FORMULATION OF GASTRORETENTIVE MICROSPHERES OF REPAGLINIDE FOR STOMACH-SPECIFIC DELIVERY: STATISTICAL OPTIMIZATION, *IN-VITRO* AND *IN-VIVO* EVALUATION

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Abstract
During the study repaglinide encapsulated floating microspheres were formulated and characterized for enhancing residence time of drug in git and thereby increasing its bioavailability. Floating microspheres of Eudragit RL100 loaded with repaglinide were prepared by solvent evaporation technique. During process optimization various parameters were studied such as: concentration of polymer and concentration of emulsifier. Selected optimized formulation was studied for scanning electron microscope (SEM), entrapment, floating behaviour, drug release and kinetics. In-vivo floating ability (X-ray) study and in-vivo antidiabetic activity were performed on alloxan induced diabetic rats. Microspheres prepared with different concentrations of polymer were spherical shaped with smooth surface. Size of microspheres was in the range of 109.3 - 241.0 um. Good entrapment and buoyancy were observed for more than 12 hr. X-ray image showed that optimized formulation remained buoyant for more than 10 hr. Optimized formulation treated group shows significant ($p < 0.01$) reduction in blood glucose level as compared to pure drug treated group. Repaglinide loaded floating microspheres expected to give new choice for safe, economical and increased bioavailable formulation for effective management of diabetes mellitus.

Keywords Repaglinide, Eudragit RL100, Floating microspheres, Solvent evaporation technique.

Introduction
The majority of the traditional oral drug delivery systems have few impediments which are mainly associated to gastric residence of dosage form.[1] Control drug delivery systems aimed not just to extend the release of drug but also to lengthen its retention in its absorption site.[2] This can be achieved by designing several gastro retentive dosage forms like mucoadhesion, expandable systems, low density and high-density systems etc.[3,4]
Gastroretentive drug delivery system is the system which is retained in the stomach for a longer period of time and improves the bioavailability of drugs that are preferentially absorbed from upper gastrointestinal tract. Reported methods for the design of gastroretentive systems include mucoadhesion, Floatation, sedimentation, Swelling and expanding drug delivery system.[5]
Microencapsulation is a useful method for prolonging drug release from dosage form and reducing adverse effect.[6] Recently, dosage forms that can precisely control the release rates and target drugs specific body site have made an enormous impact in the formulation and development of novel drug delivery system. Microspheres form an important part of such novel drug delivery system.[7,8]
Microspheres are one of the multiparticulate delivery systems and are prepared to obtain prolonged or controlled drug delivery, to improve bioavailability or stability and to target drug to specific sites.[9]
Microspheres can also offer advantages like limiting fluctuation within therapeutic range, reducing side effects, decreasing dosing frequency, and improving patient compliance.[10]
Repaglinide is an oral anti-hyperglycemic agent belonging to meglitinide class, which requires frequent dosing before meals due to short half-life (1hr) and there by imposing side effects such as skeletal muscles pain, headache and GIT effects.[11,12] Floating drug delivery system increases the effectiveness of dosage forms by releasing the drug in control manner and thus maintaining its

concentration for longer duration.[13] Due to short lasting action, fast clearance, enzymatic stability and absorption window in upper GIT (stomach), makes repaglinide a suitable target for developing gastroretentive dosage form.[14]

Response surface methodology (RSM) is widely practiced approach in the development and optimization of drug delivery devices. Based on the principle of design of experiments (DoEs), the methodology encompasses the use of various types of experimental designs, generation of polynomial equations, and mapping of the response over the experimental domain to determine the optimum formulation(s).[15] The technique requires minimum experimentation and time, thus proving to be far more effective and cost-effective than the conventional methods of formulating dosage forms.[16]

The current study aims at developing and optimizing microspheres of Repaglinide using RSM, as it may prove to be more productive than the conventional systems by virtue of prolongation of drug residence time in gastrointestinal tract. Further, microspheres of the drug would involve relatively more economical and less complicated technology vis-a-vis many other drug delivery devices.[17] Computer-aided optimization technique, using a full factorial design, was employed to investigate the effect of 2 independent variables (factors) (i.e., the drug-to-polymer ratio and stirring speed) on particle size, encapsulation efficiency, and drug release.

Materials and method

Materials

Repaglinide was obtained from Torrent Ltd, Mumbai, India as gift sample. Eudragit RL 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

Repaglinide floating microspheres were obtained by solvent evaporation technique. Firstly, polymer (Eudragit RL-100) was dissolved in Chloroform and then the drug (Repaglinide) was added to the above solution. The polymer drug solution so obtained was injected into the PVA solution maintained at variable speed using mechanical stirrer. Stirring was continued for required period until all the chloroform evaporated. The formed microspheres were collected by filtration and washed with n-Hexane and dried to obtain free flowing microspheres.[18,19]

Optimization of formulation

From the preliminary studies, it was found that there are two very important factors i.e concentration of polymer (Eudragit RL-100) and concentration of PVA, which effect the floating microspheres. Hence, it was decided to apply central composite design (CCD) design to study the effect of independent variables on dependent variables.

Response surface methodology is a tool to scrutinize the effect of a wide range of independent variables on response variables with the collection of statistical and mathematical techniques. The CCD was adopted to optimize the responses and to find out optimum process parameters. The concentration of polymer (X_1) and concentration of PVA (X_2) were considered as independent variables in the preparation of floating microspheres. Each factor was studied at three levels (−1, 0, and +1) as given in table 1. The CCD suggested 13 runs, composed of eight factorial points plus five centre points to determine the optimum concentrations of independent variables.

Table No.1: Levels of process variables in central composite design for repaglinide floating microspheres

Code	Independent Variable	Levels				
		-α	-1	0	+1	+α
X_1	Concentration of polymer (gm)	0.8	1	1.5	2	2.2
X_2	Concentration of PVA (%w/v)	0.4	0.5	0.75	1	1.1

	Dependent Variable
Y_1	Particle size (um)
Y_2	Entrapment efficiency (%)
Y_3	Buoyancy (%)
Y_4	Cumulative drug release (%)

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. One optimum checkpoint formulation was selected to evaluate optimization capability of models generated using central composite design. Optimized formulation (OF1) (run no. 14) was 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.

Characterization of drug

High performance liquid chromatography studies

High performance liquid chromatography (HPLC) was performed for the estimation of Repaglinide in pure form. The mobile phase comprises of mixture of ammonium acetate buffer (0.01 M pH 4.0) and methanol in the ratio of 20:80 % v/v, and was pumped at rate of 1.0 mL/min. The column temperature was maintained at 23 ± 1°C. The detection of Repaglinide was carried out by photodiode-array detection (PDA) detector at wavelength of 268 nm, column temperature was maintained at room temperature and injection volume was 20µL. The total run time was 15 minutes, and its retention time were recorded.[20]

Drug-excipients compatibility studies

Compatibility of drug with excipients was studied by Fourier Transform Infra-red spectroscopy (FT-IR). Effect of process of entrapment on crystallinity of the drug was studied by Differential Scanning Calorimetry (DSC).

Fourier transform infrared studies

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 analyzed by mixing with potassium bromide (1:10) individually and pressed to form a thin pellet by applying pressure using KBr press.[21] 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 Repaglinide, Eudragit RL-100, optimized formulation were recorded.[22,23]

Differential scanning calorimetry (DSC)

Differential scanning calorimetery (DSC) of pure Repaglinide, Eudragit RL-100 and Repaglinide loaded Eudragit RL-100 microspheres (optimized formulation) was done using Mettler Toledo differential scanning calorimeter to determine any possible Repaglinide-polymers interaction. The samples were triturated separately to obtain a fine divided powder. Approximately 3-5 mg sample was weighed into aluminium pans and hermetically sealed. The samples were heated from 40 - 200°C at 10°C/min under nitrogen atmosphere with gas flow rate 20 ml/min. A covered, empty pan was used as a reference. The results obtained from the heating were recorded.[24]

Powder x-ray diffraction (XRD)

The crystallinity of Repaglinide in formulations was assessed by XRD analysis. XRD diffraction analysis for Repaglinide, Eudragit RL-100, and Repaglinide loaded Eudragit RL-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 5º to 60º, having step width 0.03 and a scan time of 0.5 seconds per step.[24,25]

Evaluation of microspheres

Particle size analysis

The particle sizes of floating microspheres were determined by optical microscopy. Optical microscope was fitted with eye piece micrometer which was then calibrated with a stage micrometer.[25] 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 floating microspheres, and ''d'' for the mean size range.

Surface morphology

The prepared Repaglinide floating microspheres were morphologically examined for shape, size and surface morphology using scanning electron microscope. Dry floating 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. Floating 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.[26]

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.[27]

Drug entrapment efficiency

The floating microspheres equivalent to 50 mg of repaglinide were weighed accurately and crushed. The powdered microspheres were placed in 10 ml of ethanol and kept for 12 h. The solution was then filtered through Whatman filter paper No. 44.[14,28] The solution was diluted with fresh solvent and absorbance was measured at 268 nm using UV spectrophotometer (Shimadzu 1700) and the percent drug entrapped was calculated as follows:

$$\%Drug\ Entrapment\ Efficiency = \frac{Calculated\ drug\ content}{Theoratical\ drug\ content}\ x\ 100$$

***In vitro* evaluation of floating ability**

Floating microspheres (50 mg) were placed in simulated gastric fluid (pH 1.2, 100 ml) containing Tween 20 (0.02 w/v %) and stirred at 100 rpm using a magnetic stirrer. After 12 h, the layer of buoyant microparticles was pipette and separated from the settled microspheres by filtration. Particles of both the types were dried and weighed.[29] The buoyancy of microspheres was calculated by using the formula given as

$$Buoyancy\ (\%) = W_f / (W_f + W_s)\ x\ 100$$

where W_f and W_S are the respective weights of the floating and settled microparticles.

***In vitro* release studies of floating microspheres**

The drug release rate from floating microspheres was determined using paddle type six-station dissolution test apparatus (LABINDIA, DS8000). A weighed amount of floating microspheres equivalent to 16 mg drug was kept in 0.1 N HCl (1.2 pH) having Tween 20 (0.02 w/v %) maintained at 37 ± 0.5^{o}C at a rotation speed of 100 rpm. Sink condition was maintained during the study. 1 ml sample was withdrawn at 30 min time interval, passed through 5 um membrane filter and analyzed spectrophotometrically at 268 nm.[30]

Kinetic modelling of drug release

In order to understand the kinetic and mechanism of drug release, the Repaglinide 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).[31] 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.[32]

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.[33]

In the present work, chloroform was used in the preparation of repaglinide floating 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 repaglinide floating microspheres. Formulation was tested by a gas chromatograph (GC) using flame ionization detector. For this study, 10 mg of optimized floating 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.[34]

***In vivo* study**

Floating behavior (radiographic study)

Healthy albino rats, weighing 500-600 g were treated with optimized formulation and monitored through radiological method with modification.

The study was approved by Institutional Animal Ethics Committee, Indore institute of pharmacy, Indore, Madhya Pradesh (Protocol No: IIP/IAEC/2022/03). Animals were housed individually in polypropylene cages and maintained under standard conditions (12-h light and 12-h dark cycle; 25-30℃). The animals were fasted for 12 h and at first X-ray was taken to ensure absence of radio opaque material in the stomach. During the study animals were not allowed to eat food but water was provided ad libitum. Radiopaque microspheres were prepared by incorporating 500 mg of barium sulfate into polymeric solution and similar procedure by which optimized formulation was prepared was followed. At varying time intervals X-ray photographs (Siregraph-B, Siemens, Karlsruhe, Germany) of gastric region were taken for monitoring the floating behavior of microspheres [35].

Antidiabetic study

Experimental animals

At first Wister albino rats (250 - 300g) were maintained under laboratory condition. They were fed a regular laboratory meal and acclimatized 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 utilization 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.[36]

Experimental design

The rats were divided into 4 groups comprising 6 animals in each group as shown in table 2.

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 (Pure Repaglinide). Diabetic rats were treated with 2.5 mg/kg body weight of pure Repaglinide.

Group IV (Optimized formulation). Diabetic rats were treated with 2.5 mg/kg body weight of Repaglinide containing 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 Repaglinide 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.[37]The blood glucose level was estimated in all groups, before and after treatment on 3rd, 10th, 17th and 24th day from the overnight fasted rats.

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 No. 2: Animal required experimental study

Sr No.	Drug	Animal	Age	No. required	Study
1.	Group I (Negative control)	Wister albino rat	7-8weeks	06	Antidiabetic study
2.	Group II (Positive control)	Wister albino rat	7-8weeks	06	Antidiabetic study
3.	Group III (Pure Repaglinide)	Wister albino rat	7-8weeks	06	Antidiabetic study
4.	Group IV (Optimized formulation)	Wister 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 Repaglinide tablets (Eurepa® (2mg); Torrent). Dissolution profile was conducted in acidic medium (0.1 N HCl, pH 1.2, volume 900 ml) maintained at 37 ± 0.5^0C by using USP type II dissolution test apparatus (LABINDIA, 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 analyzed for % drug release by using UV spectrophotometer at 268 nm.

Stability study

Stability studies were performed to check the effect of environmental conditions or storage conditions on the microspheres. The optimized formulation were kept in glass bottle and stored for 6 months at 25°C ± 2°C + 60% ± 5% RH (Relative humidity) and 40°C ± 2°C + 75% ± 5% RH in stability chamber.[38] The samples were analyzed at periodically for their physical appearance, entrapment efficiency, total floating time and in vitro drug release. Obtained results for each interval of stability study were compared with zero-month results.

Results and discussion

Preparation and optimization of hollow microspheres

The hollow microspheres are multiple unit drug delivery systems and are the most efficient form of delivery systems as they can be extensively distributed throughout the stomach and may provide efficient release of drugs at the target site. Floating hollow microspheres were prepared by solvent evaporation method. Using above technique, the polymeric microsphere was prepared with an internal spherical hollow cavity produced by diffusion and subsequent evaporation of solvent mixture. The effect of independent variables such as the concentration of polymer and concentration

of polyvinyl alcohol (PVA) was studied on the physicochemical properties of the hollow microspheres such as particle size, entrapment efficiency, buoyancy, and cumulative drug release employing experimental CCD. In response surface methodology, various empirical models are fitted to experimental data which depend on the experimental design.[39] In this study, the CCD was selected as an experimental design, as it adequately describes the interaction between the factors with the least number of experiments.[40] The preliminary studies were conducted to ascertain the levels of independent variables for optimization.[41]

The Design-Expert software computed the statistical parameters, and it indicated that all responses generated using experimental design were best fitted into various polynomial models. Table 3. represents the central composite design with the effect of process variables on response values.

Table No. 3: Central composite design with the effect of process variables on response values

Code	Conc of polymer (gm)	Conc of PVA (%w/v)	Particle size (um)	Entrapment efficiency (%)	Buoyancy (%)	Cumulative drug release (%)
F_1	2	0.5	241	98.27	83.71	70.24
F_2	1.5	1.10355	128.9	83.49	55.75	90.63
F_3	1.5	0.396447	229.5	92.11	81	72.14
F_4	1.5	0.75	192	87.77	66.72	82.35
F_5	1.5	0.75	188	87.82	62.6	81.09
F_6	1.5	0.75	176.2	87.11	64.04	83.53
F_7	1.5	0.75	178.9	86.41	62.82	81.11
F_8	2.20711	0.75	227.5	96.43	83.65	73.66
F_9	1.5	0.75	170.9	88.4	63.59	78.54
F_{10}	2	1	179	87.26	69.17	84.36
F_{11}	1	1	109.3	80.79	50.98	94.35
F_{12}	1	0.5	180.4	82.4	73.41	79.42
F_{13}	0.792893	0.75	132.9	80.72	63.66	89.93

The observed responses, i.e., particle size (Y_1) and entrapment efficiency (Y_2), were fitted best into linear and 2FI response surface model, respectively (p-value < 0.0001 for Y_1 and Y_2), whereas percentage buoyancy (Y_3) and percentage cumulative drug release (Y_4) are fitted best into quadratic and linear response surface model (p-value < 0.0001 for Y_3 and Y_4) with no transformations of data. The polynomial equations for all dependent variables are as follows:

$Y_1 = +181.54 + 65.83\ X_1 - 135.22X_2$

$Y_2 = + 59.05 + 25.23X_1 + 15.79X_2 - 18.80X_1X_2$

$Y_3 = +143.58 - 50.75X_1 - 102.87X_2 + 15.78X_1X_2 + 17.70X_1^2 + 28.57X_2^2$

$Y_4 = +76.64 -10.54X_1 + 27.59X_2$

The above polynomial equations indicated the correlation between process variables and responses. Table 4 represents the effect of factors on responses and their associated p-values.

Table No.4: Summary of factor effect and associated p-values for all responses

Factors	Y_1		Y_2		Y_3		Y_4	
	Effects	p-values	Effects	p-values	Effects	p-values	Effects	p-values
X_1	+66.021	< 0.0001	+25.239	< 0.0001	-50.758	< 0.0001	-10.544	< 0.0001
X_2	-137.684	< 0.0001	+15.794	< 0.0001	-102.876	< 0.0001	+27.599	< 0.0001
X_1X_2			-18.800	0.0001	+15.780	0.0386		
X_1^2					+17.704	0.0001		
X_2^2					+28.578	0.0190		

Any model was considered to be significant if the *p*-value < 0.05 at 95% confidence level. The positive and negative signs depict the synergistic effect and antagonistic effect, respectively, which is having a relative impact of each factor on the responses.

Table 5 shows the result of analysis of variance analysis on models, and the summary statistics indicated that the response surface model developed for all response variables is significant, having $p < 0.05$ with non-significant "lack of fit" ($p > 0.05$), which ensures the reliability of applied model.

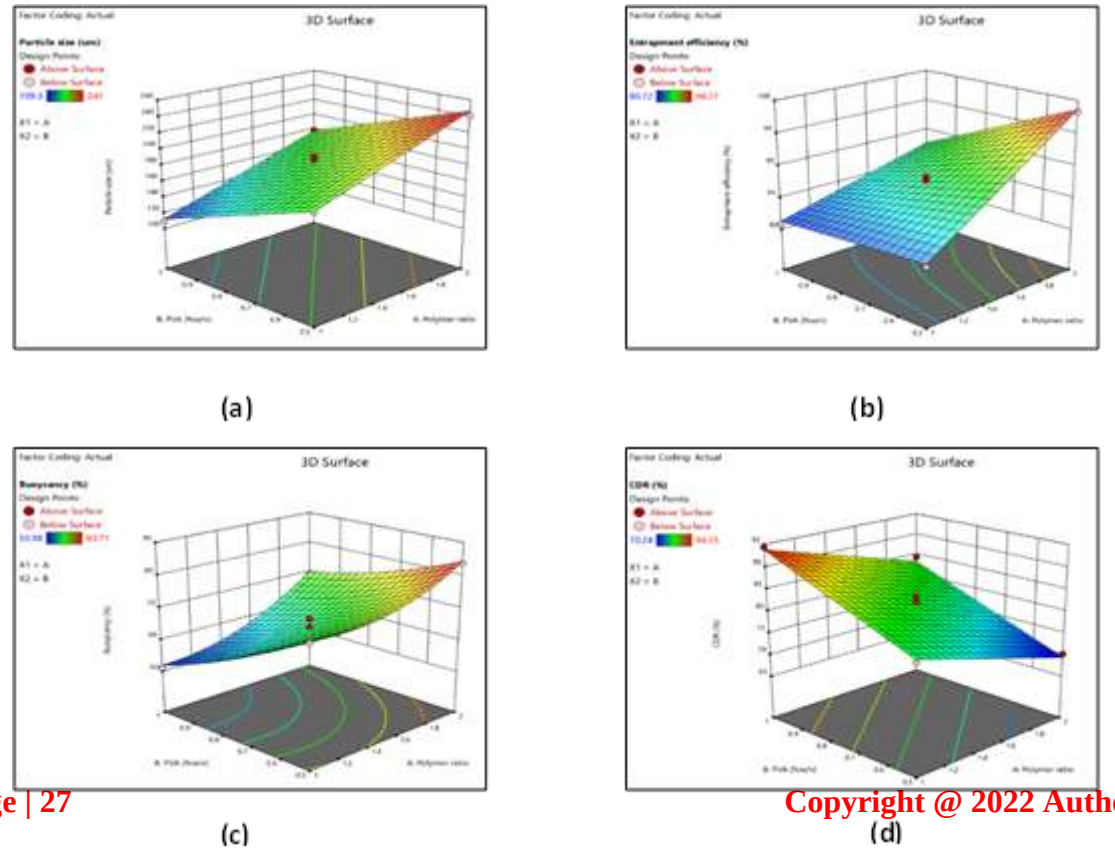
Table No.5: Detail summary of statistical analysis of response surface models

Model									Lack of fit	
Response factor	*F*-value	*P*-value	R^2	Adjusted R^2	Predicted R^2	Adeq. Prec.	C.V.	Std dev.	*F*-value	*P*-value
Y_1	249.44	<0.0001	0.9803	0.9764	0.9740	46.4847	3.36	6.04	0.15	0.9800
Y_2	204.93	<0.0001	0.9856	0.9808	0.9678	45.3336	0.8577	0.7515	0.95	0.5353
Y_3	101.63	<0.0001	0.9864	0.9767	0.9521	30.6756	2.29	1.55	0.73	0.5859
Y_4	164.59	<0.0001	0.9705	0.9646	0.9587	37.4358	1.66	1.35	0.22	0.9500

The reliability of models was denoted by higher R^2 values and good agreement between predicted R^2 and adjusted R^2. In addition to the above, adequate precision measures the signal-to-noise ratio, and the higher values of adequate precision (>4) signify those models are fitted to navigate the design space.

A three-dimensional response surface model analysis graphs were generated for the optimization of hollow microspheres by response surface methodology. The three-dimensional response surface plots for all the responses are presented in Figure 1.

Fig. 1: 3D Response surface curve for (a) Particle size (b) Entrapment efficiency (c) Buoyancy (%) (d) Cumulative drug release (%)



(a) (b)

(c) (d)

The response surface plot of particle size illustrates the interaction and effect of investigated variables as shown in Figure 1a.

The polymer ratio and concentration of PVA had a pronounced effect on particle size. The particle size decreases with the increase in the concentration of PVA, which reduces the interfacial tension more efficiently, and polymers become more uniformly dispersed in aqueous phase by stabilization of polymer droplets.[42] Concentration of polymer had a positive effect on particle size. This may be due to a greater concentration of the polymer in the formulation solution which may require greater force to breakdown the polymer into finer particles as supported by the previous studies.[43] However, the concentration of surfactant might contribute to the change in the particle size. An increase in the surfactant results in the formation of the relatively compact matrix which will help to decrease the particle size of the prepared microsphere.[44]

Figure 1b represents the response surface curve of entrapment efficiency. The equation indicates that X_1 i.e., polymer concentration had positive effect on entrapment efficiency of floating microspheres which indicates that the higher amount of Eudragit RL100 contributes to increase in entrapment of drug. Factor X_2 i.e., concentration of surfactant had negative effect on the entrapment efficiency of floating microspheres.

As the concentration of polymer increased, encapsulation efficiency is increased; this is due to the fact that higher amount of polymer would produce large size droplets with decreased surface area, such that diffusion of drug from such microsphere will be slow, resulting in higher encapsulation efficiency.[17,45] While, increase in surfactant concentration leads to decrease in encapsulation efficiency of microspheres due to the fact that increase in surfactant concentration leads to stabilization of small droplets and results in smaller microspheres.[46,47]

The effect of process variables on buoyancy can be measured as shown in Figure 1c. The *in vitro* buoyancy of microspheres can be correlated to low density and insolubility of polymers in the simulated gastric fluid (pH 1.2). As reported by Sato *et al.*, the buoyancy of particles depends on their density and size. The size of microspheres exhibited an inverse relationship to the microsphere density. Hence, the buoyancy of microspheres increased with an increase in particle size, which can be directly related to the increase in the ratio of polymers and inversely related to the increase in the concentration of PVA. The microspheres prepared with a high concentration of PVA were smaller in size and less dense than those prepared with a lower concentration of PVA. The density of Eudragit RL100 is 0.8 g/cc, which is lower than gastric fluid,[48] so the particles having a higher ratio of Eudragit RL100 are less dense and more buoyant.

Figure 1(d) illustrates the combined effect of independent variables on the cumulative release of Repaglinide. The response surface curve inferred that on increasing the relative fraction of Eudragit RL100, the percentage cumulative drug release decreases. In general, an increase in polymer concentration increased the diffusion path of drug which decreases the overall drug release from the polymer matrix. The extent of drug release was predominantly dependent on PVA levels as it increases the wettability of particles and better solvent penetration.[49,50] Typically, the formulation made with higher PVA and lower Eudragit RL100 levels exhibited a high percentage cumulative drug release. Hollow microspheres with desired parameters were accomplished by desirability approach using numerical optimization tool of Design-Expert software. The goal was to obtain the optimum values of the independent variables. Optimization was performed by setting constraints such as particle size and cumulative drug release in range and buoyancy and entrapment efficiency kept maximum. The Design-Expert software provided the single solution with the highest desirability was selected for optimized formulation. The optimized values of parameters obtained were the concentration of polymer (X_1) of 2.00 gm and the concentration of PVA (X_2) of 0.5% w/v. The results for the final optimized concentration are tabulated in Table 6.

Table No.6: Predicted values, experimental values and percentage prediction error for the responses at optimized concentration

Formulation Code	Polymer (gm)	PVA (%)	Responses	Predicted values	Experimental values	% Error
OF_1	2.00	0.5	Particle Size (µm)	240.99	239.22	0.73
			%EE	97.68	96.46	1.24
			%Buoyancy	82.56	82.42	0.16
			% CDR	70.67	69.89	1.10
Mean (SEM) of % Error						0.80

Characterization of drug

The HPLC of Repaglinide was performed and the chromatogram was obtained with the retention time of 6.104 min as shown in figure 2.

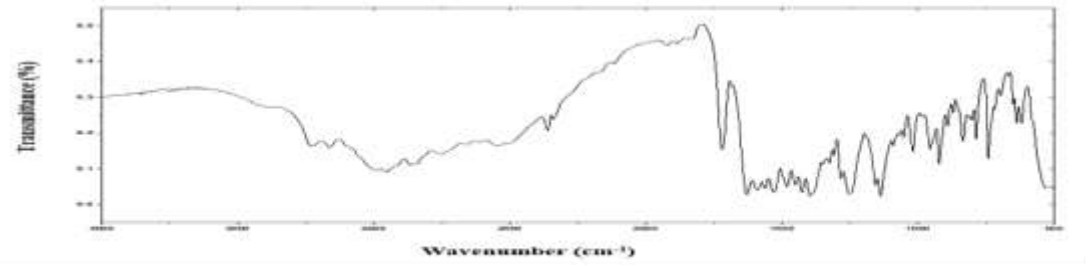


Fig. 3a

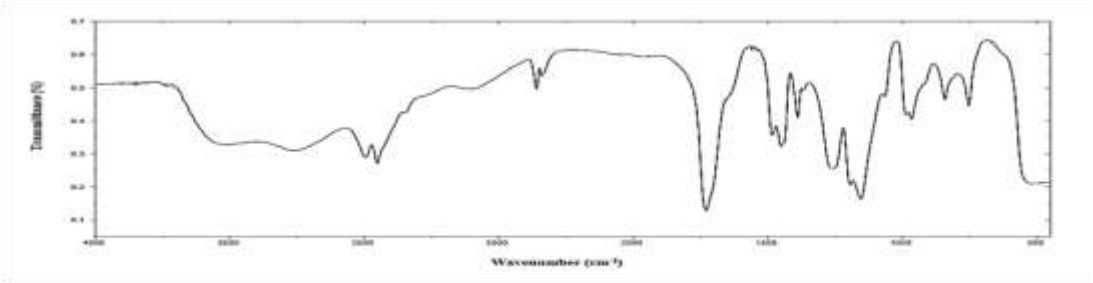


Fig. 3b

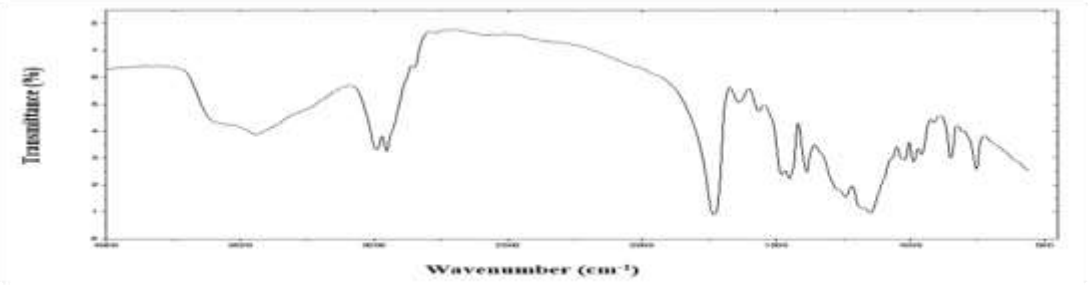


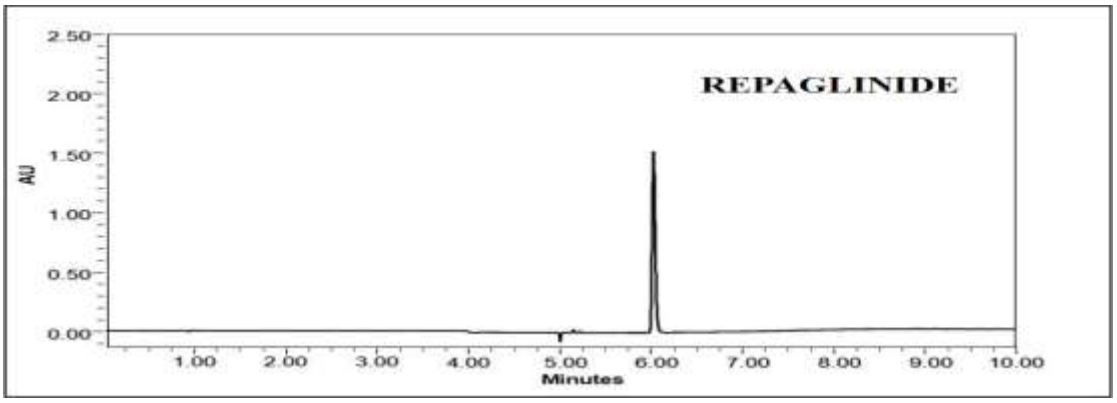
Fig. 3c

Fig. 2: HPLC chromatogram for Repaglinide

Drug excipient compatibility studies

Figure 3a, 3b and 3c depicts the FTIR peaks of Repaglinide, Eudragit RL100, and optimized formulation.

Fig. 3: FTIR of (a) Repaglinide (b) Eudragit RL-100 (c) Optimized formulation



FT-IR spectra of repaglinide, the principal peaks were found at 3236 cm^{-1} attributed to N-H stretching vibrations, 1685 cm^{-1} and 1624 cm^{-1} for >C=O stretching and amide stretching. The peak obtained at 2947 cm^{-1} is allocated to alkane C-H stretching. Furthermore, significant peaks could be detected at 1139 cm^{-1} and 1249 cm^{-1} which are related to C-O-C ether stretching and C-N amine stretching. Characteristic peak of -OH vibration of carboxylic -OH group was observed at 2841 cm^{-1} while the peaks at 1565 cm^{-1} was assigned to C=C ring stretching.

FTIR spectra of Eudragit RL-100 exhibited -OH stretching of hydrate band at 3233 cm^{-1}. The peak obtained at 1729 cm^{-1} is allocated to C=O stretching of saturated aldehyde. The peaks at 1384 cm^{-1} and 1147 cm^{-1} were assigned to N-R stretching of quaternary amine salt and C-O-C stretching of a strong band of ester.

The FTIR spectra of optimized Repaglinide-Eudragit RL100 loaded floating microspheres demonstrated overlapping of characteristic peaks of drug and polymer with slight band shifts. There were no new bands observed in the spectrum, which confirms that no new chemical bonds were formed between the drug and the polymer.

DSC thermograms of Repaglinide, Eudragit RL100 and optimized batch are presented in Figure 4a, 4b and 4c

Fig. 4: DSC of (a) Repaglinide (b) Eudragit RL-100 (c) Optimized formulation

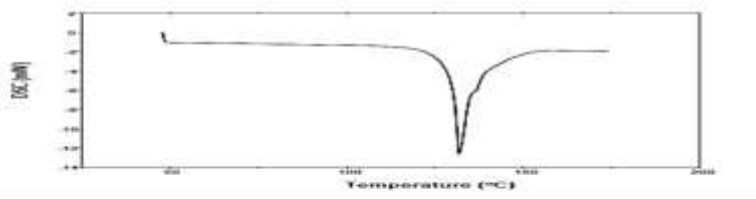


Fig. 4(a)

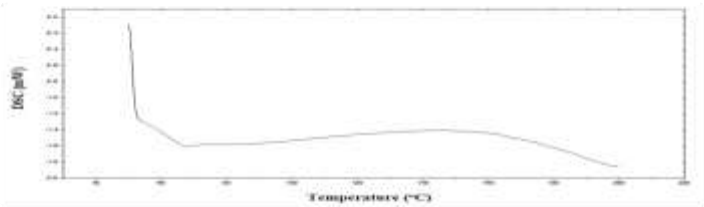


Fig. 4(b)

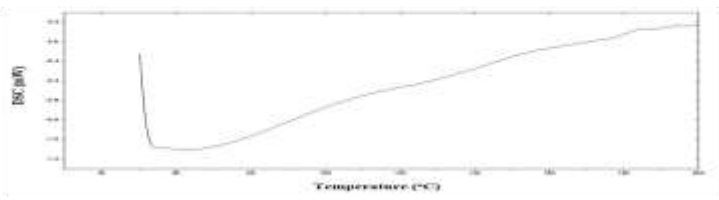


Fig. 4(c)

In the case of repaglinide, a sharp endothermic peak was observed at 132.8°C, which corresponded to its melting point in the crystalline form. Thermograms of Eudragit RL100 showed broad endothermic peaks at 68.77°C respectively, which correspond to melting process and thermal decomposition of polymer. In case of optimized floating microspheres formulation, no characteristic peak was observed at 132.8°C. It appears that there is a significant reduction of drug crystallinity in the floating microspheres. The absence of detectable crystalline domains in drug loaded floating microspheres clearly indicates that drug was dispersed completely in the formulation, thus modifying the microspheres to an amorphous, disordered-crystalline phase.

The overlay of X-ray diffraction data is listed in Figure 5.

Fig. 5 XRD of (a) Repaglinide (b) Eudragit RL-100 (c) Optimized formulation

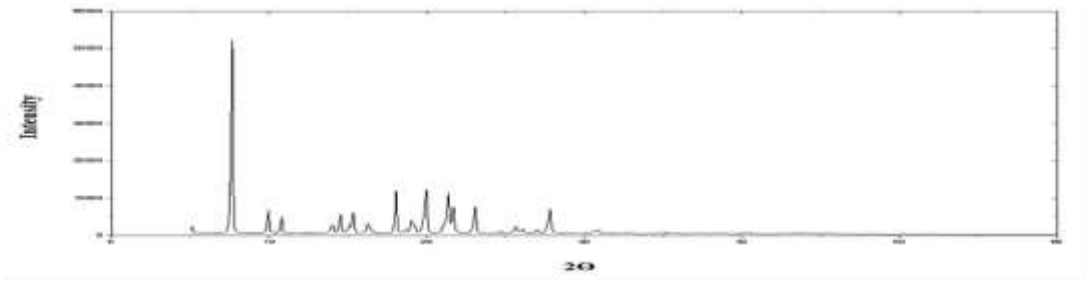


Fig. 5(a)

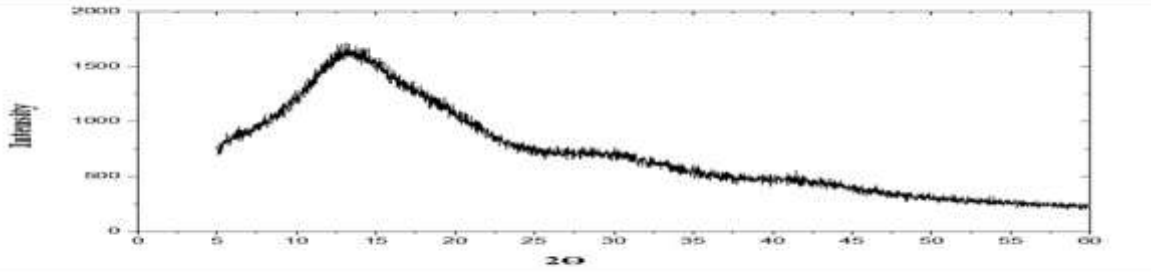


Fig. 5(b)

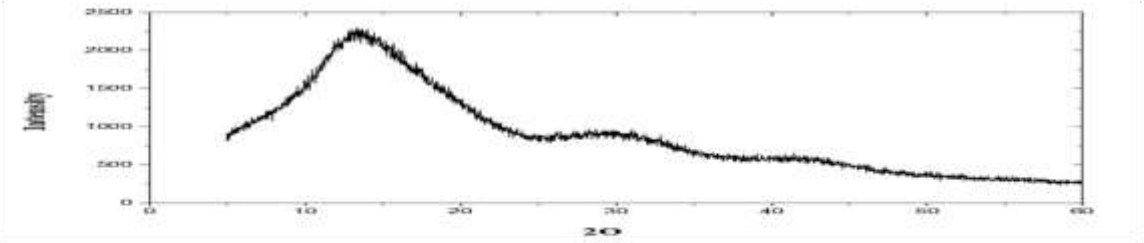


Fig. 5(c)

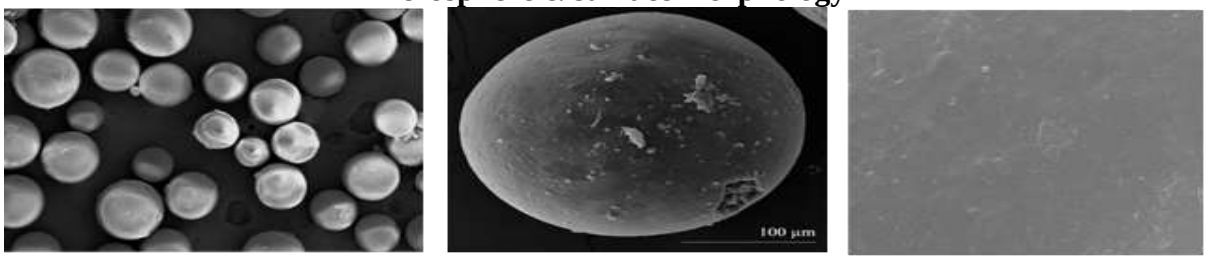
Distinct peaks in the XRD pattern of the pure drug (figure 5a) are scattered at 2θ angles at 7.66°, 9.96°, 10.83°, 14.00°, 14.56°, 15.33°, 16.25°, 18.14°, 19.99°, 21.36°, 23.15°, 25,.65°, 27.85° and 31.05° were indicated crystalline nature. The XRD pattern of Eudragit RL100 (figure 5b) showed diffused peaks, due to its amorphous nature. However, the XRD results of drug-loaded microsphere formulation (figure 5c) showed the absence of intense crystalline peaks of repaglinide and the diffraction patterns were identical to the Eudragit RL100 polymer. These results strongly proposed that the degree of crystallinity of repaglinide was decreased in the floating microsphere formulations due to the homogeneous distribution of repaglinide within the Eudragit RL-100 polymer at the molecular level.

Evaluation of hollow microspheres

The data of particle size analysis show that the average size of dried microspheres ranged from 202.4 to 383.5 µm. Depending on the concentration of PVA and ratio of polymers, the formulations F_8 and F_{11} showed the smallest and largest size of microspheres, respectively. SEM analysis was performed

to study the surface morphological and shape of microspheres. Figure 6 shows the scanning electron microscope images of hollow microspheres.

Fig. 6: SEM images of floating microspheres showing a. general appearance b. single spherical microsphere c. surface morphology



(a) (b) (c)

It could be concluded from the figure 6a, 6b and 6c that the microspheres had a smooth spherical surface having pores on them through which dissolution medium could permeate into the drug-polymer matrix and facilitate the diffusion of the drug molecule.

The type of solvent is known to influence the surface morphology of microspheres. In this study chloroform was chosen as the solvent as opposed to dichloromethane due to lesser miscibility of chloroform in water, which is 1 in 200 parts when compared to dichloromethane, which is 1 in 50 parts.[51] An increased amount of PVA is known to partition into the polymer phase when the organic solvent exhibits an increased miscibility in water because of higher hydrophilicity. Thus in order to avoid a higher deposition of residual PVA on the surface of the microspheres chloroform was used as the solvent.[52]

The percentage yield for microspheres was found to be in the range of 78.71% - 92.12% as given in table 7.

During microsphere formation, the product yield was affected by the polymers sticking to the wall of the beaker and blades of the stirrer and agglomerate formation.[53] The percentage yield was found to be independent on the amount of polymer.[54] It decreased with an increase in the concentration of PVA; the reason may be loss of small particles during filtration and washing.[55] The flow properties of the microspheres were studied as Carr's compressibility index and Hausner's ratio. The Carr's compressibility index was in the range of 6.38% - 14.29%, whereas the Hausner's ratio for all formulations was in the range of 1.07 - 1.17, which indicated good-to-excellent flow characteristics.

As shown in table 7, percentage yield increased with the increase in the concentration of emulsifier.

Table No.7: Percentage yield, and micromeritic properties of microsphere

Formulation code	Yield (%)	Bulk density (g/cm^3)	Tapped density (g/cm^3)	Hausner's ratio	Carr's compressibility index
F_1	89.31	0.250	0.270	7.50	1.08
F_2	83.05	0.233	0.256	9.30	1.10
F_3	86.16	0.250	0.278	10.00	1.11
F_4	80.19	0.213	0.227	6.38	1.07
F_5	84.04	0.263	0.294	10.53	1.12
F_6	92.12	0.244	0.278	12.20	1.14
F_7	81.66	0.270	0.294	8.11	1.09
F_8	83.67	0.250	0.278	10.00	1.11

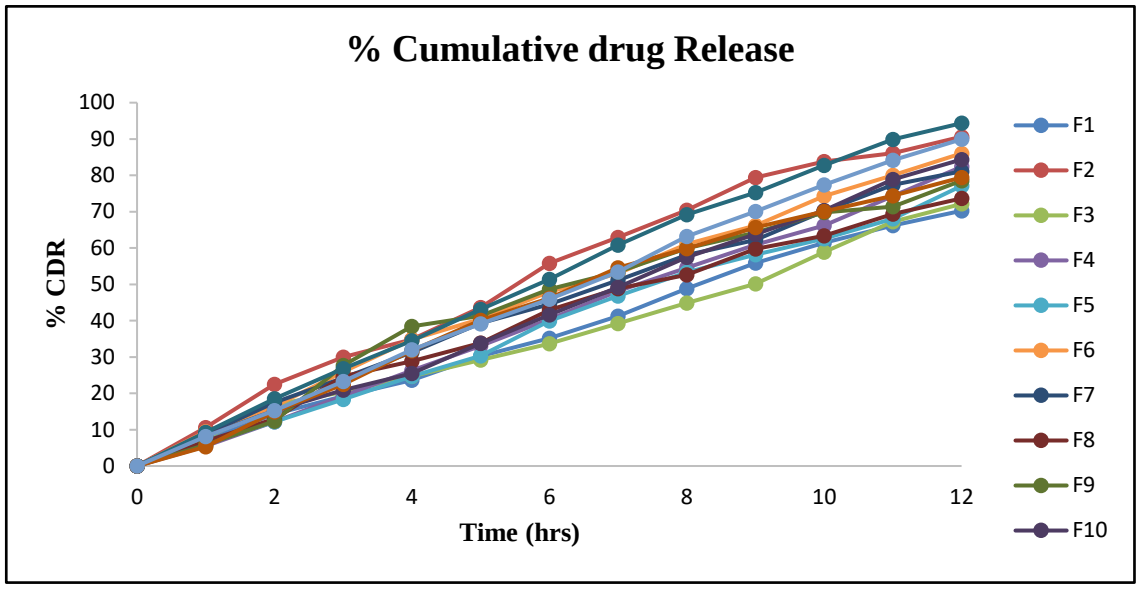
F_9	79.40	0.263	0.294	10.53	1.12
F_{10}	93.73	0.227	0.244	6.82	1.07
F_{11}	78.71	0.250	0.286	12.50	1.14
F_{12}	83.16	0.292	0.318	8.33	1.09
F_{13}	87.74	0.238	0.278	14.29	1.17

The highest entrapment efficiency was obtained at high amount of polymer and low concentration of PVA. The drug release data shows the release pattern of all the formulations prepared after applying central composite design (F_1 to F_{13}), for the optimization of Repaglinide loaded floating microspheres. The in vitro data is given in table 8 and the graphical representation of the same is given in figure 7.

Table No.8: Cumulative drug release (%) for repaglinide microspheres

Time (hr)	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{11}	F_{12}	F_{13}
0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	7.15	10.61	7.69	5.49	6.07	9.42	8.72	6.78	5.84	8.33	9.26	5.34	8.13
2	14.32	22.50	16.84	12.14	12.18	16.36	17.45	12.96	12.37	15.29	18.5	14.87	15.25
3	19.18	29.97	20.33	19.27	18.34	25.91	24.23	24.67	27.68	20.93	26.82	22.44	23.30
4	23.64	34.83	24.72	26.25	24.67	34.84	31.27	28.83	38.47	25.52	34.49	31.76	31.99
5	30.27	43.67	29.17	33.04	30.28	40.45	39.3	33.85	41.35	33.74	43.07	40.1	39.15
6	35.14	55.81	33.68	40.35	39.93	47.95	44.47	42.89	48.68	41.57	51.38	46.19	45.95
7	41.26	62.94	39.26	48.13	46.81	52.93	51.09	48.87	53.42	49.18	60.82	54.57	53.41
8	48.83	70.43	44.87	54.51	53.36	61.08	58.11	52.65	59.97	57.42	69.18	59.83	63.22
9	55.91	79.36	50.14	60.89	58.18	66.17	62.21	59.78	64.21	63.77	75.29	65.64	70.07
10	61.39	83.79	58.83	66.16	62.61	74.29	70.17	63.33	69.77	70.31	82.75	70.06	77.38
11	66.21	86.12	67.28	74.32	68.04	80.02	77.35	69.36	71.42	78.88	89.87	74.38	84.17
12	70.24	90.63	72.14	82.35	77.09	86.04	81.11	73.66	78.54	84.36	94.35	79.42	89.93

Fig. 7: Cumulative drug release (%) for repaglinide microspheres (F_1-F_{13})



The release of drug from the microspheres was varied in the range of 70.24% to 94.35% in 12 hrs study. The release study of all the batches showed the wide variation in the pattern of the drug release. This shows the significant outcome of the chosen independent variables along the drug release from the polymeric matrix.

It can be observed from the plot that 72.64% of the drug was released in a dissolution medium consisting of SGF pH (1.2) within 12 hours. To determine the mechanism of release kinetics from hollow microspheres, the release rate data were subjected to various kinetic models for the goodness of fit.

The R^2 values of release kinetic models were found to be 0.883, 0.960, 0.995, and 0.978 for zero-order, first-order, Higuchi's square root, and Korsmeyer–Peppas models, respectively. The results indicated that the *in vitro* drug release of repaglinide from microspheres was best described by Higuchi's release equation, indicating the release of drug from the insoluble matrix by the diffusion-controlled process.

Residual solvent analysis

The gas chromatogram of standard chloroform solution in dimethyl sulfoxide (DMSO) and optimized formulation dissolved in DMSO is shown in figure 8a and 8b.

Figure 8(a). GC scan of standard chloroform solution

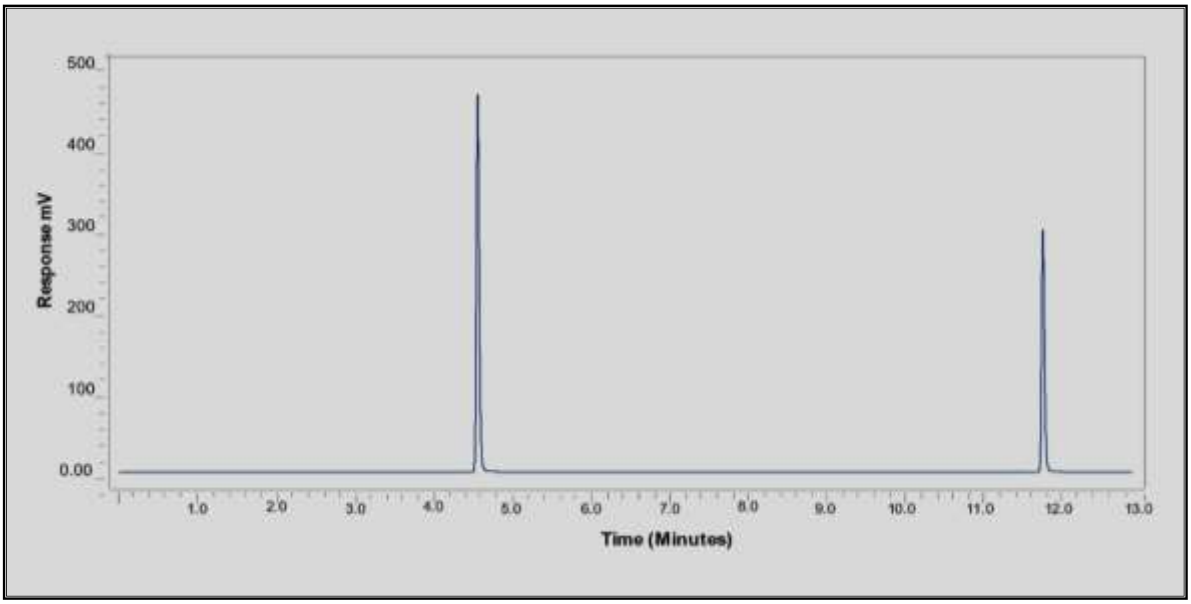
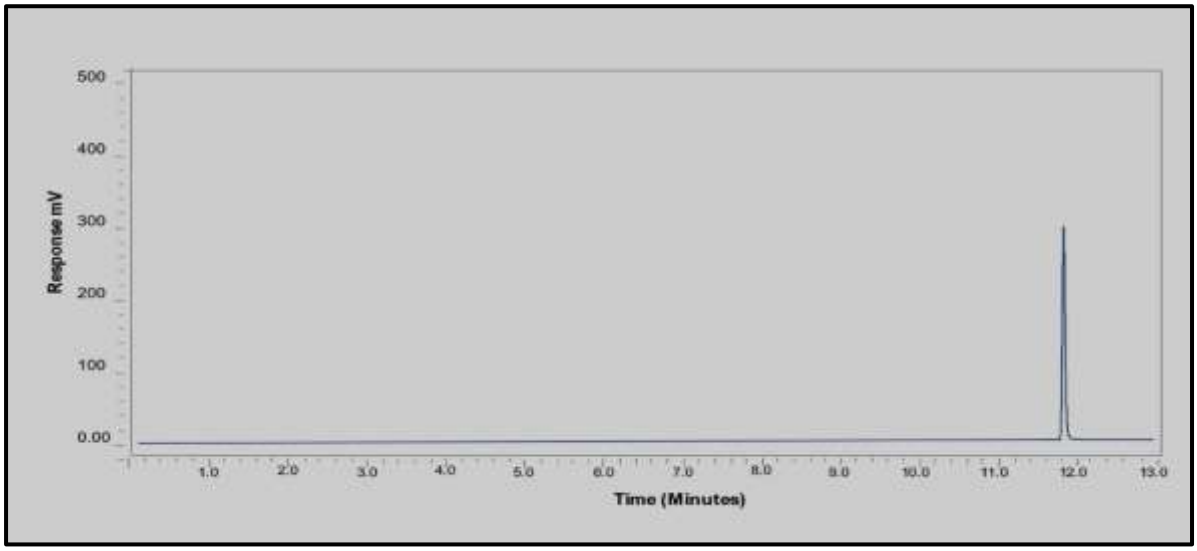


Figure 8(b). GC scan of optimized formulation prepared in DMSO solution



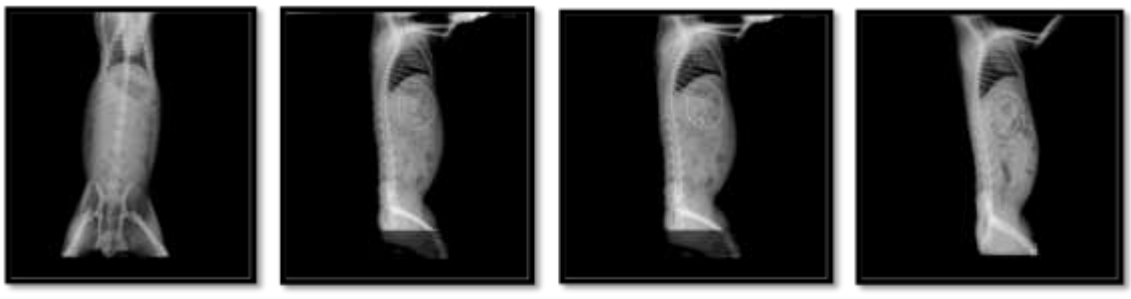
The retention time of chloroform was 4.6 min as observed in standard preparation of chloroform. The peak of DMSO was observed at 11.8 minutes in both standard and sample preparation. But, no Chloroform peak at 4.6 min was observed in sample chromatogram. Chloroform residue was within the limits, in the repaglinide loaded microspheres. Hence, the prepared formulation OF1 is considered to be safe for human use.

***In Vivo* Study**

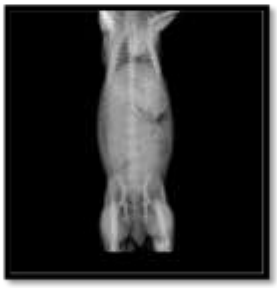
Floating Behavior (Radiographic study)

The optimized formulation of the floating microspheres (OF1) which showed good *in vitro* floating ability and enhanced drug release were selected for studying *in vivo* floating efficiency by radiological method. X-ray images of the rats were taken before giving the dosage form and they serve as the control. The radiographs obtained at 0 hour, 3 hour, 6 hours, 9 hours and 12 hours are shown in figures 9 (a), (b), (c), (d) & (e) which indicated uniform distribution of the floating microspheres in the stomach fluid and buoyancy for more than 12 hours.

Fig. 9: X-ray images of the GIT of a rat, showing the movement of an optimized floating microsphere (OF1) in the stomach (a) before administration (empty stomach), (b) 3 hr after administration, (c) at 6 hr, (d) at 9 hr and (e) after 12 hr.



a) 0 hr (b) 3 hr (c) 6 hr (d) At 9 hr



(e) At 12 hr

This study also confirmed that the prepared floating microspheres were able to retain in stomach for prolonged release of repaglinide.

Antidiabetic study

The in vivo efficiency of the optimized Repaglinide loaded floating 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, repaglinide showed a decrease in blood glucose level when compared with the alloxan group. Also, the optimized floating microsphere loaded with repaglinide 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 repaglinide 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.

Fig. 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 repaglinide and the prepared Eudragit -RL100 microsphere loaded with repaglinide, respectively, in controlling the serum blood glucose level when estimated in the given time interval.)

Comparative study of optimized v/s marketed formulation

Comparative study was done in between the optimized formulation (OF1) and marketed formulation (MF) of Repaglinide tablets (Eurepa® (2mg); Torrent). Comparative data for these two formulations is shown in table 9.

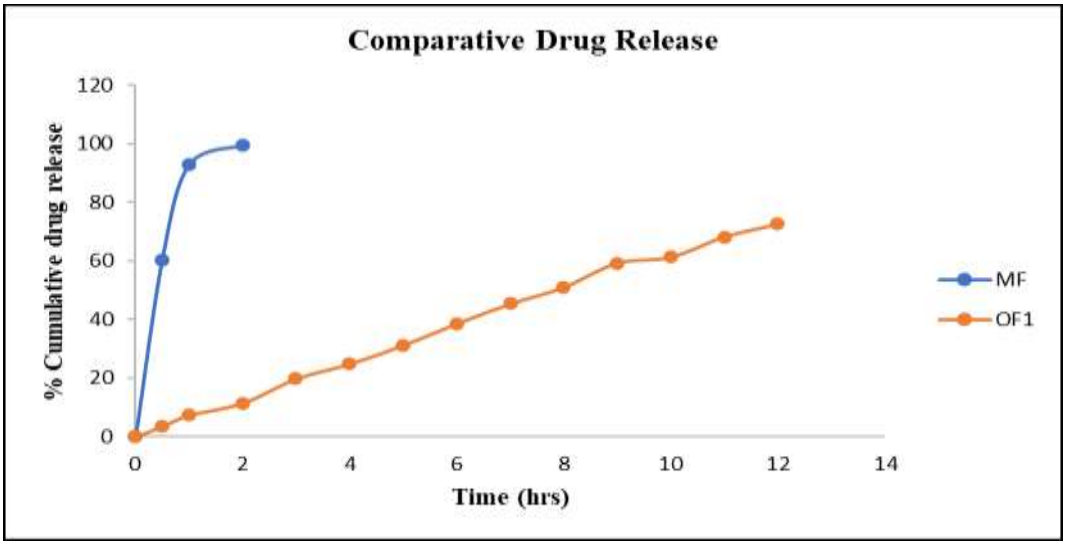
Table No.9: Comparative drug release study of marketed formulation (Eurepa®) and optimized formulation (OF1)

Time (hours)	Marketed formulation (Eurepa®)	Optimized formulation (OF1)
0	0	0
0.5	60.45	3.52
1	92.86	7.33
2	99.48	11.46
3		19.76

4		24.84
5		31.22
6		38.6
7		45.39
8		51.07
9		59.34
10		61.36
11		68.28
12		72.64

The result revealed that in comparison to the marketed product, the optimized formulation OF1 has better control over release rate of drug. Drug release in marketed product was found the 99.48% in 2 hours whereas the drug release in optimized formulation OF1 was found 72.64 % in 12 hrs. Thus, the optimized formulation was found better controlled drug release over 12 hrs, which was found for longer time than the marketed formulation. The comparative drug release of these formulations is shown figure 11.

Fig. 11: Comparative Release Profile of MF (Eurepa®) and Optimized Batch (OF1)



Stability study

After storage the formulations were withdrawn periodically and analyzed for various physical parameters, results were reported in table 10.

Table 10: Stability studies for optimized formulation (OF1)

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

Entrapment efficiency	98.23 ± 1.4	98.32 ± 1.7	97.89 ± 2.1	98.82 ± 1.2	98.20 ± 1.8
In vitro drug release at the end of 12 hrs.	78.34 ± 1.3	79.42 ± 1.5	78.68 ± 1.2	78.42 ± 1.5	78.02 ± 1.8
Total floating time	>12 hrs	>12 hrs	>12 hrs	>12 hrs	>12 hrs

The data is presented as mean value ± S.D. (n = 3)

No visible physical changes were observed in the formulation throughout the study period in both the conditions. There was no significant change observed in the entrapment efficiency, in vitro buoyancy and drug release pattern of the drug on storage for six months.

Conclusion

The results of this study emphasized that response surface methodology with CCD is a very useful statistical technique to determine the effect of selected independent variables on the dependent variables. Repaglinide microspheres were prepared successfully by emulsion solvent evaporation method using the Eudragit RL 100 polymers in various ratios. It was found that the prepared microspheres were spherical, 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, buoyancy, and percentage cumulative drug release. The low density of microspheres showed well *in vitro* floating ability and drug release profile. Hence, floating microspheres of Repaglinide prolonged the retention time in the stomach, which can ultimately result in improved bioavailability at a much lower dose. In addition, formulated microspheres can be chosen for *in vivo* antidiabetic study exhibited satisfactory results. The feasibility of the optimization procedure in developing hollow microspheres can be demonstrated by close agreement between the observed responses and predicted values of the optimized formulation.

So, from the results it can be concluded that drug retardant polymer and emulsifier concentration affect all the evaluation parameters significantly. Hence the prepared polymeric microspheres of Repaglinide might be proved to be potential candidate for safe and effective sustained drug delivery from microspheres for the treatment of type II diabetes.

References:

1. Rao GK, Mandapalli PK, Manthri R, Reddy VP. Development and in vivo evaluation of gastroretentive delivery systems for cefuroxime axetil. *Saudi Pharm J*. 2013;21(1):53-59. doi:10.1016/j.jsps.2012.01.003
2. Arora S, Ali J, Ahuja A, Khar RK, Baboota S. Floating drug delivery systems: A review. *AAPS PharmSciTech*. 2005;6(3):E372-E390. doi:10.1208/pt060347
3. Deshpande AA, Shah NH, Rhodes CT, Malick W. Development of novel controlled release system for gastric retention. *Pharm Res*. 1997;14(6):815-819. doi:10.1023/A:1012171010492
4. Ponchel G. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. *Adv Drug Deliv Rev*. 1998;34(2-3):191-219. doi:10.1016/S0169-409X(98)00040-4
5. Shakya R, Thapa P, Saha RN. In vitro and in vivo evaluation of gastroretentive floating drug delivery system of ofloxacin. *Asian J Pharm Sci*. 2013;8(3):191-198. doi:10.1016/j.ajps.2013.07.025
6. Kristmundsdóttir T, Ingvarsdóttir K. Ibuprofen microcapsules: The effect of production variables on microcapsule properties. *Drug Dev Ind Pharm*. 1994;20(5):769-778. doi:10.3109/03639049409038330

7. Capan Y, Jiang G, Giovagnoli S, Na KH, DeLuca PP. Preparation and characterization of poly(D,L-lactide-co-glycolide) microspheres for controlled release of human growth hormone. *AAPS PharmSciTech*. 2003;4(2):147-156. doi:10.1208/pt040228
8. Gohel MC, Amin AF. Formulation optimization of controlled release diclofenac sodium microspheres using factorial design. *J Controlled Release*. 1998;51(2-3):115-122. doi:10.1016/S0168-3659(97)00102-8
9. Ganesan P, Deepa John AJ, Sabapathy L, Duraikannu A. Review on Microsphere. *Am J Drug Discov Dev*. 2014;4(3):153-179. doi:10.3923/ajdd.2014.153.179
10. Metkari VB, Kulkarni LV, Patil PS, Jadhav PA, Bamane GS, Kumbhar CM. Microspheres - A New Drug Delivery System: A Review. 2014;4(2):1128-1133.
11. Gaurav A. Formulation & Evaluation of Sustained Release Matrix Tablet of Repaglinide. *Open Access Biostat Bioinforma*. 2018;1(2). doi:10.31031/OABB.2018.01.000509
12. Mandić Z, Gabelica V. Ionization, lipophilicity and solubility properties of repaglinide. *J Pharm Biomed Anal*. 2006;41(3):866-871. doi:10.1016/j.jpba.2006.01.056
13. Patel AK, Mishra MK, Gupta J, Ghoshal S, Gupta R, Kushwaha K. Guar Gum-Based Floating Microspheres of Repaglinide Using 32Factorial Design: Fabrication, Optimization, Characterization, and in Vivo Buoyancy Behavior in Albino Rats. *ASSAY Drug Dev Technol*. 2021;19(2):63-74. doi:10.1089/adt.2020.1006
14. Sharma M, Kohli S, Dinda A. Floating microspheres of repaglinide: formulation, optimization, characterization and in vitro evaluation. 2014;12(4):1259-1272.
15. NR Naveen et al. Design expert supported mathematical optimization of repaglinide gastroretentive floating tablets: In vitro and in vivo evaluation. *Res Artic*. 2017;3(2):140-147. doi:doi.org/10.1016/j.fjps.2017.05.003
16. Fukuda IM, Pinto CFF, Moreira C dos S, Saviano AM, Lourenço FR. Design of Experiments (DoE) applied to Pharmaceutical and Analytical Quality by Design (QbD). *Braz J Pharm Sci*. 2018;54(spe). doi:10.1590/s2175-97902018000001006
17. Dey S, Pramanik S, Malgope A. Formulation and Optimization of Sustained Release Stavudine Microspheres Using Response Surface Methodology. *ISRN Pharm*. 2011;2011:1-7. doi:10.5402/2011/627623
18. Subedi G, Shrestha AK, Shakya S. Study of Effect of Different Factors in Formulation of Micro and Nanospheres with Solvent Evaporation Technique. *Open Pharm Sci J*. 2016;3(1):182-195. doi:10.2174/1874844901603010182
19. Yadav VK, Rai AK, Ghosh AK. Encapsulation of repaglinide into eudragit RS microspheres and modulation of their release characteristics by use of surfactants. *Int J Pharm Sci Res*. 2017;8(9):3936-3947.
20. Pattanaik P, Panda OP, Mohanty C, Naresh P. New RP-HPLC Method for the Estimation of Repaglinide in Bulk and in Pharmaceutical Dosage Forms. 2013;3(2):189-193.
21. Yamada T, Onishi H, Machida Y. Sustained release ketoprofen microparticles with ethylcellulose and carboxymethylethylcellulose. *J Controlled Release*. 2001;75(3):271-282. doi:10.1016/S0168-3659(01)00399-6
22. Bhikshapathi DVRN, Srinivas I. Preparation and in vivo Evaluation of Solid Dispersions Using Repaglinide. *Int J Pharm Sci Drug Res*. 2018;10(05). doi:10.25004/IJPSDR.2018.100502
23. Deshmukh RK, Naik JB. Diclofenac Sodium-Loaded Eudragit® Microspheres: Optimization Using Statistical Experimental Design. *J Pharm Innov*. 2013;8(4):276-287. doi:10.1007/s12247-013-9167-9
24. Rao BP, Mathews R, Krishna KP, Baby B, Poornima null. Spray-dried gastroretentive floating microparticles: preparation and in vitro evaluation. *Pak J Pharm Sci*. 2013;26(4):707-713.
25. Jagdish C, Vaishali R, Tamizharasi S. Preparation of biodegradable microspheres containing repaglinide. *J Young Pharm*. 2009;1(2):121. doi:10.4103/0975-1483.55743

26. Jain SK, Awasthi AM, Jain NK, Agrawal GP. Calcium silicate based microspheres of repaglinide for gastroretentive floating drug delivery: Preparation and in vitro characterization. *J Controlled Release*. 2005;107(2):300-309. doi:10.1016/j.jconrel.2005.06.007
27. Jain AK, Sahu P, Mishra K, Jain SK. Repaglinide and Metformin-Loaded Amberlite Resin-Based Floating Microspheres for the Effective Management of Type 2 Diabetes. *Curr Drug Deliv*. 2021;18(5):654-668. doi:10.2174/1567201817666201026105611
28. Ahmed AB, Sengupta R, Deb R. Design, development and evaluation of hollow microspheres of Repaglinide. Published online 2014:11.
29. Pandit V, Pai RS, Yadav V, et al. Pharmacokinetic and pharmacodynamic evaluation of floating microspheres of metformin hydrochloride. *Drug Dev Ind Pharm*. 2013;39(1):117-127. doi:10.3109/03639045.2012.662503
30. M Sharma et al,. In-vitro and in-vivo evaluation of repaglinide loaded floating microspheres prepared from different viscosity grades of HPMC polymer. *Res Artic*. 2015;23(6):675-682. doi:10.1016/j.jsps.2015.02.013
31. Gibaldi M, Feldman S. Establishment of Sink Conditions in Dissolution Rate Determinations. *J Pharm Sci*. 1967;56(10):1238-1242.
32. Wagner JG. Interpretation of percent dissolved-time plots derived from in vitro testing of conventional tablets and capsules. *J Pharm Sci*. 1969;58(10):1253-1257. doi:10.1002/jps.2600581021
33. Dwivedi A. Residual Solvent Analysis in Pharmaceuticals. *Pharm Technol Eur*. 2002;14(12). Accessed August 13, 2022. https://www.pharmtech.com/view/residual-solvent-analysis-pharmaceuticals-0
34. Nogueira R, Queiroz SM, Silva GEB, et al. Determination of volatiles in pharmaceutical certified reference materials. *J Braz Chem Soc*. 2012;23(9):1636-1646. doi:10.1590/S0103-50532012005000021
35. Patel A, Modasiya M, Shah D, Patel V. Development and In Vivo Floating Behavior of Verapamil HCl Intragastric Floating Tablets. *AAPS PharmSciTech*. 2009;10(1):310-315. doi:10.1208/s12249-009-9210-9
36. Dewan I, Islam S, Rana MdS. Characterization and compatibility studies of different rate retardant polymer loaded microspheres by solvent evaporation technique: *in vitro-in vivo* study of vildagliptin as a model drug. *J Drug Deliv*. 2015;2015:1-12. doi:10.1155/2015/496807
37. Osman R, El Shamy AH, Awad G, El Mowafy E. Nanocomplexes of an insulinotropic drug: optimization, microparticle formation, and antidiabetic activity in rats. *Int J Nanomedicine*. Published online September 2014:4449. doi:10.2147/IJN.S66876
38. Bajaj S, Singla D, Sakhuja N. Stability Testing of Pharmaceutical Products. *J Appl Pharm Sci*. 2012;2(3):10.
39. Khuri AI. A General Overview of Response Surface Methodology. *Biom Biostat Int J*. 2017;5(3):87-93. doi:10.15406/bbij.2017.05.00133
40. Bhattacharya S. Central composite design for response surface methodology and its application in pharmacy. In: Kayaroganam P, ed. *Response Surface Methodology in Engineering Science*. IntechOpen; 2021. doi:10.5772/intechopen.95835
41. Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*. 2008;76(5):965-977. doi:10.1016/j.talanta.2008.05.019
42. Rohila S, Bhatt D, Ahalwat S. Fabrication of potential gastroretentive microspheres of itraconazole for stomach-specific delivery: Statistical optimization and in vitro evaluation. *J Appl Pharm Sci*. 2020;10(3):119-127. doi:10.7324/JAPS.2020.103016
43. Jagtap Y, Ranade A, Ranpise N, Bhujbal R. Effect of various polymers concentrations on physicochemical properties of floating microspheres. *Indian J Pharm Sci*. 2012;74(6):512. doi:10.4103/0250-474X.110578

44. Jabar A, Madni A, Bashir S, et al. Statistically optimized pentazocine loaded microsphere for the sustained delivery application: Formulation and characterization. Rai VK, ed. *PLOS ONE*. 2021;16(4):e0250876. doi:10.1371/journal.pone.0250876
45. Dhakar RC. From formulation variables to drug entrapment efficiency of microspheres: a technical review. *J Drug Deliv Ther*. 2012;2(6). doi:10.22270/jddt.v2i6.160
46. Sharma N, Madan P, Lin S. Effect of process and formulation variables on the preparation of parenteral paclitaxel-loaded biodegradable polymeric nanoparticles: A co-surfactant study. *Asian J Pharm Sci*. 2016;11(3):404-416. doi:10.1016/j.ajps.2015.09.004
47. Maravajhala V, Dasari N, Sepuri A, Joginapalli S. Design and evaluation of niacin microspheres. *Indian J Pharm Sci*. 2009;71(6):663. doi:10.4103/0250-474X.59549
48. Evonik Industries AG. Eudragit RL 100, Eudragit RL PO, Eudragit RS 100 and Eudragit RSPO. Published online 2012:1-7.
49. Pachuau L, Mazumder B. A study on the effects of different surfactants on Ethylcellulose microspheres. *Int J Pharmtech Res*. 2009;1(4):966-971.
50. Mouffok M, Mesli A, Abdelmalek I, Gontier E. Effect of the formulation parameters on the encapsulation efficiency and release behavior of p-aminobenzoic acid-loaded ethylcellulose microspheres. *J Serbian Chem Soc*. 2016;81(10):1183-1198. doi:10.2298/JSC160308068M
51. Francis L, Meng D, Knowles J, Keshavarz T, Boccaccini A, Roy I. Controlled Delivery of Gentamicin Using Poly(3-hydroxybutyrate) Microspheres. *Int J Mol Sci*. 2011;12(7):4294-4314. doi:10.3390/ijms12074294
52. Butreddy A, Gaddam RP, Kommineni N, Dudhipala N, Voshavar C. PLGA/PLA-Based Long-Acting Injectable Depot Microspheres in Clinical Use: Production and Characterization Overview for Protein/Peptide Delivery. *Int J Mol Sci*. 2021;22(16):8884. doi:10.3390/ijms22168884
53. Ganesan P, Soundararajan R, Shanmugam U, Ramu V. Development, characterization and solubility enhancement of comparative dissolution study of second generation of solid dispersions and microspheres for poorly water soluble drug. *Asian J Pharm Sci*. 2015;10(5):433-441. doi:10.1016/j.ajps.2015.05.001
54. Badis K, Merine H, Ramli Y, Larbi O, Memou CH. Effect of Polymers nature and Stirring Speeds on Physicochemical Properties and the Controlled Release of Allopurinol-loaded Microspheres. *J Mex Chem Soc*. 2021;66(1). doi:10.29356/jmcs.v66i1.1583
55. Khaled M. Hosny. Novel controlled release oxypentifylline microspheres; Preparation, characterization and in-vivo evaluation. *Afr J Pharm Pharmacol*. 2012;6(27):2039-2048. doi:10.5897/AJPP12.158

