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To Study Effect of Process Variables on Preparation of Desmopressin Loaded Nanoparticles Using Box-Behnken Design

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ABSTRACT

Desmopressin, a nonapeptide; mainly used in nocturnal enuresis in children and management of polyuria in central diabetes insipidus; having a very short half-life and thus required frequent administration. The present study reports preparation and optimization of desmopressin loaded polymeric nanoparticles by investigating the effect of process variables; amount of polymer, organic/ aqueous phase ratio and surfactant concentration on two dependent responses, i.e. Particle size and %entrapment efficiency using Box-Behnken design. Nanoparticles were successfully developed by double emulsion solvent evaporation method using blend of Poly lactic acid – glycolic acid (PLGA) and Poly e-caprolactone(PCL) as polymer, polyvinyl alcohol as surfactant and Dichloromethane as organic phase.The amount of Polymer and surfactant concentration showed positive contribution to both responses though organic/ aqueous phase ratio contributed to negative impact on dependent variables. All selected independent variables were found to be effective at mid level. Nanoparticles were optimized by selecting constraints of minimum particle size and maximum %entrapment efficiency. The optimized Nanoparticles showed nearly spherical shape with Particle size 213 nm, −23.7mV zeta potential with maximum % drug entrapment of 39.4%. *In-vitro* drug release behavior followed Higuchi model, showed initial burst release with prolonged drug release upto 168 hr, indicated controlled delivery of drug that can be associated for reducing the frequency of drug administration.

1. INTRODUCTION

Many proteins currently being developed are aimed at chronic conditions where therapy may be required over months or years. Alternative administration by frequent injections to keep the protein drug at effective concentrations is tedious, expensive, and has poor patient compliance. Therefore, development of sustained release injectable dosage forms becomes necessary to improve the efficacy of peptide drugs and eliminate the need for frequent administration [1-3].

Desmopressin is a synthetic cyclic nonapeptide, hormone analogue of vasopressin, having an antidiuretic action. It is indicated as antidiuretic replacement therapy in the management of central cranial diabetes insipidus and for management of the temporary polyuria and polydipsia following head trauma or surgery in the pituitary region. Though it is ineffective for the treatment of nephrogenic diabetes insipidus [4-6].

Because macromolecules, such as peptides and proteins, are very sensitive in terms of stability, encapsulation allows their protection, especially against enzymes and pH effect, when they are administered *in vivo*[7,8]. Nanoparticles (NPs) are colloidal polymeric drug carriers, formulated by biodegradable polymers like PLA, PLGA, and PCL etc. either alone or in combination. A blend of biodegradable polymers in fabrication of nanoparticles offers advantage like biocompatibility, bioavailability and variable degradation kinetics, high drug loading capability, stability and extended drug release [9-13].

Double emulsion solvent evaporation method is one of the most commonly used for protein and peptide encapsulation. It utilizes preparation of primary emulsion with consecutive addition in a large amount of external phase containing surfactant; followed by evaporation of the solvent, either by continuous magnetic stirring at room temperature or under reduced pressure. Afterward, the solidified nanoparticles can be collected by ultracentrifugation and washed with distilled water to remove additives such as surfactants; subsequently, the product is lyophilized. The organic phase acts as a barrier between the two aqueous compartments preventing the diffusion of the active principle toward the aqueous external phase. Rapid solidification of the double emulsion droplets will undoubtedly favour higher entrapment efficiency [14, 15].

Efforts have been made by researchers to develop bioadhesive microsphere [16] and liposome [17] for nasal delivery; coated microneedle by transdermal delivery [18] for sustaining the drug

release. Nanoparticles were also developed to improve oral bioavailability [19]. Marketed formulation of desmopressin are nasal solution, nasal spray, oral tablets and i.v. injection [20-23]. Available formulations are suffering from the drawback of short half-life; require frequent administration. Depot formulations have several advantages over presently available dosage forms such as less frequent administration, improved patient compliance, more predictable absorption, reduced medicinal workload.

In present study, an attempt has been made to prepare sustained release injectable nanoparticles using blend of biodegradable polymers loaded with desmopressin for controlled drug delivery; and to study the influence of process parameters in fabrication of nanoparticles with suitable particle size, high encapsulation efficiency, and sustained drug release over a long period, with minimal burst release.

Desmopressin loaded PLGA NPs were formulated using double emulsion solvent evaporation approach and the effects of related process parameters were analyzed using Box- Behnken design. Response surface methodology (RSM) is a tool used for the process of optimization. Several designs are available under RSM such as central composite, Box- Behnken, and D-optimal design. In the present study, Box-Behnken design was employed for process optimization as it generates fewer runs as compared to a central composite design with 3 variables [24, 25].

2. MATERIALS AND METHODS

2.1 Materials

Desmopressin acetate was purchased from Hemmo Pharmaceuticals, Mumbai, India. Poly (lactide -co-glycolide) PLGA (50:50) Resomer® RG502H; and Polycaprolactone (PCL) were obtained as gift sample from Boehringer Ingelheim, Germany. Poly vinyl alcohol (PVA) and other chemicals were purchased from S.D. Fine Chem., Mumbai, India.

2.2 Preparation of Nanoparticles

The nanoparticles were prepared by double emulsion- solvent evaporation technique based on the formation of a W/O/W-multiple emulsion as described by Herrmann et al. (1998). Oil phase was prepared by dissolving PLGA (50:50) and PCL in ratio of 85:15 using dichloro methane (3.0

ml). Briefly, an aqueous solution of Desmopressin (0.5 ml) (water phase) was emulsified into a solution of the water and oil phases were vigorously mixed on vortex at 2800 rpm. Primary emulsion was subjected to sonication at 55 amplitude, 4 kHz frequency for 100 sec. Prepared emulsion was re-emulsified into 50 ml of aqueous solution of 1%w/v PVA with mechanical stirrer at 1500 rpm to form the double emulsion [(W1/O)/W2]. Stirring was continued for 4-5 hr to allow the evaporation of DCM and solidification of nanoparticles. The nanoparticles were collected by centrifugation, rinsed thrice with water and lyophilized (LABCONCO, TriadTM).

2.3 Experimental Design

Box-Behnken design was employed to study the independent and interactive effect of process variables on polymeric nanoparticles by selecting three independent variables at three levels. The design and statistical analysis were performed by Design – Expert® Software Version 9.0 and Minitab 15 Software. Independent factors and responses were chosen as shown in Table 1.

Table 1. Factors and factor levels studied in a Box–Behnken experimental design Levels

Factors		**Level**		
		Low (-1)	**Medium (0)**	**High (+1)**
X1	Amount of Polymer (mg)	50	100	150
X2	Organic phase: Aqueous phase (ml)	4:1	6:1	8:1
X3	PVA concentration (%)	0.5	1	1.5

2.4 Characterization of Nanoparticles

Particle size

The size analysis of nanoparticles was performed using a Zetasizer (Nano ZS, Marlvern instrument, UK). Each sample was suitably diluted with filtered distilled water (up to 2ml) to avoid multi-scattering phenomena and placed in a small disposable zeta cell.

Zeta Potential

Zeta potential distribution was measured with a Zeta-nano particle electrophoresis analyzer setup equipped with a 5-mV He-Ne laser (633nm). Each sample was suitably diluted 5 times with

filtered distilled water and placed in a small disposable zeta cell. Zeta limits ranged from -200 to +200mV.

Determination of entrapped Desmopressin

The amount of drug entrapped was estimated by dispersing 10 mg of nanoparticles in DCM and water in 3:1 ratio, under vigorous shaking for 1hr, the resultant solution was centrifuged. Both layers were separated. As the desmopressin acetate was soluble in water but not in DCM, the drug content in aqueous solution was analyzed by using HPLC at 220 nm with further dilutions against appropriate blank. The amount of the drug entrapped in the nanoparticles was calculated using following formula:

% EE = (Actual weight of drug in sample/ Theoretical weight of drug in the sample) x 100.

A reverse phase chromatography method was used for evaluation of desmopressin using gradient HPLC system (Shimadzu- LC 20AD, Japan) equipped with a UV- Visible detector, manual injector with 20 µl loop, Shim-pack XR-ODS (Shim-pack XR-ODS II 150 mm x 3 mm x 5 µm id) and LC solution software. Optimized mobile phase was prepared by mixing Phosphate buffer saline pH 7.4: acetonitrile (85:15, v/v). The flow rate was set to 1.8 ml/min. Injection volume of 20 µl was made and the column eluents were monitored at 220 nm over a run time of 10 min.

Differential scanning calorimetry (DSC)

A differential scanning calorimeter was used to evaluate the thermal behavior of all materials used in the NP formulations. Thermograms were obtained using 2910 MDSC instrument (V4.4E model, UK.). 4 to 8 milligrams of sample was sealed in a standard aluminum pan with a lid. The sample was purged with nitrogen. The adopted scanning temperature range was from 20 to 200°C at a heating rate of 10°C /min [26].

Scanning electron microscopy (SEM)

The surface morphology of NPs was assessed by a scanning electron microscope (Mira Tescan, Czech Republic). The Nanoparticles were spread on a stub and dried at 25°C and then spattered with gold using a sputter coater (BAL-TEC, Switzerland)[27].

2.5*In Vitro* drug release studies

Lyophilized NPs were evaluated for *in vitro* release in phosphate buffer saline (pH = 7.4) by using dialysis bags. Firstly, NPs were resuspended and dispersed by sonication in 1 mL of buffer and then poured into dialysis bags. The dialysis bag was soaked in distilled and deionized water for 12 h before use. The bag was placed into the bottle containing 25 mL of release medium (buffer, pH = 7.4) at temperature 37 ± 2°C under magnetic stirring at 50 rpm[28]. At each selected time point, an aliquot of 1 mL was withdrawn and the same volume was replaced by fresh release medium. The sample was analyzed for drug content by HPLC at 220nm.

2.6 Stability study

Stability study was conducted for 3 months under storage conditions mentioned in ICH guidelines. The optimized formulation was stored at room temperature (~25°C) and refrigerator (4° to 8°C), over a period of 3 months in stoppered glass vials. The sample was evaluated for particle size and drug content on selected point.

3. RESULTS AND DISCUSSION

Using Box-Behnken factorial designs, seventeen batches of PLGA nanoparticles were prepared by double emulsion-solvent evaporation technique (Table 2). The effects of formulation variables on Nanoparticles were studied and optimized formula was proposed by design expert software upon selecting constraints. Optimized formulation was subjected to various evaluation parameters.

Table 2. Run parameters and responses for three-level three-factorial Box–Behnken experimental design

Batch no.	Independent variables			Dependent variables	
	X1	X2	X3	Y1	Y2
F1	-1	-1	0	257	21.9
F2	+1	-1	0	304	34.6
F3	-1	+1	0	271	19.3
F4	+1	+1	0	322	29.2
F5	-1	0	-1	249	21.2
F6	+1	0	-1	284	29.8
F7	-1	0	+1	261	23.5
F8	+1	0	+1	283	33.4
F9	0	-1	-1	235	27.6
F10	0	+1	-1	253	25.8
F11	0	-1	+1	262	30.3
F12	0	+1	+1	274	28.4
F13	0	0	0	213	39.2
F14	0	0	0	213	39.2
F15	0	0	0	213	39.2
F16	0	0	0	213	39.2
F17	0	0	0	213	39.2

3.1 Effect of process variables on Particle Size

Analysis of data from ANOVA test exhibited that amount of Polymer, O/W ratios, and PVA concentration had significant effects on particle size ($p < 0.05$).

The minimum particle size was achieved by operating the experiment at the midpoint of each independent variable. Analysis of independent factors showed that amount of polymer (X1) had more prominent impact on particle size than O/W ratios (X2) and PVA concentration (X3) (Figure1).

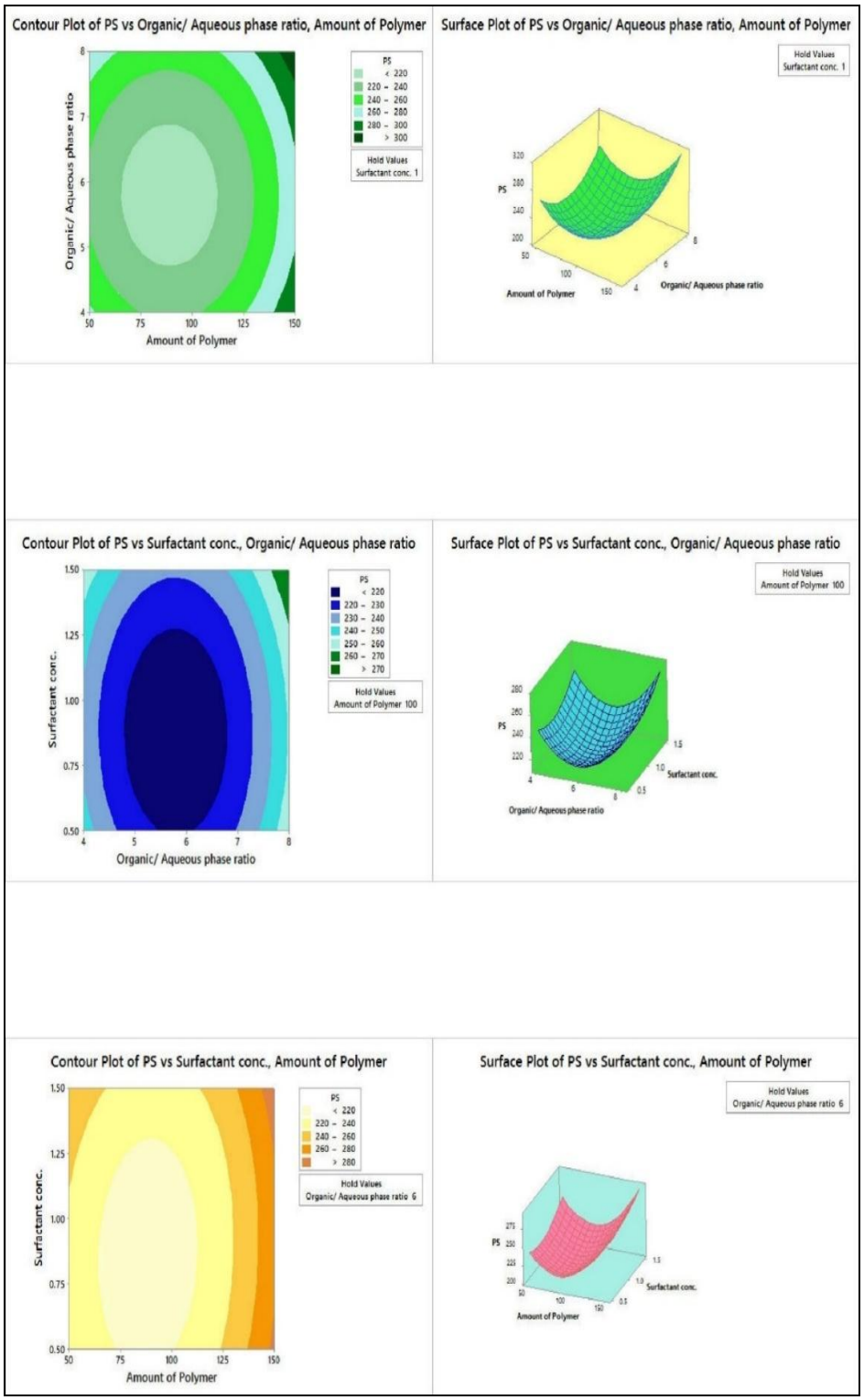

Figure 1. Contour plot and a 3D surface plot showing the effect of Amount of Polymer (X1), O/W phase (X2) and PVA concentration (X3) on Particle size.

Quantitative estimation of significant models indicated that amount of polymer (X1) and polynomial model of the amount of polymer ($X1^2$) had the prime influence on Particle size for its large positive coefficient value 19.38 and 43.81 respectively, suggesting that increasing this variable resulted in the formation of larger particle.The possible reason for an increase in particle size could be that, during emulsification, an increase in polymer concentration led to an increase in the viscosity of the organic phase which led to the formation of nanodroplets with a larger size at the interface. While the aqueous volume was kept constant, the enhanced viscosity of the polymer solution led to the formation of larger polymer/solvent droplets [29-31].

Increase in particle size was observed with increase in o/w phase ratio from 4:1 to 8:1.

At lower level, Particle size was not significantly affected but enormously increase in particle size was observed at higher level; which could be due to small amount of aqueous phase volume available at the time of emulsification for hydrophilic molecule desmopressin. As the volume of organic phase increases, greater time requires for evaporation; may lead to an increase in particle size.

The PVA concentration has a significant role in emulsification process, the stabilization of emulsion and the fortification of the droplets from coalescence [32]. At lower concentration, PVA was less effective in reducing interfacial tension between the aqueous phase and an oil phase, resulted in coarser particles. At mid-level, Particle size was reduced significantly. At higher concentration of PVA, the viscosity of aqueous phase increases and thereby leads to aggregation of droplets, which consequences in the coarser particles.

3.2 Effect of process variables on Entrapment Efficiency

The EE of NPs in different formulations is represented in Table 2. Data analysis of this response proved the significant effect of all independent variables ($p < 0.05$).

The maximum Entrapment efficiency of 39.2% was achieved by operating the experiment at the midpoint of each independent variable. Polynomial equation exhibited Positive effect of Amount of Polymer (X1) and PVA concentration (X3); and negative effect of O/W ratios (X2) on entrapment efficiency (Figure 2).

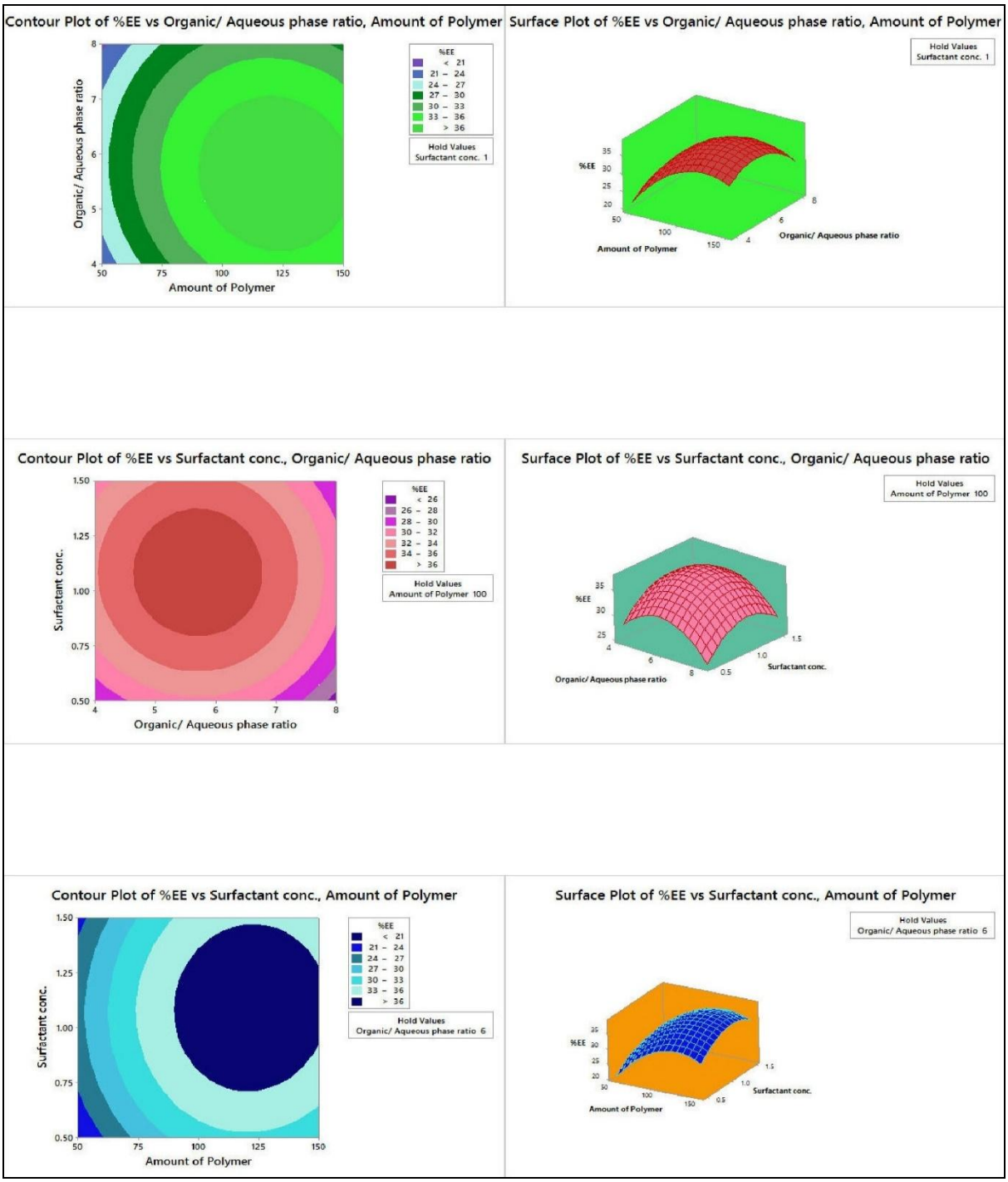

Figure 2. Contour plot and 3D surface plot showing the effect of Amount of Polymer (X1), O/W phase (X2) and PVA concentration (X3) on %Entrapment efficiency.

As mentioned in double emulsion solvent evaporation method, the low %EE of small and hydrophilic drug molecules into the polymer is an important challenge. The higher %EE values

gained by the higher polymer amounts can be explicated by the better coverage of drug molecules within the polymeric matrix [33]. In addition, the initial amount of dissolved drug in the internal phase showed a great influence on %EE.

The results showed that the increase in o/w ratio caused covering of aqueous droplets more efficiently during first emulsion preparation, this larger amount of organic phase required much more time to evaporate. So, the drug molecules had greater opportunity to escape from the inner to outer phase in formulations with higher O/W ratios (>midpoint). Slower solidification of larger particles allowed more drug diffusion to the external phase, which again resulted in the lower entrapment of the drug into the NPs. Employing higher concentration of PVA in the external aqueous phase resulted in higher %EE values [34-35]. PVA reduces surface tension and thereby helps to improve entrapment.

3.3 Optimization

The optimum batch of polymeric nanoparticles was selected by applying constraints; minimum particle size and maximum %Entrapment efficiency. Point prediction was used to determine the optimized NPs on the basis of closeness of desirability factor (close to 1), which predicted the optimized process parameters to be $X1$ 100.9 mg, $X2$ 5.70:1 mL and $X3$ 0.96%. The optimized formulation was developed and characterized for particle size and % drug entrapment. The experimental value for responses of optimized formulation was found in good agreement (<10% difference) with the predicted values generated by the RSM and the result assures the validity of RSM model (Table 3).

Table 3. Comparison of actual and predicted properties of optimized nanoparticles

Responses	Predicted value	Observed value	%Error
Particle size (nm)	212.9	217.2	2.0
%Entrapment efficiency	39.2	39.7	1.3

3.4 Characterization

Particle size and Zeta Potential

Zeta potential for nanoparticles preparation could help to assess the stability of colloidal systems. The surface charge on the particles could control the particles stability of the nanoparticulate formulation through strong electrostatic repulsion of particles with each other. In addition, from the zeta potential measurement, the dominated component on the particles surface was predicted as PLGA. PLGA being negatively charged polymer imparts anionic nature to nanoparticles where zeta potential values were found to be -23.7 mV (Figure 3). Particle size of drug loaded nanoparticles was found to be 217.2 nm with narrow size distribution (Figure 3).

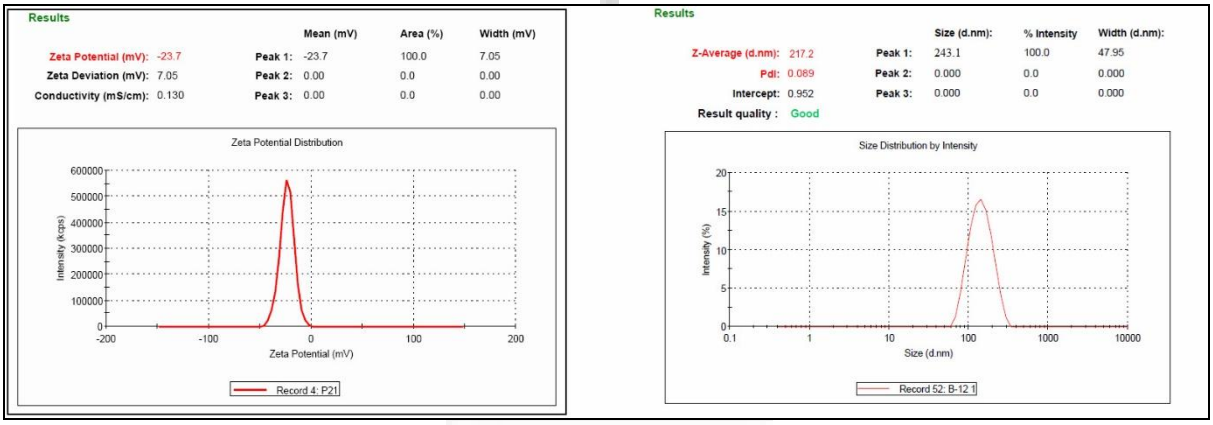

Figure 3. Zeta Potential and Particle size distribution graph of drug loaded Nanoparticles

Differential scanning calorimetry (DSC)

Thermal analysis is a supportive tool for determining the dispersion of the drug in polymeric materials. DSC thermograms of the pure drug, PVA, PLGA and desmopressin loaded nanoparticles are represented in Figure 4. The pure drug showed high endothermic peak indication of its melting peak at ~ 168-170°C which was absent in NPs.

The endothermic peak of desmopressin disappeared in the thermogram of drug loaded NPs, which indicated the absence of crystalline drug in the nanoparticles. So, it can be assumed that encapsulated drug was in an amorphous state or a molecular dispersion throughout the polymer matrix after fabrication of NPs.

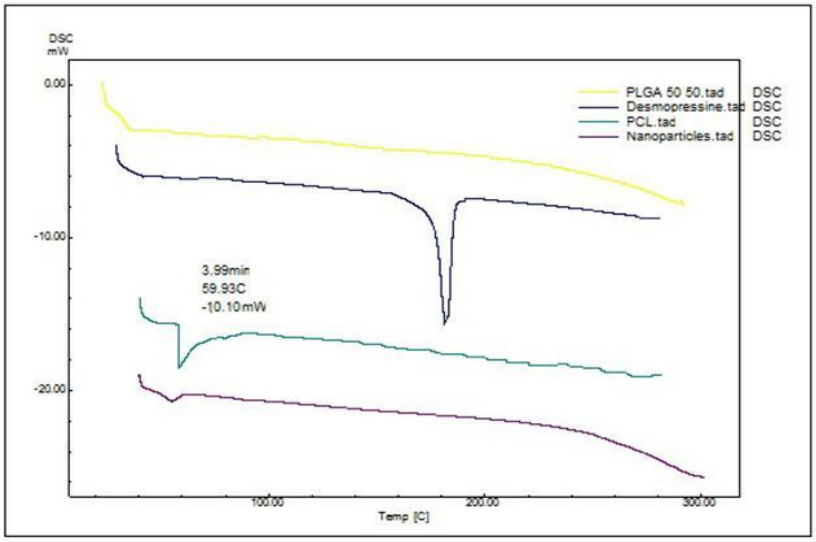

Figure 4. DSC thermogram of PLGA (50:50), Desmopressin, PCL and Nanoparticles

Scanning electron microscopy (SEM)

SEM micrographs showed that uniform polymeric NPs were successfully prepared by using the double emulsion solvent evaporation method. As shown in Figure 5, smooth surface and spherical shape of nanoparticles were observed which can be explained by stability of primary emulsion that the polymer had adequate time to form the condensed matrix around the drug molecules before particle formation.

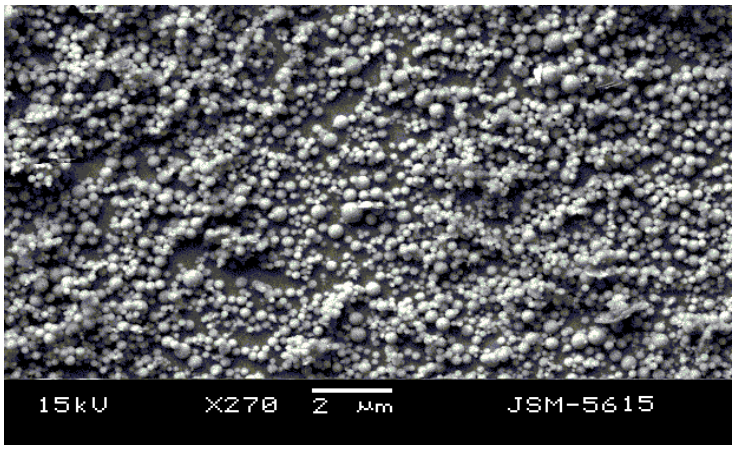

Figure 5. SEM image of optimized formulation of nanoparticles

3.5 *In vitro* drug release study

Regarding the *in vitro* release studies, depiction of the percent drug released versus time yielded a profile with two nearly different phases (Figure 6). Within the first phase of the release profile, an immediate release behavior was observed (around 30% of the drug was released within the first 24 h), while the second phase confirmed to the controlled-release nature of the system.

The prepared nanoparticles were also evaluated in terms of the drug release kinetics as well as the drug diffusion mechanism. The results showed the sustained release of drug from Nanoparticles. The release profile followed biphasic with an initial burst attributed to the drug associated near particles surface, followed by a linear release phase. The drug release profile of optimized formulation confirmed to the Higuchi model (R^2= 0.9907), suggesting the drug release to be a diffusion controlled process based on the Fick's law in which the diffusion coefficient depends upon both the concentration and the time.

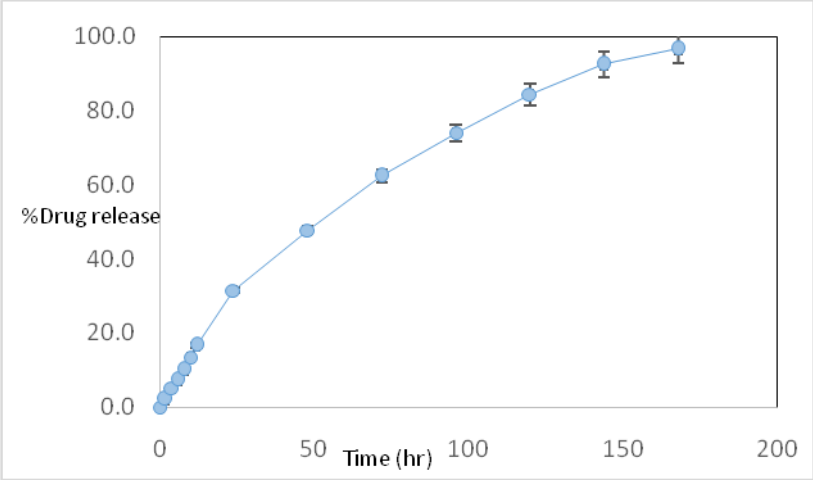

Figure 6. *In vitro* drug release profile from optimized nanoparticles

3.6 Stability study

From the results (Table 4); it was found that particle size of nanoparticulate dispersion increases significantly at room temperature (25 ± 5 °C, 60 ± 5% RH) than freeze temperature (2-8°C). Stability of Desmopressin was assessed by its assay study; showed that at room temp., drug degrades faster in comparison to freeze temp. Desmopressin was found more stable in lyophilized NPs stored at freeze temperature. Thus, to avoid increase in particle size and

Desmopressin degradation, nanoparticle dispersion should be lyophilized. Further, increase in particle size and Desmopressin degradation in lyophilized NPs was found to b6e less at freeze temperature than at room temperature. Hence, it can be concluded that lyophilized NPs should be stored at 2-8°C.

Table 4. Results of stability study of nanoparticles

Months (After Preparation)	Evaluation Parameter			
	Particle size (nm)		Entrapment Efficiency (%)	
	Freeze temp.	Room temp.	Freeze temp.	Room temp.
0	213 ± 0.3	216 ± 0.5	39.29 ± 0.76	38.15 ± 0.37
1	215 ± 0.5	228 ± 0.6	38.74 ± 0.96	37.85 ± 0.37
2	218 ± 0.4	239 ± 0.7	38.16 ± 1.12	36.52 ± 0.73
3	221 ± 0.3	252 ± 0.5	37.62 ± 0.76	35.15 ± 0.37

3.7 CONCLUSION

Desmopressin loaded nanoparticles were prepared by double emulsion solvent evaporation method. The Box- Behnken design was employed to qualify the effect of several processing variables and thereby, minimize the number of experimental trials. All three independent variables had a great influence on the characteristics of polymeric nanoparticles. Particle size and %Entrapment efficiency were greatly affected by the amount of polymer and surfactant concentration. Moreover, O/W phase ratio was found to have a negative impact on %Entrapment efficiency. Checkpoint analysis confirmed optimized formulation in close agreement with predicted points by selecting constraints with the desirability of minimum particle size and maximum entrapment efficiency. The drug release kinetic for optimized nanoparticles followed Higuchi model, showed biphasic release pattern with initial burst release followed by sustained release. Prepared nanoparticles were found to be stable at freeze temperature. This work can be better supported by extending it to animal experiment.

Additionally, nanoformulation is expected to deliver drug in a controlled manner for a longer period of time than its present single dose formulation.

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