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FORMULATION DESIGN, OPTIMIZATION AND EVALUATION OF POROUS MICROSPHERES CONTAINING ANTIDIABETIC DRUG FOR CONTROLLED DRUG DELIVERY

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Abstract:

Objective: The objective of the study was to develop, evaluate and optimize the formulation of porous microspheres containing anti-diabetic drug (Vildagliptin) using polymer (Eudragit S-100) and porogen (Ammonium bicarbonate).

Methods: The double emulsion solvent evaporation technique was used for the preparation of microspheres and the 3^2 full factorial design was employed for optimization of microspheres. The developed microspheres were characterized for percent yield, entrapment efficiency, particle size, in vitro release study, in vivo antidiabetic study, surface morphology, and stability study.

Results: The studies conducted in the present research work gave promising results. 3^2 full factorial design was used for the optimization of the formulation. The optimized batch exhibited 94.02% drug entrapment efficiency, 79.42 µm particle size and drug release from the porous microspheres were also sustained for more than 12 h. The surface morphology analysis showed porous nature of the microsphere that could be useful to increase the surface area and dissolution. Porous structures were uniform on the surface as well as inside the microspheres, and interconnected open-pore structures were observed.

Conclusion: Experimental responses of the optimized batch have close proximity with the predicted value and stability study of the optimized formulation proved the formulation is stable for a long period of time; hence, it is an excellent alternative over the conventional delivery system.

Introduction:

Type II diabetes mellitus is a chronic metabolic disorder and its occurrence has been increasing steadily all over the globe, particularly in poorly developed countries. World Health Organization (WHO) reports refer India as the potential diabetic capital of the world, with the number of patients of the disease expected to increase from three to six crores by 2030. Type II Diabetes mellitus (T2DM) is associated with the dysfunctioning of islets cells (α and β) leading to the

development of resistance against insulin.[1] Historical data from clinical studies have indicated that the loss of beta cells in T2DM patients was associated with poor management of glycemic control.[2] Additionally, it has also been reported that alpha islet cells also had compromised functioning and had a critical role in the catastrophe of management of T2DM. The studies have shown that incretin analogs/regulators have a characteristic role in the successful management of glycemic control.[3] Previous studies have reported that the incretin hormones, including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) have association with pancreatic islet α and β cells for their responsiveness against glucose level in the body.[4] Moreover it has been reported that dipeptidyl peptidase-4 (DPP-4) has vital role in the degradation of GLP-1 and GIP, thus leading to the decreased responsiveness of pancreatic islet α and β cells against glucose and dwindled secretion of insulin.[5]

Vildagliptin (VG) is one of the very effective incretin enhancers that functions as a potent inhibitor of DPP-4. The inhibition of DPP-4 conversely restrict the rapid degradation of GLP-1 and GIP thus improve the insulin secretion through enhancement of sensitivity of pancreatic α and β cells against inappropriate glucose levels.[6]

Optimization has been defined as the implementation of systematic approaches to achieve the best combination of product and/or process characteristics under a given set of conditions.[7] Optimization using factorial designs is a highly efficient and systematic tool that shortens the time required for the development of pharmaceutical dosage forms and helps in improvement of research and development work. Factorial designs are one of the major application of optimization where all the factors are studied in all possible combinations are considered as the most efficient in estimating the influence of individual variables and their interactions using minimum experiments.[8]

The current study aims to develop, optimize and evaluate porous microspheres containing anti-diabetic drug (Vildagliptin) using a suitable polymer (Eudragit S-100) and porogens (Ammonium bicarbonate) which offer large specific surface area, low density. The formulation is optimized by using factorial design with the help of design expert software. A 3^2 full factorial design (two variables in three levels) was employed to evaluate the combined effect of the selected independent variables on dependent variables such as particle size, drug entrapment efficiency and drug release.

Materials and Methods

Materials

Vildagliptin was obtained from Macleods Pharmaceuticals Ltd, Baddi, India as gift sample. Eudragit S 100 was procured from Evonik Catalysts India Pvt Ltd, Mumbai, India. Chloroform and Polyvinyl alcohol (PVA) were obtained from Loba Chemie Pvt Ltd., Mumbai, India used as dispersion media and emulsifying agent. n-Hexane (Ranbaxy Fine Chemical Ltd., New Delhi, India) was a washing agent. All chemicals received were of analytical grade and were used as such.

Method

Vildagliptin loaded porous microspheres were obtained by water-in-oil-in-water ($W_1/O/W_2$) double emulsion solvent evaporation technique. Firstly, the drug pre-dissolved in 2 ml distilled water as internal aqueous phase (W_1) along with the porogen solution was emulsified in 10 ml solution of chloroform containing Eudragit S-100 polymer as oil phase (O). Magnesium stearate was dispersed in the polymer solution. This solution was then subjected to magnetic stirring for 10 minutes which resulted in formation of primary W_1/O emulsion. This primary (W_1/O) emulsion was then injected using a syringe into a 100 ml aqueous PVA solution as external aqueous phase (W_2) at a variable speed using mechanical stirrer at room temperature to produce $W_1/O/W_2$ double emulsion. Stirring was continued for 3.5 hours for the purpose of complete evaporation of chloroform. The resultant microspheres were collected by filtration and washed with n-hexane and air dried to obtain free flowing microspheres. [9]

Experimental design

From the preliminary studies, it was found that there are two very important factors i.e concentration of polymer (Eudragit S-100) and concentration of porogen, (Ammonium bicarbonate) which effect the porous microspheres.[10] Hence, it was decided to apply full factorial design to study the effect of independent variables on dependent variables.

A 3^2 full factorial design was applied for the optimization of porous microspheres of Vildagliptin. The two different factors at three different levels were evaluated. The independent variable here are amount of polymer and porogen. The responses like particle size, entrapment efficiency and drug release (%) after 12 h were considered as dependant variable. Design-Expert® software (Version 8.0.6, Stat-Ease) was used to apply the design and total 9 runs were formulated. Levels

of process parameters in 3^2 full factorial design for vildagliptin porous microspheres are mentioned in Table 1. Coded and actual values of batches of porous microspheres of vildagliptin, prepared by applying 3^2 full factorial design is given in Table 2.

Table 1: Levels of process parameters in 3^2 full factorial design for vildagliptin porous microspheres

Independent Variable	Level		
	Upper level	Medium Level	Lower level
Eudragit S-100 (X_1)	2 gm	1.5 gm	1 gm
Porogen (X_2)	3 %	2 %	1 %
Dependent Variables – Y1 - Particle Size Y2 - % Entrapment Efficiency Y3 - % Cumulative drug release after 12 hrs			

The low and high levels of factors were adopted from the preliminary studies and the medium levels were set as the midpoint of low and high levels.

Table 2: Coded and actual values of the batches prepared by applying 3^2 full factorial design for vildagliptin porous microspheres

Formulation codes	Coded Value		Actual Value	
	X1	X2	X1 (gm)	X2 (%)
V1	1	0	2	2
V2	-1	0	1	2
V3	0	0	1.5	2
V4	0	-1	1.5	1
V5	1	-1	2	1
V6	-1	1	1	3
V7	-1	-1	1	1
V8	0	1	1.5	3
V9	1	1	2	3

The actual composition of the prepared batches is given in Table 3.

Table 3: Composition of vildagliptin loaded porous microspheres prepared by applying 3^2 factorial design

Ingredients	V1	V2	V3	V4	V5	V6	V7	V8	V9
Vildagliptin (mg)	100	100	100	100	100	100	100	100	100
Eudragit S-100 (gm)	2	1	1.5	1.5	2	1	1	1.5	2
Porogen (% w/v)	2	2	2	1	1	3	1	3	3
Polyvinyl alcohol (%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Chloroform (ml)	10	10	10	10	10	10	10	10	10
Magnesium Stearate (mg)	100	100	100	100	100	100	100	100	100

Characterization of drug

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) was performed for the estimation of Vildagliptin in pure form. The mobile phase comprises of 0.1 M phosphate buffer and acetonitrile in the ratio of 85:15 % v/v, and was pumped at rate of 1.0 mL/min. The detection of vildagliptin was carried out by PDA detector at wavelength of 210 nm, column temperature was maintained at room temperature and injection volume was 25µL. The total run time was 7 minutes, and its retention time were recorded. [11]

Drug-excipient compatibility studies

Compatibility of drug with excipients was studied by Fourier Transform Infra-red spectroscopy (FTIR). Effect of process of entrapment on crystallinity of the drug was studied by Differential Scanning Calorimetry (DSC) and Powder X-ray diffraction (XRD)

Differential scanning calorimetry (DSC)

Differential scanning calorimetery (DSC) of pure Vildagliptin, Eudragit S-100 and Vildagliptin loaded Eudragit S-100 microspheres (optimized formulation) was done using Mettler Toledo differential scanning calorimeter to determine any possible vildagliptin-polymers interaction. The samples were triturated separately to obtain a fine divided powder. They were then sealed in an aluminum pan and heated from 40–200 °C at 10 °C/min. Nitrogen flow was set at 20 ml/min.[12]

Fourier Transform Infrared Studies (FTIR)

The compatibility between pure drug and polymer is detected by Fourier Transform Infrared Spectroscopy (FTIR). The spectra for the samples were recorded using a Bruker Vertex 70 FTIR spectrophotometer by KBr pellet method. The samples were analysed by mixing with potassium bromide (1:10) individually and pressed to form a thin pellet by applying pressure using KBr press. The formed pellets were placed within the sample holder. Spectral scanning was taken in the wavelength region between 4000-400 cm^{-1}. The samples of pure drug, polymer and optimized formulation were subjected to analysis separately. FTIR scans of Vildagliptin, Eudragit S-100, optimized formulation were recorded.[13]

Powder X-ray diffraction (XRD)

The crystallinity of Vildagliptin in formulations was assessed by XRD analysis. XRD diffraction analysis for Vildagliptin, Eudragit S-100, and Vildagliptin loaded Eudragit S-100 microspheres (optimized formulation) was performed on X-ray diffractometer (Bruker, D-8 Advance) with scintillation detector by exposing the samples to CuK2α rays with voltage of 40 kV and a current of 40 mA in flat plate θ/2θ geometry, where 2θ ranges 5 to 60, having step width 0.03 and a scan time of 0.5 seconds per step**.**[9]

Evaluation of prepared porous microspheres

Particle size analysis

The particle sizes of porous microspheres were determined by optical microscopy. Optical microscope was fitted with eye piece micrometer which was then calibrated with a stage micrometer. About 100 microspheres were randomly selected from each formulations and their diameters were measured and then the average particle size was determined by the Edmondson's equation:

$$Average\ particle\ size = \frac{\Sigma nd}{\Sigma n}$$

where ‘‘n’’ stands for the number of porous microspheres, and ‘‘d’’ for the mean size range.[14]

Surface Morphology

The prepared vildagliptin porous microspheres were morphologically examined for shape, size and surface morphology using scanning electron microscope. Dry porous microspheres were mounted on a metal stub using double-sided adhesive tape and sputter-coated with gold for 80 seconds under vacuum, then placed into the specimen chamber. Porous microspheres were imaged with a JEOL JSM-840 scanning electron microscope (JEOL USA, Inc.) using a 5 kV accelerating voltage, and 10 mm working distance.[15]

Determination of Percentage yield of microspheres

The prepared microspheres were completely dried and then weighed. The percentage yield was calculated by:

$$\%\ Yield = \frac{Weight\ of\ Microspheres}{weight\ of\ drug+polymer}\ x\ 100$$

Determination of flow properties of microspheres

The prepared microspheres were evaluated for flow properties including bulk density, tapped density, Carr’s index, Hausner ratio and angle of repose.[16]

Drug Entrapment efficiency

Drug entrapment efficiency of porous microspheres of vildagliptin was performed by accurately weighed 50 mg of porous microspheres were suspended in 100 ml of phosphate buffer pH 7.4±0.1. The resulting solution was kept for 24 hours. Next day it was stirred for 15 min and subjected to filtration. After suitable dilution, vildagliptin content in the filtrate was analysed spectrophotometrically at 210nm.[17] The drug entrapment efficiency was determined using following relationship:

$$\%\ Entrapment\ Efficiency = \frac{Theoretical\ Entrapment}{Practical\ Entrapment} x100$$

***In vitro* release studies of porous microspheres**

In vitro drug release test of vildagliptin porous microspheres was conducted out for initial 2 hr in acidic medium (0.1 N HCl, pH 1.2, volume 900 ml) followed by phosphate buffer (pH 7.4, volume 900 ml) upto 12 h, maintained at 37±0.5 °C by using USP type II dissolution test apparatus (Lab India DS 8000) at 50 rpm under sink conditions. An accurately weighed, vildagliptin loaded porous microspheres equivalent to 100 mg of vildagliptin was placed in the muslin cloth and introduced into the dissolution medium. At a predetermined time intervals (0-12 hour), about 5 ml of sample was withdrawn and replenished with equal amount of fresh dissolution medium to maintain the sink condition. The withdrawn samples were analysed for drug content by using UV spectrophotometer at 210 nm.

Kinetic modelling of drug release

In order to understand the kinetic and mechanism of drug release, the vildagliptin loaded microspheres were treated in different mathematical models. The result of *in vitro* drug release study of microspheres were fitted with various kinetic equation like zero order (cumulative percentage of drug release v/s time), first order (log percentage of drug remaining v/s time), Higuchi's model (cumulative percentage of drug release v/s square root of time), Korsemeyer Peppas model (log cumulative percentage of drug release v/s Log time), Hixson Crowell model (cube root of percentage drug remaining v/s time). The release data was plotted. $R^2$and slope values were calculated from the linear portions of curve obtained by regression analysis of the above plots.[18]

Residual Solvent Analysis

There are many volatile organic chemicals which are used in the preparation of pharmaceutical preparations. Some amount of those organic solvents might remain in the final formulation, which is called as residual solvents. These residual solvents are classified into three classes based on their potential risk to human health. The ICH guidelines "Q3C" for the residual solvents, has given the permitted daily exposure (PDE) and concentration limit in ppm for such solvents.

In the present work, Chloroform was used in the preparation of vildagliptin porous microspheres. The chloroform belongs to class 2 residual solvents and its amount in the finished formulation should be within limit (up to 60 ppm). Hence, the Gas Chromatographic technique was applied to determine the amount of Chloroform (limit is upto 60ppm) in the optimized vildagliptin porous microspheres. Formulation was tested by a gas chromatograph (GC) using flame ionization detector. For this study, 10 mg of optimized porous microspheres, were dissolved in 5 mL of

Dimethyl sulfoxide (DMSO) and transferred to the GC system. For calculations, a standard solution of chloroform in DMSO (20 ppm) was also analysed.

***In Vivo* Antidiabetic Study**

Experimental Animals.

At first Wistar albino rats (250- 300g) were maintained under laboratory condition. They were fed a regular laboratory meal and acclimatised for seven days in a controlled environment with 55 percent humidity, a temperature of 20-26°C, and a 12 hour dark/light cycle.

Induction of Experimental Diabetes.

Rats were turned into diabetic ones via single intraperitoneal injection of alloxan (100 mg/kg) i.p. in 0.2 ml Tween 80. Alloxan, a -cell cytotoxin, caused chemical diabetes in a variety of animal species by causing damage to the insulin-secreting pancreatic cell (-cell). This causes a decrease in endogenous insulin secretion, which leads to impaired glucose utilisation by the tissues. After 48 hrs of Alloxan administration, blood glucose levels were estimated in rats following overnight fasting. The study comprised diabetic rats with a plasma glucose level of more than 150 mg/dl.

Experimental Design.

The rats were divided into 4 groups (Table 4.) comprising 6 animals in each group as follows.

Group I (Vehicle Control). Normal rats were treated with 0.1 ml saline daily and served as the vehicle control.

Group II (Diabetic Control). Animals were treated with single dose of Alloxan (100 mg/kg) by the intraperitoneal route to induce diabetes and served as a positive control.

Group III (Marketed Repaglinide Formulation). Diabetic rats were treated with 2.5 mg/kg body weight of marketed Vildagliptin formulation.

Group IV (Optimized formulation). Diabetic rats were treated with 2.5 mg/kg body weight of Vildagliptin containing porous microsphere.

Treatment:

Four groups of animals containing 6 rats in each group were divided as group I, II, III, and IV. Group I which is the Negative control treated with saline, Group II would result out the efficacy of the Alloxan (100mg/kg. b.w.) on the experimental rats. Group III animals were served with Vildagliptin drug (2.5 mg/kg b. w.) which was compared with the Group II, and Group IV. Group IV for which optimized formulation of dose (2.5 mg/kg b.w.) were given respectively.[19]

Collection of Blood from the Rats.

From the tail vein of rats, blood samples were taken and checked for plasma glucose level using Accu-check active glucose strips in Accu-check active test meter.

Table 4: Animal Required

Sr No.	Drug	Animal	Age	No. required	Study
1.	Group I	Wistar albino rat	7-8weeks	06	Antidiabetic study
2.	Group II	Wistar albino rat	7-8weeks	06	Antidiabetic study
3.	Group III	Wistar albino rat	7-8weeks	06	Antidiabetic study
4.	Group IV	Wistar albino rat	7-8weeks	06	Antidiabetic study

Comparative study of Optimized v/s marketed formulation

Comparative study was done in between the optimized formulation (OF1) and marketed formulation (MF) of Vildagliptin tablets (Galvus® (50mg); Norvartis). Dissolution profile was conducted in acidic medium (0.1 N HCl, pH 1.2, volume 900 ml) maintained at 37 ± 0.5 °C by using USP type II dissolution test apparatus (Lab India DS 8000) at 50 rpm under sink conditions. At a predetermined time intervals, about 5 ml of sample was withdrawn and replenished with equal amount of fresh dissolution medium to maintain the sink condition. The withdrawn samples were analysed for drug content by using UV spectrophotometer at 210 nm.

Stability Study

Stability study of optimized formulation was carried out to point out any visual physical or chemical changes made in the formulation after storing it at elevated temperature and humidity conditions. The optimized formulation was studied for stability profile at normal and accelerated conditions as per ICH guidelines. The formulation was placed separately in a coloured glass container and stored at normal room temperature (25±2 °C/60±5% RH), and for accelerated testing at oven temperature (40±2 °C/75±5% RH) in the stability chamber. The samples were analysed for 3 month and 6 month interval for their physical appearance, particle size, entrapment efficiency and drug release. Obtained results for each interval of stability study were compared with zero month results.[20]

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Validation of Model

A total of nine runs were generated by the Design-Expert software for the 3^2 full factorial design. Statistical validation of the equation was established using ANOVA. The models were evaluated using statistically significant terms and R^2 value. An intensive grid search was conducted to find out the composition of the optimized formulation having a higher value of responses. Optimum checkpoint formulation were selected to evaluate optimization capability of models generated using 3^2 full factorial design. Checkpoint formulations were prepared using the optimal formulation variables setting and evaluated for the responses. The resultant experimental value was quantitatively compared with predicted value and the prediction error was calculated.

Results and discussion:

Statistical analysis

Full factorial design as the response surface methodology (RSM) requires nine batches. The responses of all the prepared formulations were simultaneously fit to quadratic model using Design Expert 8.06. The quadratic model showed a best fit for the responses. Polynomial equations generated by Design Expert were established on the basis of ANOVA. The statistical analysis suggests that the independent variables significantly affected dependent variables.

The full factorial analyses, describes the quadratic effects of the variables on the responses. A statistical model incorporating interactive and polynomial terms were utilized to evaluate responses. The polynomial equation generated under 3^2 full factorial design using Design expert software is as follows:

$$\mathbf{Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2}$$

Where, Y is the dependent variable, b_0 is the intercept, and b_1 to b_{22} are regression coefficient. The master effects (X_1 and X_2,) represent the average result of changing one element at a time from its low to high value. X_1X_2, represents the interaction terms show how the response changes when two factors are simultaneously changed and X_1^2 and X_2^2 represents quadratic effect. The results of statistical analysis of experimental design batches obtained by Design Expert software are shown below. The model was proved to be significant after observing the P value for the response parameters. Model simplification was carried out by eliminating non-significant terms ($p > 0.05$) in polynomial equations. The P value for particle size, entrapment efficiency, and

cumulative drug release in 12 hrs (CDR12) were found to be 0.0050, 0.0167 and 0.0013, respectively, which is less than 0.0500 indicating the significance of model terms.

To validate the model, all the points were selected and observed their experimental and predicted value for the responses. Table 5 showed the model summary statistics of responses. It can be concluded that the model is best suitable because of the difference between experimental and predicted value is very low.

Table 5: Model summary statistics for dependent variables

Response	Model	SD	R^2	Adjusted R^2	Predicted R^2	Press	Significance
Particle Size	Linear	3.32	0.8795	0.8394	0.7542	134.86	
	2FI	3.63	0.8802	0.8082	0.4669	292.55	
	Quadratic	1.54	0.9870	0.9654	0.8545	79.86	Suggested
	Cubic	1.20	0.9974	0.9789	0.5191	263.90	
EE%	Linear	6.43	0.4694	0.2925	-0.0306	482.23	
	2FI	7.02	0.4727	0.1563	-0.8937	886.04	
	Quadratic	2.14	0.9706	0.9216	0.7301	126.28	Suggested
	Cubic	2.95	0.9814	0.8512	-2.3897	1586.03	
%CDR	Linear	10.67	0.4306	0.2408	-0.1989	1438.61	
	2FI	11.67	0.4327	0.0924	-1.8004	3360.22	
	Quadratic	1.45	0.9947	0.9859	0.9542	54.96	Suggested
	Cubic	2.17	0.9961	0.9687	0.2870	855.56	

Effect on particle size.

The mean particle size of all the selected 9 formulations was in the range 73.68–98.30 μm depending upon the variation in the independent variables. Formulation V9 shows maximum particle size (98.30μm) while the minimum particle size was achieved in formulation V7 (73.68μm) as shown in Table 7.3.10. ANOVA analysis indicates that there was a significant effect of various independent variables on particle size. Data in Table 6 showed the coefficient estimate and P values of each factor for particle size. Table 7 depicts the actual and predicted values obtained for Particle Size.

Table 6: Summary of ANOVA results for measured response parameters

Source	Degree of freedom	Coefficient of estimate	Adequate precision	F value	P value (prob>F)
(a)For Particle size					
Model	5	92.70	18.255	45.68	0.0050
X_1-Polymer	1	8.44	--	180.15	0.0009
X_2-Porogen	1	3.04	--	23.36	0.0169
X_1X_2	1	0.29	--	0.15	0.7272
X_1^2	1	-4.90	--	20.26	0.0205
X_2^2	1	-2.30	--	4.47	0.1249
(b)For Entrapment efficiency					
Model	5	92.73	13.362	19.82	0.0167
X_1-Polymer	1	4.85	--	30.79	0.0115
X_2-Porogen	1	-3.62	--	17.12	0.0256
X_1X_2	1	0.62	--	0.34	0.6003
X_1^2	1	-10.65	--	49.49	0.0059
(c)For Cumulative drug release					

Model	5	91.96	32.554	113.00	0.0013
X_1-Polymer	1	8.05	--	184.05	0.0009
X_2-Porogen	1	4.62	--	60.53	0.0044
X_1X_2	1	-0.80	--	1.21	0.3514
X_1^2	1	-15.78	--	235.84	0.0006
X_2^2	1	-9.38	--	83.35	0.0028

Table 7: Actual and predicted values obtained for Particle Size.

Formulation Codes	Actual Value	Predicted Value	Residual	Prediction error in %
V1	95.50	96.23	-0.73	-0.76
V2	79.29	79.36	-0.068	-0.09
V3	93.50	92.70	0.80	0.86
V4	88.29	87.36	0.93	1.05
V5	90.30	90.60	-0.30	-0.33
V6	80.50	79.80	0.70	0.87
V7	73.68	74.31	-0.63	-0.86
V8	91.70	93.43	-1.73	-1.89
V9	98.30	97.27	1.03	1.05

Prediction error (%) = (actual value – predicted value)/actual value x 100.

The Model F-value of 45.68 implies the model is significant. The difference between "Pred R-Squared" and "Adj R Squared" value was found to be less than 0.2, which is desirable. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 18.255 indicates an adequate signal. This model can be used to navigate the design space. The other information obtained from ANOVA table was about the variables. The P value for both factors, X_1 and X_2 was found to be 0.0009 and 0.0169, respectively, indicating their significance in the model. Interaction effect and quadratic effect of X_1 were found to be insignificant, whereas quadratic effect of X_2 was significant. The fitted final equation can be used to make predictions

about the response for given levels of each factor. The equation for the response percentage yield was as follow:

$$\textbf{Mean Particle size} = +92.70 + 8.44\,X_1 + 3.04X_2 + 0.29X_1X_2 - 4.90X_1^2 - 2.30\,X_2^2$$

The equation indicates that the magnitude of coefficient of both X_1 and X_2 shows positive and significant effect on particle size of porous microspheres. As the concentration of Eudragit S-100 and ammonium bicarbonate increased, the particle size of the porous microspheres increased. Linear correlation plots between the actual and predicted response for particle size is given in Figure 1(a). Figure 1(b) shows the three dimensional response surface plot and Figure 1(c) shows contour plots showing the effect of independent variables on the particle size.

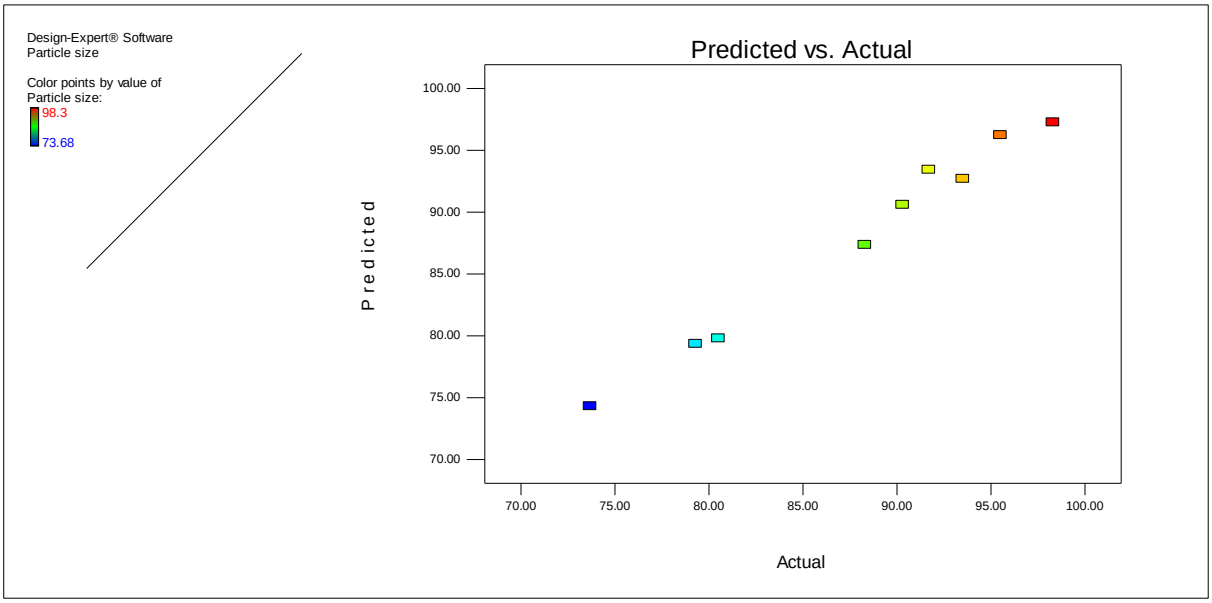

Figure 1(a) Actual and predicted response for particle size

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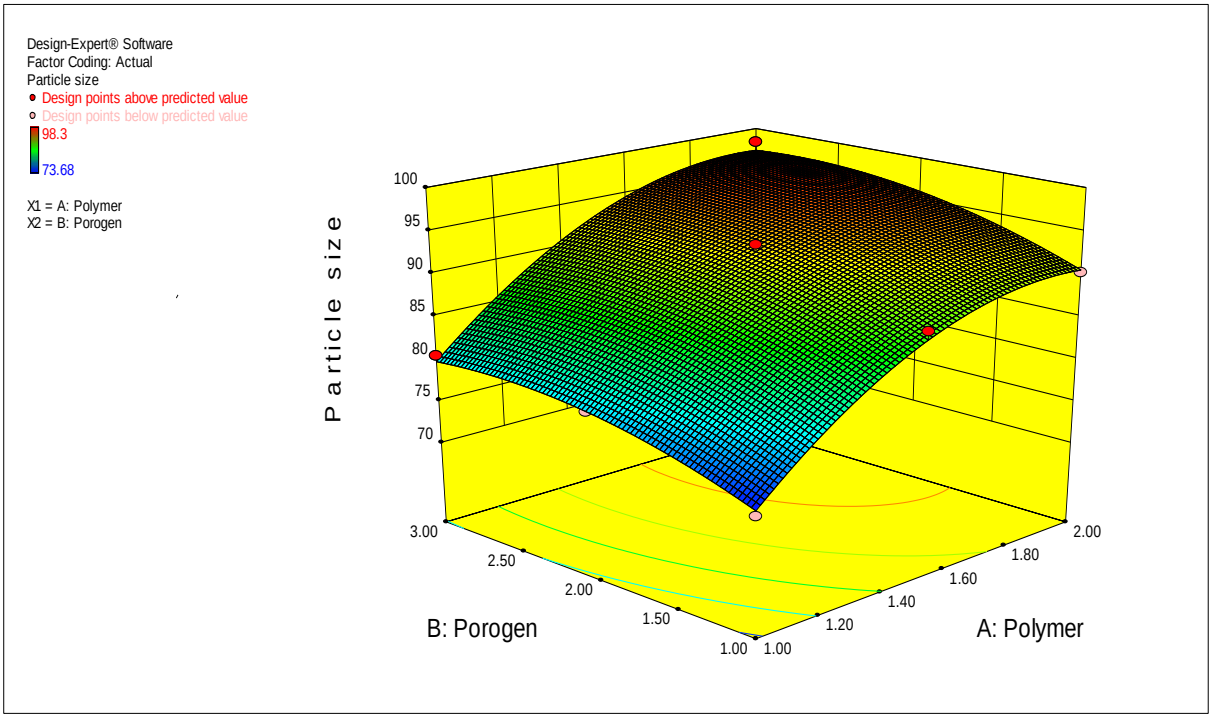

Fig. 1(b): Three dimensional response surface plots for particle size

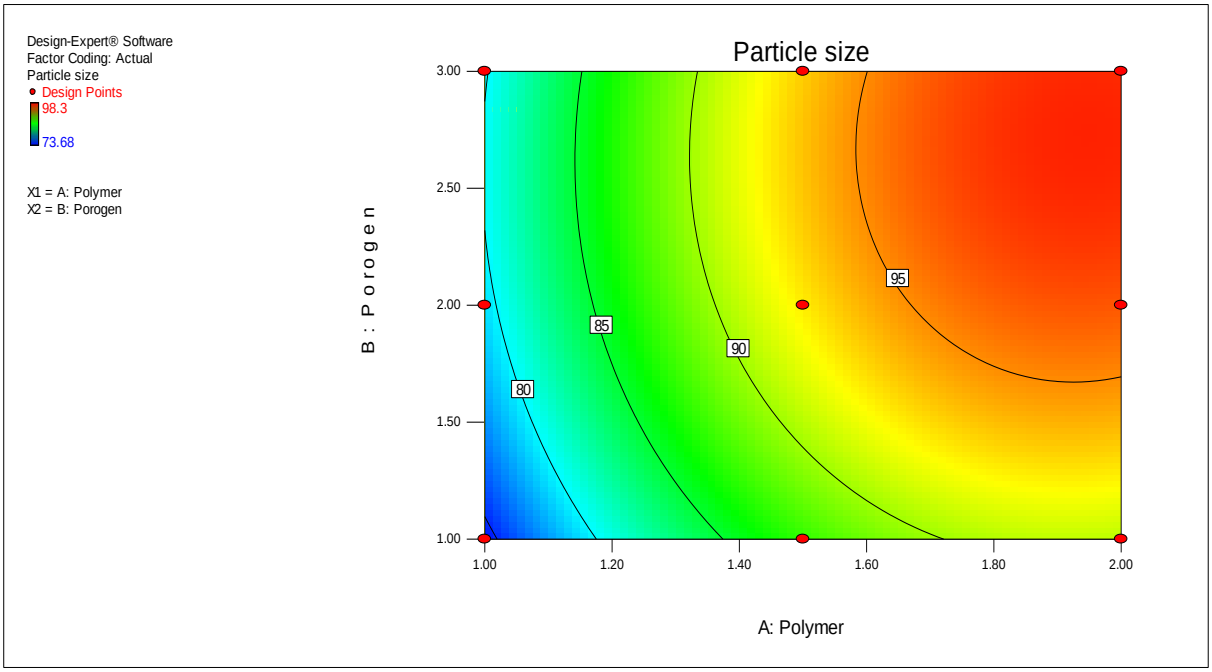

Fig. 1(c): Contour plots for particle size

7.4.2 Effect of Independent Variables on Entrapment Efficiency

The percent entrapment efficiency was found to be in the range of 70.9 to 94.7%. R^2 was found to be equal to 0.9563. Data in Table 6 showed the coefficient estimate and P values of each factor

for entrapment efficiency. Table 10 depicts the actual and predicted values obtained for entrapment efficiency.

Table 10: Actual and predicted values obtained for Entrapment Efficiency.

Formulation Codes	Actual Value	Predicted Value	Residual	Prediction error in %
V1	85.50	86.93	-1.43	-1.67
V2	76.70	77.23	-0.53	-0.69
V3	94.70	92.73	1.97	2.08
V4	92.40	94.60	-2.20	-2.38
V5	89.50	88.17	1.33	1.49
V6	70.90	71.24	-0.34	-0.48
V7	80.60	79.72	0.88	1.09
V8	87.60	87.37	0.23	0.26
V9	82.30	82.19	0.11	0.13

The Model F-value of 19.82 implies the model is significant. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 13.362 indicates an adequate signal. This model can be used to navigate the design space. The other information obtained from ANOVA table was about the variables. The P value for both factors, X_1 and X_2 was found to be 0.0115 and 0.0256, respectively, indicating their significance in the model. Interaction effect and quadratic effect of X_2 were found to be insignificant, whereas quadratic effect of X_1 was significant. The fitted final equation can be used to make predictions about the response for given levels of each factor.

The equation for the response percentage entrapment efficiency was as follow:

$$\% EE = + 92.73 + 4.85X_1 - 3.62X_2 + 0.62X_1X_2 - 10.65X_1^2 - 1.75X_2^2$$

The equation indicates that X_1 i.e. polymer concentration had positive effect on entrapment efficiency of porous microspheres which indicates that the higher amount of Eudragit S-100 contributes to increase in entrapment of drug. Factor X_2 i.e. concentration of porogen had

negative effect on the entrapment efficiency of porous microspheres. However, an antagonistic significant quadratic effect of concentration of Eudragit S-100 was observed, which means that optimal levels of X are not in the extremes of the experimental region but inside it. The 3D response surface plot unveiling the influence of an independent variable on drug entrapment efficiency is shown in Figure 2(b). These effects were further illustrated in contour plots (Figure 2(c). The contour plots were found to be nonlinear. This signifies that there is no direct linear relationship among the selected independent variables. It is evident from the contour that medium level of X_1 and minimum level of X_2 favours the entrapment efficiency of porous microspheres of vildagliptin. Linear correlation plots between the actual and predicted response for entrapment efficiency is given in Figure 2(a)

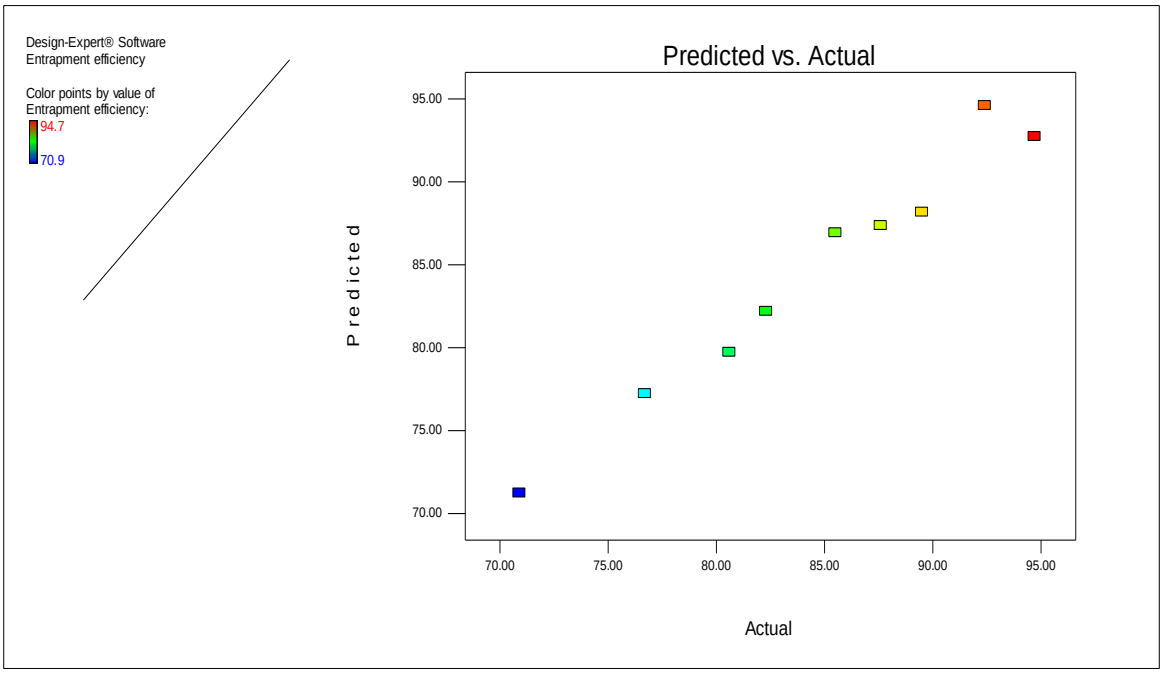

Figure 2(a) Actual and predicted response for entrapment efficiency

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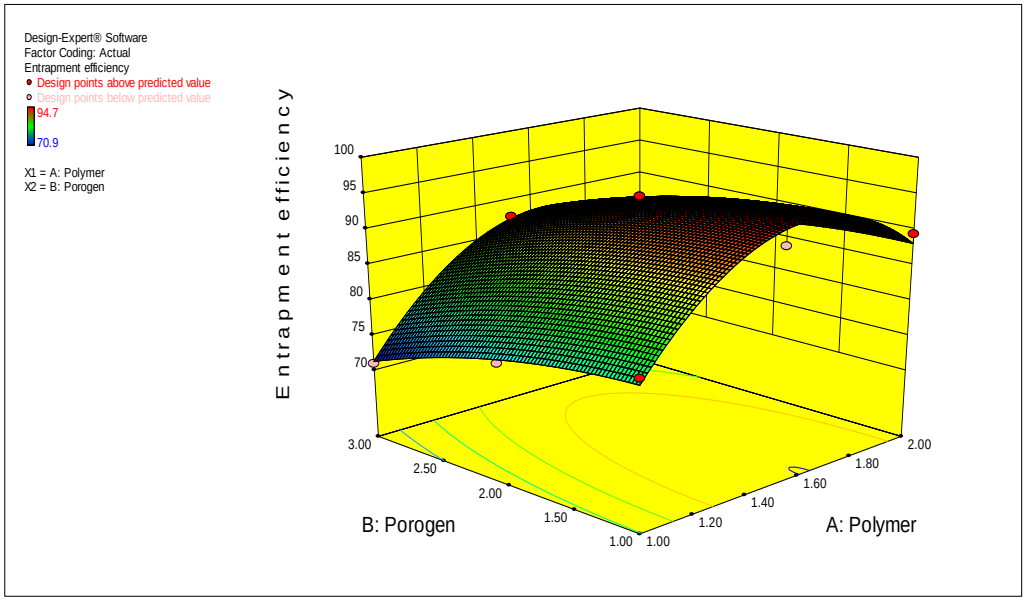

Fig. 2(b): Three dimensional response surface plots for % entrapment efficiency

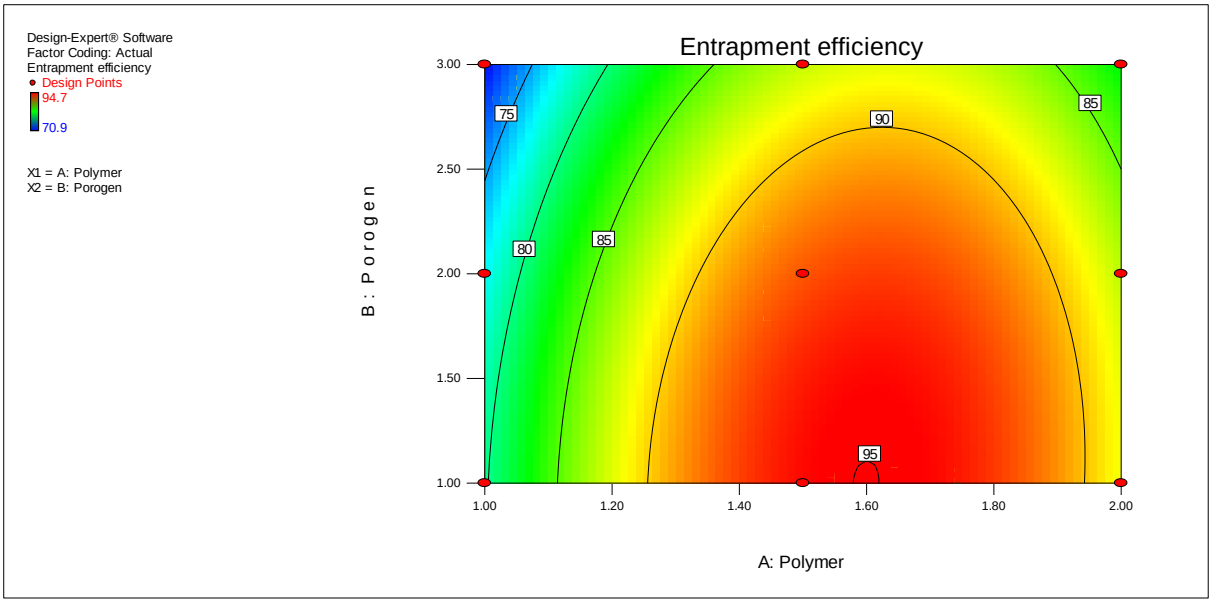

Fig. 2(c): Contour plots for entrapment efficiency

7.4.3 Effect Cumulative Drug Release (CDR 12 hrs)

Porous microspheres offer the controlled release of the drug. The percent CDR was found to be in the range of 54.2 to 93.4%. R^2 was found to be equal to 0.9542. Data in Table 9 showed the coefficient estimate and P values of each factor for cumulative drug release. Table 11 depicts the actual and predicted values obtained for cumulative drug release.

Table 11: Actual and predicted values obtained for Cumulative drug release

Formulation Codes	Actual Value	Predicted Value	Residual	Prediction error in %
V1	84.10	84.22	-0.12	-0.14
V2	66.80	68.12	-1.32	-1.98
V3	93.40	91.96	1.44	1.54
V4	76.80	77.96	-1.16	-1.51
V5	71.30	71.02	0.28	0.39
V6	64.60	64.16	0.44	0.68
V7	54.20	53.32	0.88	1.62
V8	86.90	87.19	-0.29	-0.33
V9	78.50	78.66	-0.16	-0.20

The Model F-value of 113.00 implies the model is significant. The "Pred R-Squared" of 0.9542 is in reasonable agreement with the "Adj R-Squared" of 0.9859. The difference between "Pred R-Squared" and "Adj R Squared" value was found to be less than 0.2, which is desirable. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 32.554 indicates an adequate signal. This model can be used to navigate the design space.

The other information obtained from ANOVA table was about the variables. The P value for factors, X_1 was found to be 0.0009, indicating its significance in the model, whereas the P value for X_2 was found to be insignificant. Interaction effect and quadratic effect of X_1 and X_2 were found to be significant. The fitted final equation can be used to make predictions about the response for given levels of each factor.

The equation for the response % CDR 12 was as follow:

$$\% \text{ CDR12} = + 91.96 + 8.05X_1 + 4.62X_2 - 0.80 X_1X_2 - 15.78 X_1^2 - 9.38X_2^2$$

The equation indicates that X_1 i.e. polymer concentration had positive effect on % CDR 12 of porous microspheres which indicates that the higher amount of Eudragit S-100 contributes to

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increase in % CDR 12. The highest magnitude of significant antagonistic quadratic effect was observed for the X_1 factor, which means that optimal levels of X are not in the extremes of the experimental region but inside it. These effects were further illustrated in between actual and predicted (Figure 3a), three dimensional surface response (Figure 3b), and contour plots (Figure 3c). The contour plots were found to be nonlinear. This signifies that there is no direct linear relationship among the selected independent variables. It was determined from the contour plot that a higher value of percent CDR12 (≥90%) could be obtained with medium levels of both X_1 and X_2.

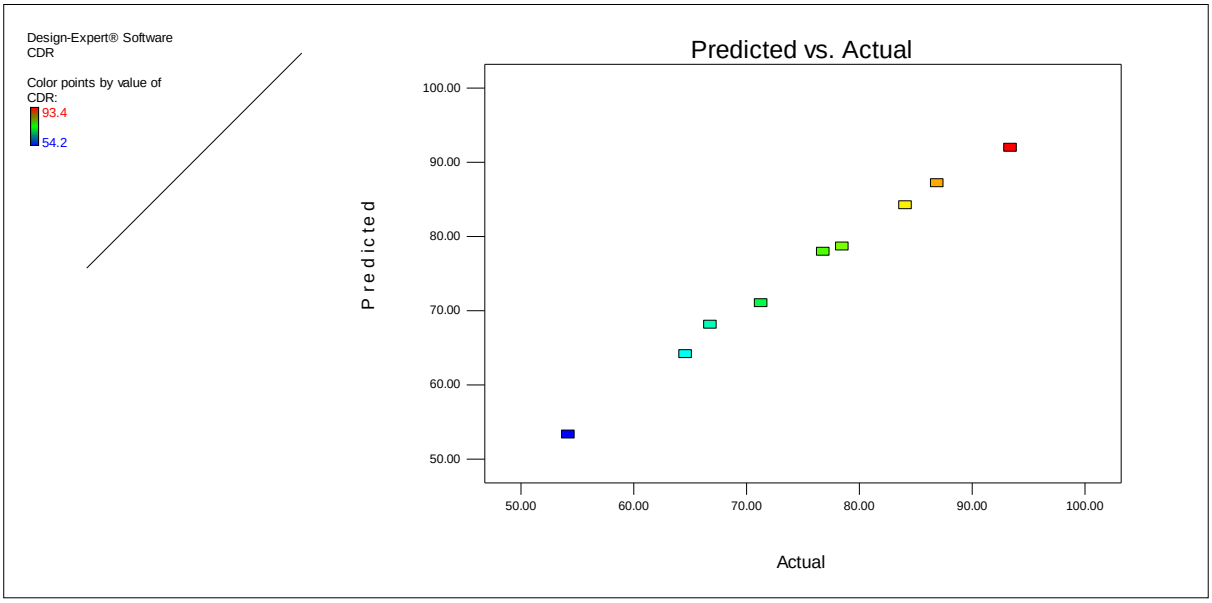

Figure 3(a) Actual and predicted response for cumulative drug release

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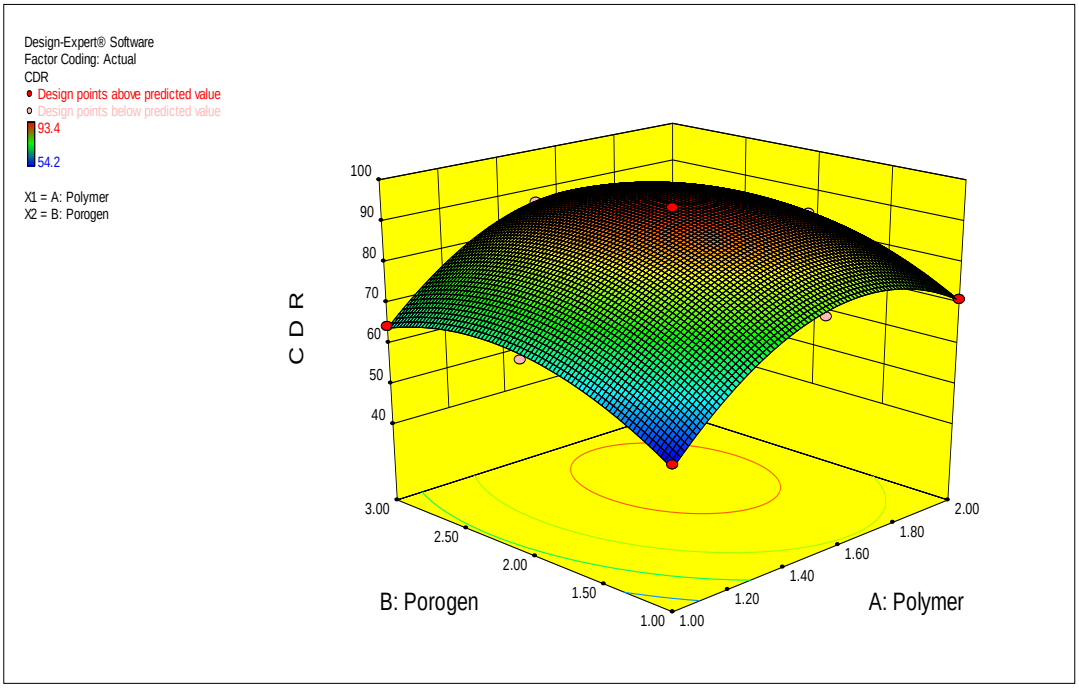

Figure 3(b): Three dimensional response surface plots for cumulative drug release

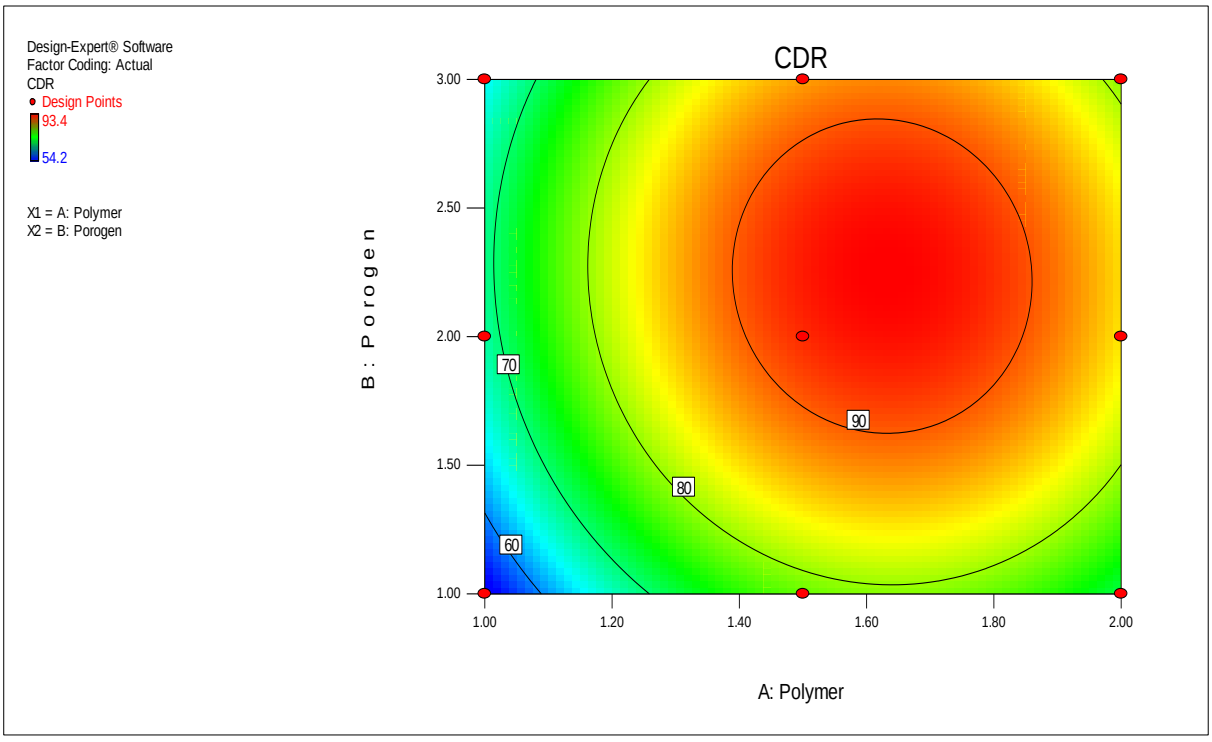

Fig. 3(c): Contour plots for cumulative drug release

7.4.4 Evaluation and Validation of the Optimized Formulation

To optimize all the above responses with different targets, a numerical optimization technique by the desirability function and a graphical optimization technique by the overlay plot was used (Figure 4 and Figure 5). The optimized formulation was obtained by applying constraints on dependent variable responses and independent variables. The constraints for the responses Y_1, Y_2, and Y_3 were found to be in the range $73.68 \leq Y_1 \geq 98.3$ µ m, $70.9 \leq Y_2 \geq 94.7\%$, and $70.0 \leq Y_3 \geq 93.4\%$, as depicted in Table 12.

Table 12: Constraints for responses

Name	Goal	Lower limit	Upper limit
Particle Size	Minimize	73.68	98.3
Entrapment Efficiency	Maximize	70.9	94.7
CDR %	In range	70.0	93.4

Constraints were set according to the formulation of porous microspheres using the minimum amount of excipients, which would give desired response values i.e., minimum particle size, maximum entrapment efficiency, and maximum drug release at 12 hrs. Based on the prediction, three formulations were selected and the responses of particle size, entrapment efficiency, and % cumulative drug release were evaluated. The validation for RSM involving all the three checkpoint formulations was found to be within limits. The composition of optimum checkpoint formulations, their predicted and observed values for all the responses, and the percentage error are shown in Table 13.

Table 13: Comparison of experimental results with predicted responses of ES 100 based Vildagliptin loaded porous microsphere formulations

Formulation Code	Polymer (gm)	Porogen (%)	Response	Predicted Value	Experimental value	% Error
OF1	1.26	1.00	Particle Size (µm)	82.28	79.42	-3.47
			%EE	90.08	94.02	4.37
			% CDR	70	72.64	3.77

OF2	1.30	1.00	Particle Size (µm)	83.32	81.08	-2.68
			%EE	91.21	90.42	-0.86
			% CDR	71.89	72.53	0.89
OF3	1.58	1.00	Particle Size (µm)	88.47	89.24	0.87
			%EE	94.99	90.65	-2.5
			% CDR	78.93	78.86	-0.08
Mean (SEM) of % Error						0.31

The recommended concentrations of the independent variables were calculated by the Design Expert software from the above plots which has the highest desirability near to 1.0.Using design expert software, optimized batch of vildagliptin porous microspheres were obtained from the overlay plot, with the level of X_1 and X_2 as 1.26 and 1.00 respectively (Formulation OF1). The theoretical values of Y_1, Y_2, and Y_3 were found to be 82.28%, 90.08%, and 70.00%, respectively were found to be in close agreement with the practical values.

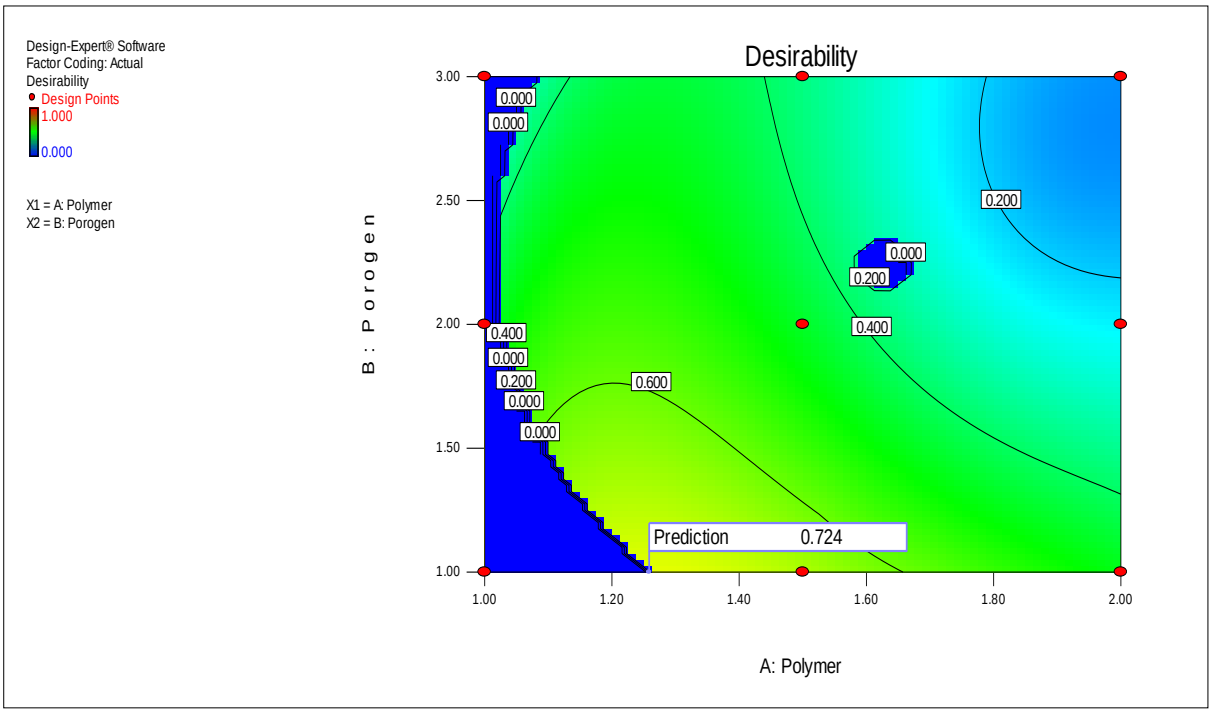

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Figure 4: Desirability plot for optimized formulation

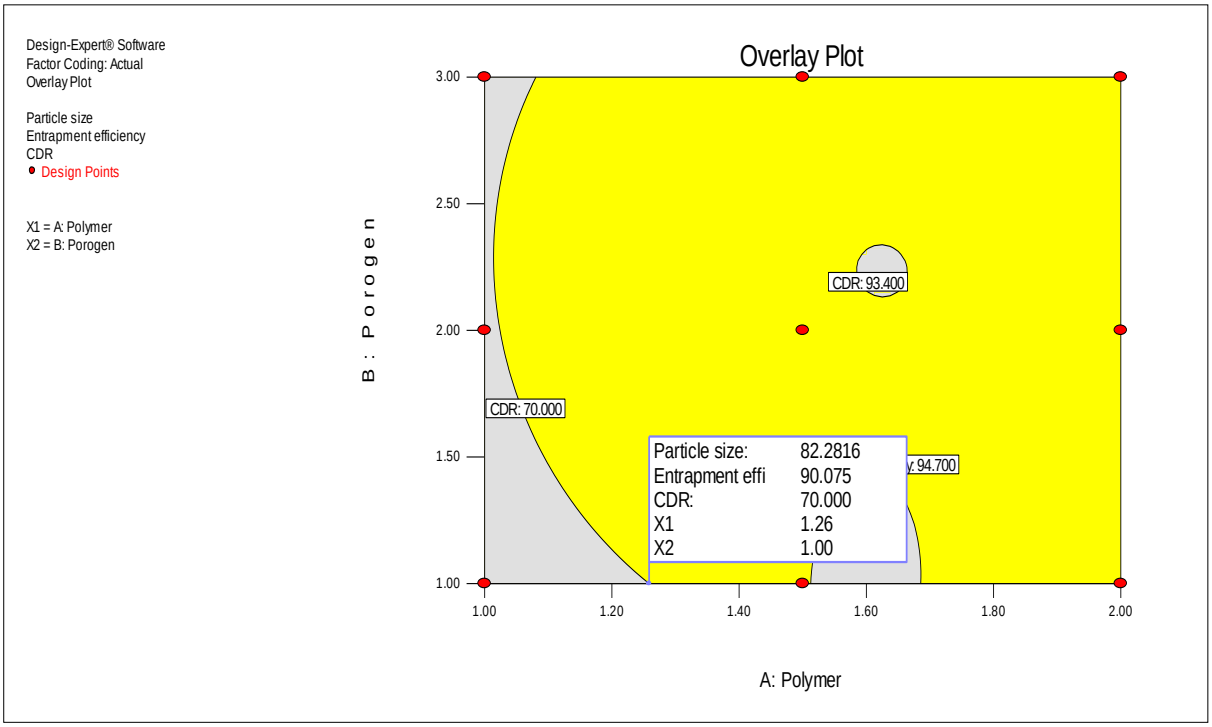

Figure 5: Overlay plot for optimized formulation

The statistically optimized formulation of vildagliptin porous microspheres (OF1) fulfilled all the physicochemical criteria. The formulation was evaluated for finding the experimental values of all the dependent variables to confirm the theoretical estimate. The relative errors (%) between the predicted and experimental values for each response were calculated and the values found to be within 1%. The experimental values were in agreement with the predicted values confirming the predictability and validity of the model. This formulation was considered to be the optimized formulation of vildagliptin porous microspheres.

High Performance Liquid Chromatography analysis of vildagliptin

The HPLC of Vildagliptin was performed and the chromatogram was obtained which showed the retention time of 3.08 min.

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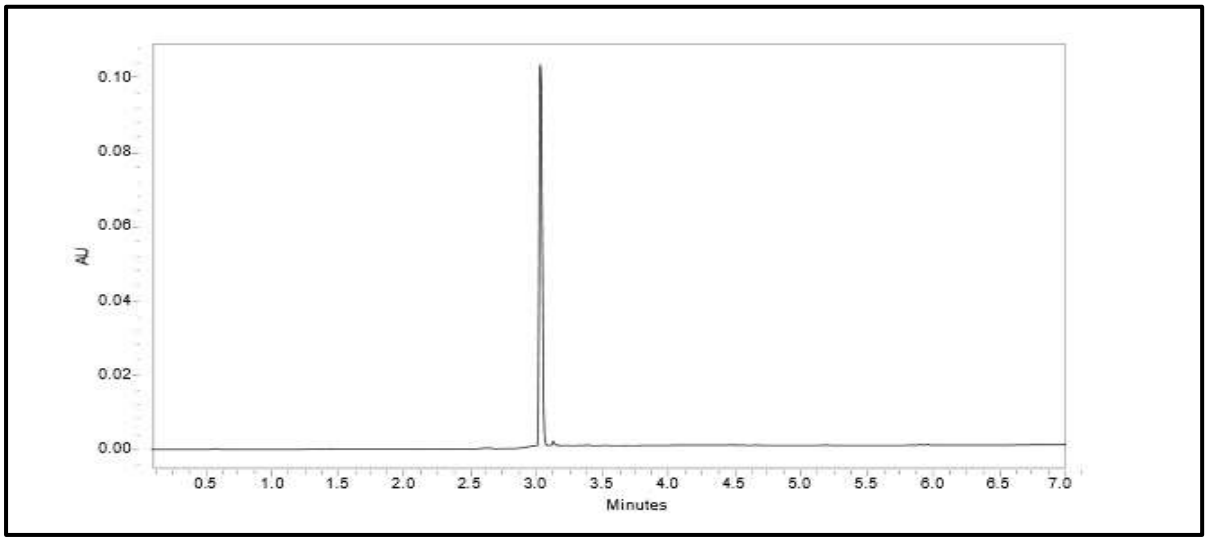


Figure 6: Chromatogram for Vildagliptin

Differential scanning calorimetry (DSC)

Figure 7: DSC spectra of Vildagliptin

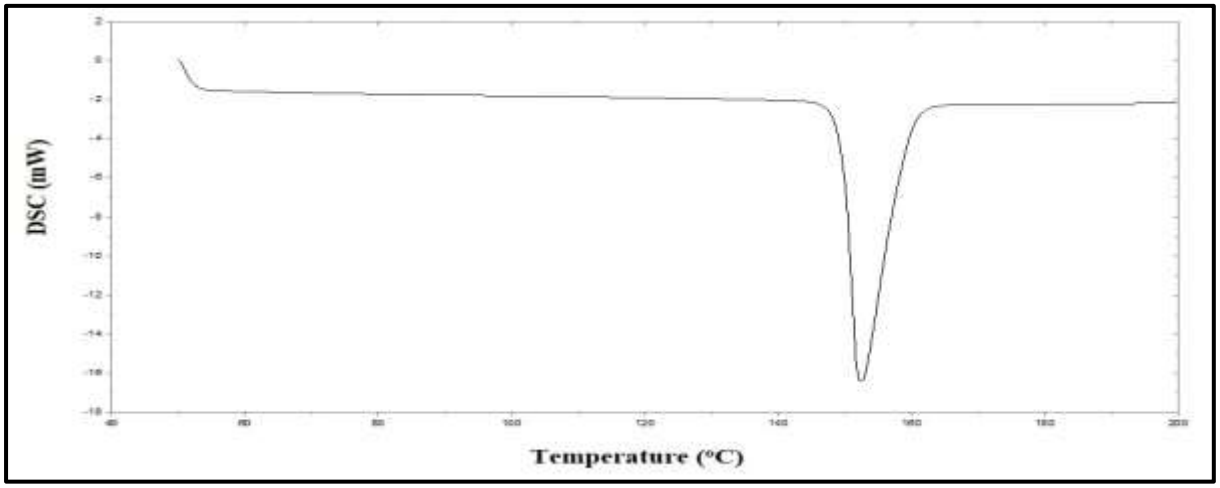


Figure 8: DSC spectra of Eudragit RL-100

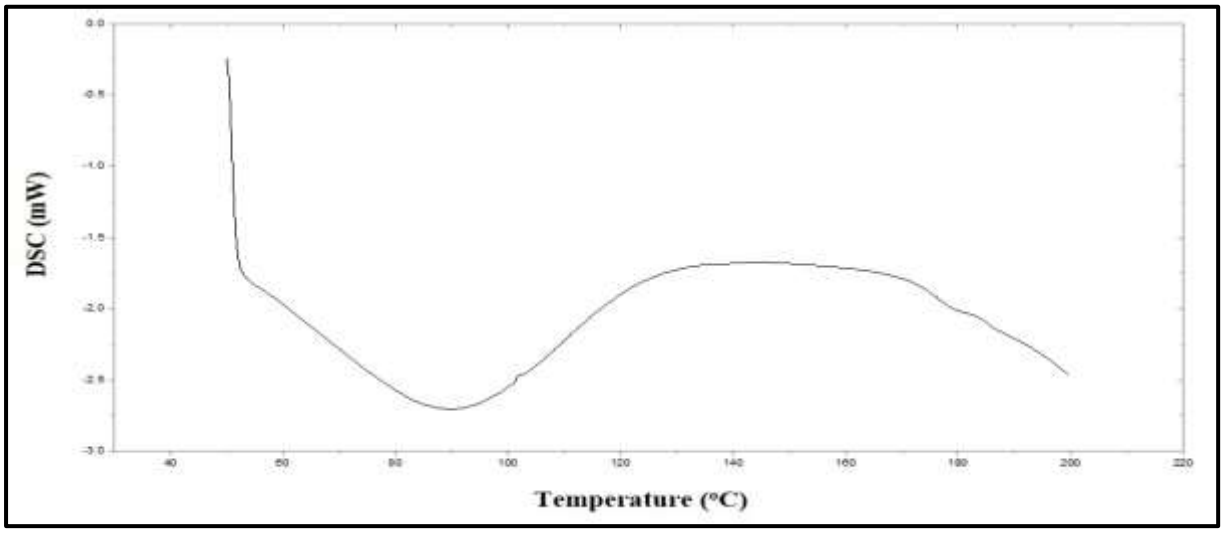
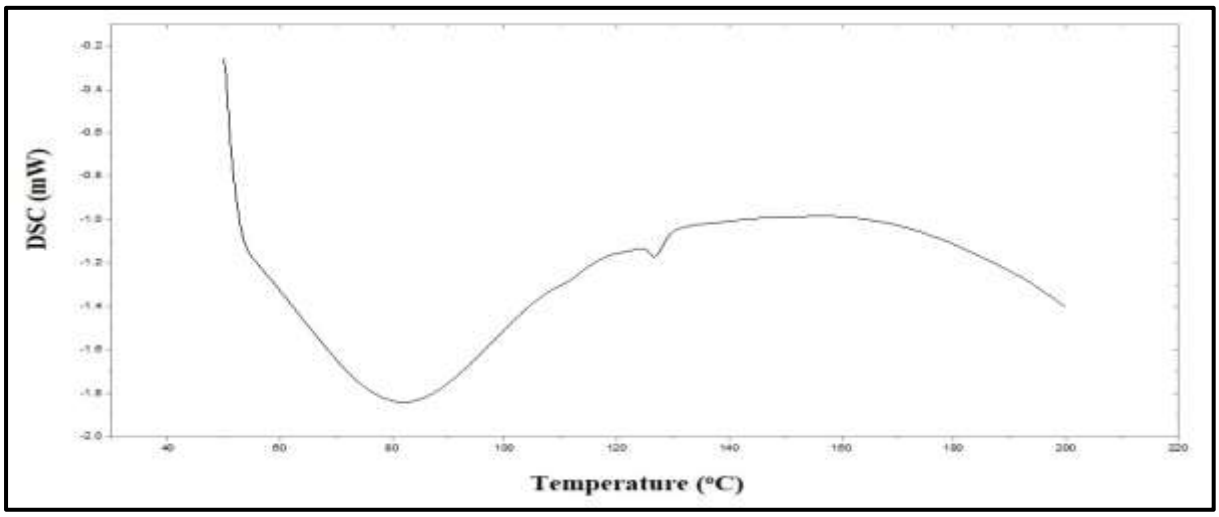


Figure 9: DSC spectra of Vildagliptin optimized formulation



DSC is a fast and reliable method for understanding polymorphic transitions when screening drugs and polymers for compatibility, obtaining information about possible interactions. According to the differential scanning calorimetry thermogram, Vildagliptin exhibited a sharp endothermic peak at 152.57°C corresponding to its melting point in the crystalline form (Figure 7) while (Figure 8) represents theromogram of Eudragit S-100. In case of optimized porous microspheres formulation, no characteristic peak was observed at 152.57 °C (Figure 9). It appears that there is a significant reduction of drug crystallinity in the floating microspheres. The absence of detectable crystalline domains in drug loaded porous microspheres clearly indicates that drug was dispersed completely in the formulation, thus modifying the microspheres to an amorphous, disordered-crystalline phase.

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Fourier Transform Infrared Studies

FTIR spectrum of pure drug Vildagliptin, polymer Eudragit S-100 and Vildagliptin loaded porous microspheres were shown in figures 10, 11 and 12. The characteristic peaks as obtained are listed in the table below which confirm the presence of the main groups of the drug. There is no considerable change in drug characterization peaks and the results obtained with drug-drug and drug–excipients showed good compatibility.

Figure 10: FTIR spectra of Vildagliptin

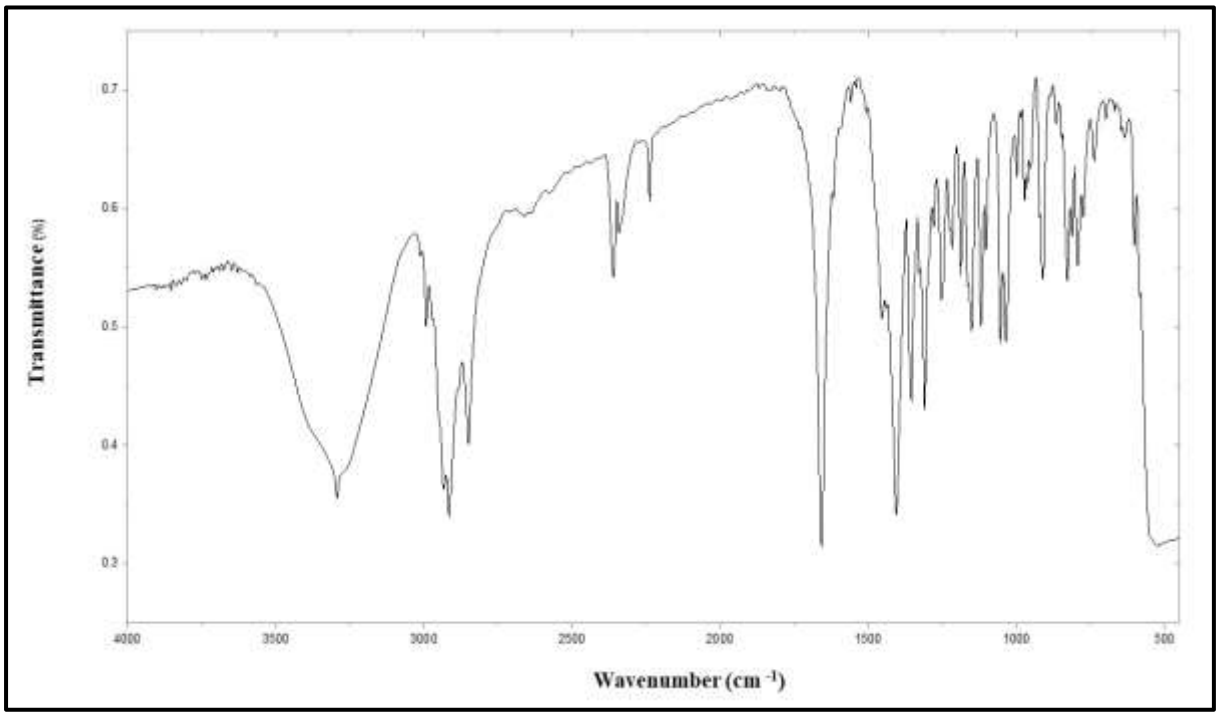

Figure 11: FTIR spectra of Eudragit S-100

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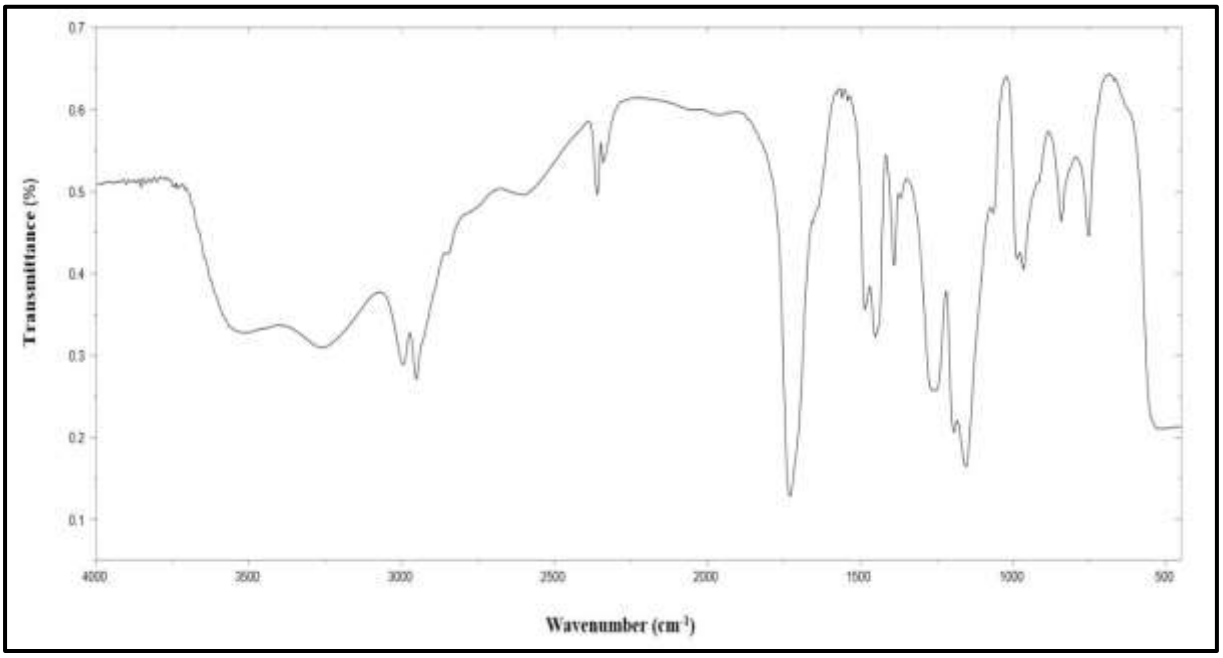
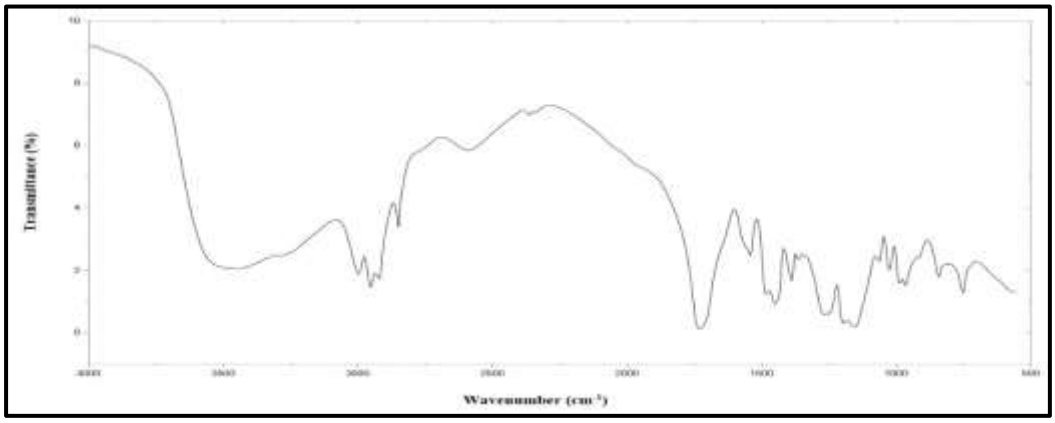


Figure 12: FTIR spectra of Vildagliptin loaded microspheres



Powder X-ray diffraction (XRD) study

Figure 13: XRD spectra of Vildagliptin

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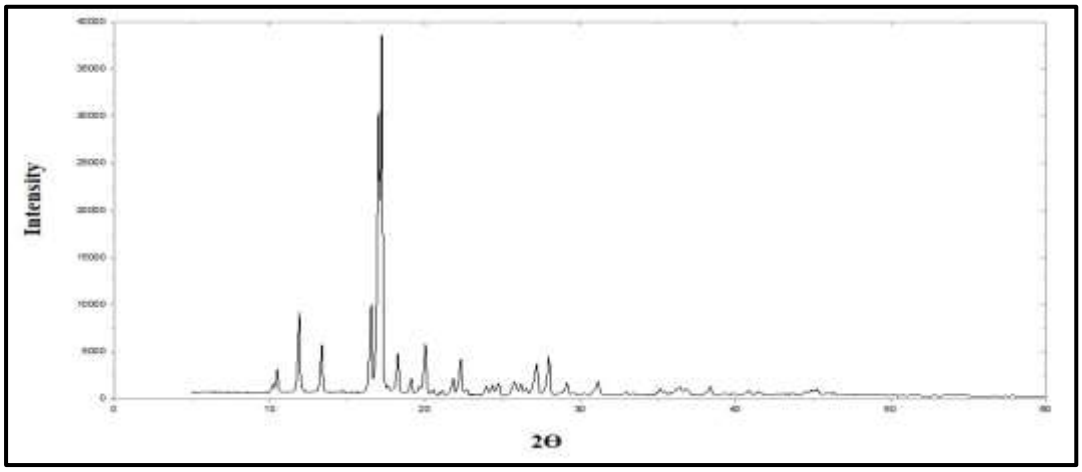


Figure 14: XRD spectra of Eudragit S-100

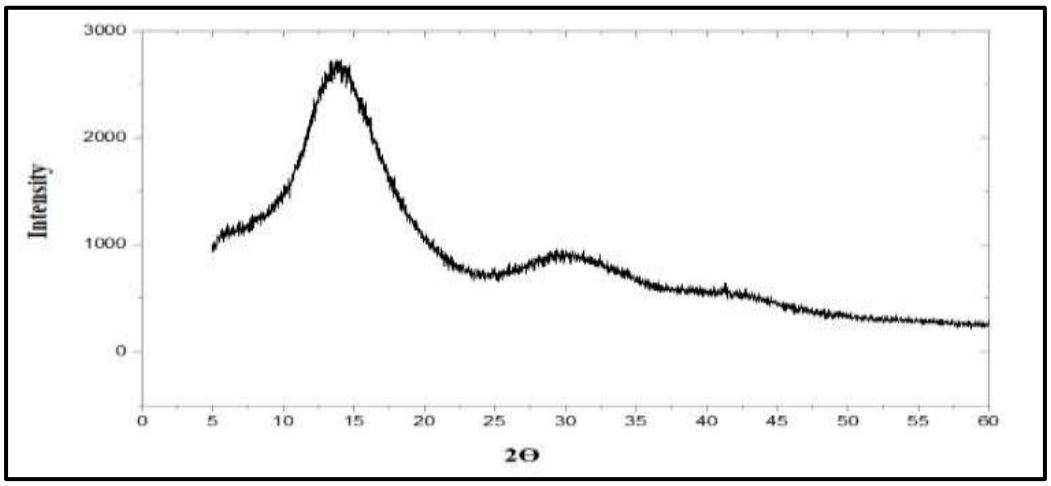


Figure 15: XRD spectra of Vildagliptin loaded microspheres

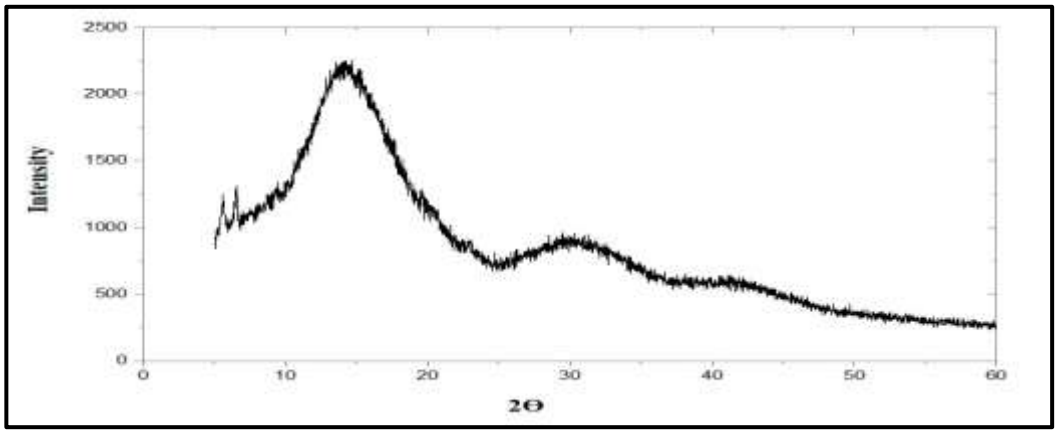


Figure 13,14 and 15 showed XRD patterns of the pure drug Vildagliptin, Eudragit S-100, and Vildagliptin-loaded porous microsphere formulation respectively. Distinct peaks in the XRD

pattern of the pure drug (a) are scattered at 2θ angles at 10.47°, 11.90°, 13.44°, 16.60°, 17.27°, 18.29°, 19.21°, 20.64°, 21.82°, 22.38°, 25.30°, 27,.34° and 28.05° were indicated crystalline nature.

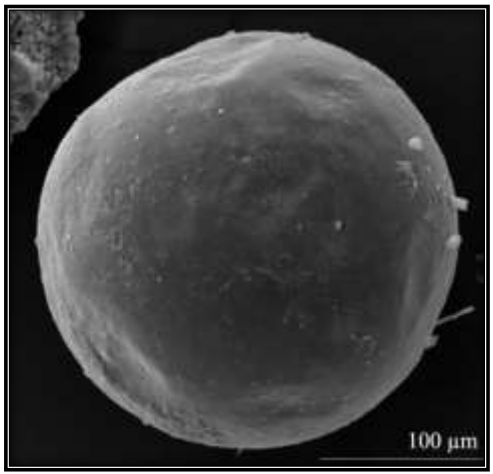
The XRD pattern of Eudragit S-100 (b) showed diffused peaks, due to its amorphous nature. However, the XRD results of drug-loaded porous microsphere formulation showed the absence of intense crystalline peaks of vildagliptin and the diffraction patterns were identical to the Eudragit S-100 polymer. These results strongly proposed that the degree of crystallinity of vildagliptin was decreased in the porous microsphere formulations due to the homogeneous distribution of vildagliptin within the Eudragit S-100 polymer at the molecular level.

Particle size analysis

Particle size distribution was in the range of 73.68 µm to 98.30 µm. Particle-size distribution is affected by the interaction between the dispersed phase and the dispersion medium. In our study, the mean particle size increases with the increase in polymer concentration due to increase in relative viscosity. Also it was because of the fact that polymer available at a higher concentration was in more amount thereby increasing polymer wall thickness which led to the greater size of porous microspheres.

Surface Morphology

Prepared batches of vildagliptin loaded porous microspheres were subjected to scanning electron microscopy (SEM) analysis for assessing their morphology and surface topography. The captured SEM images of porous microspheres are shown in Figures 16,17,18 and 19. Scanning electron micrographs of the porous microspheres taken at different magnifications reflected that porous microspheres formed were predominantly spherical, highly porous and smooth surface. The surface morphology analysis showed porous nature of the microsphere that could be useful to increase the surface area and dissolution. Porous structures were uniform on the surface as well as inside the microspheres, and interconnected open-pore structures were observed. Also the cross-sectional images of all the microspheres, depicts the presence of pores in the microspheres.



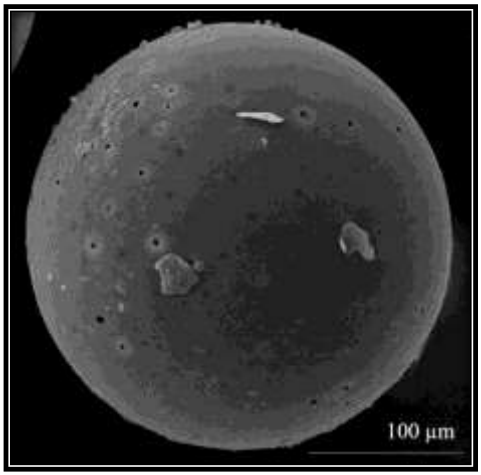


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Figure 16: SEM image of microsphere without porogen

Figure 17: SEM image of microsphere with porogen

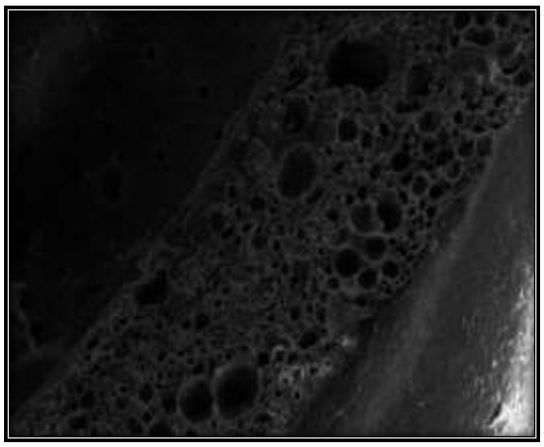

Figure 18: SEM image of porous microsphere at 1000 magnification

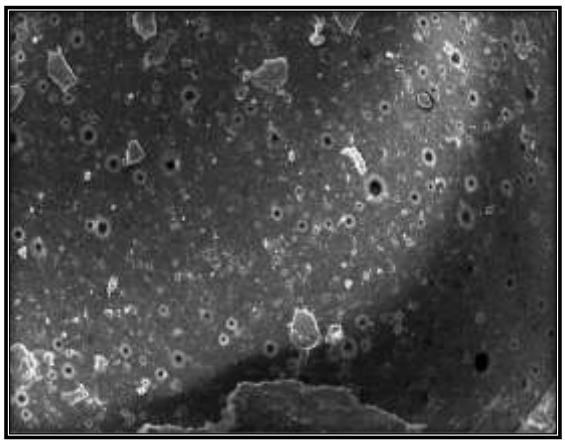

Figure 19: SEM image of porous microsphere at 500 magnification

Percentage yield of microspheres

Percentage yield of all the formulations prepared from double emulsion solvent evaporation technique was found in the range 64.13 to 93.14 % which is sufficiently high (Table 14). During porous microspheres formation, the product yield might be affected due to sticking of polymers to the wall of the beaker and blades of the stirrer and agglomerate formation also there may be loss of small particles during filtration and washing. As experimental results revealed percentage yield value was directly related to polymeric concentration.

Flow properties of porous microspheres

Flow properties of batches were evaluated by measuring the angle of repose, Hausner's ratio and Carr's index. The values of each parameter for all the formulations are mentioned in the Table 14. The porous microspheres showed the desired flowability due to the optimal presence of moisture, spherical shape, and diminished cohesiveness.

The angle of repose of all the formulations showed excellent flow-ability and ranged from 16.69 to 23.30°. The bulk density and the tapped density of all the formulations were within the range of 0.208 g/cm^3 to 0.250 g/cm^3 and 0.227 g/cm^3 to 0.286 g/cm^3 respectively. The Carr's index of all the formulations exhibited excellent flow properties and ranged from 8.33 to 14.29. Hausner's ratio of the formulated porous microspheres exhibited good flow properties that ranged from 1.09 to 1.17.

Thus, the angle of repose and compressibility index is indicative of good flow-ability of microspheres, showing no need for the addition of glidants to enhance flow-ability. The better flow property of porous microspheres indicated that the microspheres produced were nonaggregated. Excellent flow properties of prepared porous microsphere suggested less poly-dispersity, complete drying and particle size uniformity.

Determination of Entrapment Efficiency

The drug entrapment efficiency is important variable for assessing the drug loading capacity of porous microspheres. This parameter is dependent on the process of preparation, physicochemical properties of drug, and formulation variables. From the above results, it was found that % entrapment efficiency was in the range of 70.09 % to 94.7%. All batches show percentage entrapment efficiency more than 50%. This study suggests the increment in the polymer concentration results in an increment in entrapment efficiency. This observation might be due to the availability of a greater amount of polymer for the complete encapsulation of the Vildagliptin molecule. Drug encapsulation efficiency did not attain 100%. It was because of some drug dissolve in aqueous phase or solvent used.

***In vitro* dissolution studies of porous microspheres**

The release of drug from the porous microspheres was varied in the range of 64.60% to 93.40% in 12 hrs study. The release study of all the batches showed the wide variation in the pattern of the drug release (Figure 20). This shows the significant outcome of the chosen independent variables along the drug release from the polymeric matrix.

The drug release of vildagliptin from the porous microspheres showed a biphasic release behaviour, indicating an initial burst release followed by the sustained release over a period of 12 hrs. The porous surface of the prepared microspheres may contribute to the initial release of vildagliptin by giving quick access of dissolution medium to the drug. Also the initial burst of release was attributed to the solubility of vildagliptin in water.

While the later sustained release may be attributed by the presence of release retardant polymer Eudragit S-100 in the form of the matrix that controls the penetration of the dissolution medium in the porous microspheres. It might be due to the formation of a compact matrix of the microsphere and a relatively thicker layer of the polymer on the encapsulated drug. Thus, after the initial burst, the release became slow and prolonged. As the microspheres were not eroded in the gastric juice, the release rate of vildagliptin was determined by its diffusion from the rigid matrix structure of the porous microspheres.

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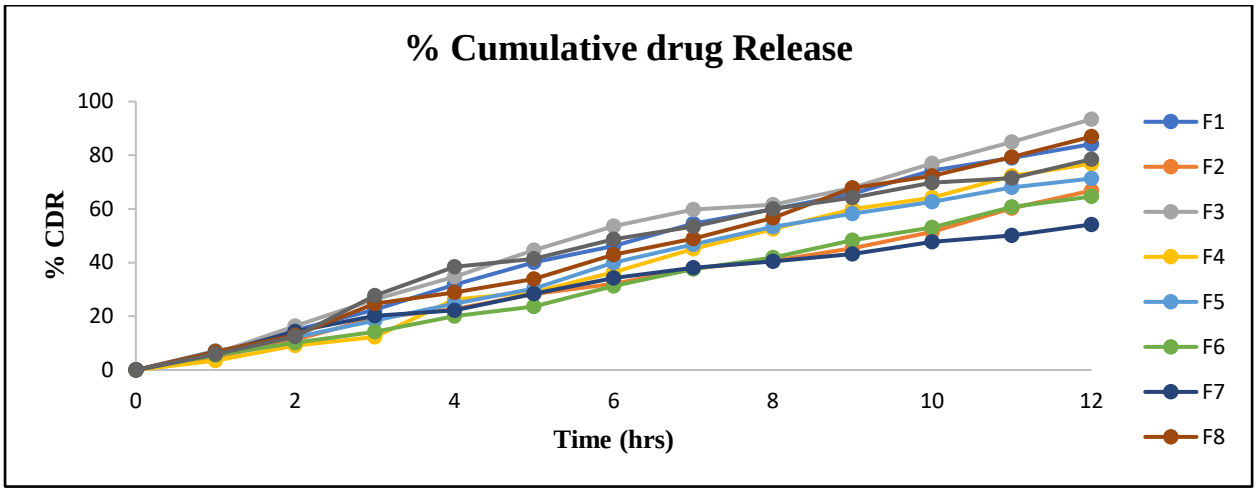

Figure 20: Cumulative drug release (%) for vildagliptin porous microspheres

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Formulation Codes	Bulk Density (gm/cm^3)	Tapped Density (gm/cm^3)	Carr's Index	Hausner Ratio	Angle of Repose (θ)	Particle Size (µm)	% Yield	% Entrapment	Cumulative drug release (%)
V1	0.244	0.270	9.76	1.11	20.21	95.50	78.37	85.5	84.10
V2	0.227	0.256	11.36	1.13	22.47	79.29	86.57	76.7	66.80
V3	0.250	0.286	12.50	1.14	19.63	93.50	88.75	94.7	93.40
V4	0.208	0.227	8.33	1.09	21.06	88.29	79.23	92.4	76.80
V5	0.216	0.242	10.81	1.12	20.13	90.30	73.54	89.5	71.30
V6	0.238	0.278	14.29	1.17	16.69	80.50	64.13	70.9	64.60
V7	0.242	0.271	10.53	1.12	20.51	73.68	83.73	80.6	54.20
V8	0.244	0.278	12.20	1.14	23.30	91.70	93.14	87.6	86.90
V9	0.234	0.270	13.16	1.15	18.30	98.30	81.45	82.3	78.50

Table 14: Evaluation of Vildagliptin loaded porous microspheres

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Drug Release Kinetics

The results for the analysis of model-dependent drug release kinetics, for the in vitro release of vildagliptin from the porous microspheres, is given in table 15. The model dependent approaches evaluated for the drug release kinetics were zero order, first order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas. The drug release from the porous microspheres of vildagliptin batches V1, V2, V3, V4, V5, V6 and V8 followed Zero Order model with R^2 value close to 1. This model is generated by plotting cumulative percentage drug release versus time. Other batches, V7 and V9 followed First order model. This data is obtained from in vitro drug release studies by plotting log percentage drug remaining versus time.

Table 15: Kinetics of drug release of all formulations

Formulation Code	Zero Order	First Order	Higuchi	Hixson-Crowell	Korsmeyer-Peppas	Best fit model
V1	0.9946	0.9665	0.9389	0.9898	0.8791	Zero Order
V2	0.9923	0.9558	0.9193	0.9744	0.883	Zero Order
V3	0.99	0.8842	0.9409	0.956	0.8612	Zero Order
V4	0.9935	0.9597	0.8966	0.9791	0.9413	Zero Order
V5	0.9938	0.9855	0.9287	0.9936	0.8648	Zero Order
V6	0.9972	0.9696	0.9031	0.9831	0.8943	Zero Order
V7	0.9786	0.9959	0.969	0.9928	0.8236	First Order
V8	0.9971	0.9247	0.9161	0.9654	0.8685	Zero Order
V9	0.9685	0.9901	0.9553	0.9928	0.8549	First Order

Residual solvent analysis

The gas chromatogram of standard chloroform solution in dimethyl sulfoxide (DMSO) and optimized formulation dissolved in DMSO is shown in figure 22 and 23. The retention time of Chloroform was 4.6 min as observed in standard preparation of chloroform. The peak of DMSO was observed at 11.8 minutes in both standard and sample preparation. But, no Chloroform peak at 4.6 min was observed in sample chromatogram. Chloroform residue was within the limits, in the Vildagliptin loaded porous microspheres. Hence, the prepared formulation OF1 is considered to be safe for human use.

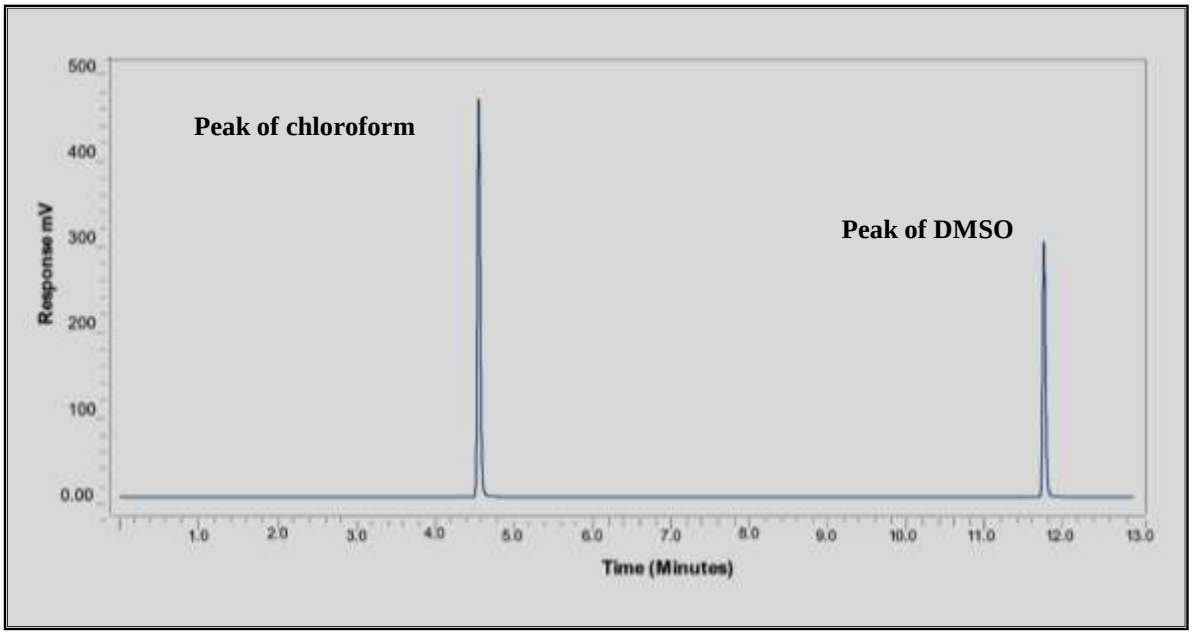

Figure 22: GC scan of standard chloroform solution

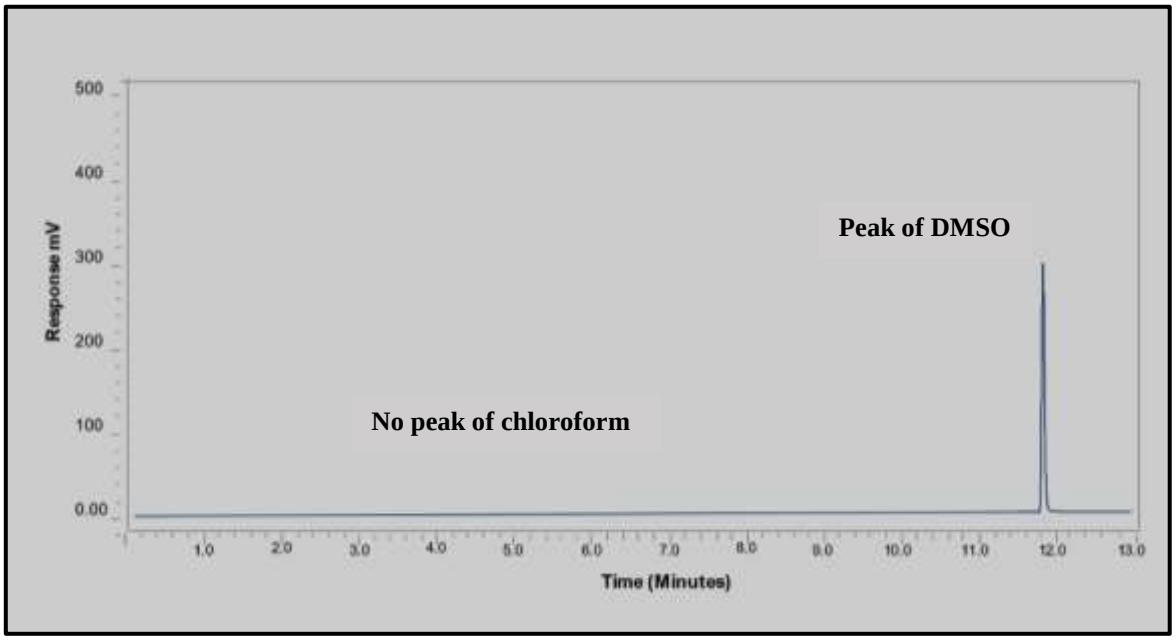

Figure 23: GC scan of optimized formulation prepared in DMSO solution

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***In Vivo* Antidiabetic Study**

The in vivo efficiency of the optimized Vildagliptin porous microsphere was performed in alloxan induced diabetic rats and was estimated by measuring the blood glucose level. It was seen that there was an increase in the glucose level after alloxan administration when compared with the normal group. As expected, vildagliptin showed a decrease in blood glucose level when compared with the alloxan group. Also, the optimized porous microsphere loaded with vildagliptin also showed a potential decrease in blood glucose level. The comparative in vivo blood glucose level along with the reduction in blood glucose level in alloxan-induced diabetic rats after oral administration of the optimized vildagliptin loaded microsphere is shown in figure 10. Treatment with formulation caused significant ($p < 0.01$) reduction in blood glucose level as compared to pure drug treated group of animals as evident from figure 10.

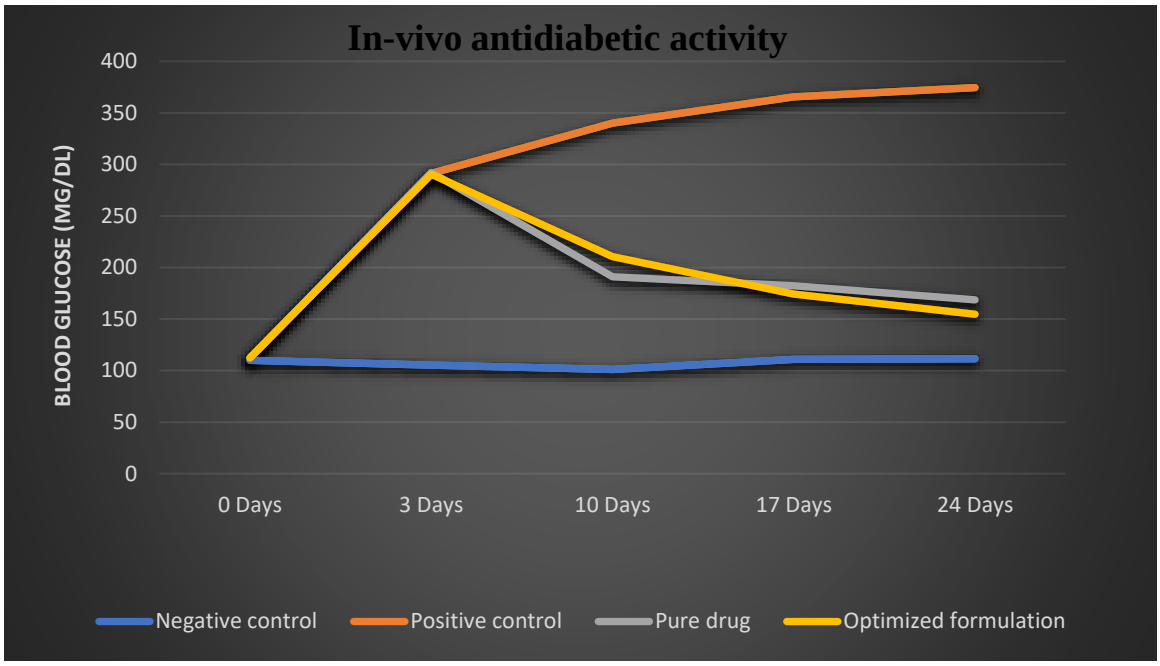

Figure 10. Comparative blood glucose level of different groups of animals

The alloxan diabetic model used for estimation of the effective nature of the prepared microsphere is resembled here. As seen in the blue line, there was no such enhancement of serum blood glucose level when estimated at different time interval of 0 days, 3 days, 10 days, 17 days and 24 days. The red line indicates the gradual increase in serum blood glucose level at different time interval for the alloxan treated group. Similarly, the grey line and the

yellow line represent the effectiveness of the standard drug vildagliptin and the prepared Eudragit -S100 porous microsphere loaded with vildagliptin, respectively, in controlling the serum blood glucose level when estimated in the given time interval.

Stability studies

Stability studies should include testing of those attributes of the drug product that are susceptible to change during storage and that are likely to influence quality, safety or efficacy.

Table 16: Stability studies

Evaluation parameters	Before stability storage	25°C ± 2°C, 60% ± 5% RH		40°C ± 2°C, 75% ± 5% RH	
		After 3 months storage	After 6 months storage	After 3 months storage	After 6 months storage
Shape	Spherical	Spherical	Spherical	Spherical	Spherical
Color	White	White	White	White	White
Particle size (µm)	79.42	79.86	79.91	79.31	79.14
Entrapment efficiency (%)	94.02	94.32	94.78	93.89	94.12
In vitro drug release at the end of 12 hrs. (%)	72.64	73.24	72.68	72.02	72.45

Physical appearance showed no significant variation in shape and colour. Similarly there was no significant change observed in the particle size, entrapment efficiency and drug release pattern of the drug on storage for six months. Thus, no visible physical changes were observed in the formulation throughout the study period in both the conditions.

Conclusion:

The results of this study emphasized that response surface methodology with 3^2 factorial is a very useful statistical technique to determine the effect of selected independent variables on the dependent variables. Vildagliptin loaded porous microspheres were prepared successfully by double emulsion solvent evaporation technique using the Eudragit S-100 polymer in various ratios. It was found that the prepared microspheres were spherical, porous, free

flowing, high percentage entrapment efficiency and high percentage yielding capacity. The method of preparation was found to be reliable and inexpensive. The results of optimization revealed that the concentration of polymer and concentration of PVA have a significant effect over response variables such as particle size, entrapment efficiency, and percentage cumulative drug release. The feasibility of the optimization procedure in developing porous microspheres can be demonstrated by close agreement between the observed responses and predicted values of the optimized formulation.

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