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Influence of Selected Formulation Variables on the Preparation of Peptide Loaded Lipospheres

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ABSTRACT

In the present study, efforts have been made to statistically optimize the formulation parameters of sustained-release lipospheres of Enalapril maleate, a water-soluble peptide, using wax and polar lipid combination. A 3-factor, 3-level Box-Behnken design was used to derive a second order polynomial equation and construct 3D surface plots to predict the effect on responses. The peptide amount (X_1), Tween® 80 concentration (X_2) and stirring speed (X_3) were selected as independent variables while the mean diameter (Y_1) and the entrapment efficiency (Y_2) of lipospheres were chosen as the dependent variables in the present investigation. Lipospheres were prepared by Water-in-oil-in-water double emulsion (w/o/w) method. The Response Surface Methodology (RSM) and multiple response optimization were used to select optimal formulation with maximum entrapment and particle size in range. The optimal formulation was subsequently characterized in terms of morphology, release kinetics and stability studies. The maximum entrapment (80.62±2.54%) was achieved with 10mg EM, 0.1% (v/v) Tween® 80 and 1000 rpm stirring speed. The observed responses coincided well with the predicted values from the RSM optimization technique. Scanning electron microscopy confirmed the smooth spherical microspheres in the size range of 23.00±0.82-34.57±1.04 μm. Kinetic models revealed that drug release followed non-Fickian release pattern. Finally, drug bioactivity was found to remain intact after microencapsulation. Thus, Box-Behnken design demonstrated the role of the derived equation and 3D surface plots in predicting the values of dependent variables for the preparation and optimization of Enlapril maleate loaded lipospheres.

Key words: Peptide, box-behnken design, liposphere, lipid microspheres, antihypertensive, optimization

INTRODUCTION

In the recent years, it has been realized that complete drug therapy for an ailment does not rely on the development of new drugs alone. In this context, a promising approach involves the development of suitable drug delivery systems. Numerous polymer based colloidal carriers have widely been studied as drug carriers in the field of drug delivery system (Singh *et al*., 2010a, b; Owlia *et al*., 2007). But the use of synthetic polymer matrix materials often goes along with detrimental effects on incorporated drug during manufacturing of the formulations or during the erosion of the polymers after application (Reithmeier *et al*., 2001). Moreover, the degradation of polymer might possibly cause systemic toxic effects through the impairment of Reticulo Endothelial

System (RES) or after phagocytosis of particles by human macrophages and granulocytes (Kumar, 2000). Therefore, alternative carrier substances have been investigated; among them lipidic materials have garnered growing attention. Numerous lipid based delivery systems such as liposomes, solid lipid nanoparticles, oily suspensions, submicron lipid emulsions, lipid implants, lipid microtubules and microcylinders, lipid microbubbles and lipid microspheres (Lipospheres) have been investigated for proteins and peptides (Rawat *et al*., 2008). Lipospheres carrier system has several advantages over other delivery systems, including emulsions, liposomes and microspheres, such as: Better physical stability, low cost of ingredients, ease of preparation and scale-up, high dispersability in an aqueous medium, high entrapment of hydrophobic drugs, controlled particle size and extended release of entrapped drug (Rawat and Saraf, 2008).

The liposphere drug delivery system is an aqueous microdispersion of solid water insoluble spherical microparticles of a particle size be Tween® 0.2 and 100 μm. The lipospheres are made of solid hydrophobic triglycerides having a monolayer of phospholipids embedded on the surface of the particle. The solid core contains the bioactive compound dissolved or dispersed in a solid fat matrix. These are generally used as carrier vehicle for hydrophobic drugs. These exhibit low entrapment of hydrophilic drugs which could be improved by using polar lipids like cetyl alcohol, stearyl alcohol and cetostearyl alcohol etc. (Esposita *et al*., 2007).

Enalapril maleate is the maleate salt of enalapril, highly water-soluble polypeptide in salt form and the prodrug of enalaprilat having strong Angiotension-converting Enzyme (ACE) inhibitor activity. It is a hydrophilic polypeptide with short half-life of 3-4 h used in the treatment of hypertension and congestive heart failure (Moncloa *et al*., 1985; Warner and Rush, 1988; Abdel-Salam *et al*., 2007). The recommended daily dose is as low as 5 mg which may allow for development of a practical dosage form for sustained release. Although it is stable in acidic pH but due to its short half life it is cleared from the circulation and hence requiring frequent administration. An induction of the therapy can be achieved by a controlled initial rapid drug release followed by a prolonged continuous release to maintain the drug at the desired concentration. Moreover, a consistent and steady supply of enalapril at the ACE site is very crucial to the hypertensive patients because of its strong dose dependency. Due to the increased uptake of LS by tissue macrophages and liver, it could serve as target specific carrier of EM at it principal site of conversion-liver (Khopade and Jain, 1997; Masters and Domb, 1998).

Controlling both the drug-loading efficiency and particle size of drug-loaded lipospheres is important to apply lipospheres to delivery of peptides. Therefore, process optimization using response surface morphology may be advantageous for the efficient entrapment of highly hydrophilic drugs like peptides as these parameters can be improved and the physicochemical properties of the microspheres such as the particle size, surface texture, morphology and drug release profile can be controlled (Brannon-Peppas and Vert, 2000; Ai-Noi *et al*., 2008). Although, EM loaded delivery vehicles have been prepared for oral, parenteral and transdermal delivery (Ahlin *et al*., 2002; Yoo *et al*., 1999; Bhavna *et al*., 2008) but based on literature cited there exists a lack of studies regarding statistical optimization of formulation parameters to enhance both the entrapment and controlled release of EM from lipospheres. In the present study, efforts have been made to prepare a sustained-release lipospheres of EM using wax and polar lipid combination. Based on the reports supporting the uptake and localization of lipospheres at inflammatory sites and liver, it can serve as an ideal candidate system for site specific delivery of selected proteins.

Box-Behnken design is an established method to study the effect of selected parameters. These use only three levels for each factor and the domain is within the original factorial shape. The

overall structure of a three-factor Box-Behnken design is represented as a cube but the experimental points are at the midpoints of the edges of the cube rather than at the corners and centers of the faces, that is, v2 or 1.414 e.u. from the center point. Each combination of the extreme values of two of the variables is examined with the third variable having a value of zero (Singh *et al*., 2010a, b; Ko *et al*., 2003).

The objective of the present study was to statistically optimize the formulation parameters of sustained-release lipospheres of Enalapril maleate, a water-soluble peptide, using wax and polar lipid combination. A 3-factor, 3-level Box-Behnken design was used to derive a second order polynomial equation and construct 3D surface plots to predict the effect on responses. The peptide amount (X1), Tween® 80 concentration (X2) and stirring speed (X3) were selected as independent variables while the mean diameter (Y1) and the entrapment efficiency (Y2) of lipospheres were chosen as the dependent variables in the present investigation.

MATERIALS AND METHODS

Materials: Enalapril maleate was kindly gifted by Alkem Pharma, Mumbai, India. Paraffin wax was purchased from Himedia labs. Cetyl alcohol, Tween® 80, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate and sodium hydroxide were purchased from S.D. Fine Chemicals Ltd. (India). All other chemicals used were of analytical grade. Preparation of Lipospheres

Preparation of lipospheres: Lipospheres were prepared by a method based on the water-in-oil-in-water double emulsion (w/o/w) method reported by Reithmeier *et al*. (2001) and Cortesi *et al*. (2002) with few modifications. EM (10, 20 or 30 mg) was solubilized in the 100 µL internal aqueous phase of a w/o/w double emulsion containing Tween® 20 (3% w/v) as stabilizer to prevent loss of EM to the external phase during solvent evaporation. This aqueous solution of peptide was emulsified in 100 mg of Paraffin wax and cetyl alcohol dissolved in 1.0 mL of methylene chloride under vigorous vortex-mixing for 10 sec. The obtained primary emulsion was further emulsified into 30 mL of a stabilizer (0.1, 0.15 or 0.2% v/v Tween® 80, 37°C) containing aqueous phase (Stirring speed- 500, 1000 or 1500 rpm) for 1 min. Hardening of the oily internal phase resulting in encapsulation of the peptide was accomplished by pouring emulsion into 100 mL of ice cold water maintained at 4°C and stirred at 300 rpm. After 3-5 h, lipospheres were isolated by filtration, washed with ice cold water and dried at room temperature (25°C) for 24 h. The final product was stored in dessicator at 2-8°C. The full experimental design and layout with coded and actual values of variables for each batch and responses are shown in Table 1. The trials were performed in random order. The other formulation and processing variables were maintained constant during the process.

Characterization

Particle size: Particle size analysis of EM-loaded lipospheres was performed by optical microscopy using a compound microscope (Labomed, India). A small amount of dry lipospheres was suspended in purified water (10 mL). The slide containing lipospheres was mounted on the stage of the microscope and 300 particles were measured using a calibrated ocular micrometer and photographed at a magnification of ×400. The process was repeated for each batch prepared.

Morphology: The surface morphology and shape of the lipospheres were analyzed by scanning electron microscopy for selected batches (Leo, VP-435, Cambridge, UK). Photomicrographs were observed at ×303 magnification operated with an acceleration voltage of 15 kV and working

Table 1: Box-Behnken design layout with coded levels and actual values of variables

F. code	X_1 EM (mg)	X_2 Tween® 80 (%v/v)	X_3 Stirring speed (rpm)
ELS1	20 (0)*	0.15 (0)	1000 (0)
ELS2	10 (-1)	0.1 (-1)	1000 (0)
ELS3	30 (+1)	0.1 (-1)	1000 (0)
ELS4	20 (0)	0.15 (0)	1000 (0)
ELS5	20 (0)	0.1 (-1)	500 (-1)
ELS6	10 (-1)	0.2 (+1)	1000 (0)
ELS7	20 (0)	0.1 (-1)	1500 (+1)
ELS8	10 (-1)	0.15 (0)	500 (-1)
ELS9	20 (0)	0.2 (+1)	500 (-1)
ELS10	20 (0)	0.15 (0)	1000 (0)
ELS11	30 (+1)	0.2 (+1)	1000 (0)
ELS12	30 (+1)	0.15 (-1)	500 (-1)
ELS13	20 (0)	0.2 (+1)	1500 (+1)
ELS14	30 (+1)	0.15 (0)	1500 (+1)
ELS15	10 (-1)	0.15 (0)	1500 (+1)
ELS16	20 (0)	0.15 (0)	1000 (0)

*Value in parenthesis indicates coded levels of the variables

distance of 10 mm was maintained. Lipospheres were mounted on the standard specimen-mounting stubs and were coated with a thin layer (20 nm) of gold by a sputter-coater unit (VG Microtech, Uckfield, UK).

Drug content: Twenty milligrams of the dried lipospheres were accurately weighed and added to 5 mL of ethyl acetate. The EM separated in phosphate buffer (pH-7.4) was analyzed by HPLC system (Shimadzu LC-10AT vp, binary gradient) equipped with detector (Shimadzu UV-visible SPD-10A vp), software (Spinchrom CFR V. 2.2, Spincotech Pvt. Ltd., Chennai) and Column (Phenomenex Luna, C-18, 5 µm, 25 cm×4.6 mm i. d.) (Walily *et al.*, 1995). Results were expressed as Mean±SD of 3 experiments. The measured responses are shown in Table 2.

***In vitro* release:** *In vitro* release of EM from lipospheres was evaluated in both acidic buffer (pH-1.2) and phosphate buffer (pH-7.4). Amount of lipospheres equivalent to 20 mg of EM were transferred to the pre-warmed dissolution media (20 mL) and maintained at 37±0.5°C under stirring at 50 rpm. Samples were withdrawn every h up to 12 h and the volume was replaced immediately by fresh phosphate buffer. The sample withdrawn was centrifuged (3000 rpm, 15 min). The EM concentration in the supernatant solution was analyzed by HPLC system as given in drug content Results were expressed as Mean±SD of 3 experiments.

Experimental design: A Box-Behnken experimental design was employed to statistically optimize the formulation parameters of EM microsphere preparation for maximum entrapment and controlled drug release. The Box-Behnken design was specifically selected since it requires fewer treatment combinations than other design in cases involving three or four factors. The Box-Behnken design is also rotable and contains statistical missing corners which may be useful when the experimenter is trying to avoid combined factor extremes. This property prevents a potential loss of data in those cases. Generation and evaluation of the statistical experimental design was performed with the STAT-EASE, design expert, 7.1.1. A design matrix comprising of

Table 2: Responses with actual and predicted values

		MD (μm) (Y_1)			EE (%)Y_2			
F. code	(%) Yield	Exp	Pred	% Bias	Exp	Pred	% Bias	(%) Loading
ELS1	83.51±3.69	29. 21	29.09	-0.41	69.22	69.39	0.244	10.63±1.48
ELS2	84.09±3.44	23.48	23.53	0.21	78.93	80.93	2.470	10.14±1.26
ELS3	74.54±3.87	23.00	23.39	1.66	65.87	66.59	1.081	14.63±1.39
ELS4	83.62±3.24	29.14	29.09	-0.17	69.46	69.39	-0.100	10.35±1.22
ELS5	78.44±3.78	29.33	29.91	1.93	70.81	68.55	-3.290	12.36±1.46
ELS6	82.33±3.70	29.46	29.07	-1.34	74.46	73.74	-0.976	6.44±1.21
ELS7	76.87±4.60	28.39	27.37	-3.72	67.85	67.40	-0.660	12.04±1.06
ELS8	81.26±3.92	32.43	31.80	-1.98	63.26	63.52	0.409	8.75±1.45
ELS9	79.54±3.65	34.57	35.59	2.86	58.82	59.27	0.759	8.36±1.33
ELS10	83.41±3.20	29.00	29.09	0.30	68.87	69.39	0.749	10.28±1.24
ELS11	70.12±4.16	29.05	29.00	-0.17	62.36	60.36	-3.313	8.06±1.95
ELS12	69.00±3.07	32.72	31.75	-3.05	53.94	55.49	2.793	11.43±1.47
ELS13	77.35±2.95	33.44	32.86	-1.76	60.98	63.24	3.573	7.10±1.20
ELS14	69.92±3.31	28.43	29.06	2.16	51.33	51.07	-0.509	11.02±1.02
ELS15	82.24±4.24	28.26	29.23	3.31	72.31	70.76	-2.190	7.33±1.42
ELS16	83.90±3.45	29.03	29.09	0.20	70.00	69.39	-0.879	10.15±1.08

16 experimental runs was constructed. An interactive second order polynomial model was utilized to evaluate both the response variables:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_2X_3 + b_6X_1X_3 + b_7X^2_1 + b_8X_2^2 + b_9X^2_3 \quad (1)$$

where, b_0-b_9 are the regression coefficients, X_1-X_8 the factors studied and Y_i is the measured response associated with each factor level combination. To assess the reliability of the model, a comparison be Tween® the experimental and predicted values of the responses is also presented in terms of %Bias in Table 2.

Bias was calculated by Eq. 2:

$$\%\text{Bias} = \frac{\text{Predicted value} - \text{Experimental value}}{\text{Predicted vaue}} \times 100 \quad (2)$$

Storage stability: Stability studies were conducted to find out stable product under storage as per ICH guidelines (Q1AR2) for new drug product and Q5C for stability testing of Biotechnological/ Biological products (CPMP/ICH/138/95) (Grimm, 1998).

RESULTS

Experimental designing: For the response surface methodology involving Box-Behnken design, a total of 16 experiments were performed for three factors at three levels each. Table 1 summarizes the experimental runs, their factor combinations and the levels of experimental units used in the study.

Effect of selected formulation variables: In order to determine the levels of factors which yielded maximum entrapment, mathematical relationships were generated be Tween® the dependent and independent variables.

For estimation of coefficients in the approximating polynomial function (Eq. 1) applying uncoded values of factor levels, the least square regression method was used. A suitable polynomial equation involving the individual main effects and interaction factors was selected based on the estimation of several statistical parameters such as the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicted residual sum of squares (PRESS) provided by the design expert software 7.1.1 (Table 3). The mean diameter (Y_1) and entrapment efficiency (Y_2) of lipospheres from the sixteen experiments were used to generate predictor equations for lipospheres with independent variables as peptide amount (X_1), Tween® 80 concentration (X_2) and stirring speed (X_3). Limit for these variables were selected from preliminary trials. The results of multiple regression analysis and Analysis of Variance (ANOVA) are summarized in Table 4.

As presented in Table 3, the quadratic model was selected as a suitable statistical model for optimized formulation with maximum entrapment because it had the smallest value of PRESS (91.42 for Y_1 and 395.63 for Y_2). PRESS is a measure of the fit of the model to the points in the design. The smaller the PRESS statistic, the better the model fits to the data points (Segurola *et al*., 1999). From the p-values presented in Table 3, it can be concluded that for both responses the cross product contribution (2FI) of the model was not significant indicating the absence of interaction effects. Furthermore, Mean Diameter (MD) and the percent drug entrapment of EM lipospheres showed R^2 values of 0.9598 and 0.9687 (Table 4), respectively; indicating good fit and it was concluded that the second order model adequately approximated the true surface.

For estimation of significance of the model, the Analysis of Variance (ANOVA) was applied. Using 5% significance level, a model is considered significant if the p-value is less than 0.05. The results of multiple regression analysis and Analysis of Variance test (ANOVA) are also summarized in Table 4.

Table 3: Summary of results of (a) model analysis (b) lack of fit (c) R-square analysis for measured responses

	(MD) Y_1		(% EE) Y_2	
Source	Sum of squares	p>F	Sum of squares	p>F
Model analysis				
Mean vs total	13744.05	0.0236	70022.42	0.0110
Linear vs. mean	76.16	1.0000	478.50	0.7422
2FI vs. linear	0.014	0.0013	40.77	0.0014
Quadratic vs. 2FI	61.01	0.0006	264.90	0.0073
Cubic vs. quadratic	5.71		24.65	
Residual	0.029		0.68	
Total	13886.96		70831.93	
Lack fit				
Linear	66.73	<0.0001	330.33	0.0007
2FI	66.72	<0.0001	289.56	0.0005
Quadratic	5.71	0.0006	24.65	0.0073
Cubic	0.00		0.00	
Pure error	0.028		0.68	
R-square analysis	Adjusted R-square	PRESS	Adjusted R-square	PRESS
Linear	0.4161	141.04	0.4889	658.76
2FI	0.2216	347.87	0.4024	1342.68
Quadratic	0.8996	91.42	0.9218	395.63
Cubic	0.9990		0.9958	

Table 4: Regression analysis data for measured responses

	(MD)Y_1		(EE)Y_2	
Coefficients	Full model	Reduced model	Full model	Reduced model
b_0	29.09	29.31	69.39	66.15
b_1	-0.054	-0.054	-6.93	-6.93
b_2	2.79	2.79	-3.36	-3.36
b_3	-1.32	-1.32	0.70	0.70
$b_1 b_2$	0.017	-	0.24	-
$b_2 b_3$	-0.048	-	1.28	-
$b_1 b_3$	-0.030	-	-2.92	-
b_1^2	-1.91	-	-1.69	-
b_2^2	-0.94	-	2.71	-
b_3^2	3.28	-	-7.48	-
R^2	0.9598	0.5329	0.9687	0.5911
p-value	0.0016	0.0236	0.0008	0.0110
F	15.94	4.56	20.64	5.78

Table 5: Standardized main effects of the factors on the responses and associated p-values

	(MD)Y_1			(EE)Y_2		
Factor	Coefficient estimate	p-value	SME	Coefficient estimate	p-value	SME
X_1	-0.054	0.8816	-0.15	-6.93	< 0.0001	-9.49
X_2	2.79	0.0002	7.97	-3.36	0.0036	-4.60
X_3	-1.32	0.0089	-3.77	0.70	0.3693	0.95
$X_1 X_2$	0.017	0.9726	0.03	0.24	0.8230	0.23
$X_2 X_3$	-0.048	0.9258	-0.09	1.28	0.2592	1.24
$X_1 X_3$	-0.030	0.9531	-0.06	-2.92	0.0297	-2.83
X_{12}	-1.91	0.0079	-3.89	-1.69	0.1503	-1.64
X_{22}	-0.94	0.1037	-1.91	2.71	0.0386	2.63
X_{32}	3.28	0.0005	6.69	-7.48	0.0003	-7.26

*Standardized main effects (SME) were calculated by dividing the main effect by the standard error of the main effect

$$Y_1 (MD) = 29.09 + 2.79 X_2 - 1.32 X_3 - 1.91 X^2_1 + 3.28 X^2_3 \quad (3)$$

The predictor equation generated for the mean diameter was found to be significant with an F-value of 28.84 ($p<0.0001$) and R^2 value of 0.9352: The Eq. 3 generated revealed that both factors X_2 and X_3 independently exerted a significant influence on the mean diameter. The influence of the main effects on the particle size of the liposphere was further elucidated using the response surface plot (Fig. 2).

The model generated for encapsulation efficiency was found to be significant with an F-value of 26.36 ($p<0.0001$) and R^2 value of 0.9462:

$$Y_2 (5EE) = 69.39 - 6.93 X_1 - 3.36 X_2 - 2.92 X_1 X_3 + 2.71 X^2_2 - 7.48 X^2_3 \quad (4)$$

The model (Eq. 4) indicated that both X_1 and X_2 factors studied exerted independently a significant influence on the encapsulation efficiency. The 3-D plot (Fig. 3) shows that the 3 entrapment efficiency decreased with increase in drug and Tween® 80 amount.

In Table 5, factor effects of the Box-Behnken model, associated p-values and Standardized Main Effects (SME) values for both responses are presented. A factor is considered to influence the response if the effects significantly differ from zero and the p-value is less than 0.05. Coefficient signs also give an indication of the effect produced (Table 5).

Particle size and yield: All the lipospheres prepared with in the experimental design yielded smooth spherical structures with size in the range of 23.00±0.82-34.57±1.04 μm (Fig. 1; ELS2). The yields of all trials of lipospheres were upto 85% (most of the formulations had yields of more than 65%) which reflects a good efficiency of the preparation method (Table 2).

EM and Tween® 80 at low level (X_1, -1; X_2, -1) and stirring speed at medium level (X_3, 0) yielded microspheres with highest drug entrapment (78.93±1.36%) with 23.48 μm mean diameter of lipospheres.

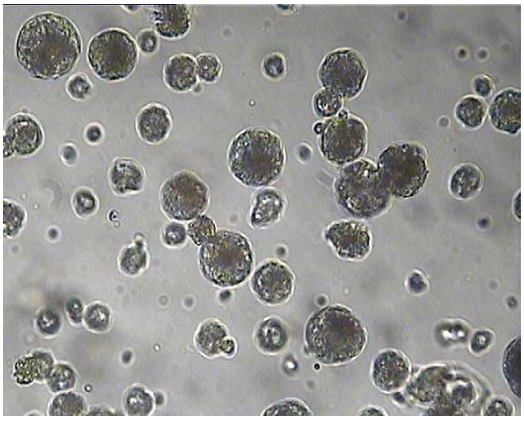

Fig. 1: Photomicrograph of EM lipospheres

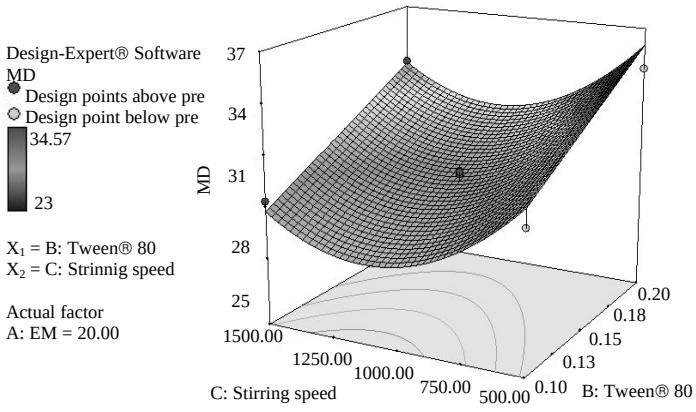

Fig. 2: 3D surface curve for the effect of selected variables (X_2, X_3) on the mean diameter of Microspheres (X_2, -1)

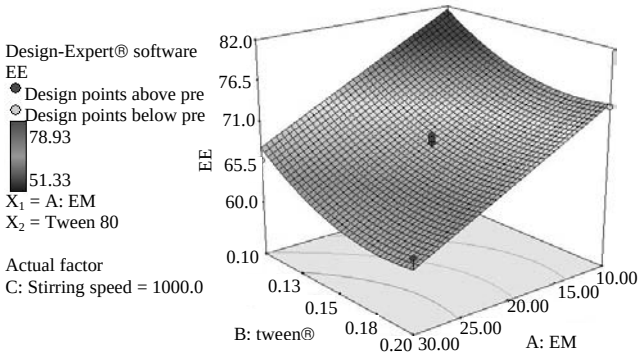

Fig. 3: 3D surface curve for the effect of selected variables (X_1, X_2) on the entrapment of Microspheres (X_1, 0)

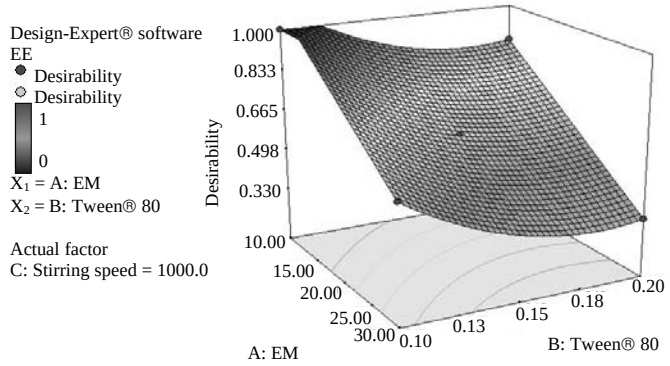

Fig. 4: 3D surface curve for the effect of selected variables (X_1, X_2) with medium level of X_3 for the desirable response in terms of maximum entrapment and diameter in optimum range

A positive sign indicates a synergistic effect while a negative sign represents an antagonistic effect of the factor on the selected response. SME values were calculated by dividing the main effects by the standard error of the main effects. The SME values in case of Y_1 response indicated that the peptide (EM) had insignificant effect on size of lipospheres whereas Tween® 80 concentration (SME = 7.97) and stirring speed (SM E= -3.77) significantly affected the size of lipospheres. In case of entrapment efficiency, factor X_1 (SME = -9.49) and X_2 (SME = -4.60) played major role with insignificant effect of factor X_3 (SME = 0.95) (Table 5). This was further investigated by the study of ANOVA. The breakup of source sum of squares (Source SS) in ANOVA indicated that the contribution of factor X_1 (EM) (SSY_2-384.48) is much higher than factor X_2 (Tween® 80) (SSY_2-90.05) and X_3 (Stirring speed) (SSY_3-3.98) for optimizing the entrapment efficiency. The contribution of factor X_2 (SSY_1-62.27) was higher on the mean diameter of lipospheres than factor X_1 (SSY_1-0.023) and X_3 (SSY_1-13.86).

Factor X_3 affected liposphere size significantly with X_2 (Tween® 80) whereas factor X_1 affected entrapment efficiency. Tween® 80 affected both size and entrapment efficiency of lipospheres. The interaction terms X_1X_2, X_2X_3, X_1X_3 and the polynomial terms X_1X_1, X_2X_2 and X_3X_3 indicated

insignificant values of individual source sum of squares. In addition, three dimensional response plots were presented to estimate the effects of the independent variables on each response by keeping one factor at constant level.

Using the model generated with both responses (Eq. 3 and 4), the optimization tool in the experimental design software was used to identify a formulation with a maximum entrapment (Fig. 4). It predicted a maximum entrapment of 80.73 and MD of 24.30 μm with a formulation comprising of 10.53 mg EM concentration, 0.1% v/v Tween® 80 and 1041.45 rpm stirring speed. To confirm the validity of the model, three batches of lipospheres were prepared using this formulation and entrapment was determined. The actual experimental entrapment obtained was 80.62±2.54%. The predicted response and residual value performed at optical values investigated in this study was found to be 80.73% and -0.11, respectively, validating the model generated in this study.

***In vitro* release study:** *In vitro* release behavior of optimized lipospheres formulation with more than 60% entrapment was investigated in phosphate buffer (pH 7.4) for duration of 12 h. Figure 5, 6 display the release profile of EM from lipospheres. In the prepared formulation, an initial burst of 20.88±1.24% was observed in the first hour due to the drug located on or near the surface of the lipospheres (Fig. 5, 6). All formulations showed an initial burst from 20.88±1.24% to 27.75±1.14% in one hour with additional 73.34±1.02% to 94.68±3.90% in next 12 h. Thus, the formulation could protect the peptide from gastric degradation and would release its drug load slowly at pH 7.4. Release of EM from lipospheres formulation in phosphate buffer (pH 7.4) was faster than that into acidic buffer (pH 1.2) reflecting differences in extent to which the peptide dissolved in the two fluids. A maximum drug release of 15.32±1.06% was observed for optimized formulation in acidic buffer (pH 1.2) after 4 h whereas in phosphate buffer an initial burst followed by sustained release of EM was observed. On the other hand, more than 85% of EM was rapidly released from these formulations within 12 h in phosphate buffer (pH-7.4) and complete release occurred in about 24 h.

Release models such as first order model, Higuchi model and Ritger-Peppas empirical model were applied to the release data (Dredan *et al.*, 1996). Results revealed that peptide was released

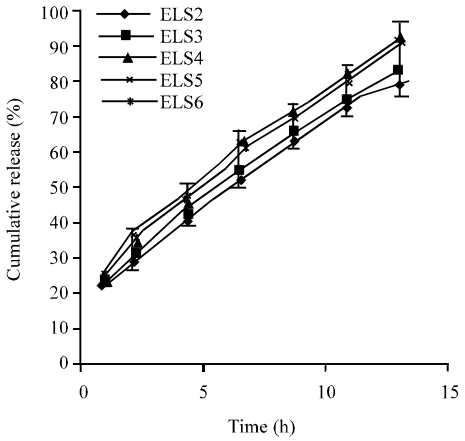

Fig. 5: *In vitro* release profiles of EM from Lipospheres (ELS2, ELS3, ELS4, ELS5 and ELS6)

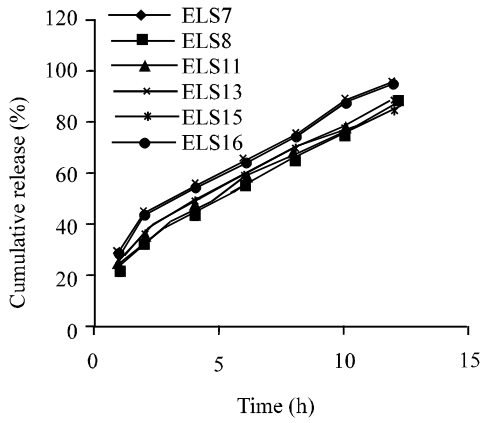

Fig. 6: *In vitro* release profiles of EM from Lipospheres (ELS7, ELS8, ELS11, ELS13, ELS15 and ELS16)

from lipospheres by a diffusion controlled mechanism following Higuchi matrix model. The value of coefficient of determination (R^2) in First order, Higuchi and Ritger-Peppas equation was found to be >0.9 which indicates the diffusion-controlled release.

***In vitro* stability:** HPLC chromatogram of free drug and drug released from lipospheres showed almost identical peaks and pattern similar to free drug indicating stability and intact nature of drug.

Storage stability: Results of stability studies showed that lipospheres lost around 2-4% of protein content in first month under room temperature and around 6-7% in 6 months. This loss was also marginal in case of accelerated conditions where system lost more than 3% drug in 1 month and around 10-12% in 6 months.

DISCUSSION

Lipid based carriers were selected to eliminate the toxic effects associated with the use of polymers as carriers (Rudra *et al.*, 2011). Melt dispersion technique is commonly used for preparation of lipospheres but w/o/w double emulsion method was considered with the aim to possibly reduce the exposure to high temperature of thermolabile compounds, such as proteins and peptides (Baimark, 2009).

When the lipid solution in methylene chloride was used, the aqueous phase coalesced rapidly, especially when the emulsion was prepared by vortex-mixing. So, stabilizer was used to improve the emulsion stability and the encapsulation efficiency in case of the w/o/w-solvent evaporation method. Tween® 20 and 80 were used as stabilizers in inner and outer aqueous phase, respectively for liposphere formation and emulsion stabilization.

Cetyl alcohol itself exhibits emulsifying capability further stabilizing the primary emulsion (Kamble *et al.*, 2004). Moreover, it also imparts sphericity with smooth surface and modifies the release of the entrapped drug. As being polar lipid, it improves the entrapment of hydrophilic drugs (Maheshwari *et al.*, 2003). The slight loss of solids could be attributed to the losses occurring during various steps of processing such as sticking of the lipid solution, adsorption on the glass wall during solidification or loss of lipospheres during the washing step etc.

The paraffin wax due to its physical properties and behaviour in the intestinal lumen was used to prepare gastro-resistant SLS formulations using the adopted technique (Shivakumar *et al.*, 2007). Since lipospheres produced with paraffin wax alone resulted in poor drug entrapment and release, efforts were made to enhance drug release from the lipospheres by incorporating a polar wax modifier like cetyl alcohol. Study citations reveal that cetyl alcohol has been successfully employed as a wax modifier to modulate drug release from wax microspheres (Maheshwari *et al.*, 2003). Tween® 80 was used to stabilize the oil in water emulsion by reducing the interfacial tension be Tween® the hydrophobic wax dispersion and the external aqueous phase, producing an emulsified oily dispersion which resulted in drug loaded lipospheres on cooling. Fatty alcohols like cetyl alcohol and stearyl alcohol have been reported to improve release and entrapment of hydrophilic peptide due to their polar hydrophilic nature (Nasr *et al.*, 2008).

On the basis of above results, factor X_1 (EM) is found to be the main influential factor on the entrapment and factor X_3 (Stirring speed) on the size of liposphere. Factor X_2 exerted combined effect on both size and entrapment efficiency of lipospheres.

Factor X_1 exerted negative influence on entrapment, also supported by the sign of coefficients in the fitted model (Eq. 4). The significant decrease in entrapment with increase in EM concentration may be because of the increase in viscosity of the inner aqueous phase. The increase in EM may improve coagulation of primary emulsion droplets by the increase in viscosity of inner water phase which will accelerate the leakage of inner to outer water phase leading to increase in size with reduced drug load (Ito *et al.*, 2007). Moreover, this effect might also be due to increase in the ratio of EM: Polymer with insufficiency of polymer to effectively coat the drug.

Tween® 80 concentration (X_2) exerted positive influence on particle size and negative effect on drug entrapment of lipospheres. As for Tween® 80, its CMC is ~0.014 mol L^{-1}. So, possible reason for decreased drug entrapment at both medium and high level might be due to formation of sphere shaped micelle at higher concentration of Tween® 80 than its Critical Micelle Concentration (CMC), whereby sphere shaped micelles are further transformed into cylinder shaped micelle structure also supported by Zhang and Zhu (2004).

Factor X_3 exerted significant effect on size of lipospheres as compared to entrapment efficiency. With the increase in stirring speed from 500 to 1000 rpm, size decreased but with further increase size increased might be due to increased surface free energy of small particles leading to aggregate formation and clumping. At both low and high level of stirring speed (X_3), lower entrapment was found and maximum entrapment was found at medium level.

The findings of release pattern are in agreement with those of Adeyeye and Price (1994) and Giannola and De Caro (1997) who reported that rapid drug release (such as phenytoin and diclofenac sodium) from fatty acid or alcohol-wax microspheres would be expected due to the hydrophilicity and leaching characteristics. Early studies reported that the drug release from matrix systems was affected by the particle size and drug: Polymer ratio (Kirn *et al.*, 1998). These are matrix systems in which the drug molecules are dispersed throughout the particles.

CONCLUSION

The optimized formulation for enalapril maleate was obtained with EM, Tween® 80 and stirring speed using response surface methodology based on a Box-Behnken design. It was found that the optimized formulation was achieved with 10.53 mg EM concentration, 0.1% v/v Tween® 80 and 1041.45 rpm stirring speed. The observed responses were close to the predicted values for the optimized formulation. Microencapsulation doesn't affect the integrity of entrapped drug as

determined by HPLC chromatograms. In conclusion, controlled release biocompatible polar lipid based oral delivery system for hydrophilic peptide was successfully developed. Further parameters can be identified by systemic approach for optimum formulation in terms of better long-term stability and to study the therapeutic effects of these particles *in vivo*.

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