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Optimization of Formulation Parameters of Simvastatin Loaded PLGA Nanoparticles by Using 3^3 Factorial Design

CM Detroja[1*], LD Patel[2], PR Vasanani[3]

[1]*School of Pharmacy, RK University, Rajkot, Gujarat, India*
[2]*Sharda School of Pharmacy, Pethapur, Gandhinagar, Gujarat, India*
[3]*Amneal Pharmaceutical, Ahmedabad, Gujarat, India*

ABSTRACT

Factorial design enables researchers to study and understand how multiple factors affect a dependent variable, both independently and jointly. In present report, 3^3 factorial design was used to study the combined influence of three independent variables in preparation of Simvastatin loaded Poly (D, L Lactide -co- Glycolide) (PLGA) nanoparticles. Nanoparticles were prepared by nanoprecipitation method. The process variables like rate of addition of organic phase to aqueous phase, temperature, speed of magnetic stirrer and time to evaporate organic phase were kept constant throughout the investigation. The formulation variables like concentration of stabilizer (Polyvinyl alcohol), drug (Simvastatin): polymer ratio (PLGA), and organic (acetone): aqueous phase ratio were selected as independent variables. Prepared nanoparticles were evaluated for particle size (PS) and entrapment efficiency (EE). PS and EE were selected as dependent variables. The coded values of independent variables were subjected to multiple regressions to derive a second order polynomial equation (full model). After neglecting the non-significant terms from full model, F-Statistics was applied to set up reduce polynomial equation. Among the three independent variables, value of coefficient of drug: polymer ratio was found to be maximum. This revealed that major contributing variable for PS and EE in nanoparticles is drug: polymer ratio. Two dimensional contour plots and three dimensional response surface plots were established by varying levels of two factors and keeping the third factor at fixed level at a time. Thus the derived equation, surface response plot and contour plot helps in predicting the value of independent variables for optimum PS and EE in preparation of Simvastatin loaded PLGA nanoparticles.

Keywords: Factorial design, Simvastatin, PLGA nanoparticles, contour plots, surface response plot.

INTRODUCTION

Numerous methods have been explored by researchers to overcome the poor aqueous solubility of drug candidates in the research and development of oral formulations. These methods include changing the chemical structure of drug candidate, pro-drug approach to various formulation techniques. The formulation techniques which can overcome the problem of solubility are generally salt formation, use of co-solvent, use of surfactant, complexation, micronization, use of particulate system like liposomes, nanoparticles microemulsion, polymeric micells etc. [1] Several particulate systems have been reported as effective carriers of therapeutic agents administered orally because they have less limitations compared to

***Corresponding author: Mr. Chetan Detroja,**
School of Pharmacy, RK University, Kasturbadham, Rajkot – Bhavnagar Highway, Rajkot – 360020, Gujarat, India; **E-mail:** chetan14386@yahoo.co.in
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other approaches. Among the particulate systems employed in last two decades each carries some advantages and disadvantages e.g. liposome formulations can carry lipid soluble as well as water soluble drug but are readily disrupted by intestinal detergents, such as bile salts, and are subject to degradation by intestinal phospholipases. Microemulsion offers several advantages compared to emulsion, such as, thermodynamic stability, high solubilization capacity, ease of preparation. Many times castor oil based formulations, has triggered adverse events, most frequently renal dysfunction, hypertension, and hepatotoxicity. [2]

PLGA nanoparticles are often explored by researchers to enhance the bioavailability of poorly water soluble drugs. PLGA is mostly widely used polymer due to its property of biocompatibility and biodegradability. It degrades through natural pathways into non-toxic lactic acid and glycolic acid in the body. [3-4]

Simvastatin, an inactive lactone, is cholesterol and lipid lowering agent developed synthetically from a fermentation product of *Aspergillus terreus*. It is indicated for the treatment of hypercholesterolemia and for the reduction in the risk of cardiac heart disease mortality and cardiovascular events. It acts by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), which is the rate-limiting enzyme in cholesterol biosynthesis. [5-6] It is listed in the World Health Organization's List of Essential Medicines. [7] It is a class-II drug according to the Biopharmaceutical Classification System and, therefore, has a dissolution rate-limited absorption in *vivo* and, hence, suboptimal oral bioavailability. It is practically insoluble in water. It shows only 5% bioavailability.[8] Traditional approaches of optimization, performed by varying one variable at a time, neglect the impact of each variable and their interaction in the formulation giving inaccurate output. Factorial design is an efficient optimization technique for learning the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. [9-10] It establishes the relationship between independent variables and dependent variables or responses. However, prior knowledge and understanding of the process and the process variables under investigation are necessary for achieving a more realistic model. The contour plots and surface plots give graphical representation of the values of the response. [11]

The aim of present study is to optimize the formulation parameters in the process of preparation of Simvastatin loaded PLGA nanoparticles by effective use of 3^3 factorial design. PS and EE were selected as dependent variables due to its significant impact on the quality or performance of the formulation. The formulation variables which have been predicted to play a substantial role in formulation such as concentration of stabilizer (Polyvinyl alcohol), drug (Simvastatin): polymer ratio (PLGA), and organic (acetone): aqueous phase ratio were selected as independent variables. 2D contour plots and 3D surface response plots were constructed and used to study the main and interaction effect of the independent variables on the dependent variables.

MATERIALS AND METHODS

Simvastatin was obtained as gift sample from Alembic Ltd, Vadodara, India. Poly (D, L Lactide-co-Glycolide) (PLGA 50:50) was received as gift sample from Sun Pharmaceutical Industries Ltd. Vadodara, India. Polyvinyl alcohol (PVA, MW 30,000–70,000 Da; hydrolyzed 87–89%) was purchased from BASF, Mumbai, India. Acetone, acetonitrile and methanol were purchased from S.D. Fine chem., Mumbai, India.

Preparation of Simvastatin loaded PLGA nanoparticles

Simvastatin loaded nanoparticles were prepared by using nanoprecipitation method. [12] The organic phase was prepared by dissolving 10mg Simvastatin (fix quantity) and appropriate quantity of PLGA in appropriate volume of acetone. The aqueous phase was prepared using the stabilizer and water. The organic phase was added drop wise into 10ml of aqueous phase (fix volume) on Remi magnetic stirrer at slow speed. Nanoparticles were formed immediately with spontaneous diffusion of acetone into water. Nanoparticles were recovered by centrifugation at 20,000 rpm for 30 minutes by using Cooling centrifuge. The prepared nanoparticles were washed twice with distilled water to remove excess stabilizer.

Optimization of Simvastatin nanoparticles by using 3^3 factorial designs

Primary objective of optimization is to achieve Simvastatin loaded nanoparticles with maximum EE and minimum/optimum PS. Single factor investigation revealed that drug: polymer ratio (it is amount of PLGA as amount of Simvastatin is fixed), concentration of PVA and organic: aqueous phase ratio (it is volume of acetone as that of the aqueous phase is constant) have profound effect on PS and EE of nanoparticles. So in present investigation further optimization with these three identified factors were performed by using 3^3 factorial designs. Briefly, 27 batches were prepared by varying the drug: polymer ratio (1:5, 1:7.5 and 1:10), concentration of PVA (0.5, 1.0 and 1.5% w/v) and organic: aqueous phase ratio (2.5, 3.3 and 5 mL corresponding to acetone to water ratios of 1:4, 1:3 and 1:2) and evaluated for PS and EE responses.

Independent variables:

1. X_1: Concentration of PVA (0.5, 1.0 and 1.5% w/v)
2. X_2: Simvastatin: PLGA ratio (1:5, 1:7.5 and 1:10), and
3. X_3: Organic: aqueous phase ratio (2.5, 3.3 and 5 mL corresponding to acetone to aqueous phase ratios of 1:4, 1:3 and 1:2)

Dependent variables:

1. Particle size (PS)

2. Entrapment efficiency (EE)

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

$$x_i = \frac{(X_i - X_i^x)}{X\Delta_i} \quad (1)$$

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^{x_i} is the natural value of the i^{th} independent variable at the centre point and $X\Delta_t$ is the step change value.

Three independent variables as stated above are coded as shown in Table 1. Mathematical modeling was carried out by using below equation 2 to obtain a second order polynomial equation which describes the relationship of the PS and EE with X_1, X_2 and X_3. [13]

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

Where Y is the dependent variable (PS/EE) while b_0 is the intercept, b_i (b_1, b_2 and b_3), bij (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.

Table 1: Coded values of formulation parameters

Coded values	Actual values		
	Concentration of PVA	Simvastatin: PLGA ratio	Organic: aqueous phase ratio
	X_1 (%w/v)	X_2 (mg)	X_3 (mL)
-1	0.5	50 (1:5)	2.5
0	1.0	75 (1:7.5)	3.3
1	1.5	100 (1:10)	5

Table 2: 3^3 full factorial layout

Batch	X1	X2	X3	PS (±SD)	EE(±SD)
NP1	0.5	50	2.5	170.3 (4.8)	55.3 (1.8)
NP2	0.5	50	3.3	154.2 (5.8)	51.6 (2.1)
NP3	0.5	50	5	139.0 (5.1)	64.3 (1.4)
NP4	0.5	75	2.5	183.9 (3.5)	64.4 (1.6)
NP5	0.5	75	3.3	173.6 (4.3)	60.1 (1.5)
NP6	0.5	75	5	153.8 (4.4)	70.3 (2.0)
NP7	0.5	100	2.5	199.3 (6.2)	68.5 (1.3)
NP8	0.5	100	3.3	181.1 (4.5)	69.3 (2.2)
NP9	0.5	100	5	169.8 (3.8)	52.6 (0.6)
NP10	1	50	2.5	158.5 (5.2)	52.6 (1.7)
NP11	1	50	3.3	142.6 (4.9)	50.9 (2.3)
NP12	1	50	5	128.7 (4.6)	48.1 (1.5)
NP13	1	75	2.5	170.6 (5.4)	63.6 (1.4)
NP14	1	75	3.3	158.2 (3.7)	61.5 (1.4)
NP15	1	75	5	143.3 (5.5)	59.3 (1.8)
NP16	1	100	2.5	184.9 (4.1)	71.5 (1.9)
NP17	1	100	3.3	171.8 (4.1)	69.3 (1.1)
NP18	1	100	5	155.4 (3.7)	68.9 (2.1)
NP19	1.5	50	2.5	153.6 (3.4)	49.5 (1.7)
NP20	1.5	50	3.3	135.1 (3.0)	45.3 (1.9)
NP21	1.5	50	5	122.6 (3.5)	44.3 (1.8)
NP22	1.5	75	2.5	169.2 (4.2)	60.9 (2.2)
NP23	1.5	75	3.3	156.3 (4.2)	56.3 (2.3)
NP24	1.5	75	5	138.8 (3.7)	55.3 (1.9)
NP25	1.5	100	2.5	180.6 (2.9)	68.9 (1.7)
NP26	1.5	100	3.3	166.6 (3.0)	65.3 (0.9)
NP27	1.5	100	5	150.9 (3.9)	63.1 (1.1)

Values are represented as mean ± SD, n=3

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 PS and EE, dependent variables and the results are recorded in Table 2. A full and reduced model for both PS and EE was established by putting the values of regression coefficients in polynomial equation. Statistical soundness of the polynomial equations was established on the basis of analysis of variance (ANOVA) statistics.

Two dimensional contour plots and three dimensional response surface plots were established by varying levels of two factors and keeping the third factor at fixed levels at a time. [14] In this way, they are more helpful in understanding the actual interaction amongst the varying factors on the response parameter and are more meaningful. The 2-D contour plots and 3-D response surface graphs were constructed using the NCSS 9 software (Trial version).

The experimental design and the reduced polynomial equation for the optimization of formulation were validated for their utility by performing check point analysis. 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 PS and EE were calculated by substituting the values in the reduced polynomial equation. Statistical comparison between the predicted values and average of three experimental values of the response parameters was performed to derive percentage error and to evaluate significant difference between these values.

For simultaneous optimization of PS and EE desirability function (multi-response optimization techniques) was applied and total desirability was calculated using Design Expert software. The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. The total desirability is defined as a geometric mean of the individual desirability for PS and EE. [15]

$$D = (d_{PS} X\, d_{EE})^{1/2}$$

Where, D is the total desirability, and d_{PS} and d_{EE} are individual desirability for PS and EE. If both of the quality characteristics reach their ideal values, the individual desirability is 1 for both. Consequently, the total desirability is also 1.

Determination of Particle size

The size of Simvastatin nanoparticles was determined by particle size analyzers based on laser light scattering principle. A particle size analyzer model Zetatrac (Microtrac Ltd., U.K.) equipped with an argon laser was utilized for evaluating the particle size. Light scattering was monitored at 90° angle and 25°C. The mean droplet size was calculated from intensity, volume and bimodal distribution assuming spherical particles.

Determination of Entrapment efficiency

The amount of drug entrapped in Nanoparticles was estimated by UV spectrophotometer. One milliliter of Nanoparticles dispersion was added to acetonitrile and subjected to shaking using vertex mixer. The resultant suspension was subjected to centrifugation at 10,000

rpm for 15 min to remove precipitated components. The supernatant was diluted appropriately and absorbance was recorded at 238 nm by using UV Visible spectrophotometer. All tests were performed in triplicate.

RESULT AND DISCUSSION

Optimization of Simvastatin loaded nanoparticles

Twenty seven batches of Simvastatin Nanoparticles were prepared by using 3^3 factorial experimental design, varying three independent variables, concentration of PVA, drug: polymer ratio (X_2), and Organic: aqueous phase ratio (X_3) as shown in Table 2. All batches were prepared in triplicate and evaluated for PS and EE. Results are recorded in Table 2. Mathematical modeling was carried out as per Equation 2 to obtain a second-order polynomial equation (full model) which describes the relationship of the PS and EE with X_1, X_2 and X_3. [16]

PS full model equation

$$Y_{PS} = 158.45 - 8.41X_1 + 14.21X_2 - 14.92X_3 + 3.93X_{11} - 1.69X_{22} - 0.32X_{33} + 0.083X_{12} - 0.01X_{13} + 0.283\ X_{23} - 0.0625X_{123} \quad (3)$$

EE full model equation

$$Y_{EE} = 61.344 - 2.501X_1 + 9.294X_2 - 2.127X_3 - 1.583X_{11} - 1.583X_{12} + 0.5166X_{33} + 0.6X_{12} - 0.525X_{13} + 0.45X_{23} - 0.5875X_{123} \quad (4)$$

The PS and EE (dependent variables) 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 dependent variable changes when two or more independent variables were simultaneously changed. A value of PS varies from 122.6 nm to 199.3 nm while that of EE varies from 44.3 to 70.3 % among twenty seven batches. This is reflected by wide range of coefficients of the terms in equation 3 and 4 respectively.

Small values of coefficients (p value greater than 0.01) are regarded as least contributing and non-significant in the optimization process. Omitting non-significant terms from the full model equations established reduced model equations for PS (Equation 5) and EE (Equation 6).

PS reduced model equation

$$Y_{PS} = 157.11 - 8.4055X1 + 14.21X2 - 14.92X3 + 3.9277X11 \quad (5)$$

EE reduced model equation

$$Y_{EE} = 61.689 - 2.51X1 + 9.294X2 - 2.128\ X3 - 1.583\ X11 - 1.583X22 \quad (6)$$

The predicted and observed values of response parameter are shown in Table 3. Low values of %RE implied that there was a reasonable agreement between the predicted and observed values. This indicates suitability of the model.

The significance of each coefficient of equation 3 and 4 was determined by 'student t' test and p-value which are listed in tables 4 and 5 for PS and EE respectively. The larger the magnitude of the t value and smaller the p value, more significant is the corresponding coefficient. [17] This reveals that for PS quadratic main effect of concentration of PVA, Drug: polymer ratio and Organic: aqueous phase ratios are significant. The second order main effect of Concentration of stabilizer is also significant while all interaction effects are found to be non-significant as evident from their p-values. For EE, quadratic main effect of Concentration of stabilizer, Drug: polymer ratio and Organic: aqueous phase ratio are significant. The second order main effect of Concentration of stabilizer and Drug: polymer ratio is significant while all other interaction effects are found to be non-significant as evident from their p-values. Tables 6 and 7 represent ANOVA of full model and reduced model for PS and EE respectively.

F-Statistic value obtained from the results of ANOVA confirmed omission of non-significant terms of equations. Since the calculated F value, as shown in Tables 6 and 7, was less than the tabled F value for PS as well EE, it was concluded that the neglected terms do not significantly contribute in the prediction and hence reduced model can be applied. For equations 3 and 4, sign of the coefficients explains the nature of effect while magnitudes determine extent of effect for variables. Among the three independent variables X_1, X_2 and X_3, value of coefficient of X_2 was found to be maximum in equation 3 and 4. This reveals that X_2 was major contributing variable for PS and EE in nanoparticles. The goodness of fit of the model was checked by the determination coefficient (R^2).

For PS, the values of the determination coefficients (R^2 = 0.9944 for full model and 0.9923 for reduced model) indicated that over 99% of the total variations are explained by the model. For EE, values of the determination coefficients (R^2 = 0.9934 for full model and 0.9854 for reduced model) indicated that over 98% of the total variations are explained by the model. The values of adjusted determination coefficients (PS: adjusted R^2 = 0.9909 for full model and 0.9909 for reduced model, EE: adjusted R^2 = 0.9894 for full model and 0.9820 for reduced model) are also very high which indicates a high significance of the model.

The optimum formulation offered by software based on desirability was found at 0, 1, and 1 level of X_1, X_2 and X_3 respectively. The calculated desirability factor for offered formulations was 1.00 indicating suitability of the designed factorial model. All the above considerations indicate an excellent adequacy of the regression model.

Contour Plots

Contour plots are used for graphical presentation of Nanoparticles optimization process. Contour plots drawn at -1, 0 and 1 level of X_1 for predefined PS values are shown in Figure 1A, B and C and for predefined

values of EE are shown in Figure 1 D, E and F respectively. Plots for PS at -1 (1A) level of X_1 were found to be nonlinear for all predefined values of PS. This explains nonlinear relationship between X_2 and X_3 variables. It was determined that desirable PS (≤160nm) could be obtained with X_2 at range 50 mg to 88mg and X_3 at range 2.9 to 5mL. It was concluded from the contour plot that to obtain desirable PS lower amount of polymer concentration and higher amount of organic phase was required when 0.5% stabilizer concentration was used.

Plots for PS at 0 level of X_1 (1B) were also found to be nonlinear for all predefined values of PS. This shows nonlinear relationship between X_2 and X_3 variables. It was observed that desirable PS could be obtained with X_2 at range 60 mg to 100mg and X_3 at range 2.5 to 4.2 ml. This revealed that to obtain desirable PS higher amount of PLGA and lower amount of organic phase was required when 1.0% stabilizer concentration were employed for Nanoparticles preparation.

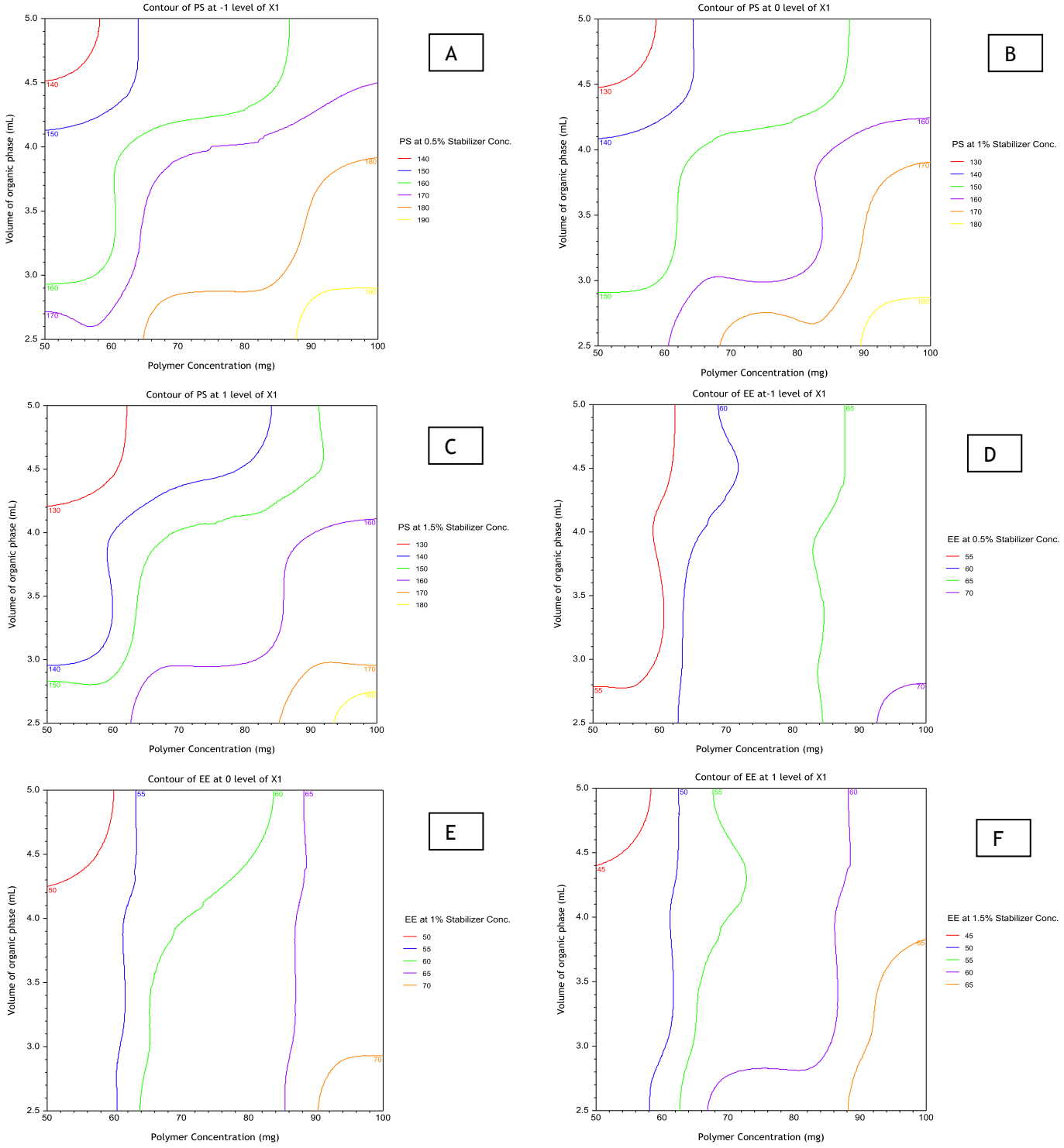

Fig. 1: Contour plots showing effect of X_2 and X_3 on PS at -1 level of X_1 (A), 0 level of X_1 (B) and 1 level of X_1 (C); Effect of X_2 and X_3 on EE -1 level of X_1 (D), 0 level of X_1 (E) and 1 level of X_1(F).

Table 3: Observed and predicted values of response (PS and EE).

Batch	Response					
	PS			EE		
	Observed	Predicted	%RE	Observed	Predicted	%RE
NP1	170.3	172.4	1.210	55.3	55.36	0.108
NP2	154.2	153.0	0.791	51.6	52.34	1.434
NP3	139	140.5	1.086	50.2	49.87	0.657
NP4	183.9	185.0	0.571	64.3	66.01	2.659
NP5	173.6	170.4	1.838	64.4	65.35	1.475
NP6	153.8	154.4	0.358	60.1	61.27	1.947
NP7	199.3	197.3	0.993	70.3	72.98	3.812
NP8	181.1	181.5	0.221	68.5	65.78	3.971
NP9	169.8	170.3	0.294	69.3	70.65	1.948
NP10	158.5	159.3	0.505	52.6	51.21	2.643
NP11	142.6	144.2	1.122	50.9	50.22	1.336
NP12	128.7	125.3	2.642	48.1	50.32	4.615
NP13	170.6	171.2	0.352	63.6	62.58	1.604
NP14	158.2	158.6	0.253	61.5	60.35	1.870
NP15	143.3	141.9	0.977	59.3	60.35	1.771
NP16	184.9	185.1	0.108	71.5	73.35	2.587
NP17	171.8	172.3	0.291	69.3	70.35	1.515
NP18	155.4	155.1	0.193	68.9	67.35	2.250
NP19	153.6	155.5	1.237	49.5	50.35	1.717
NP20	135.1	139.2	3.035	45.3	46.35	2.318
NP21	122.6	123.1	0.408	44.3	45.35	2.370
NP22	169.2	170.2	0.591	60.9	62.35	2.381
NP23	156.3	155.3	0.640	56.3	58.65	4.174
NP24	138.8	141.3	1.801	55.3	56.44	2.061
NP25	180.6	181.3	0.388	68.9	69.35	0.653
NP26	166.6	167.8	0.720	65.3	64.41	1.363
NP27	150.9	151.2	0.199	63.1	64	1.426

[%RE = % relative error = (Observed –predicted) X 100/observed)

Table 4: Model coefficients estimated by multiple linear regressions for PS

Factor	Coefficients	t Stat	P-value
Intercept	158.4519	175.0526	1.081E-27*
X1	-8.4056	-20.0605	9.134E-13*
X 2	14.2111	33.9158	2.479E-16*
X3	-14.9222	-35.6130	1.146E-16*
X11	3.9278	5.4120	5.758E-05*
X22	-1.6889	-2.3271	3.341E-02
X33	-0.3222	-0.4440	6.630E-01
X12	0.0083	0.0162	9.872E-01
X13	-0.0167	-0.0325	9.745E-01
X23	0.2833	0.5521	5.885E-01
X123	-0.0625	-0.0994	9.220E-01

*Significant (p value < 0.01)

Table 5: Model coefficients estimated by multiple linear regressions for EE

Factors	Coefficients	t Stat	P-value
Intercept	61.3444	140.4812	3.64E-26*
X1	-2.5056	-12.3951	1.28E-09*
X 2	9.2944	45.9801	2.00E-18*
X3	-2.1278	-10.5262	1.34E-08*
X11	-1.5833	-4.5223	3.47E-04*
X22	-1.5833	-4.5223	3.47E-04*
X33	0.5167	1.4757	1.59E-01
X12	0.6000	2.4236	2.76E-02
X13	-0.5250	-2.1206	4.99E-02
X23	0.4500	1.8177	8.79E-02
X123	-0.5875	-1.9376	7.05E-02

*Significant (p value < 0.01)

Plots for PS at 1 level of X_1 (1C) were found to be curved representing nonlinear relationship between X_2 and X_3 variables. Desirable PS could be obtained with X_2 at range 62 mg to 100mg and X_3 at range 2.5 to 4.2 ml. It was concluded from the plot that to obtain desirable PS higher amount of PLGA and lower amount of organic phase were required when 1.5% stabilizer concentration was employed for Nanoparticles preparation.

Table 6: Analysis of variance (ANOVA) for PS for full and reduced model

		df	SS	MS	F	R^2
Regression	FM	10	9026.372	902.637	285.620	0.9972
	RM	4	9007.636	2251.909	714.891	0.9961
Error	FM	16	50.564 (E_1)	3.160		
	RM	22	69.3 (E_2)	3.150		

Where df, Degree of freedom; E1 and E2, Sum of squares of error of full and reduced model respectively; FM, full model; F, Fischer ratio; MS, Mean squares; RM, reduced model; SS, Sum of squares

Number of parameters omitted (N) = 6.

F calculated = $[(SSE_2–SSE_1)/N]/MS$ of error for FM = [69.3-50.56/6]/3.16 = 0.988

F tabulated = 2.74 (α = 0.05, V1 = 6, and V2 = 16).

Table 7: Analysis of variance (ANOVA) for EE for full and reduced model

		df	SS	MS	F	R^2
Regression	FM	10	1793.958	179.395	243.911	0.996
	RM	5	1779.538	355.907	285.396	0.992
Error	FM	16	11.767 (E_1)	1.135		
	RM	23	26.188 (E_2)	1.247		

Where df, Degree of freedom; E1 and E2, Sum of squares of error of full and reduced model respectively; FM, full model; F, Fischer ratio; MS, Mean squares; RM, reduced model; SS, Sum of squares

Number of parameters omitted (N) = 5.

F calculated = $[(SSE_2–SSE_1)/N]/MS$ of error for FM = [26.188-11.767/5]/1.247 = 2.5411

F tabulated = 2.85 (α = 0.05, V_1 = 5 and V_2 = 16)

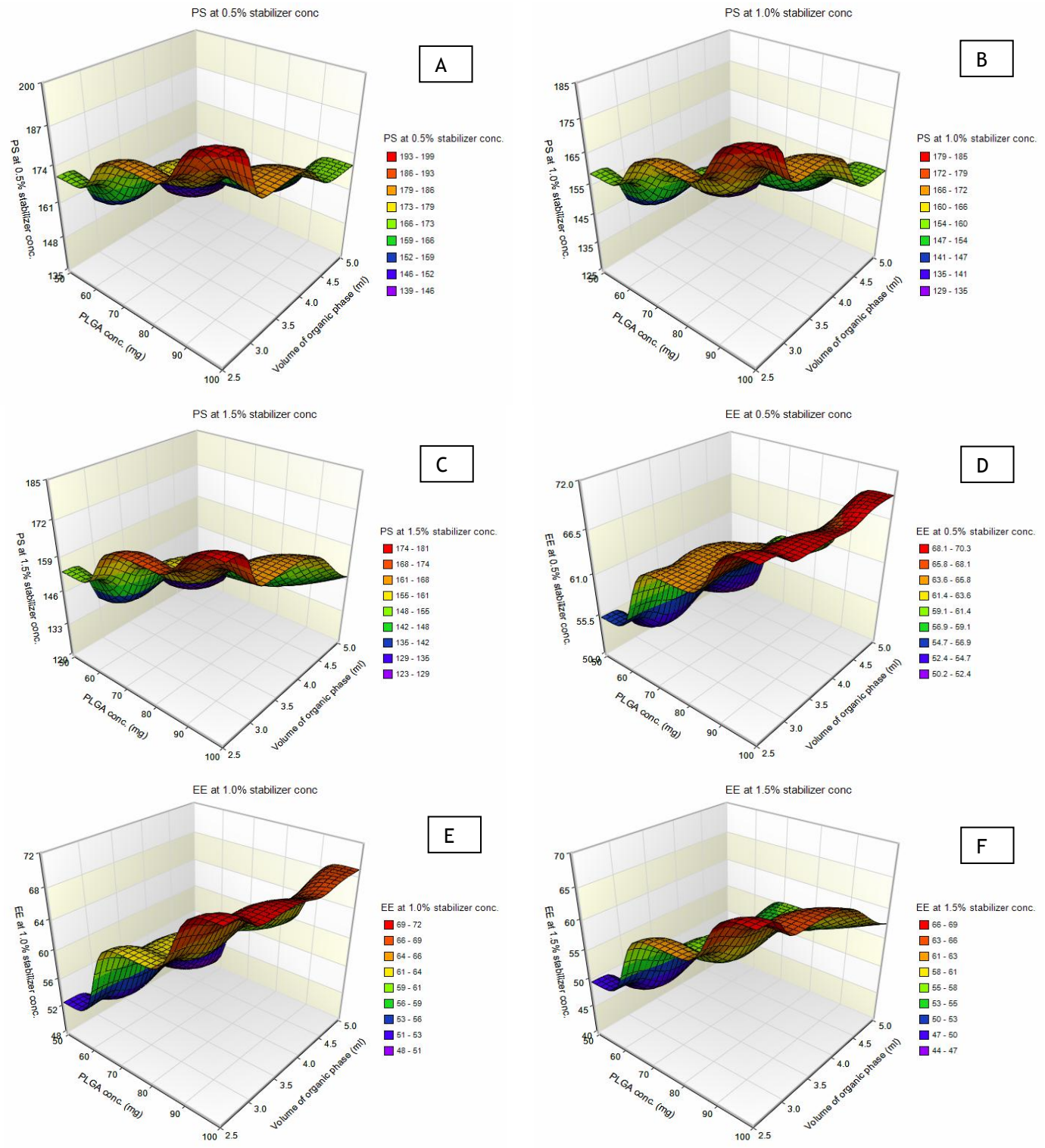

Fig. 2: 3D Surface response plots showing effect of X_2 and X_3 on PS at -1 level of X_1 (A), 0 level of X_1 (B) and 1 level of X_1 (C); Effect of X_2 and X_3 on EE -1 level of X_1 (D), 0 level of X_1 (E) and 1 level of X_1(F).

Contour plots drawn at different levels of stabilizer concentration revealed that desirable PS was achievable at all levels of stabilizer concentration but with different values of polymer concentration and volume of organic phase.

Plots for EE at -1, 0 and 1 level of X_1 (1D, E and F respectively) were found to be nonlinear for all predefined values of EE. This explains nonlinear relationship between X_2 and X_3 variables. It was determined that desirable EE (≤60nm) could be obtained with X_2 at range 67 mg to 100 mg and X_3 at all levels. The vertical curves signify that X_3 contribute considerably lesser than X_2 for EE.

Contour plots drawn at different levels of stabilizer concentration revealed that desirable EE was achievable at all levels of stabilizer concentration and volume of organic phase with value of polymer concentration 67mg and above.

Overlay of contours is one of the techniques for optimizing multiple responses. The overlay of PS and

EE contour at 0 level of X_1(overlay of 1B and 1E) is shown in Figure 3. It is observed from the figure that area formed by crossing of line of 160 nm for PS and 60% for EE, marked with arrow, is optimum at 0 level of X_1. Such optimum area can be found with -1 and 1 level of X_1.

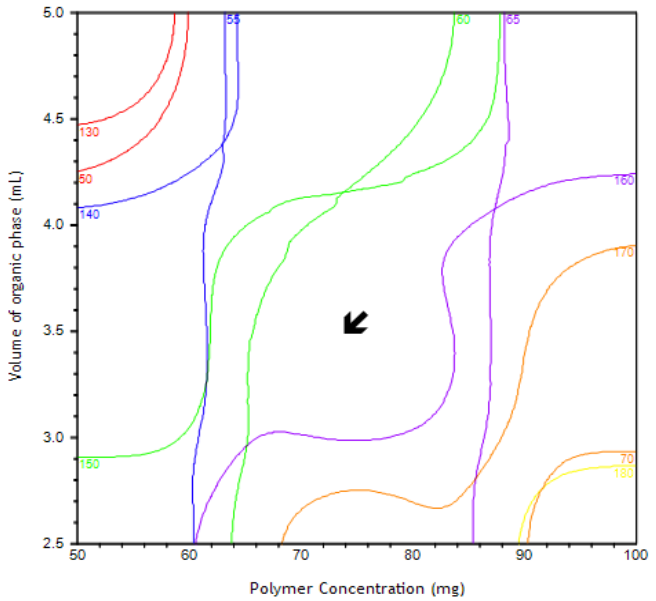

Fig. 3: Overlay of contour plots for PS and EE at 0 level of stabilizer concentration (X_1).

Response surface plots

Response surface plots were generated using NCSS software. These plots were generated at fixed level (-1, 0 and 1) of X_1. Figure 2A, B and C show response surface plots obtained as a function of X_2 Vs X_3 at -1, 0 and 1 level of X_1 for PS. Figure 2D, E and F show response surface plots obtained as a function of X_2 Vs X_3 at -1, 0 and 1 level of X_1 for EE.

Plots for PS illustrate increase in PS with increase in polymer concentration and decrease in volume of organic phase. Plots for EE show linear relationship between EE and polymer concentration and also depict least or minor effect of volume of organic phase on EE.

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