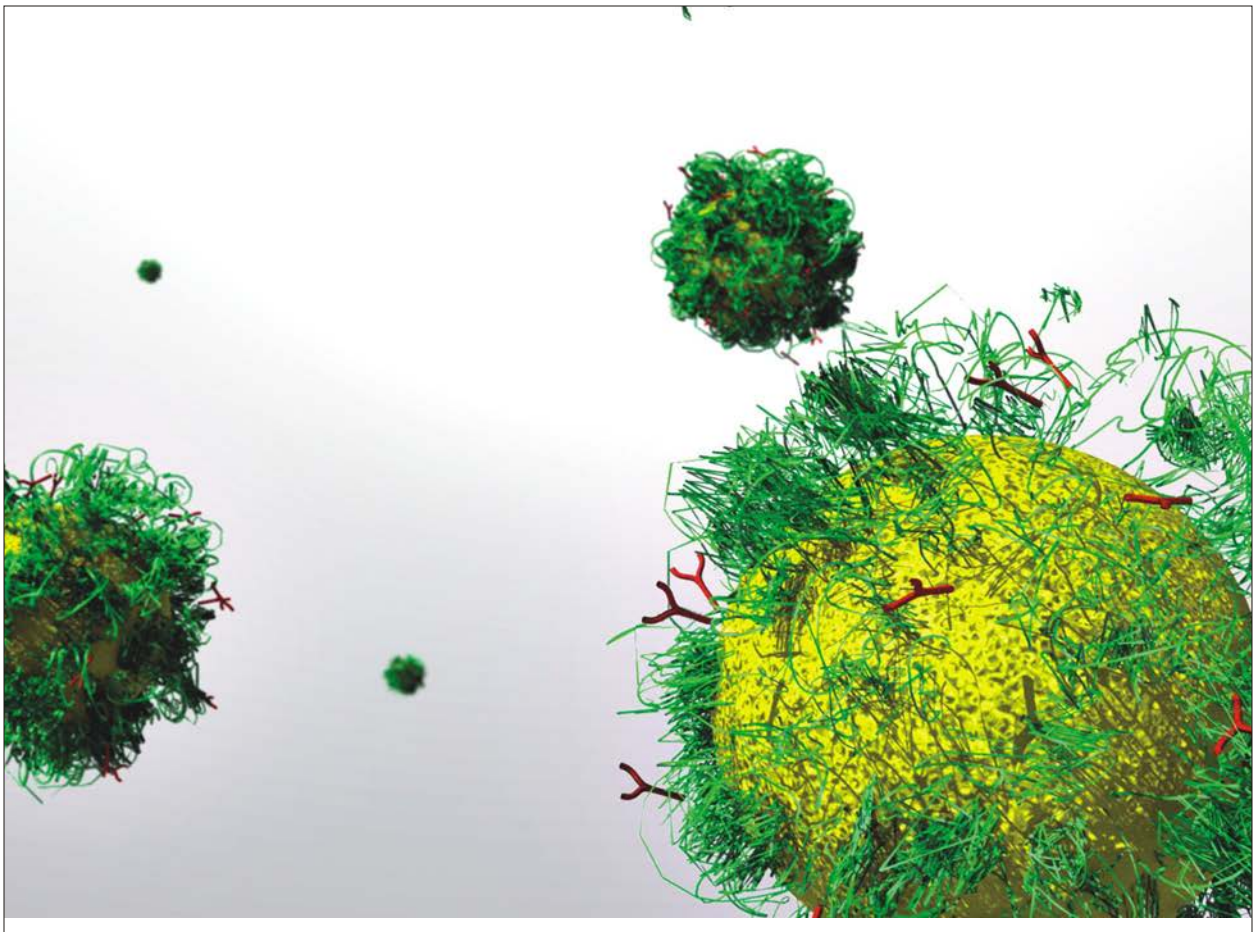

American Journal of Drug Discovery and Development

ISSN 2150-427X

Academic Journals Inc.

www.academicjournals.com

American Journal of Drug Discovery and Development 1 (3): 174-187, 2011
ISSN 2150-427x / DOI: 10.3923/ajddd.2011.174.187

Formulation Optimization of Controlled Delivery System for Antihypertensive Peptide using Response Surface Methodology

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ABSTRACT

The aim of present study was to apply experimental design methodology in the development and optimization of carrier system for highly water-soluble polypeptide in salt form. Enalapril Maleate (EM), developed for hypertension and congestive heart failure, was used as a model peptide. A three-level three-factorial Box-Behnken experimental design was used to characterize and optimize three physicochemical parameters, i.e., drug concentration (10, 20 and 30 mg), External Aqueous Phase volume (EAP) (50, 100 and 150 mL) and stabilizer (Tween 80) concentration (0.1, 0.15 and 0.2% v/v) on the entrapment and size of Eudragit S100 microspheres prepared by modified double emulsion solvent evaporation technique. The Response Surface Methodology (RSM) and multiple response optimization utilizing the polynomial equation were used to select optimal formulation with maximum entrapment and particle size in range. The maximum entrapment (80.62±1.56%) was achieved with 10 mg drug, 50 mL EAP and 0.15% v/v Tween 80 and the observed responses coincided well with the predicted values from the RSM optimization technique. The drug release from formulations showed a controlled-release pattern following Higuchi Kinetics. HPLC chromatograms of drug released from microspheres showed the intact nature of peptide. In conclusion, a novel, oral controlled-release delivery system for highly water soluble peptide was successfully developed by experimental design methodology. It is an economical way of obtaining the maximum amount of information in a short period of time and with the fewest number of experiments.

Key words: Optimization, Box-Behnken design, water-soluble polypeptide, microspheres, Eudragit S100, experimental designing

INTRODUCTION

Polymeric microspheres have widely been studied as drug carriers in the field of drug delivery system (Gombotz and Pettit, 1995; Rawat and Saraf, 2007; Surini *et al*., 2009). A variety of microencapsulation techniques are used for effective encapsulation of drugs (Benita, 1996; Rudra *et al*., 2011). Among the various techniques, the double emulsion method (w/o/w) has been widely accepted as an alternative method for the encapsulation of hydrophilic drugs (Rawat and Reader, 2009). This method involves the dispersion of an aqueous solution of bioactive molecule into an organic solution containing the polymer followed by emulsification. This primary emulsion (w/o) is then dispersed in a second aqueous phase containing a suitable emulsifier. Solid microspheres are collected following the complete removal of the volatile organic solvent. However,

it is associated with the limitation of low encapsulation of water-soluble drugs due to solubility of the drug in the two aqueous phases of the microparticles (Singh *et al.*, 2008).

Controlling both the drug-loading efficiency and particle size of drug-loaded microspheres is important to apply microspheres to Drug Delivery Systems (DDS). Therefore, process optimization 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 by altering the preparative conditions (Singh *et al.*, 2010; Brannon-Peppas and Vert, 2000).

In the present study, efforts have been made to prepare oral sustained release system for these peptides by modification in the double emulsion method. Enalapril Maleate (EM) and Eudragit S100 were used as model highly water-soluble peptide and water insoluble polymer, respectively for the preparation of microspheres using response surface methodology combined with Box-Behnken design for oral delivery. 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). It has also been reported to be effective in hepatic injury (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 (Warner and Rush, 1988; Yoo *et al.*, 1999).

Although, EM loaded delivery vehicles have been prepared for oral and parenteral delivery (Ahlin *et al.*, 2002; Yoo *et al.*, 1999) 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 Eudragit S100 microspheres.

Variables selected were EM concentration (X_1), External Aqueous Phase volume (EAP) (X_2) and Tween® 80 (X_3) concentration and the response variables were the entrapment efficiency (Y_1) and the Mean Diameter (MD) of microspheres (Y_2). The levels for these variables were determined from the preliminary trials. Furthermore, formulations were evaluated for *in-vitro* release studies and peptide stability. The object of the present study was to statistically optimize the formulation parameters of sustained-release Eudragit S100 microspheres of Enalapril maleate, a water-soluble peptide.

MATERIALS AND METHODS

Materials: Enalapril maleate (Alkem Pharma, Mumbai, India) and Eudragit S100 (Rohm Pharma, Germany), Tween® 80 and Tween® 20 were purchased from Himedia Laboratories, Mumbai, India. All other chemicals used in the study were of analytical grade.

Preparation of EM loaded microspheres: EM loaded microspheres were prepared by modified double emulsion solvent evaporation technique reported by De Rosa *et al.* (2000). Briefly, EM (10, 20 or 30 mg) was dissolved in Tween®20 (3% w/v) aqueous solutions (W1). Primary emulsion was made with 0.5 mL of Internal Aqueous Phase (IAP) into 5 mL of organic phase containing 100 mg of Eudragit S100 by mechanical stirring for 1 min (2500 rpm) (Laboratory stirrer, 1NL-2116, Remi

Motors Ltd., Mumbai, India). The temperature was maintained at 4°C using an ice bath. The organic phase consisted of Eudragit S100 in 5 mL of DCM (Dichloromethane): ethanol: IPA (Isopropyl alcohol) in a ratio of 5: 6: 4. The resulting primary emulsion (W1/O) was added drop by drop to respective volume of External Aqueous Phase (EAP) (50, 100 and 150 mL with ratio between EAP:IAP as 100:1; 200:1 or 300:1) of Tween® 80 solution (0.1; 0.15 or 0.2% v/v). Emulsification was continued by stirring at 1200 rpm for 5 min to form multiple emulsion (W1/O/W2). The resulting (W1/O/W2) emulsion was stirred at Room Temperature (RT) for 5-8 h for solvent evaporation. The collected microspheres were washed three times with demineralized water (about 500 mL) by centrifugation at 10,000 g for 10 min. The microspheres were resuspended in distilled water and lyophilized for 24 h (Heto power dry LL 3000 Lyophilizer, Denmark). The final product was stored in dessicator at 2-8°C. The full factorial 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.

Characterization of the microspheres

Particle size: Particle size analysis of peptide loaded Eudragit S100 microspheres was performed by optical microscopy using a compound microscope (Erma, Tokyo, Japan). A small amount of dry microspheres was suspended in purified water (10 mL). The suspension was ultrasonicated for 5 sec. A small drop of suspension was placed on a clean glass slide. The slide containing Eudragit S100 microspheres was mounted on the stage of the microscope and 300 particles were measured using a calibrated ocular micrometer. The process was repeated for each batch prepared.

Morphology: The surface morphology and shape of the microspheres were analyzed by scanning electron microscopy for selected batches (Leo, VP-435, Cambridge, UK). Photomicrographs were observed at 1.84 KX magnification operated with an acceleration voltage of 15 kV and working distance of 9 mm was maintained. Microspheres were mounted on the standard specimen mounting stubs and were coated with a thin layer (20 nm) of gold by sputter coater unit (VG Microtech, UK).

Table 1: Full factorial experimental design layout with coded levels and actual values of variables

F.code	X_1 EM	X_2 EAP	X_3 Tween® 80	Y_1 EE (%)	Y_2 MD (µm)	Yield (%)
EM1	30 (+1)*	100 (0)	0.2 (+1)	52.34±1.23	15.67±2.31	68.34±1.87
EM2	20 (0)	50 (-1)	0.1 (-1)	79.85±2.45	10.92±2.05	69.54±1.93
EM3	20 (0)	150 (+1)	0.2 (+1)	63.90±1.90	18.02±1.54	67.68±1.85
EM4	10 (-1)	100 (0)	0.1 (-1)	69.54±2.76	14.36±1.04	68.60±1.67
EM5	20 (0)	150 (+1)	0.1 (-1)	65.04±2.65	17.97±1.76	72.34±1.34
EM6	30 (+1)	150 (+1)	0.15 (0)	49.02±2.89	16.08±1.65	78.37±1.33
EM7	30 (+1)	100 (0)	0.1 (-1)	61.63±3.02	14.41±1.66	80.42±1.28
EM8	20 (0)	100 (0)	0.15 (0)	70.15±2.80	15.45±1.34	66.12±2.09
EM9	10 (-1)	100 (0)	0.2 (+1)	68.32±2.44	16.65±1.62	75.30±1.76
EM10	30 (+1)	50 (-1)	0.15 (0)	59.05±2.75	9.69±1.00	70.18±2.98
EM11	20 (0)	50 (-1)	0.2 (+1)	72.05±1.97	10.99±1.56	68.89±2.83
EM12	20 (0)	100 (0)	0.15 (0)	71.16±3.08	15.21±1.43	65.04±1.54
EM13	10 (-1)	50 (-1)	0.15 (0)	80.62±3.31	9.32±1.07	74.56±2.34
EM14	20 (0)	100 (0)	0.15 (0)	70.30±2.67	15.46±1.56	71.46±2.40
EM15	20 (0)	100 (0)	0.15 (0)	70.17±2.88	15.02±1.22	69.74±2.54
EM16	10 (-1)	150 (+1)	0.15 (0)	70.02±2.56	15.20±1.70	70.69±2.88

*Value in parenthesis indicates coded levels of the variables

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, 7.0.3. A design matrix comprising of 16 experimental runs was constructed. An interactive second order polynomial model was utilized to evaluate both the response variables:

$$Y_i = b_0 + b_1X_0 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_2X_3 + b_6X_1X_3 + b_7X^2_1 + b_8X^2_2 + b_9X^2_3 \quad (1)$$

where, b_0 is intercept and b_1-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.

Encapsulation efficiency: Ten milligrams of the dried microspheres were accurately weighed and added to 2 mL of DCM. 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). The calibration curve of peptide was linear (10-100 μg mL^{-1}) and content was determined using the formula:

$$y = 15.81x + 162.9 \ (R^2 + 0.0991) \quad (2)$$

Results were expressed as (Mean±SD) of 3 experiments.

The yield of microspheres thus formed was calculated:

$$(\%)\text{Yield} = \frac{\text{Practical mass of microspheres}}{\text{Theoretical mass (Polymer + drug)}} \times 100 \quad (3)$$

Encapsulation efficiency (%) was calculated using the following formula:

$$\text{Encapsulation efficiency}\ (\%) = \frac{\text{Actual EM loading}}{\text{Theoretical EM loading}} \times 100 \quad (4)$$

The measured responses are shown in Table 1.

Enteric nature of microspheres: Ten milligrams of microspheres were soaked in 5 mL of 0.1 N HCl that was equilibrated at 37±0.5°C in a water bath. After the immersion of the microspheres for 2 h, the sample was centrifuged (3000 rpm, 15 min). The EM concentration in the supernatant solution was analyzed by HPLC system (Walily *et al.*, 1995).

***In vitro* drug release:** *In vitro* release of EM from microspheres was evaluated in phosphate buffer (pH 7.4). Twenty milligrams of microspheres containing EM were transferred to the 100 mL

prewarmed dissolution media (phosphate buffer (pH 7.4) and maintained at 37±0.5°C under stirring at 50 rpm. Samples (1 mL) were withdrawn every hour up to 18 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.

RESULTS

Preliminary studies: 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 as well as the entrapment and mean diameter obtained for each factor combination. In order to determine the levels of factors which yielded maximum entrapment, mathematical relationships were generated between the dependent and independent variables.

Influence of selected formulation variables: All the microspheres prepared with in the experimental design yielded smooth spherical microspheres with size in the range of 9.32±0.24-18.02±0.49 μm (Fig. 1). The yields of all trials of microspheres were up to 50% (most of the formulations had yields of more than 50%) which reflects a good efficiency of the preparation method (Table 1).

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 7.0.3 (Table 2).

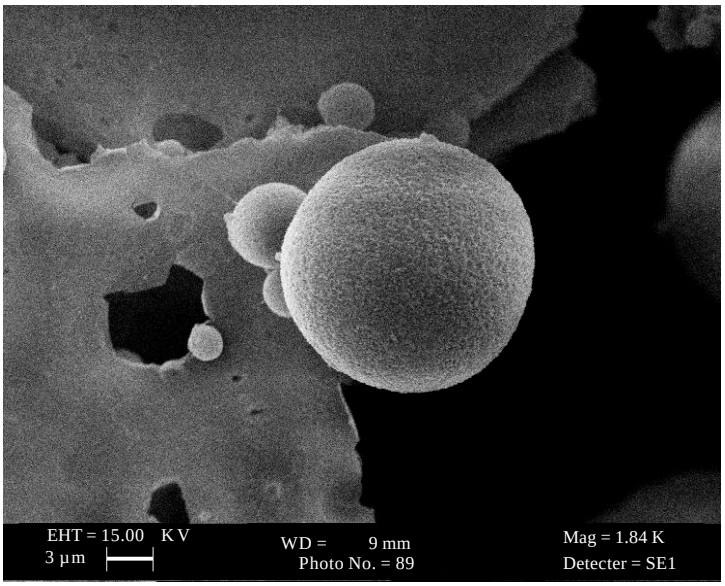

Fig. 1: Scanning electron micrograph of EM13 microspheres with maximum entrapment

Table 2: Summary of results of a) model analysis b) lack of fit c) R-square analysis for measured responses

	(% EE) Y1		(MD) Y2	
Source	Sum of squares	P>F	Sum of squares	P>F
Model analysis				
Mean vs total	71979.52	0.0004	3318.34	0.0002
Linear vs. mean	836.92	0.7747	88.49	0.9866
2FI vs. linear	27.45	0.0174	0.33	0.0034
Quadratic vs. 2FI	175.55	0.0032	19.56	0.0194
Cubic vs. quadratic residual	44.60	2.47	0.69	0.1300
Total	73064.74		3429.32	
Lack of fit				
Linear	247.60	0.0011	22.36	0.0035
2FI	220.15	0.0008	22.03	0.0020
Quadratic	44.60	0.0032	2.47	0.1940
Cubic	0.00		0.00	
Pure error	0.69		0.13	
R-square analysis	Adjusted R-square	PRESS	Adjusted R-square	PRESS
Linear	0.714	714.91	0.7466	44.41
2FI	0.6608	965.05	0.6671	102.5
Quadratic	0.8956	480.16	0.9413	39.82
Cubic	0.9968		0.9940	

As presented in Table 2, the quadratic model was selected as a suitable statistical model for optimized formulation with maximum entrapment because it had the smallest value of PRESS (480.16 for Y_1 and 39.82 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). The model showed a statistically insignificant lack of fit as shown in Table 2. From the p-values presented in Table 2, 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, the percent drug entrapment and Mean Diameter (MD) of EM microspheres showed R^2 values of 0.9583 and 0.9765 (Table 3), 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 3.

The resultant equations for both responses Y_1 and Y_2 are presented below:

$$Y_1 (\%\ EE) = 70.45 - 8.31X_1 - 5.45X_2 - 2.43X_3 + 0.14X_1X_2 + 1.66X_2X_3 - 2.02X_1X_3 - 6.51X^2_1 + 0.74X^2_2 - 0.98X^2_3 \quad (5)$$

$$Y_2 (MD) = 15.28 + 0.040X_1 + 3.29X_2 + 0.46X_3 + 0.13X_1X_2 - 5.0X_2X_3 - 0.26X_1X_3 - 0.96X^2_1 + 1.75X^2_2 - 0.95X^2_3 \quad (6)$$

EM and EAP at low level (X_1,-1; X_2,-1) and Tween® 80 at medium level (X_3, 0) yielded microspheres with highest drug entrapment (80.62%) with 9.32 μm mean diameter of microspheres.

Table 3: Regression analysis data for measured responses

	Y_1		Y_2	
Coefficients	Full model	Reduced model	Full model	Reduced model
b_0	70.45	67.07	15.28	14.40
b_1	-8.31	-8.31	0.04	0.04
b_2	-5.45	-5.45	3.29	3.29
b_3	-2.43	-2.43	0.46	0.46
b_4	0.14	-	0.13	-
b_5	1.66	-	-5.00	-
b_6	-2.02	-	-0.26	-
b_7	-6.51	-	-0.96	-
b_8	0.74	-	-1.75	-
b_9	-0.98	-	0.95	-
R^2	0.9583	0.7712	0.9765	0.7973
p-value	0.0017	0.0004	0.0003	0.0002
F	15.30	13.480	27.700	15.730

Table 4: Standardized main effects of the factors on the responses and associated p-values

	Y_1			Y_2		
Factor	Coefficient estimate	p-value	SME*	Coefficient estimate	p-value	SME
X_1	-8.31	0.0001	-8.567	0.04	0.8694	0.173
X_2	-5.45	0.0014	-5.618	3.29	<0.0001	14.30
X_3	-2.43	0.0464	-2.505	0.46	0.0966	2.00
$X_1 X_2$	0.14	0.9208	0.102	0.13	0.7123	0.3939
$X_2 X_3$	1.66	0.2711	1.211	-5.00	0.9884	-15.15
$X_1 X_3$	-2.02	0.1924	-1.474	-0.26	0.4644	-0.7878
X_1^2	-6.51	0.0032	-4.751	-0.96	0.0272	-2.909
X_2^2	0.74	0.6083	0.540	-1.75	0.0018	-5.303
X_3^2	-0.98	0.5035	-0.715	0.95	0.0285	2.878

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

In Table 4, 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 4).

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 large negative SME (8.567 for Y_1) of EM (X_1) for Y_1 response indicated that the drug concentration was the main influential factor on the entrapment efficiency of microspheres, whereas for mean diameter of microspheres, EAP (X_2) was the main influential factor demonstrated by large SME value (14.30 for Y_2).

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) (SSY1 - 552.12) is much higher than factor X_2 (EAP) (SSY_1-237.51) and X_3 (Tween® 80) (SSY_1-47.29) for optimizing the drug entrapment. The contribution of factor X_2 (SSY2 -86.79) was higher on the mean diameter of microspheres than factor X_1 (SSY2 -0.013) and X_3 (SSY2 -1.68).

Factor X_3 affected entrapment efficiency as well as microsphere size but not so significantly as other selected variables. 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 (Fig. 2-5).

On the basis of above results, factor X_1 (EM) is found to be the main influential factor on the entrapment and factor X_2 (EAP) on microsphere size.

Using the model generated with both responses (Eq. 5 and 6), the optimization tool in the experimental design software was used to identify a formulation with a maximum entrapment.

It predicted a maximum entrapment of 80.65 and MD of 13.57 μm with a formulation comprising of 13.57 mg EM concentration, 50 mL EAP volume and 0.12% v/v Tween® 80 concentration. To confirm the validity of the model, three batches of microspheres 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 80.65% and -0.046, respectively, validating the model generated in this study.

Enteric nature of microspheres: Enteric nature of the coatings for prepared formulations was evaluated. Absolute enteric coating could not be achieved as 6-7% of the peptide was released in 0.1N HCl medium (pH 1.2) in two hours. This could be attributable to the adsorption of EM on the surface of microspheres.

***In vitro* release studies:** *In vitro* release behavior of microspheres was investigated in phosphate buffer (pH 7.4) for duration of 18 h. Figure 6 and 7 display the release profile of EM from Eudragit S100 microspheres. All the microspheres exhibited almost similar release profile of initial burst followed by sustained release of EM (Parashar *et al.*, 2010).

Minimum initial burst was observed in EM13 microspheres with 22.21±2.46% release in the first hour, due to low EAP and drug concentration.

The release rates were analyzed by least square linear regression method. Release models such as first order model, Higuchi model and Ritger-Peppas empirical model were applied to the release data (Table 5) (Dredan *et al.*, 1996; Peppas, 1985). Microspheres are matrix systems in which the drug molecules are dispersed throughout the particles. Results revealed that peptide was released from microspheres by a diffusion controlled mechanism. The release of drug from these microspheres followed Higuchi matrix model indicating diffusion release pattern supporting integrity of the system. 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 (Table 5).

Substituting the release values in the Ritger-Peppas equation, the value of coefficient of determination was about 0.9 in each case. But the value of n obtained (0.352 to 0.458) was confusing as value of n = 0.43 indicates Fickian (case I) release; >0.43 but <0.85 for non-Fickian (anomalous) release and >0.89 indicates super case II type of release. Case II generally refers to the erosion of the polymeric chain and anomalous transport (non-Fickian) refers to a combination of both diffusion and erosion controlled drug release (Rawat *et al.*, 2008). The results showed that the Ritger-Peppas model was not suitable for estimating the release kinetics might be due to the immediate release of EM during first two hours.

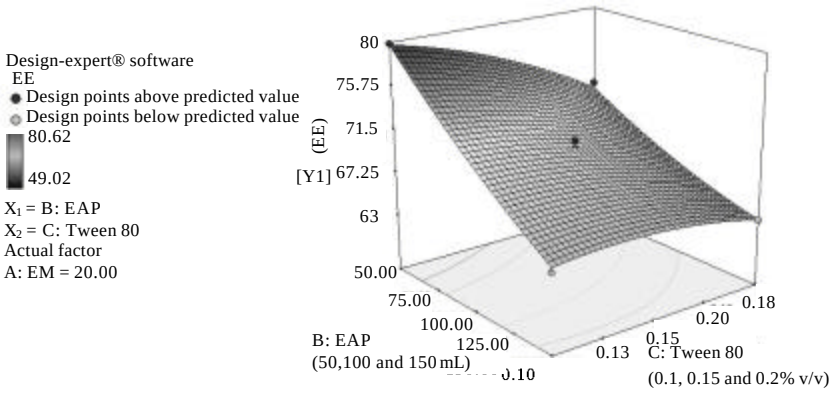

Fig. 2: 3D surface curve for the effect of selected variables (X_2, X_3) on the entrapment of Microspheres (X_1, 0)

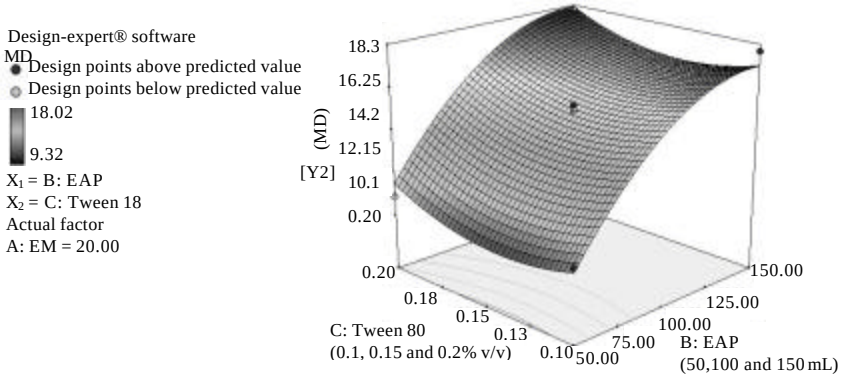

Fig. 3: 3D surface curve for the effect of selected variables (X_2, X_3) on the MD of Microspheres (X_1, 0)

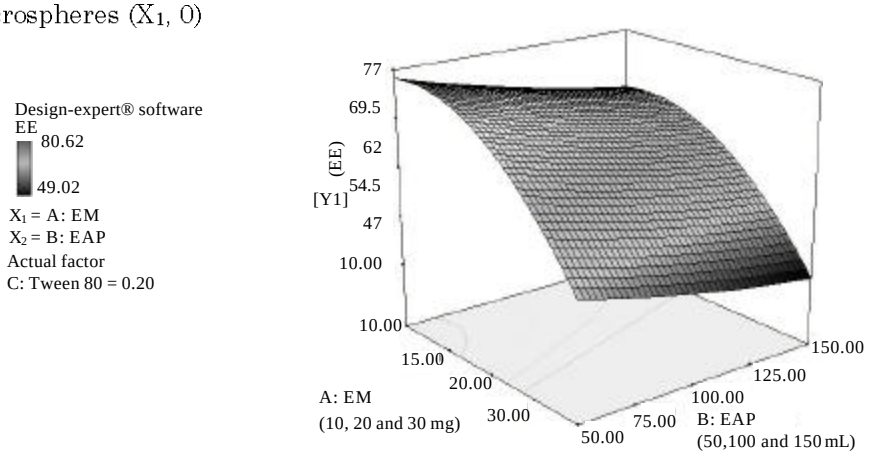

Fig. 4: 3D surface curve for the effect of selected variables (X_1, X_2) on the entrapment of Microspheres (X_3, +1)

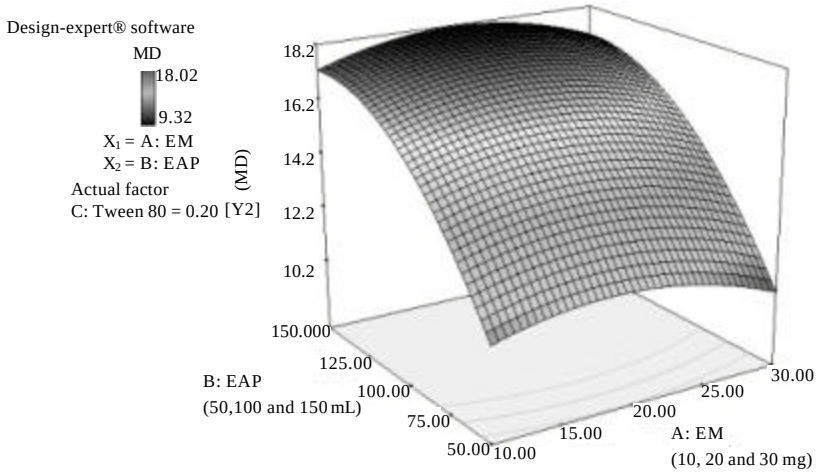

Fig. 5: 3D surface curve for the effect of selected variables (X_1, X_2) on the MD of Microspheres (X_3, +1)

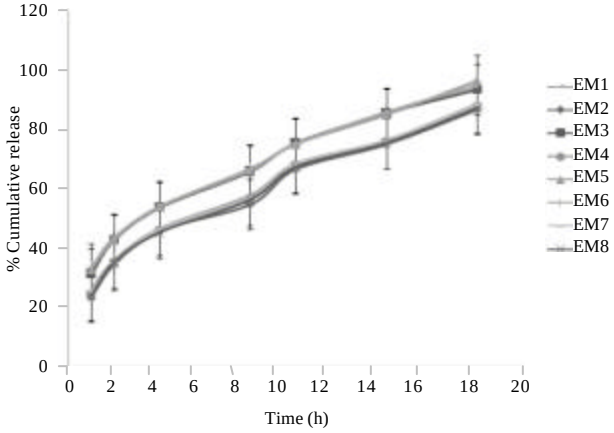

Fig. 6: *In vitro* release profiles of EM from Eudragit S100 microspheres (EM1-EM8)

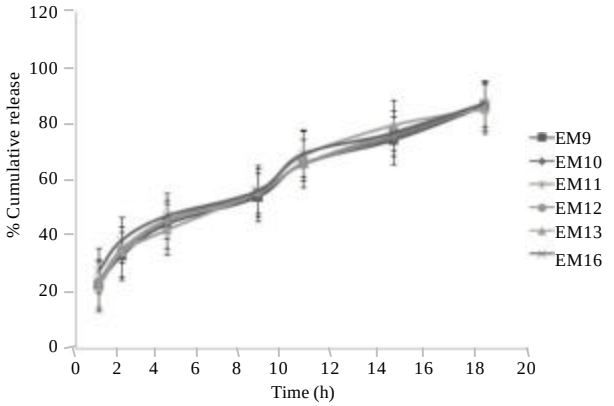

Fig. 7: *In vitro* release profiles of EM from Eudragit S100 microspheres (EM9-EM16)

Table 5: Release behavior of EM in phosphate buffer (pH 7.4)

Formulation code	First order		Higuchi		Ritger-Peppas	
	k	R^2	K	R^2	n	R^2
EM1	0.101	0.975	18.64	0.993	0.410	0.994
EM2	0.094	0.973	18.59	0.989	0.429	0.989
EM3	0.128	0.976	18.85	0.995	0.370	0.996
EM4	0.099	0.972	18.72	0.992	0.422	0.993
EM5	0.151	0.916	18.98	0.996	0.362	0.995
EM6	0.138	0.959	18.56	0.997	0.352	0.966
EM7	0.099	0.971	18.69	0.993	0.416	0.994
EM8	0.096	0.975	18.69	0.992	0.426	0.992
EM9	0.094	0.973	18.78	0.990	0.438	0.991
EM10	0.094	0.972	18.50	0.990	0.425	0.990
EM11	0.096	0.977	18.70	0.992	0.430	0.991
EM12	0.096	0.978	18.69	0.991	0.424	0.990
EM13	0.099	0.990	19.66	0.989	0.458	0.989
EM16	0.094	0.973	17.67	0.985	0.378	0.985

***In vitro* stability studies:** HPLC chromatogram of free drug and drug released from optimized microspheres showed almost identical peaks and pattern similar to free drug indicating stability and intact nature of peptide (Reithmeier *et al*., 2001; Al-Omari *et al*., 2001).

DISCUSSION

Rapid diffusion of solvents into the continuous aqueous phase is responsible for complete precipitation of the polymer present in the microdroplets. The process of diffusion is dependent on the solubility of the organic solvent in the aqueous phase. The droplets may remain in liquid state if the solvent has very low aqueous solubility and this condition results in greater leaching of water-soluble drug in the outer aqueous phase. The solubility of the dichloromethane is 2% w/w that is reported to be significant for rapid diffusion of solvent and better polymer precipitation (Yamakawa *et al*., 1992). Mixture of solvents was selected instead of single solvent, as a part of the DCM could also move with isopropyl alcohol and ethanol into the outer aqueous phase, contributing to faster precipitation of polymer and greater drug loading (Sah, 1997). The rapid diffusion of isopropyl alcohol into the aqueous phase causes a remarkable decrease in interfacial tension between organic and aqueous phase and hence finer microspheres are obtained (Niwa *et al*., 1994). Eudragit S100 dissolved completely at selected level of organic solvents.

At least one stabilizer is necessary for the microsphere formation and suspension stabilization. Tween® 80 was used as emulsifier due to its better emulsification capability. Limit for Tween® 80 solutions was selected in the range of 0.1-0.2% v/v through preliminary trials.

Factor X_1 exerted negative influence on entrapment and positive on particle size, also supported by the sign of coefficients in the fitted model Eq. 5 and 6. 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 a 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; Denkbas *et al*., 1999). Moreover, this effect might also be due to increase in the ratio of EM: Polymer with insufficiency of polymer to effectively coat the drug.

EAP concentration (X_2) also exerted negative influence on drug entrapment and positive on particle size of microspheres. This might be due to drug leakage in the large volume of continuous aqueous phase with increasing EAP leading to increase in size of outer droplet and decrease in entrapment (Jain *et al.*, 2005).

Factor X_3 also exerted the similar mixed but not so significant effect. Its influence was found to be more on entrapment efficiency as compared to size of microspheres. At both low and high level of Tween® 80 (X_3), lower entrapment was found and maximum entrapment was found at medium level. The viscosity of 0.1, 0.15 and 0.2% v/v Tween® 80 were found to be 1.30, 1.32 and 1.34 mPa, respectively (Brookefield viscometer, LVDV-I+, USA). So, possible reason for decreased drug loading at low level is decreased viscosity and at higher-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). As for Tween® 80, its CMC is ~0.014 mol L^{-1}.

Early studies reported that the drug release from polymeric microspheres was affected by the particle size and drug: polymer ratio (Sanders *et al.*, 1986; Sung *et al.*, 1998; Kirn *et al.*, 1998). In the present investigation, *in vitro* drug release increased with the increase in EM concentration and EAP volume.

HPLC chromatograms showed identical peaks for free drug and drug released from optimized microspheres indicating stability and intact nature of peptide.

CONCLUSION

The optimized formulation was found with 13.57 mg EM concentration, 50 mL EAP and 0.12% v/v Tween® 80 concentration and 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 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*.

ACKNOWLEDGMENTS

The authors are thankful to M/s Alkem Pharma Mumbai, India, for the gift sample of Enalapril maleate; SIF, AIIMS, New Delhi, India, for Scanning electron micrography and Director, University Institute of Pharmacy, Pt. Ravishankar Shukla University, Raipur (C.G.) India for providing all necessary facilities for carrying out this work and Chhattisgarh council of science and technology for financial assistance (CGCOST/MRP/650).

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