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Development of Delivery Cargoes for Debriding Enzymes Effective in Wound Healing

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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 Serratiopeptidase by modified double emulsion solvent evaporation technique. A three-level three-factorial Box-Behnken experimental design was used to characterize and optimize three physicochemical parameters-Eudragit RS100 concentration, external aqueous phase volume and stirring speed of primary emulsion on the entrapment and size of microspheres. 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±1.96%) was achieved with 300 mg Eudragit RS100, 100 mL EAP and 2000 rpm as stirring speed and the observed responses coincided well with the predicted values from the RSM optimization technique. *In vitro* proteolytic activity confirmed the bioactivity of peptide after microencapsulation. The drug release from formulations showed a similar sustained release showing an initial burst followed by diffusion. In conclusion, a novel, controlled-release delivery system for peptide drug was successfully developed by experimental design methodology with the fewest number of experiments.

Key words: Optimization, box-behnken design, eudragit RS100, microspheres, serratiopeptidase

INTRODUCTION

Wound is one of the oldest suffering associated with the mankind and its history is as old as humanity. Chronic wounds generate tremendous physical, psychological and financial burdens for the patient, family and health care community (Martin, 1997). Wound healing is an intricate, biological progression involving contraction and closure of wound and restoration of a functional barrier (Singer and Clark, 1999). Wound healing becomes more difficult in case of delayed wound as local or systemic antibiotics offer little therapeutic benefit due to extravasation of fibrous material into the wound site (Guo and Dipietro, 2010). Thus, the first goal of wound care is debridement as necrotic tissue can be life threatening. It also helps in the cleaning of dead and senescent cells that hinder the path of healing. Among various techniques, enzymatic hydrolysis by proteases is the most efficient, selective and least traumatic means of dissolving this coagulum (Yaakobi *et al*., 2004). Being protein in nature, these bioactive agents exhibit increased biochemical and structural complexity. Moreover, repeated and high dose administration of these enzymes causes toxicity, limiting their therapeutic potential. This necessitates effective formulation design for their controlled delivery with effective tissue repair and treatment of pain and inflammation.

Wound healing process relies essentially on an inflammatory reaction involving overlapping phases of inflammation, proliferation and remodeling (Martin, 1997). Among this category, Serratiopeptidase (STP) offers a powerful treatment for pain and inflammation with widespread use in wound debridement, arthritis, fibrocystic breast disease, chronic bronchitis, sinusitis, atherosclerosis and carpal tunnel syndrome (Kee *et al*., 1989; Majima *et al*., 1990). However, oral bioavailability of these peptide drugs is generally very low, owing to the acidic conditions of the stomach and poor permeability across intestinal mucosa (Rawat *et al*., 2007). Alternative routes like topical, nasal and parenteral could be exploited for maintaining its therapeutic effectiveness.

There is an increased surge of interest in polymeric microspheres and microcapsules for the delivery of therapeutically useful proteins in a controlled way (Gombotz and Pettit, 1995; Rudra *et al*., 2011). A variety of microencapsulation techniques are used for effective encapsulation of drugs (Tice and Gilley, 1985; Benita, 1996). Among the various microencapsulation techniques, the double emulsion method (w/o/w) has been widely accepted as an alternative method for the encapsulation of hydrophilic and labile drugs (Ogawa *et al*., 1988). But, it limits encapsulation of water-soluble drugs due to solubility of the drug in the two aqueous phases of the microparticles. However, the encapsulation efficiency 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 (Brannon-Peppas and Vert, 2000). Statistical models are extensively used nowadays in diversified areas to strengthen the art of drug formulation. 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 (Gupta *et al*., 2001; Ko *et al*., 2003). Therefore, process optimization may be advantageous for the efficient entrapment of water-soluble labile drugs like therapeutic enzymes (Rawat *et al*., 2007).

In the present study, topical microparticulate system for controlled release of debriding agent in the alkaline media was developed as open wounds tend to have a neutral or alkaline pH, predominantly in the range of 6.5-8.5.

MATERIALS AND METHODS

Materials: Serratiopeptidase (MW 52 kDa; Advanced Enzyme Technologies Ltd. Nasik, India) and Eudragit RS100 (Rohm Pharma, Germany). PVA of molecular weight 30,000 was from Loba chemicals, Mumbai, India. All other chemicals used in the study were of analytical grade.

Preparation of STP loaded eudragit RS100 microspheres: STP loaded eudragit RS100 microspheres were prepared by modified double emulsion solvent evaporation technique (Blanco-Prieto *et al*., 1996). Briefly, STP was dissolved in 1ml of a PVA 0.5% aqueous solution (W_1) and Eudragit RS100 (X_1) with drug: Polymer ratio of 1:1; 1:3; 1:6 was dissolved in 5 mL of dichloromethane (DCM) (O). Both phases were mixed by mechanical stirring for 1 min (1000, 1500 or 2000 rpm) (X_3) to form a primary emulsion. This inner emulsion was then poured under vigorous stirring to External Aqueous Phase (EAP) (X_2) containing 1% w/v of PVA, 20% v/v Glycerol and 6% w/v NaCl using a magnetic stirrer for 2 min (Singh *et al*., 2008). The resulting double emulsion

Table 1: Full factorial experimental design layout with coded levels and actual values of variables for STP loaded eudragit RS100 MS (SE1-SE16)

Formulation code	X_1 Polymer (mg)	X_2 EAP (mL)	X_3 Stirring speed (rpm)
SE1	300 (0)*	100 (-1)	1000 (-1)
SE2	100 (-1)	200 (0)	1000 (-1)
SE3	600 (+1)	200 (0)	1000 (-1)
SE4	300 (0)	300 (+1)	1000 (-1)
SE5	100 (-1)	100 (-1)	1500 (0)
SE6	600 (+1)	100 (-1)	1500 (0)
SE7	300 (0)	200 (0)	1500 (0)
SE8	300 (0)	200 (0)	1500 (0)
SE9	300 (0)	200 (0)	1500 (0)
SE10	300 (0)	200 (0)	1500 (0)
SE11	100 (-1)	300 (+1)	1500 (0)
SE12	600 (+1)	300 (+1)	1500 (0)
SE13	300 (0)	100 (-1)	2000 (+1)
SE14	100 (-1)	200(0)	2000 (+1)
SE15	600 (+1)	200 (0)	2000 (+1)
SE16	300 (0)	300 (+1)	2000 (+1)

*Value in parenthesis indicate coded levels

was stirred for at least 3 h under Room Temperature (RT) to allow solvent evaporation and microspheres formation. After preparation, the microspheres were isolated by centrifugation 7000×g for 10 min, washed with distilled water and freeze-dried. The trials were performed in random order. The full factorial design layout is given in Table 1.

Particle size: The particle size was measured directly by optical microscopy using a compound microscope (Erma, Tokyo, Japan) on 300 microspheres (Qian *et al*., 2004). 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 RS100 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 morphology and surface appearance of microspheres were examined by Scanning Electron Microscopy (SEM) (Leo, VP-435, Cambridge, UK). Photomicrographs were observed at 300x magnification operated with an acceleration voltage of 15 kV and working distance of 19 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).

Entrapment efficiency: Twenty milligrams of the dried microspheres were accurately weighed and dissolved in DCM. After the microspheres dissolved completely, 5 mL of phosphate buffer (pH 7.4) was added to this solution and mixed thoroughly. The resulting solution was filtered using whattman filter (0.45 μm pore size) and analyzed for STP content by measuring absorbance in UV-spectrophotometer (Shimadzu UV-1700, Pharmaspec, Tokyo, Japan) at 229.5 nm by first derivative spectrophotometric method using phosphate buffer (pH 7.4) and DCM mixture (1:1) as blank (Saudagar *et al*., 2007). Results were expressed as (Mean±SD) of 3 experiments. Encapsulation efficiency (%) was calculated using the following formula:

$$\text{Encaspsulation efficiency}(\%) = \frac{\text{Actual STP loading}}{\text{Theoretical STP loading}} \times 100 \quad (1)$$

Box-Behnken design: A Box-Behnken experimental design was employed to statistically optimize the formulation parameters of Eudragit RS100 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:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1^2 + b_5X_2^2 + b_6X_3^2 + b_7X_1X_2 + b_8X_1X_3 + b_9X_2X_3 \quad (2)$$

where, b_0-b_9 are the regression coefficients, X_1 - X_3 the factors studied and Y_i is the measured response associated with each factor level combination.

***In vitro* drug release:** Microsphere formulations exhibiting more than 70% entrapment were subjected to *in vitro* release studies due to the need of prolonged drug action. Weighed quantities of microspheres were suspended in 50 mL of isotonic phosphate buffer (pH 7.4, 37±0.5°C). The dissolution medium was agitated at 50 rpm and maintained at a constant temperature of 37±0.5°C in a water bath. Samples were periodically removed at predetermined time intervals and the volume was replaced immediately by fresh phosphate buffer. The samples withdrawn were centrifuged (3000 rpm, 15 min, at room temperature). The supernatant was analyzed for STP content using UV-Vis spectrophotometer (Shimadzu UV-1700, Pharmaspec, Tokyo, Japan) at 229.5 nm by first derivative spectrophotometric method using phosphate buffer (pH 7.4) as blank (Saudagar *et al*., 2007). Results were expressed as (Mean±SD) of 3 experiments.

***In vitro* proteolytic activity:** Prepared STP loaded microspheres were placed in 5 mL of phosphate buffer saline (PBS, pH 7.4) separately maintained at 37±0.5°C and stirred constantly at 100 rpm. After two hrs, samples were recovered by centrifugation at 3000 rpm for 15 min at room temperature (n = 3). The proteolytic activity was determined as per the method reported in Food and Chemical Codex (2003). The assay was based on a 30 min proteolytic hydrolysis of casein at 37°C and pH 7.0. Unhydrolyzed casein was removed by filtration and the solubilized casein was determined spectrophotometrically at wavelength of 275 nm. In this method, the protease activity is expressed as PC units of preparation derived from *Bacillus subtilis* var. and *Bacillus licheniformis* var. One bacterial protease unit (PC) is defined as quantity of enzyme that produces 1.5 μg mL^{-1} equivalent of L-tyrosine per minute under the condition of the assay. Activity of enzyme was calculated by equation:

$$\frac{PC}{g} = \left(\frac{A_u}{A_s}\right)\left(\frac{1.5}{30w}\right) \quad (3)$$

where, A_u is the value obtained by subtracting blank reading from test reading, A_s absorption of standard solution, 1.5 is the final volume in mL of reaction mixture, 30 is the time of the reaction in minutes and w is the weight of the original sample in 'g'.

RESULTS

Preliminary studies: A total of 16 experiments (SE1-SE16) 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: The effect of formulation variables on yield, mean diameter and encapsulation efficiency of STP loaded Eudragit RS100 microspheres (SE) are shown in Table 2. Microspheres with smooth surface and spherical morphology were obtained for all batches of formulation (SE1-SE16) (Fig. 1). Microsphere yield and entrapment efficiency was relatively low with emulsifier alone in the EAP. This could be due to rapid diffusion of hydrophilic drug into the continuous aqueous phase leading to decreased entrapment and rapid loss of drug. So, 20% v/v Glycerol concentration and NaCl concentration of 6% w/v were selected from our previous studies (Singh *et al.*, 2008).

Further entrapment and release was controlled by optimizing other parameters like polymer concentration (X_1), external aqueous phase volume (X_2) and stirring speed of primary emulsion (X_3). All the trials of microspheres yielded smooth spherical microspheres with size in the range of 18.65±0.84 to 39.44±0.65 μm (Table 2). %Yield of microspheres obtained was more than 50% in almost all microspheres. Polymer concentration influenced the yield of microspheres.

In order to determine the levels of factors which yielded maximum entrapment, mathematical relationships were generated between the dependent and independent variables. For estimation of coefficients in the approximating polynomial function (Eq. 2) 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

Table 2: Experimental responses obtained for the studied parameters

Formulation code	%Yield	MD (μm)	E.E (%)
SE1	66.96±2.67	29.50±1.56	70.21±1.46
SE2	55.12±1.32	20.46±2.73	63.64±2.98
SE3	687.70±1.54	39.44±0.65	64.70±2.24
SE4	65.66±1.35	36.84±2.54	45.08±0.52
SE5	46.42±2.53	18.65±0.84	75.86±2.02
SE6	74.01±3.57	33.86±0.65	74.68±3.16
SE7	67.12±3.14	32.98±1.39	70.02±1.85
SE8	67.24±0.16	32.84±1.80	69.98±2.08
SE9	66.99±2.62	32.49±1.86	69.89±2.42
SE10	67.22±1.28	30.80±1.88	69.95±2.40
SE11	49.03±1.02	20.64±1.50	60.34±1.75
SE12	72.15±1.54	37.79±2.00	61.00±2.04
SE13	70.42±2.14	26.05±0.42	80.12±1.96
SE14	53.87±1.93	20.53±1.08	73.18±1.64
SE15	68.52±1.47	36.06±1.43	73.87±2.50
SE16	58.66±1.99	34.92±0.46	58.53±1.32

*Values are shown as representative of Mean±SD for three independent determinations ($p<0.05$)

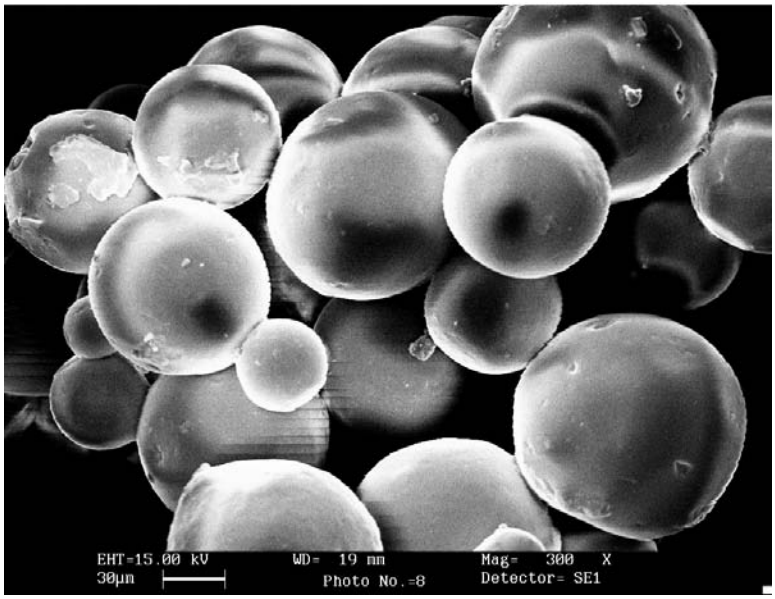

Fig. 1: Scanning electron micrograph of STP microspheres (SE13) with smooth surface

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

	(Mean diameter) Y_1		(% Entrapment efficiency) Y_2	
Source	Sum of squares	P>F	Sum of squares	P>F
Model analysis				
Mean vs total	14631.93	73041.82		
Linear vs. mean	572.14	0.0001	942.53	<0.0001
2FI vs. linear	3.37	0.9713	6.47	0.9314
Quadratic vs. 2FI	114.23	0.0056	96.14	0.0472
Cubic vs. quadratic	15.48	0.1071	39.26	<0.0001
Residual	3.04		9.00	
Total	15340.18		74126.23	
Lack fit				
Linear	133.07	0.0247	141.87	<0.0001
2FI	129.70	0.0147	135.40	0.0002
Quadratic	15.48	0.1071	39.26	<0.0001
Cubic	0.000		0.000	
Pure error	3.04		9.000	
R-square analysis	Adjusted R-square	PRESS	Adjusted R-square	PRESS
Linear	0.7598	250.42	0.8365	279.16
2FI	0.6876	541.27	0.7919	619.89
Quadratic	0.9346	251.48	0.9095	622.83
Cubic	0.9786		1.0000	

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.

As presented in Table 3, the linear model was selected as a suitable statistical model for optimized formulation with maximum entrapment and optimum size because it had the smallest value of PRESS (250.42 for Y_1 and 279.16 for Y_2) signifying role of single factor. PRESS is a measure of the fit of the model to the points in the design. The smaller the PRESS statistic is, the better the model fits to the data points. 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.

The Mean Diameter (MD) and percent drug entrapment of STP microspheres showed R^2 values of 0.9876 and 0.9638 (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.

The resultant equations for both responses Y_1 and Y_2 (fitted model) are presented below:

$$Y_1(MD) = 34.17 + 8.36X_1 + 2.79X_2 - 5.52X_1^2 \quad (4)$$

$$Y_2(\%EE) = 69.92 - 9.40X_2 + 5.24X_3 - 3.68X_2^2 \quad (5)$$

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). A positive sign indicates a synergistic effect, while a negative sign represents an antagonistic effect of the factor on the selected response. Signs indicate the significant positive effect of Eudragit RS100 (X_1) on size of microspheres, EAP exerted negative effect on entrapment efficiency. The large SME of Eudragit RS100 (X_1) for mean diameter indicated that the polymer concentration was the main influential factor on the size of microspheres whereas EAP showed large negative SME of entrapment efficiency indicating negative effect of EAP on entrapment. This

Table 4: Regression analysis data for measured responses

	Y_1 (MD)		Y_2 (%EE)	
Coefficients	Full model	Reduced model	Full model	Reduced model
b_0	34.17	31.31	69.92	67.60
b_1	8.36	7.78	0.15	0.32
b_2	2.79	2.19	-9.40	-9.49
b_3	-1.16	-0.51	5.24	5.26
$b_1 b_2$	0.38	-	0.88	-
$b_2 b_3$	-0.80	-	0.88	-
$b_1 b_3$	-0.91	-	-0.20	-
b_1^2	-5.52	-	1.72	-
b_2^2	-0.92	-	-3.68	-
b_3^2	0.47	-	-2.80	-
R^2	0.9876	0.7754	0.9638	0.8692
p-value	0.0004	0.0003	0.0012	< 0.0001
F	24.83	13.81	17.74	26.57

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

	Y_1 (MD)			Y_2 (%EE)		
Factor	Coefficient estimate	p-value	SME[a]	Coefficient estimate	p-value	SME
X_1	8.36	<0.0001	13.450	0.15	0.8706	0.1690
X_2	2.79	0.0043	4.440	-9.40	<0.0001	-10.2900
X_3	-1.16	0.1127	-1.850	5.24	0.0012	5.7300
$X_1 X_2$	0.38	0.8060	0.256	0.88	0.5130	0.6950
$X_2 X_3$	-0.80	0.6784	0.435	0.88	0.5149	0.6918
$X_1 X_3$	-0.91	0.3958	-0.914	-0.20	0.8769	-0.1615
X_1^2	-5.52	0.0010	-5.969	1.72	0.2473	1.2814
X_2^2	-0.92	0.3359	-1.046	-3.68	0.0283	-2.8738
X_3^2	0.47	0.6127	0.533	-2.80	0.0713	-2.1879

[a]Standardized main effects (SME) were calculated by dividing the main effect by the standard error of the main effect

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 (Eudragit RS100) (SSY_1-558.95; SSY_2-0.19) is much higher than factor X_2 (EAP) (SSY_1-61.01; SSY_2-693.58) and X_3 (stirring speed of primary emulsion) (SSY_1-10.64; SSY_2-215.30) for optimizing the mean diameter of microspheres whereas EAP and stirring speed contributed significantly for entrapment.

Polymer at medium level (X_1, 0), EAP at low level (X_2, -1) and stirring speed of primary emulsion at high level (X_3,+1) yielded microspheres with highest drug entrapment 80.12±1.96% with 26.05±0.42 μm mean diameter of microspheres. In Table 5, factor effects of the Box-Behnken model associated p-values and Standardized Main Effects (SME) values for both responses are presented.

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 entrapment efficiency by keeping one factor at constant level (Fig. 2-4).

Using the model generated with both responses (Eq. 4 and 5), the optimization tool in the experimental design software was used to identify a formulation with a maximum entrapment. It predicted a maximum entrapment of 80.12±1.96% and MD 26.05±0.42 μm with a formulation comprising of 300 mg Eudragit RS100 concentration, 100 mL EAP with 2000 rpm as stirring speed of primary emulsion (SE13).

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 80.12±1.96%. The predicted response and residual value performed at optical values investigated in this study was found to be 77.46% and 2.66 respectively, validating the model generated in this study.

***In vitro* release study:** *In vitro* release behavior of microspheres exhibiting entrapment more than 70% (SE1, SE5, SE6, SE7, SE13, SE14 and SE15) was investigated in phosphate buffer (pH 7.4) for 5 days (Fig. 5). All formulations exhibited almost similar release pattern with initial burst followed by nearly sustained release for 5 days. Variation was in terms of initial burst and found to be dependent on polymer concentration. Polymer concentration exhibited negative effect whereas EAP showed positive effect on initial burst.

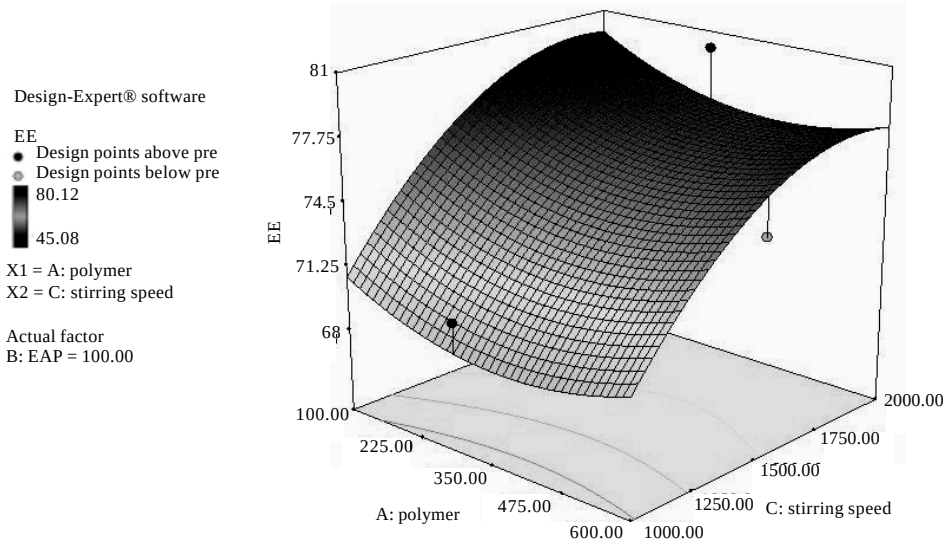

Fig. 2: 3D surface curve for the effect of selected variables (X_1, X_3) on the entrapment of Microspheres (X_2, -1)

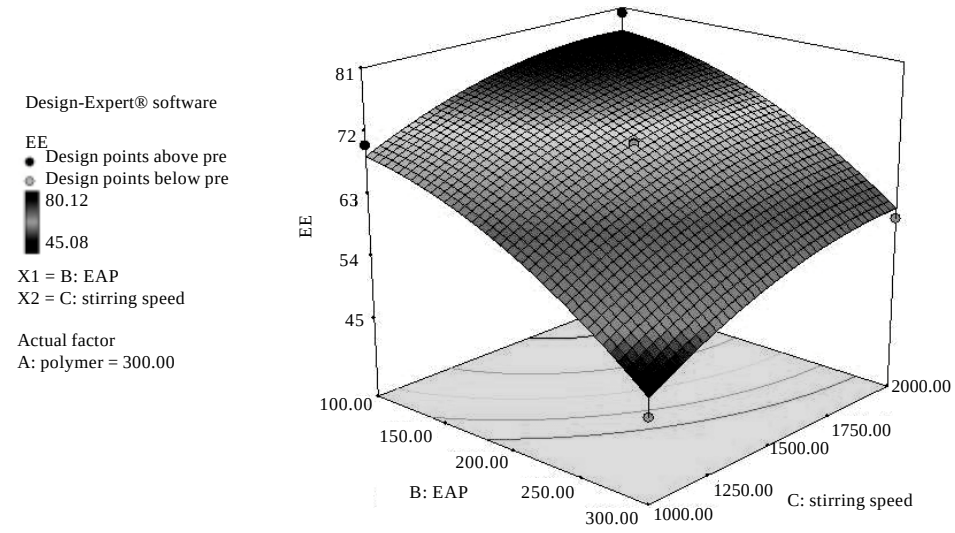

Fig. 3: 3D surface curve for the effect of selected variables (X_2, X_3) on the entrapment of Microspheres (X_1, 0)

Formulation with maximum entrapment (SE13) showed an initial burst of 18.24±2.56% observed in the first hour due to the drug located on or near the surface of microspheres. At the end of the 5 th day test period the formulation (SE13) showed 97.15±4.41% drug release. In order to investigate the release mechanism of present drug delivery system, the release data of prepared

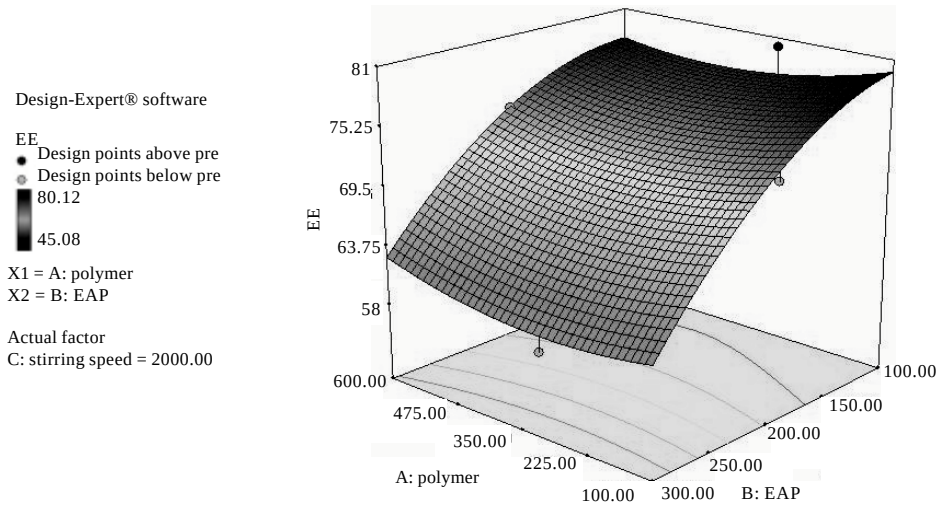

Fig. 4: 3D surface curve for the effect of selected variables (X_1, X_2) on the entrapment of Microspheres (X_8 +1)

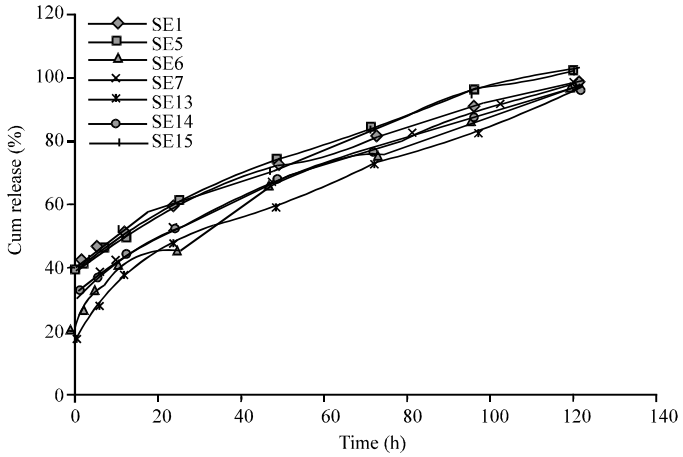

Fig. 5: *In vitro* release profiles of STP from Eudragit RS100 microspheres

STP loaded Eudragit RS100 microspheres with entrapment more than 70% in phosphate buffer (pH 7.4) were fitted to classic drug release kinetics models (Table 6).

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 6) (Dredan *et al*., 1996; Peppas, 1985). The coefficient of determination (R^2) of equation for release of STP from all microspheres in phosphate buffer was>0.9 for all models studied.

***In vitro* proteolytic activity:** Proteolytic activity of free and formulation with maximum entrapment (SE13) was evaluated separately before and after treating them for 2 h in phosphate

Table 6: Release behavior of STP in phosphate buffer (pH 7.4) from (SE1, SE5, SE6, SE7, SE13, SE14 and SE15)

	First order		Higuchi		Ritger-Peppas	
Formulation code	k	R^2	k	R^2	n	R^2
SE1	0.0207	0.961	6.052	0.996	0.210	0.961
SE5	0.0299	0.911	6.205	0.993	0.199	0.929
SE6	0.0253	0.895	7.550	86.40	0.310	0.967
SE7	0.020	0.958	5.918	0.998	0.201	0.963
SE13	0.0230	0.900	7.690	0.995	0.347	0.986
SE14	0.0184	0.952	6.373	0.991	0.231	0.931
SE15	0.020	0.880	6.501	0.985	0.242	0.929

*K: Release rate constant, R^2: Coefficient of determination, n: Release exponent

buffer saline (phosphate buffer, pH 7.4). STP in Eudragit RS100 microspheres (SE13) showed about 4.21-1.34% loss of proteolytic activity in basic medium whereas free STP showed around 18.65±0.89% loss of activity. Microspheres exhibited much better retention of proteolytic activity as compared to plain solution.

DISCUSSION

The objective of the present study was to optimize the formulation of Eudragit RS100 microspheres loaded with STP in terms of uniform spherical shape, size, maximum entrapment and controlled release of debriding agent with low initial burst. Role of wound debridement using enzymes is well reported and established for complete wound healing (Ajlia *et al*., 2010). The potential of STP in wound healing has been supported in various reports (Rath *et al*., 2011). But, being protein in nature these bioactives exhibit high structural and biochemical instabilities. All these factors demand effective formulation design for safe and effective delivery of proteases. We selected Eudragit RS100 as the polymer to effectively release the enzyme at alkaline wound site as natural polymer based systems have been reported to show variation in predicted release (Owlia *et al*., 2007).

The variables selected were Eudragit RS100 concentration (X_1) [drug (STP): Polymer ratio as 1:1; 1:3; 1:6], external aqueous phase volume (X_2) (100, 200 or 300 mL) [internal aqueous phase to external aqueous phase volume as 1:200; 1:400 or 1:600] and stirring speed of primary emulsion (X_3) (500, 1500 or 2000 rpm). The levels for these parameters were determined from the preliminary trials. Eudragit RS100 was chosen for controlled release of debriding agent in the alkaline media as open wounds tend to have a neutral or alkaline pH. Double emulsification method is the commonly utilized method for encapsulation of hydrophilic drugs particularly proteins and peptides. However, hydrophilic drugs get partitioned in the aqueous phases leading to low entrapment. External Aqueous Phase (EAP) volume with respect to internal phase volume (IAP) was selected as another variable, which is reported to affect the encapsulation efficiency and initial burst (Jain *et al*., 2005).

On the basis of above results, factor X_1 (Eudragit RS100 concentration) was found to be the main influential factor on the microsphere size. Although it exerted positive effect on both mean diameter and entrapment of microspheres, but effect on size was more as compared to entrapment, also supported by the positive coefficients in the fitted model Eq. 4 and 5.

This significant increase in size may be because of the increase in the viscosity of the droplets. Increase in entrapment with the increase in the polymer concentration might be due to increase

in the thickness of barrier separating two aqueous phases (Ito *et al*., 2007). But it was not consistent at higher concentration of polymer. External aqueous phase (X_2) exerted positive effect on mean diameter and negative on entrapment efficiency of microspheres. Effect on entrapment efficiency is more significant as compared to size of microspheres. It is also evident by factor effects and their signs. Low entrapment at higher levels of EAP might be due to drug leakage in the large volume of continuous aqueous phase and instability of droplet globules due to wide variation between outer to inner aqueous phase volume leading to decreased entrapment.

Stirring speed (X_3) exerted almost negligible effect on size of microspheres whereas it exerted positive effect on entrapment efficiency of microspheres. High loading efficiency have been reported by Shiomori *et al*. (2000) from smaller primary emulsions (Shiomori *et al*., 2000). This might be due to decrease in the ratio of droplet diameter of primary emulsion against that of secondary emulsion. Increase in stirring speed of primary emusion decreases the size of primary emulsion droplet leading to increase in ratio of secondary emulsion droplet diameter to that of primary emulsion. This signifies the thick oil layer with reduced leakage of inner water phase to outer water phase.

STP entrapped in Eudragit RS100 microspheres exhibited much better retention of proteolytic activity as compared to plain STP solution. Possible explanation for the improved physical and chemical stability of proteolytic enzyme in microsphere may be due to reduced mobilization and effective protection of enzyme from acidic environment.

CONCLUSION

Box-Behnken design was used to investigate the effects of selected formulation variables and to optimize the formulation of Eudragit RS100 microspheres loaded with acid labile-STP for maximum loading and controlled topical release for prolonged period. This statistical technique allows scientists to examine more than one independent variable at a time. The microsphere size and entrapment was highly dependent on the selected variables. Eudragit RS100 (X_1) had positive effect on size of microspheres, EAP (X_2) exerted negative effect on entrapment efficiency whereas stirring speed (X_3) effected both responses with negative effect on size and positive on entrapment efficiency. Thus STP loaded Eudragit RS100 microspheres were successfully prepared for sustained release upto 5 days with retention of its proteolytic activity. Further studies are required to establish optimum formulation in terms of improved long-term stability and *in vivo* therapeutic effects.

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REFERENCES

Ajlia, S.A.S.H., F.A.A. Majid, A. Suvik, M.A.W. Effendy and H.S. Nouri, 2010. Efficacy of papain-based wound cleanser in promoting wound regeneration. Pak. J. Biol. Sci., 13: 596-603.

Benita, S., 1996. Microencapsulation: Methods and Industrial Applications. Marcel Dekkar, New York.

Blanco-Prieto, M.J., E. Leo, F. Delie, A. Gulik, P. Couvreur and E. Fattal, 1996. Study of the influence of several stabilizing agents on the entrapment and *in vitro* release of pBC 264 from poly latide co-glycolide microspheres prepared by w/o/w solvent evaporation method. Pharm. Res., 13: 1127-1129.

Brannon-Peppas, L. and M. Vert, 2000. Polylactic and Polylactic and Polyglycolic Acids as Drug Delivery Carriers. In: Handbook of Pharmaceutical Controlled Release Technology, Wise, D.L. (Ed.). Marcel Dekker, New York, pp: 99-130.

Dredan, J., I. Antal and I. Racz, 1996. Evaluation of mathematical models describing drug release from lipophilic matrices. Int. J. Pharm., 145: 61-64.

Gombotz, W.R. and D.K. Pettit, 1995. Biodegradable polymers for protein and peptide drug delivery. Bioconjugate Chem., 6: 332-351.

Guo, S. and L.A. Dipietro, 2010. Factors affecting wound healing. J. Dent. Res., 89: 219-229.

Gupta, V.K., M.W. Assmus, T.E. Beckert and J.C. Price, 2001. A novel pH- and time-based multi-unit potential colonic drug delivery system. II. Optimization of multiple response variables. Int. J. Pharm., 213: 93-102.

Ito, F., H. Fujimori and K. Makino, 2007. Incorporation of water-soluble drugs in PLGA microspheres. Coll. Surf. B Biointerfaces, 54: 173-178.

Jain, D., A.K. Panda and D.K. Majumdar, 2005. Eudragit S100 entrapped insulin microspheres for oral delivery. AAPS PharmSciTech, 6: E100-E107.

Kee, W.H., S.L. Tan, V. Lee and Y.M. Salmon, 1989. The treatment of breast engorgement with serrapeptase: A randomized double blind controlled trial. Singapore Med. J., 30: 48-54.

Ko, J.A., H.J. Park, Y.S. Park, S.J. Hwang and J.B. Park, 2003. Chitosan microparticle preparation for controlled drug release by response surface methodology. J. Microencapsul., 20: 791-797.

Majima, Y., K. Hirata, K. Takeuchi, M. Hattori and Y. Sakakura, 1990. Effects of orally administered drugs on dynamic viscoelasticity of human nasal mucus. Am. Rev. Respit. Dis., 141: 79-83.

Martin, P., 1997. Wound healing aiming for perfect skin regeneration. Science, 276: 75-81.

Ogawa, Y., M. Yamamoto, H. Okada, T. Yashiki and T. Shimamoto, 1988. A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid. Chem. Pharm. Bull., 36: 1095-1103.

Owlia, P., L. Sadeghzadeh, F. Orang, M. Rafienia and S. Bonakdar, 2007. Evaluation of ceftriaxone releasing from microspheres based on starch against *Salmonella* spp. Biotechnology, 6: 597-600.

Peppas, N.A., 1985. Analysis of fickian and non-fickian drug release from polymers. Pharm. Acta Helv., 60: 110-111.

Qian, Z., S. Li, Y. He, H. Zhang and X. Liu, 2004. Preparation of biodegradable polyesteramide microspheres. Coll. Polym. Sci., 282: 1083-1088.

Rath, G., E.S. Johal and A.K. Goyal, 2011. Development of serratiopeptidase and metronidazole based alginate microspheres for wound healing. Artificial Cells Blood Substitutes Biotechnol., 39: 44-50.

Rawat, M., S. Saraf and S. Saraf, 2007. Influence of selected formulation variables on the preparation of enzyme-entrapped eudragit S100 microspheres. AAPS PharmSciTech, 8: 289-297.

Rudra, A., K. Santra and B. Mukherjee, 2011. Poly [D, L-lactide-co-glycolide] microspheres as a delivery system of protein ovalbumin used as a model protein drug. Trends Applied Sci. Res., 6: 47-56.

Saudagar, R.B., M. Rawat, D. Singh, S. Saraf and S. Saraf, 2007. Development and validation of derivative spectrophotometric method for determination of serratiopeptidase in pharmaceutical formulation. Oriental. J. Chem., 23: 1057-1060.

Shiomori, K., Kawano, Y., Kiyoyama, S., Yoshizawa, H., Uemura, Y. and Y. Hatate, 2000. Effective entrapment of protein into polylactide microcapsule by sol- vent evaporation of W/O/W emulsion. Kagaku Kogaku Ronbunshu, 26: 50-55.

Singer, A.J. and R.A.F. Clark, 1999. Cutaneous wound healing. N. Engl. J. Med., 341: 738-746.

Singh, D., S. Saraf, V.K. Dixit and S. Saraf, 2008. Formulation optimization of gentamicin loaded Eudragit RS100 microspheres using factorial design study. Biol. Pharm. Bull., 31: 662-667.

Tice, T.R. and R.M. Gilley, 1985. Preparation of injectable controlled-release microcapsules by a solvent-evaporation process. J. Controlled Release, 2: 343-352.

Yaakobi, T., D. Roth, Y. Chen and A. Freeman, 2004. Streaming of proteolytic enzyme solutions for wound debridement: A feasibility study. Wounds, 16: 201-205.