levels of X_3 for obtaining maximum PDE.

Checkpoint Analysis:

At fixed levels of -1, 0 and 1 of independent variable X_3, three check points were selected each on three plotted contours (Table 6). The computed PDE values from the contours at -1, 0 and 1 level were found to be 80 %, 83 % and 75 % respectively. Cytarabine liposomes at these three checkpoints were prepared experimentally using the same procedure keeping the other process variables as constants with the amounts of X_1 and X_2 at the selected check points. The experiment was repeated three times and the experimentally obtained mean PDE values were shown in Table 6. when both experimentally obtained and theoretically computed PDE values were compared using student 't' test, the difference was found to be non significant ($p>0.05$). This proves the role of a derived reduced polynomial equation and contour plots in the preparation of cytarabine liposomes of predetermined PDE.

Response Surface Plots:

Response surface plots are very helpful in learning about both the main and interaction effects of the independent variables. These plots were plotted by keeping the factor X_3 at different fixed levels (levels of X_3 were fixed at -1, 0 and 1 in Figure 2A, 2B and 2C respectively).

Figure 2A shows the response surface plot obtained as a function of Drug / Lipid ratio Vs PC /Chol ratio, while the third independent variable (volume of hydration medium) maintained at low level (-1). An increase in PDE with increase in the Drug / Lipid ratio Vs PC /Chol ratio was observed.

Figure 2B shows the response surface plot obtained as a function of Drug / Lipid ratio Vs PC /Chol ratio, while the third independent variable (volume of hydration medium) maintained at medium level (0). Almost a linear relationship was noticed.

Figure 2C shows the response surface plot obtained as a function of Drug / Lipid ratio Vs PC /Chol ratio, while the third independent variable (volume of hydration medium) maintained at high level(1). An increase in PDE with increase in the Drug / Lipid ratio Vs PC /Chol ratio was noticed. There was a non linear relationship between the two variables after the PDE value of 70 %.

CONCLUSIONS

This work has demonstrated the use of 3^3 factorial design, derived reduced polynomial equation, two dimensional contour plots and response surface plots in optimizing formulation variables in the preparation of cytarabine liposomes by thin film hydration method. By using the factorial design we could achieve a maximum drug entrapment of 83.5 % with less number of experiments and could predict the PDE for various combinations of the formulation variables using the contour plots and response surface plots. Similar methodology can be used in optimizing the process variables for hydrophilic or ampiphilic drugs. These methodologies could therefore be employed successfully to any process which involves the effects and interactions of many experimental variables. Thus desirable goals can be achieved by systematic formulation approach in shortest possible time with reduced number of experiments and thereby reducing the cost of development of the formulations.

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Table 1: Coded Values of the formulation parameters of Cytarabine liposomes

Coded Values	Actual Values		
	X_1	X_2	X_3
-1	1 : 7	50 : 50	1 ml
0	1 : 10	60 : 40	2 ml
1	1 : 13	70 : 30	3 ml

X_1--Drug: Lipid (molar ratio)

X_2--PC: Chol (in percent of total lipids)

X_3--Hydration volume (Distilled water)

Table 2: 3^3 Full factorial design layout.

Batch No.	X_1	X_2	X_3	X_1^2	X_2^2	X_3^2	X_1X_2	X_2X_3	X_1X_3	$X_1X_2X_3$	PDE* ($\pm$ SEM)
1	-1	-1	-1	1	1	1	1	1	1	-1	43.5 (0.463)
2	0	-1	-1	0	1	1	0	1	0	0	52.1 (0.235)
3	1	-1	-1	1	1	1	-1	1	-1	1	77.8 (0.142)
4	-1	0	-1	1	0	1	0	0	1	0	63.1 (0.045)
5	0	0	-1	0	0	1	0	0	0	0	59.6 (0.012)
6	1	0	-1	1	0	1	0	0	-1	0	78.6 (0.044)
7	-1	1	-1	1	1	1	-1	-1	1	1	70.5 (0.110)
8	0	1	-1	0	1	1	0	-1	0	0	64.1 (0.097)
9	1	1	-1	1	1	1	1	-1	-1	-1	80.4 (0.152)
10	-1	-1	0	1	1	0	1	0	0	0	70.4 (0.045)
11	0	-1	0	0	1	0	0	0	0	0	66.9 (0.104)
12	1	-1	0	1	1	0	-1	0	0	0	77.2 (0.336)
13	-1	0	0	1	0	0	0	0	0	0	70.6 (0.043)
14	0	0	0	0	0	0	0	0	0	0	69.2 (0.066)
15	1	0	0	1	0	0	0	0	0	0	83.5 (0.174)
16	-1	1	0	1	1	0	-1	0	0	0	71.3 (0.049)
17	0	1	0	0	1	0	0	0	0	0	67.8 (0.128)
18	1	1	0	1	1	0	1	0	0	0	77.4 (0.084)
19	-1	-1	1	1	1	1	1	-1	-1	1	70.1 (0.106)
20	0	-1	1	0	1	1	0	-1	0	0	64.2 (0.025)
21	1	-1	1	1	1	1	-1	-1	1	-1	59.2 (0.071)
22	-1	0	1	1	0	1	0	0	-1	0	64.5 (0.022)
23	0	0	1	0	0	1	0	0	0	0	58.7 (0.059)
24	1	0	1	1	0	1	0	0	1	0	75.4 (0.132)
25	-1	1	1	1	1	1	-1	1	-1	-1	59.7 (0.067)
26	0	1	1	0	1	1	0	1	0	0	60.2 (0.038)
27	1	1	1	1	1	1	1	1	1	1	71.3 (0.105)

* n = 3

Table 3: Observed responses and Predicted values.

Batch No	Observed PDE	Predicted PDE	Residual Value	% Error
1	43.5	45.86	-2.36	5.42
2	52.1	53.60	-1.50	2.89
3	77.8	76.84	0.96	1.23
4	63.1	60.28	2.82	4.47
5	59.6	61.91	-2.31	3.87
6	78.6	79.03	-0.43	0.54
7	70.5	70.05	0.45	0.64
8	64.1	65.57	-1.47	2.29
9	80.4	76.57	3.83	4.76
10	70.4	66.62	3.78	5.37
11	66.9	64.50	2.40	3.59
12	77.2	77.86	-0.66	0.86
13	70.6	71.52	-0.92	1.30
14	69.2	69.14	0.06	0.09
15	83.5	82.25	1.25	1.49
16	71.3	71.27	0.03	0.05
17	67.8	69.14	-1.34	1.98
18	77.4	82.00	-4.60	5.94
19	70.1	72.21	-2.11	3.00
20	64.2	60.21	3.99	6.22
21	59.2	63.70	-4.50	7.61
22	64.5	67.58	-3.08	4.78
23	58.7	61.20	-2.50	4.25
24	75.4	70.30	5.10	6.77
25	59.7	58.32	1.38	2.32
26	60.2	57.54	2.66	4.42
27	71.3	72.25	-0.95	1.33

Table 4: Model coefficients estimated by Multiple linear regression.

Factor	Coefficient	Computed t-value	p-value
Intercept	69.141	40.742	0.000000*
X_1	5.366	6.769	0.000005*
X_2	2.323	2.930	0.009813*
X_3	-0.356	-0.453	0.656601
X_1^2	7.745	5.678	0.000034*
X_2^2	-2.322	-1.702	0.108040
X_3^2	-7.590	-5.509	0.000048*
X_1X_2	-0.253	-0.252	0.804281
X_2X_3	-3.658	-3.806	0.001553*
X_1X_3	-4.008	-4.170	0.000722*
$X_1X_2X_3$	5.863	4.980	0.000136*

* Very significant at $p< 0.01$

Table 5: Analysis of Variance (ANOVA) of full and reduced models.

		DF	SS	MS	F	R	R^2	Adj. R^2
Regression	FM	10	1980.817	198.082	17.866	0.9580	0.9178	0.8664
	RM	7	1944.702	277.814	24.723	0.9493	0.9011	0.8646
Error	FM	16	177.390 (E1)	11.087				
	RM	19	213.504 (E2)	11.237				

SSE2-SSE1 = 213.504 – 177.390 = 36.114

No. of parameters omitted = 3

MS of Error (full model) = 11.087

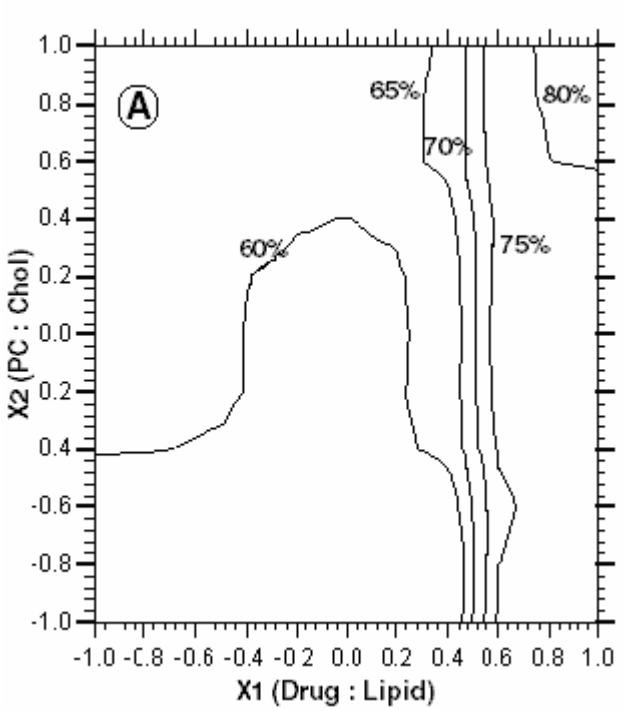
F calculated = (36.114/3)/ 11.087 = 1.086

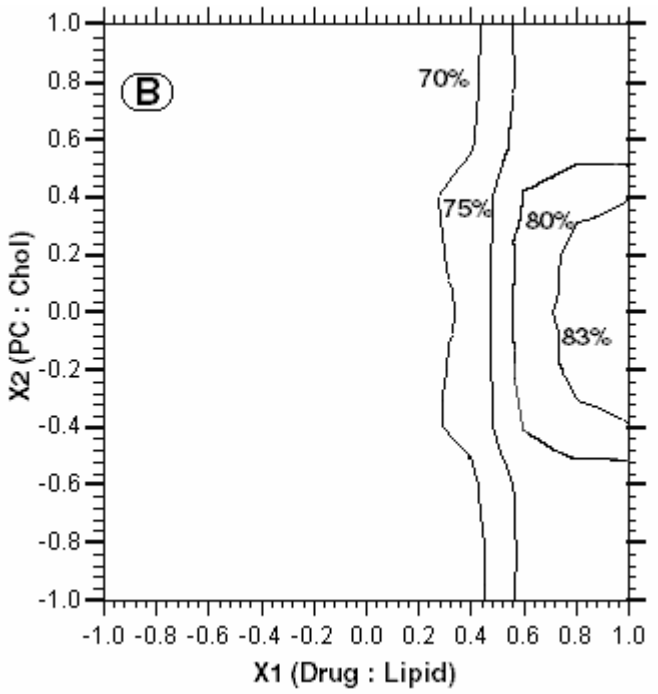
Table 6: Checkpoint Analysis

Hydration volume (X_3)	Values from Contour plots		Calculated PDE	Experimentally obtained PDE* (± SEM)
	X_1 level Drug : Lipid	X_2 level PC : Chol		
(-1) 1 ml	1 : 12.22	68.8 : 31.2	80	80.3** (0.945)
(0) 2 ml	1 : 12.22	61.2 : 38.8	83	83** (1.245)
(1) 3 ml	1 : 12.28	60.8 : 39.2	75	74.81** (1.412)

*n = 3

** Difference from the calculated PDE value not significant ($p>0.05$)





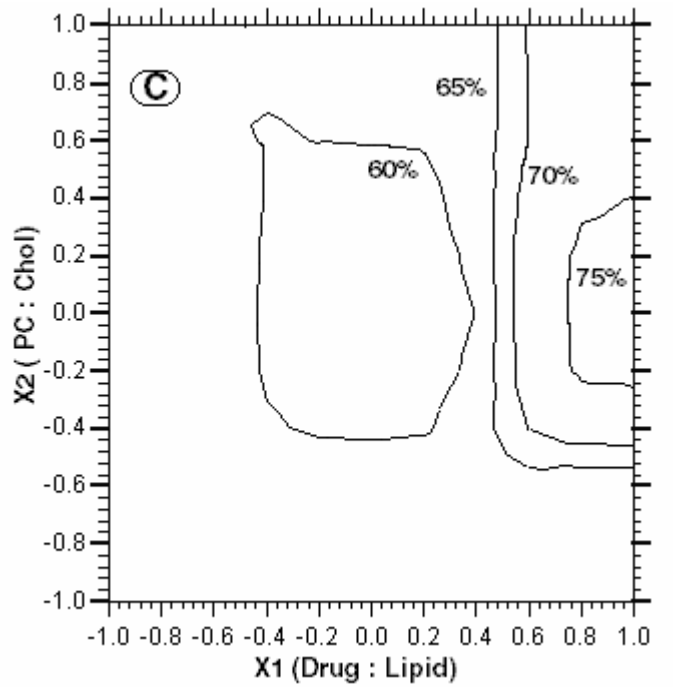


Figure 1: Contour plots
(A) at -1 level of variable X_3
(B) at 0 level of variable X_3
(C) at 1 level of variable X_3

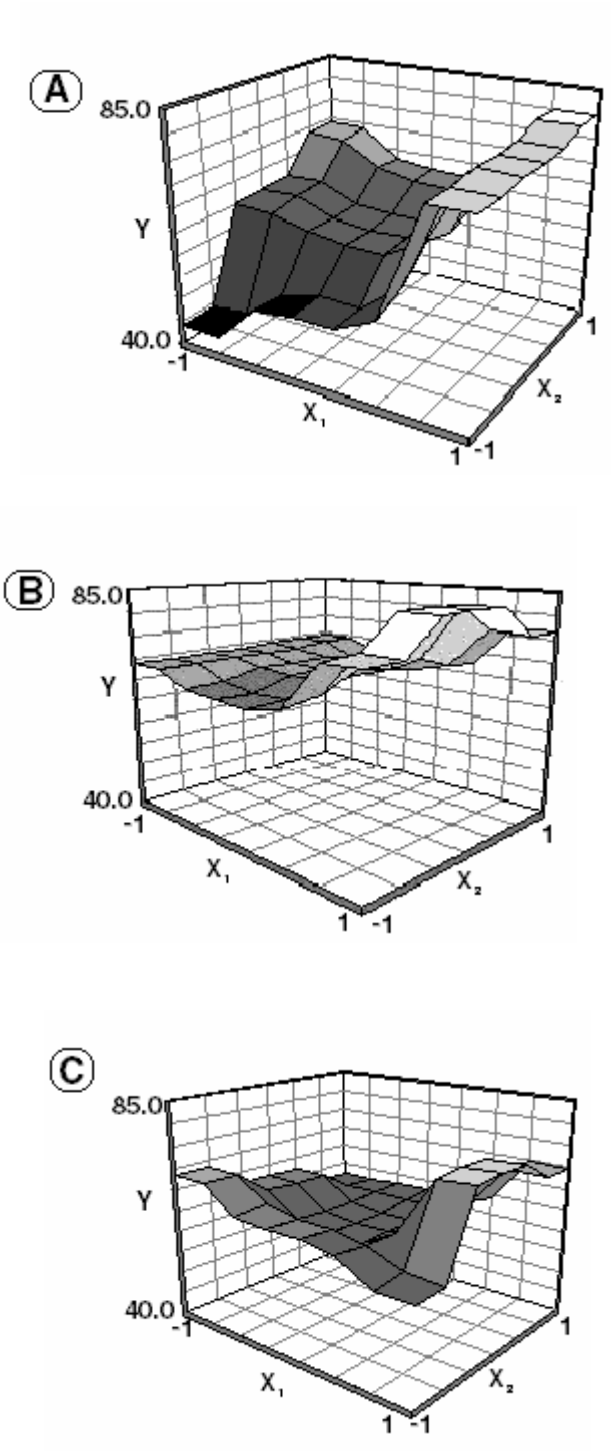


Figure 2: Response surface plots
(A) at -1 level of variable X_3,
(B) at 0 level of variable X_3,
(C) at 1 level of variable X_3.