Research Article

Chitosan Based Microspheres of Glibenclamide Incorporated in *in-situ* Gel for Nasal Delivery: Optimization and *in-vitro* Evaluation

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Abstract

The purpose of research work was to develop and optimize mucoadhesive microspheres of glibenclamide incorporated in *in-situ* gel for nasal delivery with the aim to enhance the residence time and improve therapeutic efficacy. Chitosan (mucoadhesive) based microspheres of glibenclamide were prepared by emulsification-crosslinking method. Glutaraldehyde was used as crosslinking agent. A 3^2 factorial design was employed for preparation of microspheres wherein the concentration of polymer and crosslinking agent were selected as independent variables while particle size of microspheres, *in vitro* mucoadhesion and % cumulative drug permeation were the dependent variables. Microspheres were evaluated with respect to the production yield, particle size, entrapment efficiency, swelling property, *in vitro* mucoadhesion and % cumulative drug release. Formulation F8 was found to be optimized. The optimized formulation F8 was then formulated into thermo reversible *in-situ* gel. The prepared micro particulate *in-situ* nasal gel was mucoadhesive in nature which adhere onto the mucus and increase the residence time within the nasal cavity.

INTRODUCTION

The nose is considered as an attractive route for needle-free vaccination and for systemic drug delivery, especially when rapid absorption and effect are desired. In addition, nasal delivery may help address issues related to poor bioavailability, slow absorption, drug degradation, and adverse events in the gastrointestinal tract and avoids the first-pass metabolism in the liver [1]. However, when considering nasal delivery devices and mechanisms, it is important to keep in mind that the prime purpose of the nasal airway is to protect the delicate lungs from hazardous exposures, not to serve as a delivery route for drugs and vaccines [2]. Nasal cavity possesses various advantages as a site for drug delivery, such as it provides much vascularized epithelium, large surface area for drug absorption, lower enzymatic activity compared with the gastrointestinal tract and liver and the direct drug transport into the systemic circulation, thereby avoiding hepatic first pass metabolism and irritation of gastrointestinal membrane [3]. Nasal route is non-invasive, therefore it reduces the risk of infection. Furthermore, ease of use and self-medication possibility result in improved patient compliance [3]. The range of compounds investigated for possible nasal application greatly from very lipophilic drugs to polar, hydrophilic molecules including peptides and proteins. The nasal dosage forms involved include solutions, sprays, gels, liposomes and microspheres [4,5].

In nasal drug delivery, the most important limitation factor is rapid mucociliary clearance, which is the cause of a limited contact period allowed for drug absorption through the nasal mucosa. Thus, mucoadhesive nano and micro-particles have been formulated to overcome the rapid mucociliary clearance, thereby increasing drug absorption through nasal cavity [6]. Chitosan is a natural polymer that has mucoadhesive properties because of its positive charges at neutral pH, which enable an ionic interaction with the negative charges of sialic acid residues on the mucus [7]. This highly mucoadhesive characteristics of chitosan provide a longer contact period for drug transport through nasal mucosa

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and prevents the clearance of the formulation via mucociliary clearance mechanism. Therefore, chitosan microspheres have been extensively evaluated as a drug delivery system. In this study, we aimed to formulate glibenclamide-loaded bioadhesive microspheres with chitosan and to investigate feasibility of glibenclamide nasal delivery with chitosan microspheres [8,9].

Chitosan microspheres have received considerable attention as nasal drug delivery systems. Chitosan, being biodegradable, biocompatible, and non-toxic and bioadhesive polymer is a suitable excipient for use in biomedical and pharmaceutical formulations [10,11]. Chitosan is a cationic polysaccharide, derived by the deacetylation of chitin. Chitosan is positively charged due to its amino group and able to interact strongly with the negatively charged mucus layer of the nasal epithelium. This is to provide a longer contact time for drug transport across the nasal membrane, before the formulation is cleared by the mucociliary clearance mechanism. In addition, chitosan has been shown to increase the paracellular transport of polar drugs by transiently opening the tight junctions between the epithelial cells [12,13].

Glibenclamide is a second-generation sulfonylurea used in the treatment of noninsulin-dependent diabetes. It is one of the most prescribed long-acting anti-hyperglycemic agents. Glibenclamide is classified as BCS class II drug, which means it has high permeability and poor water solubility. It has a short biological half life (3 to 5 h), with a log *p* value of 4.7 (o/w). Thus, it has poor aqueous solubility and undergoes oxidative hepatic first-pass metabolism to yield metabolites having no hypoglycemic activity [14-16].

In the present study chitosan microspheres intended for nasal delivery of glibenclamide were prepared by emulsification cross linking technique using glutaraldehyde (GLA) as the cross linking agent. Microspheres were characterized in terms of the particle size, morphological properties, production yield, drug content, encapsulation efficiency, and *in vitro* drug permeation.

MATERIALS AND METHODS

Materials

Glibenclamide was received as a kind gift from Sun Pharmaceutical Industries Ltd. (Sikkim, India). Chitosan was provided by Sigma Aldrich, Steinheim, Japan. All other ingredients used were of analytical grade and were used without further purification. Spectrophotometric studies were carried out by using double-beam UV-spectrophotometer, Shimadzu, Pharma Spec 1700, Kyoto, Japan.

Methods

Preparation of mucoadhesive microspheres: Microspheres were prepared by the emulsification crosslinking technique. For the preparation of microspheres, 1%-3% w/v solutions of chitosan were prepared in acetic acid. Then drug and cross-linking agent were added in each chitosan solution to prepare a uniform dispersion. The drug dispersion was then extruded with the help of syringe and needle (24G) into liquid paraffin containing span 80 being kept under 100 rpm stirring. After 1 hr, the microspheres were separated by filtration and

washed with petroleum ether to remove traces of paraffin oil. The microspheres were dried overnight in an oven at 40°C [7].

Experimental design: A 3^2 full factorial was applied to design the experiments. Concentration of chitosan and volume of glutaraldehyde was used as independent variables, whereas particle size, % mucoadhesion and % cumulative drug release were kept as dependent variables. Formulation F1-F10 was prepared using three different levels of chitosan and glutaraldehyde and response parameters were evaluated (Table 1).

Evaluation of glibenclamide loaded microspheres

Particle size: The optical microscope (Hicon^R, Grover Enterprises, Delhi) was used for measurement of particle size of microspheres and the mean particle size was calculated by measuring more than 100 particles with the help of a calibrated ocular micrometer (Hicon^R, Grover Enterprises, Delhi).

Determination of percentage yield: Percentage yield of glibeclamide containing microspheres was calculated by dividing actual weight of product after drying to total amount of glibenclamide and excipients used for the preparation of microspheres and are represented by following formula:

Percentage Yield =

$$\frac{\text{Actual weight of microspheres after drying}}{\text{Total weight of the drug and excipients}} \times 100$$

Determination of entrapment efficiency: 10 mg of microspheres were crushed and dissolved in required quantity of phosphate buffer pH6.4 and kept overnight. The samples were assayed for drug content by UV- spectrophotometer at 300 nm. The drug loading and entrapment efficiency was calculated using following formula:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Mactual}}{\text{Mtheoretical}} \times 100$$

Where M_{actual} is the actual glibenclamide content in weighed quantity of powder of microspheres and $M_{theoretical}$ is the theoretical amount of glibenclamide in microspheres calculated from the quantity added.

Swelling ability of microspheres: The swelling ability of microspheres was determined by allowing them to swell to their equilibrium in phosphate buffer of pH 6.4. Swelling was determined in triplicate by using the following formula:

$$\alpha = \frac{Ws - Wo}{Ws}$$

Where α is degree of swelling, W_o is initial weight of microspheres and Ws is the weight of microspheres after swelling.

***In vitro* mucoadhesion study:** The mucoadhesive property was determined by falling liquid film technique. A freshly cut piece, 5 cm long, of goat nasal mucosa obtained from a local abattoir was cleaned by washing with isotonic saline solution. An accurate weight of microspheres was placed on mucosal surface, which was attached over a glass plate that fixed in an angle of 45°C relative to the horizontal plane and phosphate buffer of pH 6.4

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warmed at 37°C was drawn at a rate of 5 mg/min over the tissue from the burette. One hour after administration of microspheres, the concentration of the drug in the collected perfusate was spectrophotometrically determined. The microspheres amount corresponding to the drug amount in perfusate was calculated. The adhered microspheres amount was estimated from the differences between the applied microspheres and the flowed microspheres amount. The ratio of the adhered microspheres was computed as percentage mucoadhesion [17].

In vitro drug permeation: The permeation of drug from microspheres was determined by using a Franz diffusion apparatus which consists of donor and receptor compartments. Goat nasal mucosa was used to keep the microspheres on the donor side, which allowed free diffusion of drug to the receptor compartment containing 10 ml phosphate buffer solution pH 6.4 (within the pH range in nasal cavity) and maintained at 37 ± 0.05°C. The receptor compartment was stirred with a magnetic stirring bar. At predetermined time intervals, aliquots (1 ml) were withdrawn from receptor cell and replaced with the same volume of fresh medium. The samples were diluted appropriately and assayed spectrophotometrically at 300 nm to analyze the drug permeation. Graph was plotted between percent cumulative drug permeation versus time. The release data generated was subjected to zero order, first order and Higuchi's model(s) to understand the release kinetics of glibenclamide from microspheres. The model with highest correlation coefficient was considered to be the best fitted for release profile.

Selection of optimized formulation

The optimized formulation was selected on the basis of minimum particle size, maximum mucoadhesion and maximum cumulative drug permeation at 12 h, and was subjected to characterization studies.

Characterization of optimized formulation

Scanning electron microscopy: The surface morphology of the glibenclamide, placebo and glibenclamide loaded microspheres were examined by scanning electron microscopy. The samples were mounted directly onto the SEM sample holder using double sided sticking tape and images were recorded at the 100X magnification at the acceleration voltage of 10 kv.

Differential scanning calorimetry: Differential Scanning Calorimetry was performed on placebo microspheres, drug-loaded microspheres and Glibenclamide using differential scanning calorimeter instrument (NETZSCH DSC 200F3 240-427-L, Japan). Samples were heated at the rate of 10°C min-1 over the temperature range of 30-300°C. An inert atmosphere was maintained by purging with nitrogen at the flow rate of 70 mL/min.

Fourier transform infrared spectroscopy (ft-ir spectroscopy): Glibenclamide, Chitosan physical mixture and optimized formulation (F8) was examined using FTIR Spectrophotometer (Shimadzu FTIR-8400S Kyoto, Japan). The test sample diluted with KBr to get a final dilution of 1:10 was mounted into the instrument. The measurements were made in transmittance mode in the range of 400-4000 cm⁻¹ against the background spectra of pure KBr by setting resolution of 4 cm⁻¹ and 50 times accumulation.

Development of microspheres loaded in situ gel: Optimized formulation F8 was developed as micro particulate loaded in situ gel (thermoreversible) by cold method. In this method, specified amount of carbopol 934P was stirred in the calculated amount of distilled water at room temperature. The dispersion was cooled to 4°C by keeping it in a refrigerator. Pluronic F127 was added in different concentrations with continuous stirring at 50 rpm by Remi stirrer (thermostatically controlled magnetic stirrer, S.M. Scintific Instruments (P), Ltd, India). Dispersion was then stored in a refrigerator overnight to clear solution. The microspheres containing 50 mg equivalent Glibenclamide were incorporated into the in situ gel. Thereafter, gel formulation was stored in a refrigerator so that it remains in sol form.

Evaluation of glibenclamide loaded microspheres incorporated in in situ gel

Clarity: The clarity of all formulations was determined by visual inspector under black and white background.

Gelling temperature: It is defined as the temperature at which liquid phase makes the transition to a gel. 2ml of sol were transfer to a test tube, immersed in water bath. The temperature of water bath was increased in increment of 1°C and left to equilibrate for five minute at each new setting. The sample was examined for gelation which was said to have occurred when the meniscus would have no longer move upon tilting test tube through 90°C.

pH and viscosity: One ml quantity of gel formulation was transferred to the 10ml volumetric flask and diluted by using distilled water to make volume up to 10ml. the pH of resulting solution was determined by using digital pH meter, model 111 E(HICON, New Delhi, India). The viscosity of in situ gel was measured using Brookfield viscometer DV-II + Pro coupled with S-94 spindle at 100 rpm at 4 ± 1°C.

Drug content: One ml of gel was extracted with 10 ml phosphate buffer pH 6.4 by vortexing (vortex shaker, HICON, India). Further dilution were made with phosphate buffer pH 6.4 and analyzed by UV spectrophotometer at 300 nm.

Gel strength: Gel strength was measured by placing 50 g of formulation in a 100ml graduated cylinder and gelled at 37°C using thermostat. A weight of 35 g was placed on to the gelled solution and allowed to penetrate 5 cm in the gel. Time taken by weight to sink 5 cm was measured.

Ex-vivo permeation: The in vitro permeation study was performed using goat nasal mucosa. The nasal mucosa was collected from local slaughter house and washed three times with phosphate buffer pH 6.4. The extraneous tissues were removed with surgical blade to get the mucosa. The prepared nasal mucosa was inserted in Franz diffusion cell having permeation area of 3.14 cm². Stabilization was performed by stirring the receiving medium with magnetic stirrer for 30 minute in phosphate buffer pH 6.4. After stabilization the phosphate buffer was replaced with fresh media i.e. 18 ml of phosphate buffer pH 6.4 at 34°C. Then the formulation equivalent to 5mg of glibenclamide was replaced in the donor chamber. At predetermined time points i.e. at 0,1........8h, 1ml sample were withdrawn from the acceptor compartment and replaced with an equal volume of the phosphate

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buffer pH 6.4. the amount of permeated drug was determined by using UV spectrophotometer at 300 nm.

HISTOLOGY

Histological studies were conducted to determine the effect of formulation on nasal mucosa. Goat nasal mucosa was collect from the local slaughter house and transported to laboratory in normal saline at cold condition within 1h of slaughtering. The nasal mucosa was incubated in optimized G5, phosphate buffer saline pH6.4 as negative and 75% isopropyl alcohol as positive control for 30minute. The mucosa washed with phosphate buffer pH6.4 then immediately fixed with 10% v/v formalin solution for 24h, their after embedded in paraffin. Paraffin sections (7 μm) were cut on glass slides and stained with hematoxylin and eosin. Then to detect any damage to the tissue, sections were observed through photomicrograph (HICON enterprises, New Delhi, India) at 100X magnification.

RESULT AND DISCUSSION

Characterization of glibenclamide loaded microspheres

Glibenclamide loaded cross linked chitosan microspheres were prepared with spray-drying method. The characteristics of the microspheres are presented in Table (3).

Particle Size: The largest particle size was found in formulation F7 (75.66 μm) (Table 3). F3 showed least particle size (38.66 μm) followed by F2 (40.33 μm). A particle size more than 10 μm and less than 100 μm is desirable for nasal deposition thus all the formulations were suitable for nasal delivery. Formulation F7 exhibited larger particle size (75.66 μm) than F4 (52.66 μm) because of higher concentration of polymer. Analysis of the effect of independent variables on the particle size revealed that on increasing the concentration of the polymer (chitosan) the particle size increased, as high concentration of chitosan increases the viscosity of polymeric dispersion and larger droplets are formed. As the concentration of cross linking agent was increased, the particle size decreased due to formation of rigid network structure.

Percentage yield: Percentage yield of microspheres ranged within 68.66 to 84.57% (Table 3) indicating efficiency of the process. Considering the design as the concentration of polymer increased, % yield increased.

***In vitro* mucoadhesion:** The mucoadhesion of glibenclamide loaded nasal microspheres closely varied between 67.9 ± 0.675 to 87.20 ± 1.101 (Table 3) and was dependent on polymer concentration. The percent mucoadhesion was found to decrease from 87.2 ± 1.101 to 67.9 ± 0.675 with gradual decrease in concentration of chitosan from 3 to 1% w/v. F3 showed lowest mucoadhesion (64.3 ± 0.650) containing more concentration of cross linking agent but F8 exhibited highest mucoadhesion (87.2 ± 1.101) followed by F3 (64.3 ± 0.650). As the concentration of chitosan increased the mucoadhesion ability of the microspheres increases due to enhanced binding of chitosan molecules with Mucin. As a result the formulation will be able to resist physiological process of mucociliary clearance *in vivo*. The mucoadhesive property of microspheres decreased on increasing the concentration of cross-linking agent. Pre-requisite for a good mucoadhesion is the high flexibility of polymer backbone structure and its polar functional groups. Such flexibility of the polymer chain is reduced if the polymer molecules are cross-linked either with each other or with cross-linking agent. The

Table 1: 3^2 Full Factorial Design Layout for Preparation of Glibenclamide loaded microspheres.

Formulation code	Glibenclamide (mg)	Chitosan (%w/v) (X1)	GLA (ml) (X2)	Dependent variables
F1	100	1(-)	1(-)	
F2	100	1(-)	2(0)	
F3	100	1(-)	3(+)	
F4	100	2(0)	1(-)	
F5	100	2(0)	2(0)	Particle size (Y1)
F6	100	2(0)	3(+)	% Mucoadhesion (Y2)
F7	100	3(+)	1(-)	% CDP (Y3)
F8	100	3(+)	2(0)	
F9	100	3(+)	3(+)	
F10*	100	1.5	1.5	
*extra design check point formulation				

Table 2: Formulation composition of Glibenclamide loaded microparticulate *in situ* gel.

Formulation code	Concentration of Poloxamer 407 (%w/v)	Concentration of carbopol 934P (%//v)
G1	14	0.5
G2	16	0.5
G3	18	0.5
G4	20	0.5
G5	22	0.5

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Table 3: Characterization of mucoadhesive glibenclamide loaded microspheres.

Code	Mean Particle size (µm)	P. Yield (%)	Drug Entrapment (%)	Entrapment Efficiency (%)	Mucoadhesion (%±SD)	Swelling Index (%±SD)	Cumulative drug permeation (%±SD)
F1	44	68.66	68.11±0.300	33.06±0.145	67.9±0.675	0.210±0.0095	57.16±0.381
F2	40.33	79.81	69.54±1.761	17.42±0.443	65.9±1.305	0.182±0.0071	67.33±0.303
F3	38.66	82.85	78.94±2.016	13.61±0.348	64.3±0.650	0.169±0.0056	61.49±0.172
F4	52.66	84.57	78.12±2.019	13.02±0.104	75.1±1.014	0.293±0.0081	64.10±0.502
F5	50.66	81.28	80.27±0.282	14.10±0.017	74.3±1.069	0.273±0.0023	77.94±2.869
F6	45	81.40	82.52±1.184	20.27±0.290	73.6±1.365	0.238±0.0037	73.27±0.479
F7	76	79.00	82.17±0.834	20.80±0.210	83.9±1.708	0.366±0.0036	65.90±0.172
F8	68	73.64	85.27±0.550	38.57±0.250	87.2±1.101	0.340±0.0050	80.81±2.205
F9	61	77.66	82.06±0.960	35.21±0.406	79.8±0.556	0.320±0.0051	75.76±0.335
F10*	54	79.78	78.63±0.571	20.74±0.149	72.7±1.20	0.250±0.0058	80.52±0.334

decrease in flexibility imposed upon polymer chain by cross-linking makes it more difficult for cross-linked polymer to penetrate the mucin network. Thus cross-linking effectively limits the polymer chain that can penetrate the mucus layer and could possibly decrease mucoadhesion strength.

***In vitro* drug permeation study:** Permeation study of glibenclamide loaded nasal microsphere of all formulation was performed in the pH of nasal cavity (phosphate buffer pH 6.4) for 12 hrs using goat nasal mucosa in Franz diffusion cell [18]. It was found that, at the end of 12hrs formulation F5 and F8 showed highest drug permeation in phosphate buffer pH 6.4 i.e 77.94 ± 2.869 and 80.81 ± 2.205 respectively (Figure 1). This shows that when particle size increased, drug permeation was decreased. In large microspheres permeation was decreased due to increase in diffusional path length which the drug molecules have to travels (Sultana et al., 2010). But F8 shows high drug permeation in comparison of F5 this may be due to less drug entrap in microspheres of F5 formulation because of less particle size. The results showed that optimized formulation showed highest and prolonged drug release as compared to other formulation and in optimized formulation drug release rate was controlled by the presence of polymer (chitosan). Target flux of microsphere formulation was obtained 17.579 µg/cm^2/hr which was achieved in optimized F8 formulation.

Data analysis: On the basis of the data obtained from the optimized formulations, a general statistical model can be depicted with respect to the above data. The model developed can be characterized by using the polynomial equation representing the respective response data. This can be given as follows:

Particle size = + 49.67 + 13.83X1 - 4.8X2 - 2.255X1X2 + 4.83X1^2 - 0.17X2^2

% Mucoadhesion = + 74.67 + 8.80X1 - 1.53X2

% Cumulative drug permeation = + 78.29 + 6.78X1 + 4.59X2 + 0.34X1X2 - 4.39X1^2 - 9.77X2^2

After removing the non-significant values the equation found out is known as transformed equation or final equation. These are as follows:

Particle size = + 49.78 + 13.83X1 - 4.8X2 - 2.255X1X2 + 4.83X1^2

% Mucoadhesion = + 74.67 + 8.80X1

% Cumulative drug permeation = + 78.29 + 6.78X1 + 4.59X2 - 4.39X1^2 - 9.77X2^2

From the above polynomial equations, 3D response surface graphs of the respective responses were generated, which were used to predict the responses of dependent variables at the intermediate levels of independent variables.

Response-surface analysis: Three dimensional response surface plots drawn for the graphical optimization of glibenclamide loaded nasal system are presented in Figure (2). Figure (2a) shows that when concentration of chitosan (X1) and volume of glutaraldehyde (X2) increases simultaneously than particle size was increases. Same effect was observed in Figure (2b) when concentration of chitosan (X1) and volume of glutaraldehyde (X2) increases simultaneously than Mucoadhesion was also increases. In case of % CDP, when concentration of chitosan increases and volume of glutaraldehyde decreases, then % CDP increases.

Validation of design: For the validation of 3^2 full factorial design one extra check point was formulated and evaluated on the basis of their particle size, % mucoadhesion, and % cumulative drug release. The results were found that the experimental values were close to the predicted values. (Table 4) lists the composition of the extra check points, the predicted and experimental values of the entire response variable and the percentage error, which is
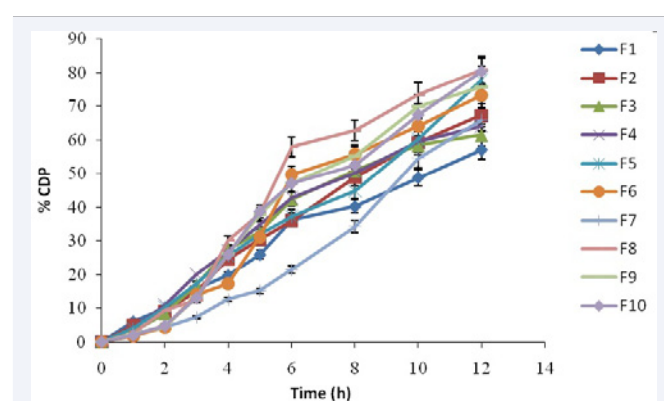
Figure 1 % Cumulative drug permeation of F1-F9 formulations.
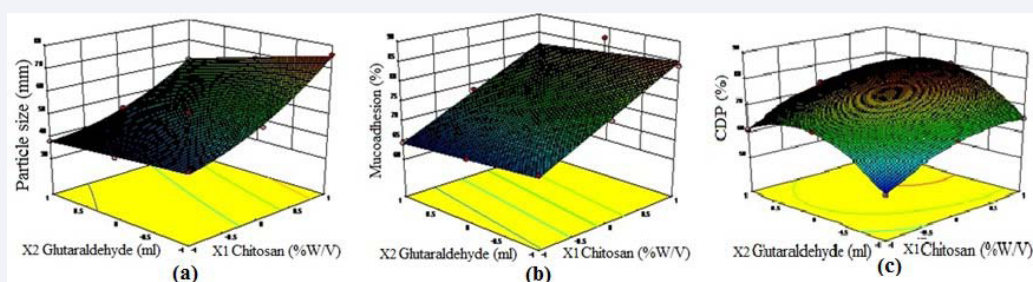
Figure 2 3D Surface response plots showing effect of independent factor on particle size (a), % Mucoadhesion (b) and % Cumulative Drug Permeation (c).

calculated by the following formula;

$$\text{Percentage error} = \frac{\text{Predicted value} - \text{Actual value}}{\text{Predicted value}} \times 100$$

Upon comparison of the predicted values to the experimental values, the predicted error was found. Thus the low magnitude of error in the current study indicates a high prognostic ability of nasal microspheres of glibenclamide and also proved that 3^2 full factorial design was validated.

Selection of optimized formulation

The formulation F8 was optimized on the basis of minimum particle size, maximum mucoadhesion and % cumulative drug permeation which were found to be 69.11 µm, 87.20 ± 1.101 % and 80.81 ± 2.205 % respectively. The *in-vitro* drug permeation data was fitted to different kinetics models namely zero order, First model, Higuchi model, Hixon-Crowell model and Peppas model. Zero order model was considered as the best fitted model with the highest value of coefficient of determination which is as $r^2 = 0.9729$ in pH 6.4 (Table 3), which shows that the drug was released in controlled manner in the simulated nasal pH.

Characterization of optimized formulation

Scanning electron microscopy: The placebo and drug loaded microspheres were nearly spherical in appearance (Figure 3). Placebo and glibenclamide microspheres were found to be discrete and with rough surface. SEM micrograph reveals no difference between the surface of placebo and drug loaded microspheres, explaining the dispersion of drug in the polymeric matrix.

Differential scanning calorimetry (DSC): DSC thermogram of the glibenclamide showed sharp peak at 172°C due to its melting, which is consistent with literature report (B.P 2004). Placebo microspheres showed an exothermic peak at 92°C. This indicated that the exotherm at 92°C corresponded to thermal decomposition of the chitosan. In glibenclamide loaded microspheres characteristic peak of glibenclamide was not observed at 172°C, suggesting that glibenclamide was molecularly dispersed in matrix (Figure 4).

Fourier transforms infrared spectroscopy (FTIR): The FTIR studies were carried out to assess any possible interaction between drug and polymer. The FTIR spectrum of glibenclamide was done by using KBR pellet technique. Glibenclamide and chitosan showed characteristic peak at range of 400-4000 cm^{-1}. Glibenclamide showed prominent peaks at wave numbers were 3315 cm^{-1} due to stretching of N-H group. Sharp peak was observed at 2910 cm^{-1} indicated C-H stretching vibration. The peak at 2350 cm^{-1} and 1714 cm^{-1} showed stretching due to O-H and C=O group. The dominant IR peak of chitosan was observed at 2920 cm^{-1} due to presence of C-H group, peak at 1310 cm^{-1} due to N-acetyl glucosamide and -CO group stretching at 1094 cm^{-1}. The spectral analysis of optimized formulation (F8) as shown in Figure (5) - showed characteristic bands of glibenclamide at 3315 cm^{-1}, 2931 cm^{-1}, 1714 cm^{-1} corresponding to -NH stretching, C-H stretching and C=O stretching respectively (Pavia et al., 2007). These characteristic stretching vibrations were also present in physical mixture (Figure 5) without any significant shift in the wave number of peaks and changes in the intensity of the peaks indicating no interaction between drug and polymer.

Table 4: Comparison of experimental results with predicted responses of glibenclamide loaded nasal microspheres formulations.

F. code	Composition		Response	Predicted Value	Experimental Value	Error (%)
	X1	X2				
F 10*	1.5	1.5	Particle size (µm)	54.81	54	1.47
			% Mucoadhesion	78.30	78.28	0.025
			% Cumulative Drug permeation	80.52	80.32	0.248

Table 5: Model fitting on the release data of glibenclamide from optimized formulation (F8).

Simulated pH	Result	Zero Order	First Order	Higuchi	Peppas	Hixon-Crowel
6.4	r^2	0.9729	0.9715	0.8812	0.9368	0.9521
	B	7.55	0.063	2.730	1.647	0.179
	A	1.78	2.068	1.865	0.324	4.781

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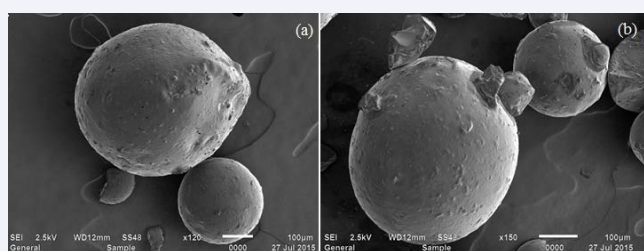*Email: manish_singh17@gmail.com*

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Figure 3 Scanning electron micrographs of placebo (a) and glibenclamide loaded microspheres of optimized formulation (b) of microspheres.
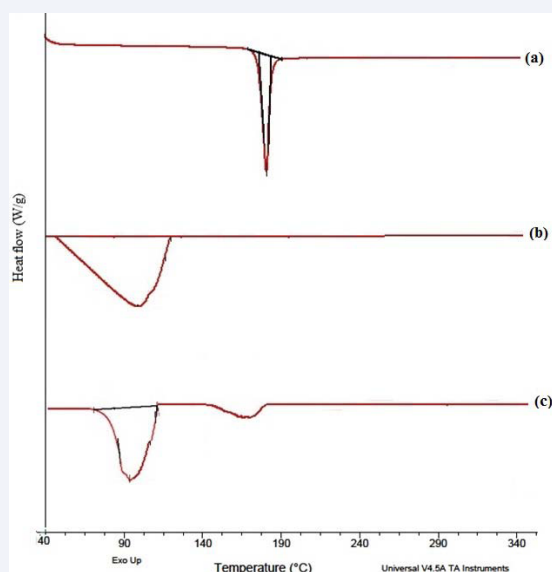

Figure 4 DSC of (a) Glibenclamide (b) Placebo microspheres and (c) Glibenclamide loaded microspheres.
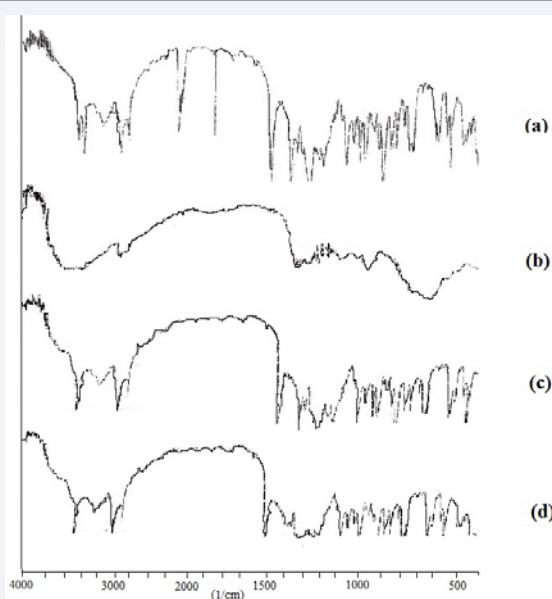

Figure 5 FTIR spectra of (a) pure drug glibenclamide (b) Chitosan (c) Physical mixture (d) Optimized formulation (F8).

Formulation of glibenclmide loaded microspheres incorporating in *in situ* gel

The optimized formulation (F8) was further incorporated into thermoreversible *in situ* gelling system with the aim to achieve more controlled release of microspheres from gelling system. The evaluation characteristics of different formulations of glibenclamide loaded microspheres incorporated in *in situ* gel (G1-G5) prepared using cold method (Table 2).

Evaluation of glibenclamide loaded microspheres incorporated in *in situ* gel

Clarity: All the formulations were found to be clear. There were no residue matters present which can harm nasal mucosa and affect the syringeability of the formulations [19].

Gelling temperature: The gelling temperature of the formulations was ranged from 27.2-35.9°C (Table 6). A gelling temperature is suitable in the range of 25°C to 37°C. The gel having gelling temperature lower than 25°C, leads to difficulty in manufacturing, handling and administering and gel having gelling temperature higher than 37°C, it would not form a gel at the temperature of the nasal cavity. The results revealed that the gelling temperature decreased with increase in the concentration of thermo sensitive polymer Poloxamer 407 due to formation of larger number of the micelles that occupy larger volume at lower temperature [20]. The mucoadhesive agent carbopol 934 P also causes lowering of gelling temperature because of its ability to bind to poly (ethylene) oxide chains present in Poloxamer 407 molecule, thus promoting dehydration and cause an increase in entanglement of adjacent molecule with more extensive intermolecular H-bonding [21]. Below the transition temperature Poloxamer 407 solutions allow a precise and comfortable delivery in the nasal cavity where thermo gelation occurs. Immediate gelling increases residence time.

pH and viscosity: pH is a very important factor for nasal formulations. The normal physiological pH of the nasal mucosa ranges from 4.5-6.5. But the nasal mucosa has capability to tolerate pH between 3 to 10. The pH of all the formulations was ranged from 5.2 ± 0.20-5.8 ± 0.26 i.e, which is within the range of nasal mucosa and can be easily tolerated and thus causes no irritation to it. As alkaline pH inactivates the lysozyme secreted by nasal cells, hence makes the nasal tissue susceptible to microbial infection and lower pH acts as hypertonic solution, which causes the shrinkage of epithelial cells and also inhibits ciliary activity. Viscosity of all the formulations lies between 45.6 ± 0.15-67.7 ± 0.15 cps. The formulations should have an optimum viscosity, which will allow its easy administration into nasal cavity, as a liquid, which will then undergo rapid sol to gel conversion and it helps in increasing the nasal residence time of the formulation by decreasing the mucociliary clearance [21].

Drug content: Drug content of all formulations was found to be in the range of 81.24-93.91% (Table 6). Formulation G5 was found to contain high drug content (93.91 ± 2.07%) when compared to other *in situ* gel formulations.

Gel strength: All the formulations showed a gel strength value in range 27.36-48.40 (Dyne/cm^2) (Table 6) which was acceptable for nasal delivery as the gel strength values between

Table 6: Evaluation parameters for glibenclamide loaded mucoadhesive microspheres incorporated in *in situ* gel.

Formulation code	Clarity	pH	Gelling temp. (°C)	Viscosity of sol (cps)	Drug content (%)	Gel streanth (dyne/cm²)
G1	Clear	5.6±0.25	35.9±0.55	45.6±0.15	85.57±2.50	27.36±0.55
G2	Clear	5.8±0.26	32.2±0.35	48.6±0.05	81.24±3.19	36.23±0.45
G3	Clear	5.5±0.26	30.5±0.80	55.6±0.20	89.07±1.50	41.23±0.30
G4	Clear	5.4±0.20	29.3±0.95	61.3±0.05	84.57±2.67	44.90±0.36
G5	Clear	5.2±0.20	27.2±0.45	67.7±0.15	93.91±2.07	48.40±0.45

25 to 50 sec. were considered sufficient. The gel strength was found to be affected by concentration of Poloxamer 407. It has been reported that the gel strength duration of less than 25 sec is not be able to retain its integrity and can erode rapidly while gels with strength greater than 50 sec are too stiff which may cause discomfort to the mucosal surfaces or may damage it [17].

***In vitro* drug permeation study:** *In vitro* drug permeation studies of glibenclamide from optimized formulation F8, G5 and control gel were studied using goat mucosa via Franz diffusion apparatus (Figure 6) [22]. The percentage cumulative drug permeation from *in situ* gel formulation (G5) at 12 h was found to be 69.27 ± 0.196. The permeation profile of G5 was compared to optimized microspheres formulation (F8) and control gel of same concentration of Poloxamer 407 (18% w/v) as compared to G5. A significant difference was observed between the permeation profiles. The permeation profile was obtained indicate the greater sustained action of developed microparticulate *in situ* gel G5 as compared to the control gel and microparticulate formulation (F8) because of presence of Poloxamer 407 in the gel which retards the drug release rate and thus permeation slightly decreases due to reduction in dimension of water channels [23].

This study also confirms the importance of chelating calcium for enhancing the permeation of glibenclamide across the mucosa. Anionic polymer carbopol is reported to demonstrate permeation - enhancing properties as it is able to bind Ca2+ of the nasal mucosa [24-28]. When carbopol concentration is increased, it results in increased concentration of ionized carboxyl group which causes conformational changes in the polymeric chain. Decoiling of the polymer chain occur due to electrostatic repulsion of the ionized carboxyl group, which
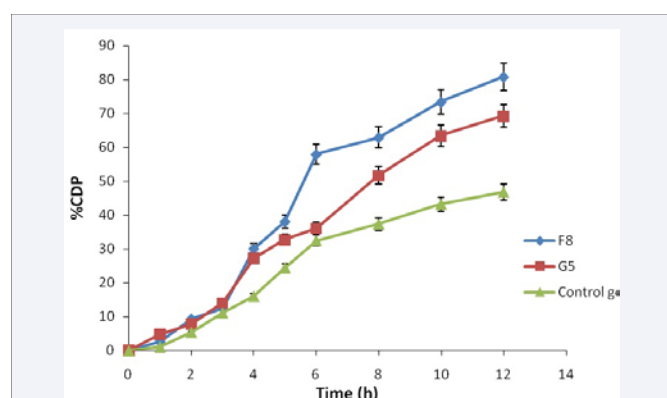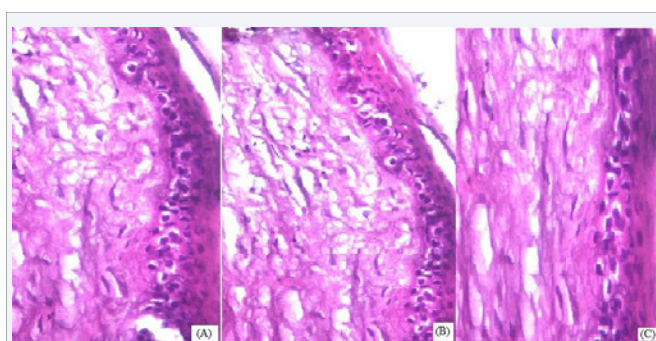
Figure 7 Histological photomicrographs of goat nasal mucosa (A) Negative control mucosa treated with phosphate buffer pH 6.4 (B) positive control mucosa treated with 75% isopropyl alcohol (C) Nasal mucosa after permeation study of glibenclamide loaded microsphere incorporated in *in- situ* gel.

results in relaxation of polymer network. During this stage, drug is rapidly dissolved and diffused from the gels due to very high swelling of the ionized carbopol [21].

HISTOLOGY

The integrity of optimized microparticulate *in situ* gel formulation (G5) was evaluated using histological sections of sheep nasal mucosa. After incubating nasal mucosa in 75 % isopropyl alcohol, an irritant damage of the epithelium from the nasal mucosa was observed. But no significant effect on the microscopic structure of mucosa was seen when microscopic observation was done for the optimized micro particulate *in situ* gel formulation (G5). As shown in Figure (7) neither cell necrosis nor removal of the epithelium from the nasal mucosa was observed after permeation of gel. There were no alterations in epithelium layer, basal membrane and superficial part of sub mucosal blood vessels. Thus it can be said that the *in situ* gel formulation can be safely administered via nasal route.

CONCLUSION

Developed glibenclamide loaded mucoadhesive microspheres incorporated in *in-situ* gel (G5) can be considered as an effective and superior doses form. It may overcome the drawbacks of commercial formulations. The developed formulation show longer duration of action (up to 12h). Furthermore, presence of poloxamer 407 as an *in situ* gelling polymer had sufficient mucoadhesive properties that result in increase residence time of drug with the nasal mucosa due to transformation of microspheres into gel which is not easily clear by mucociliary clearance [29-34].

Figure 6 Comparative Ex-vivo permeation profile of Glibenclamide from F8, G5 and control gel.

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