4.1 MATERIALS AND METHODS

4.1.1 Materials:

- Carvedilol (CRV) was a gift sample from Torrent Research Centre, Ahmedabad, India.
- Chitosan was a gift sample from Ample Effect Sdn Bhd, Selangor (Malaysia) and used without any modification and purification (Molecular weight < 600,000 Daltons, Degree of deacetylation > 85%).
- Liquid paraffin (light and heavy), glutaraldehyde (25% aqueous solution) (GA), and hexane were purchased from S. D. Fine Chemicals, Mumbai, India.
- Dioctyl sodium sulfosuccinate (DOSS) was procured from Wilson Laboratories, Mumbai, India.
- Mucin (type III, partially purified from porcine stomach, bound sialic acids ~1%) was purchased from Sigma Chemical Company, St. Louis, MO.
- A dialysis membrane (cut-off Mw 12000) was procured from Hi Media, India.
- All other chemicals and reagents used in the study were of analytical grade.

4.1.2 Equipments

- Eurostar high speed stirrer (IKA Labortechnik, Germany)
- Remi high speed magnetic stirrer (Remi, MS500, Remi equipments, Mumbai, India)
- Malvern particle size analyzer (Malvern Mastersizer 2000, Malvern Instruments, UK)
- UV-VIS spectrophotometer (Shimadzu UV1610, Japan)
- Light transmission microscope (Olympus Optical Co. Ltd., Japan)
- Scanning electron microscopy (JSM 5610 LV, Jeol Datum Ltd., Japan)
- Differential Scanning Calorimeter (Mettler Toledo DSC 822e, Japan)
- X-ray diffractometer (Bruker AXS D8 Advance, with X-ray source of Cu, Wavelength 1.5406 A° and Si(Li) PSD detector)

4.2 Preparation of Chitosan Microspheres

Chitosan microspheres were prepared by simple w/o emulsification-cross linking process using liquid paraffin (heavy and light, 1:1) as external phase (Thanoo et al., 1992). Briefly, chitosan (0.2 g) was dissolved in 2% aqueous acetic acid solution (10 mL) by continuously stirring until a homogeneous solution was obtained. Then the solution was added slowly through syringe to 100 mL of liquid paraffin (heavy and light, 1:1) containing 0.2% w/v of DOSS as stabilizer under constant stirring at 1200 rpm for 15 min using a Eurostar (IKA Labortechnik, Germany) high speed stirrer. To this W/O emulsion, 1 ml of glutaraldehyde (25% solution, as cross linking agent) was added slowly and stirring was continued for 2 h. The hardened microspheres were separated by vacuum filtration and washed several times with hexane to remove oil. Finally, microspheres were washed with distilled water to remove unreacted GA. The microspheres were air dried for 24 h and then stored in vacuum desiccator until further use.

4.3 Optimization of Formulation Parameters

Almost all procedures reported for the preparation of chitosan microspheres have been same, but the formulation conditions are varied. Hence, it was necessary to optimize formulation parameters like chitosan concentration, stirring speed, volume of GA (cross linking agent), cross linking time, aqueous to oil phase volume ratio and DOSS (Dioctyl sodium sulphosuccinate) concentration. The influence of these formulation parameters on particle size distribution of chitosan microspheres was investigated. Various batches of formulations were prepared by varying one parameter and keeping the others constant as given in Table 4.1.

Table 4.1 Different batches of chitosan microspheres

Constant parameters		Formulation variables
		Chitosan concentration
Aqueous to oil phase ratio	10:100	A1 (1%)
Stirring rate	1200 rpm	A2 (2%)
Volume of GA	1 ml	A3 (3%)
Cross linking time	2 hr	
		Aqueous to oil phase ratio
Chitosan concentration	2%	B1 (5:100)
Stirring rate	1200 rpm	A2 (10:100)
Volume of GA	1 ml	B3 (20:100)
Cross linking time	2 hr	B4 (30:100)
		Stirring rate
Chitosan concentration	2%	C1 (600 rpm)
Aqueous to oil phase ratio	10:100	C2 (900 rpm)
Volume of GA	1 ml	A2 (1200 rpm)
Cross linking time	2 hr	C3 (1500 rpm)
		Volume of GA
Chitosan concentration	2%	D1 (0.5 ml)
Aqueous to oil phase ratio	10:100	A2 (1 ml)
Stirring rate	1200 rpm	D2 (2 ml)
Cross linking time	2 hr	D3 (3 ml)
		Cross linking time
Chitosan concentration	2%	E1 (1 hr)
Aqueous to oil phase ratio	10:100	A2 (2 hr)
Stirring rate	1200 rpm	E2 (3 hr)
Volume of GA	1 ml	
		DOSS concentration
Chitosan concentration	2%	F1 (0.1 %)
Aqueous to oil phase ratio	10:100	A2 (0.2%)
Stirring rate	1200 rpm	F2 (0.3%)
Volume of GA	1 ml	
Cross linking time	2 hr	

4.4 Preparation of CRV loaded Chitosan Microspheres

Chitosan microspheres were prepared by simple w/o emulsification-cross linking process using liquid paraffin (heavy and light, 1:1) as external phase (Thanoo et al., 1992). Briefly, chitosan (0.2 g) was dissolved in 2% aqueous acetic acid solution (10 mL) by continuously stirring until a homogeneous solution was obtained. Carvedilol (0.2 g) was added in chitosan solution and the dispersion was added slowly through syringe to 100 mL of liquid paraffin (heavy and light, 1:1) containing 0.2% w/v of DOSS as stabilizer under constant stirring at 1200 rpm for 15 min using a Eurostar (IKA Labortechnik, Germany) high speed stirrer. To this W/O emulsion, 1 ml of glutaraldehyde (25% solution, as cross linking agent) was added slowly and stirring was continued for 2 h. The hardened microspheres were separated by vacuum filtration and washed several times with hexane to remove oil. Finally, microspheres were washed with distilled water to remove unreacted GA. The microspheres were air dried for 24 h and then stored in vacuum desiccator until further use.

4.5 Characterization of Microspheres

4.5.1 Particle Size Measurements

Particle size and size distribution of the microspheres were determined by laser light scattering on a Malvern particle size analyzer (Malvern Mastersizer 2000, Malvern Instruments, UK). The dispersion of microspheres was added to the sample dispersion unit containing the stirrer and stirred in order to reduce the aggregation between the microspheres and the laser obscuration range was maintained between 5 and 15%. The average volume mean particle size was measured after performing the experiment in triplicate.

4.5.2 Morphological Characterization

All batches of microspheres were preliminarily checked for shape and size by optical microscopy. A light transmission microscope (Olympus Optical Co. Ltd., Japan) at 10X magnification was employed. The samples were prepared by suspending a small amount of microspheres in paraffin oil. The surface morphology of the CRV loaded microspheres was examined by scanning electron microscopy (JSM 5610 LV, Jeol

Datum Ltd., Japan). The samples were mounted directly onto the SEM sample holder using double-sided sticking tape and images were recorded at the required magnification at the acceleration voltage of 10 kV.

4.5.3 Flow Properties

The flow properties of microspheres were investigated by determining the angle of repose, bulk density, and tapped density.

Angle of repose:

The fixed funnel method was used to find out angle of repose. In this method, a funnel was secured with its tip at a 2 cm height (H) above a graph paper that was placed on a flat horizontal surface. Microspheres were carefully poured through the funnel until the apex of the conical pile just touched the tip of the funnel. Thus, with the radius of the conical pile (R) was noted and angle of repose was calculated from following equation

$$\tan \alpha = H/R \qquad \text{..... 4.1}$$

Here α is the angle of repose (Banker and Anderson, 1991; Taylor et al, 2000).

Compressibility index:

The Carr's index (percent compressibility) was calculated as one hundred times the ratio of the difference between the tapped density and bulk density to the tapped density (Gohel and Jogani, 2002).

$$Ic = 100 \text{ X } (\rho_t - \rho_b)/ \rho_t \qquad \text{..... 4.2}$$

Here Ic is Carr's index, ρ_b is bulk density and ρ_t is tapped density. The bulk density was determined as the quotient of weight to the volume of sample. The tapped density was determined as the quotient of weight to the volume of sample after manually tapping a measuring cylinder filled with microspheres for 100 times from a height of 2 inches.

4.5.4 Determination of Entrapment Efficiency (EE)

The CRV loaded microspheres (5 mg) were added to a mixture of 10 ml of methanol and 0.1N HCl (3:2) under stirring. The mixture was filtered and the amount of CRV was determined spectrophotometrically at 242 nm on UV spectrophotometer (Shimadzu UV1610, Japan). It was confirmed from preliminary UV studies that the presence of dissolved polymers did not interfere with the absorbance of the drug at 242 nm. The percentage entrapment efficiency was calculated using following equation:

$$\% \text{ Entrapment efficiency} = \frac{\text{Actual loading}}{\text{Theoretical loading}} \times 100 \quad \text{..... 4.3}$$

4.5.5 Mucus Glycoprotein Assay

A periodic acid/Schiff colorimetric method reported by Mantle and Allen (Mantle and Allen, 1978) was used to determine the free mucin concentration in order to assess the amount of mucin adsorbed on the chitosan microspheres and its effect on the assessment of mucoadhesive behavior of chitosan microspheres. Two reagents were prepared. Schiff reagent contained 100 mL of 1% basic fuchsin (pararosaniline) aqueous solution and 20 mL of 1 M HCl. Sodium metabisulphite (0.1 g) was added to every 6 mL of Schiff reagent before use, and the resultant solution was incubated at 37°C until it became colorless or pale yellow. Periodic acid reagent was freshly prepared by adding 10 µL of 50% periodic acid solution to 7 mL of 7% (vol/vol) acetic acid solution.

Standard calibration curves were prepared from 2 mL of mucin standard solutions (0.1, 0.2, 0.3, 0.4 and 0.5 mg/2 mL). After adding 0.2 mL of periodic acid reagent, the samples were incubated at 37°C for 2 hours in a water bath. Then, 0.2 mL of Schiff reagent was added at room temperature. After thirty minutes, the absorbance of the solution was recorded at 555 nm by a UV spectrophotometer (He et al., 1998) (Shimadzu UV1610, Japan). All the samples were determined with the same procedure. The mucin content was calculated from the standard calibration curve. Each experiment was performed in triplicate and average values were calculated.

4.5.6 Adsorption of Mucin to Chitosan Microspheres

Mucin aqueous solution with different concentrations (0.1, 0.2, 0.4, 1.0 mg/mL) was prepared. Chitosan microspheres (5 mg) were dispersed in the above mucin solutions (2 mL), vortexed, and shaken at room temperature for 1 h (He et al., 1998). Then, the dispersions were centrifuged at 4000 rpm for 4 minutes, and the supernatant was used for the measurement of the mucin content by mucus glycoprotein assay. The mucin concentration was calculated from a calibration curve, and the amount of mucin adsorbed to the microspheres was calculated as the difference between the total amount of mucin added and the free mucin in the supernatant. The data obtained were interpreted using Freundlich and Langmuir equations describing the adsorption isotherms (He et al., 1998).

4.5.7 Measurement of In Vitro Mucoadhesion

The in vitro mucoadhesion of microspheres was carried out by modifying the method described by Ranga Rao et al. and others (Ranga Rao and Buri, 1989; Patil and Murthy, 2006) using sheep nasal mucosa. The microspheres were placed on sheep nasal mucosa after fixing to the polyethylene support. The mucosa was then placed in the dessicator to maintain at >80% RH at room temperature for 30 min to allow the polymer to hydrate and to prevent drying of the mucus. The mucosa was then observed under microscope, and the number of particles attached to the particular area was counted. After 30 min, the polyethylene support was introduced into a plastic tube cut in circular manner and held in an inclined position at an angle of 45^0. Mucosa was washed thoroughly for 5 min with phosphate buffer pH 6.2. Tissue was again observed under microscope to see the number of microspheres remaining in the same field area.

The adhesion number was determined by the following equation:

$$Na = N/N_0X100 \qquad \text{..... 4.4}$$

Here Na is adhesion number, N_0 is total number of particles in a particular area, and N is number of particles attached to the mucosa after washing.

4.5.8 In Vitro Drug Release

The drug release profiles of CRV loaded chitosan microspheres were evaluated using a Franz diffusion cell apparatus which consisted of donor and receptor compartments. A dialysis membrane (cut-off Mw 12000, Hi Media, India) was used to keep the microspheres on the donor side which allowed free diffusion of CRV to the receptor compartment containing 20 ml phosphate buffer solution pH 6.2 (within the pH range in nasal cavity) and maintained at 37°C ± 1°C. The receptor compartment was stirred with a magnetic stirring bar. At scheduled time intervals, aliquots were withdrawn from receptor cell and replaced with same volume of fresh medium. The samples were assayed spectrophotometrically at 242 nm. All experiments were carried out in triplicate and average values were calculated. The release kinetics was determined by fitting the release data to various kinetic equations including zero-order, first-order and Higuchi (James et al., 1994; Wu et al., 1994; Jones et al, 2004; Costa et al, 2001).

4.5.9 Differential Scanning Calorimetry (DSC) Analysis

DSC (Mettler Toledo DSC 822) was performed on placebo chitosan microspheres, drug-loaded chitosan microspheres and plain CRV. Samples were heated from 30 to 300 ^{0}C at the heating rate of 10 ^{0}C min $^{-1}$ in nitrogen atmosphere (flow rate, 20 ml/min).

4.5.10 X-ray Diffraction Studies

The XRD patterns of placebo chitosan microspheres, drug-loaded microspheres and plain CRV were recorded using X-ray diffractometer (Bruker AXS D8 Advance, with X-ray source of Cu, Wavelength 1.5406 A° and Si (Li) PSD detector). The samples were mounted on a sample holder and XRD patterns were recorded in the range of 3-50^{0} at the speed of 5^{0}/min.

4.5.11 Histology studies

The nasal mucosa tissues were carefully removed from the nasal cavity of sheep obtained from the local slaughterhouse. Tissues were immediately used after separation. Histopathological evaluation of tissue incubated in Phosphate Buffer (pH 6.2) after collection was compared with tissue incubated in the diffusion chamber of

Franz cell with microsphere formulations. Tissue was fixed in 10% buffered formalin (pH 6.2), routinely processed and embedded in paraffin (Baroda Pathology Lab, Baroda). Paraffin sections were cut on glass slides and stained with hematoxylin and eosin (HE). The sections were examined by light microscopy (Olympus microscope), to examine the morphological changes to the tissue during in vitro permeation study by a pathologist blinded to the study (Majithiya et al., 2006).

4.5.12 Stability study

Stability studies of chitosan microspheres of carvedilol were carried out as per ICH guidelines. A 3-month accelerated condition stability study was carried out on the optimized formulation. This involved the microspheres being kept in a desiccator at a temperature of 40 ^{0}C and a relative humidity of 75%. The physical characteristics, particle size and drug content of the microspheres were determined at the end of 1, 2 and 3 months and compared with the data of freshly prepared microspheres (Yang et al., 2004).

4.6 RESULTS AND DISCUSSION

4.6.1 Optimization of Formulation Parameters

4.6.1.1 Effect of chitosan concentration

The particle size of the microspheres was found to be dependent on the concentration of chitosan (Fig. 4.1). At lower concentration (1%), the mean particle size of microspheres observed was 28.26±1.14 µm and aggregation and clumping of the microparticles was observed. The mean particle size of microspheres (46.58±2.34 µm) was significantly increased when high concentration of chitosan (3%) was used. Under the same preparation conditions, the droplets formed from the higher viscosity chitosan solution will be larger in size and hence result in formation of larger microspheres (Aiedeh et al., 1997; He, Davis, & Illum, 1999). Also, at higher concentration (3%), the chitosan solution was so viscous that it was difficult to pass through syringe. At medium concentration (2%), the mean particle size was observed to be 36.43±0.85 µm.

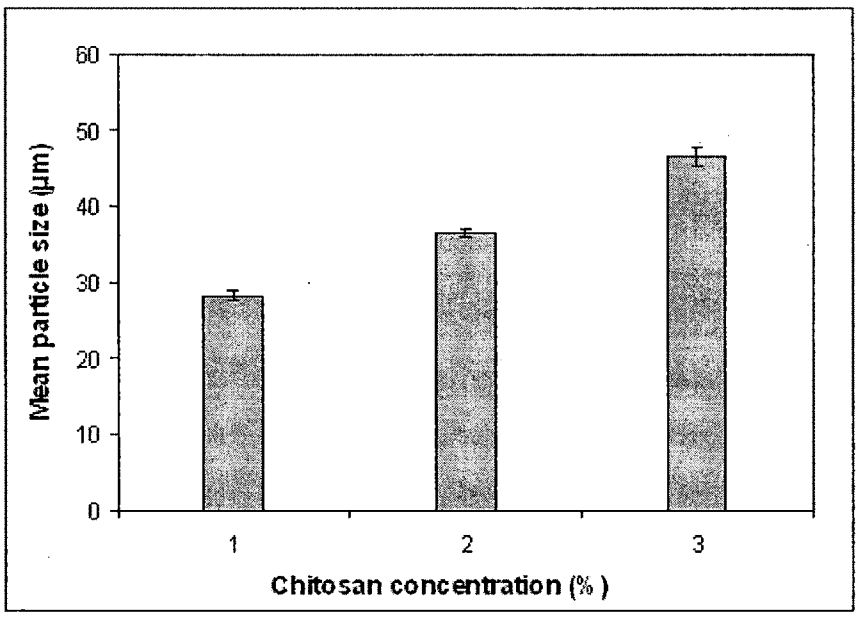

Fig. 4.1 Effect of chitosan concentration on particle size of microspheres at 1200 rpm, aqueous to oil phase ratio of 1:10, volume of GA of 1 mL and cross linking time of 2 h.

4.6.1.2 Effect of aqueous to oil phase ratio

Fig. 4.2 shows that the mean particle size of microspheres was increased with an increase in aqueous to oil phase ratio. When ratios of 5:100 and 10:100 were used, a slight increase in microsphere particle size was observed from 33.46±0.52 µm to 36.43±0.85 µm. At higher ratios of 20:100 and 30:100, a significant increase in the particle size of microspheres was observed from 58.12±2.55 to 96.85±2.62 µm and the microspheres were aggregated. As the ratio of aqueous to oil phase was increased, the mean distance between the droplets of chitosan (aqueous phase) in oil phase will be decreased, which may result in increasing the chances of coalescence between the droplets. This may lead to aggregation of microspheres and increasing the particle size. Another reason for increase in the particle size may be due to decrease in the shearing efficiency of the stirrer due to increased viscosity (Dinarvand, Mahmoodi, & Farboud, 2004).

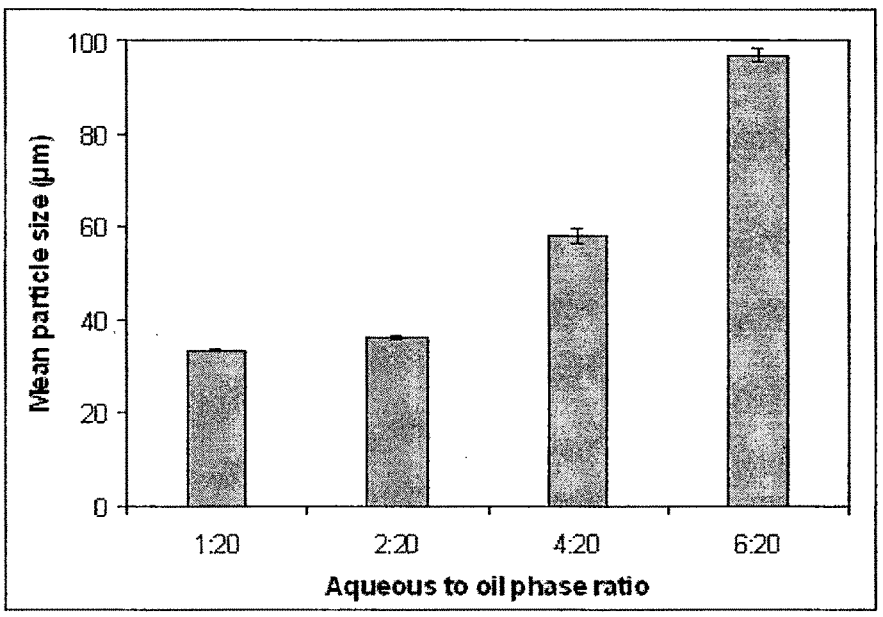

Fig. 4.2 Effect of aqueous to oil phase on particle size of microspheres at 1200 rpm, chitosan concentration of 2%, volume of GA of 1 mL and cross linking time of 2 h.

4.6.1.3 Effect of stirring rate

As shown in Fig. 4.3, the mean particle size of the microspheres decreased with increasing stirring rate. When the stirring rate was increased from 600 rpm to 1500 rpm, the particle size of microspheres was observed to decrease from 62.25±4.20 to 31.24±0.96 µm. At stirring rate of 600 rpm, along with the larger particle formation, sphericity and smoothness of the microspheres was affected. This may be due to less efficient shearing of the chitosan solution to form fine droplets at low stirring rate. At higher stirring rate of 1200 and 1500 rpm, smooth and spherical particles were obtained with mean particle size of 36.43±0.52 and 31.24±0.96 µm respectively.

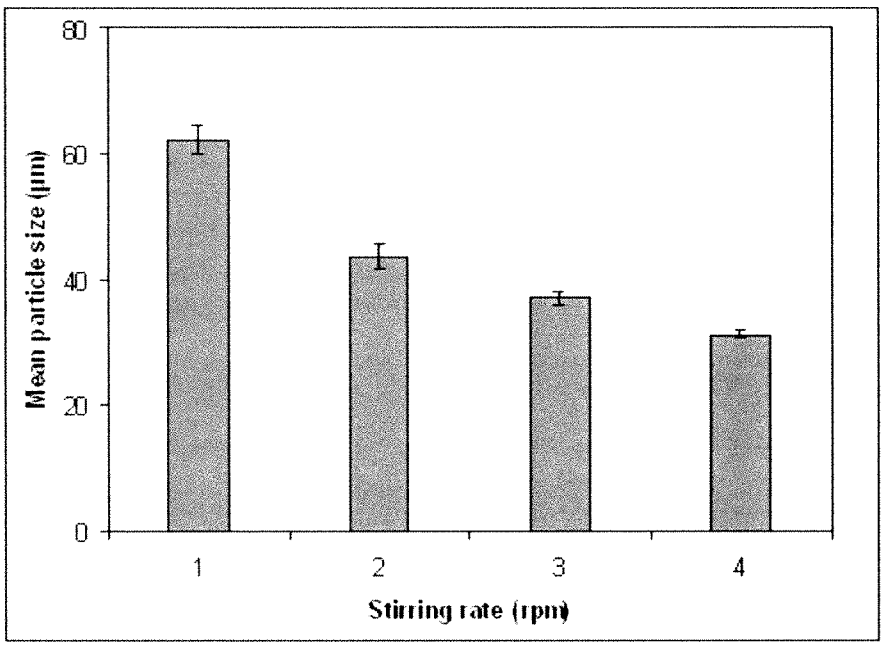

Fig. 4.3 Effect of stirring rate on particle size of microspheres at aqueous to oil phase ratio of 1:10, chitosan concentration of 2%, volume of GA of 1 mL and cross linking time of 2 h.

4.6.1.4 Effect of volume of GA

As the volume of GA was increased, very slight decrease in the particle size of microspheres was observed. The mean particle size of microspheres prepared with 0.5, 1, 2 and 3 ml of GA was 40.32±2.05, 36.43±0.85, 34.12±0.84, and 33.45±1.80 µm respectively.

4.6.1.5 Effect of cross linking time

The different cross linking times of 1, 2 and 3 hr were used. The microspheres prepared with a cross linking time of 1 hr were aggregated and improperly hardened. This may be due to insufficient time for cross linking reaction between chitosan and GA and matrix stability may not be achieved within this time. At cross linking time of 2 and 3 hr, spherical microspheres with smooth surface were obtained with mean particle size of 36.43±0.85 and 32.87±0.33 µm respectively without aggregation. A slight decrease in particle size was observed with increasing the cross linking time.

4.6.1.6 Effect of DOSS concentration

At a DOSS concentration of 0.1% w/v, the microspheres obtained were irregular in shape with mean particle size of 48.56±3.38 µm. When 2.0% w/v concentration was used, the microspheres with particle size of 36.43±0.85 µm were obtained. But at 0.3% w/v concentration, the resulting mixture of microspheres with liquid paraffin was very viscous and difficult to be filtered.

Hence the optimized formulation parameters for preparation of chitosan microspheres are as follows:

Chitosan concentration: 2%
Stirring speed: 1200 RPM
Volume of GA (cross linking agent): 1 mL
Cross linking time: 2 h
Aqueous to oil phase volume ratio: 1:10
DOSS (Dioctyl sodium sulphosuccinate) concentration: 0.2%

4.6.2 Preparation of CRV loaded microspheres

The formula for the various batches of CRV loaded microspheres is shown in Table 4.2.

Table 4.2 Various formulation parameters used in the preparation of CRV loaded microspheres

Formulation code	Drug: polymer ratio	Volume of GA (mL)	Cross linking time (h)
CHCR1	1:2	1	1
CHCR2	1:1	1	1
CHCR3	1:2	2	1
CHCR4	1:1	2	1
CHCR5	1:2	1	2
CHCR6	1:1	1	2
CHCR7	1:2	2	2
CHCR8	1:1	2	2

4.6.3 Characterization of CRV loaded Microspheres

4.6.3.1 Particle size measurements

The particle size of microspheres was in the range of 20.82–49.26 μm (Table 4.3), which is favorable for intranasal administration. The size of the microspheres was found to increase with an increase in drug loading. This increase in size of the microspheres may be due to the increase in viscosity of the internal phase caused by the increase in drug concentration as explained by Denkbas et al (Denkbas et al., 1999).

Table 4.3 Characteristics of prepared CRV loaded chitosan microspheres

Formulation Code	Particle size (μm)	Angle of repose (θ)	Compressibility Index (%)	Entrapment Efficiency (%)	In vitro mucoadhesion (%)
CHCR1	24.44±1.67	30.73±1.90	16.29±1.66	47.45±1.03	83.21±2.21
CHCR2	42.53±1.38	28.81±1.34	15.33±1.15	62.40±1.50	82.89±1.28
CHCR3	20.52±1.19	33.13±1.08	18.43±1.47	43.31±1.09	75.21±1.44
CHCR4	38.55±1.10	31.40±1.53	15.51±0.72	56.57±0.55	74.74±1.27
CHCR5	34.86±1.58	29.66±0.93	15.61±0.63	52.35±1.59	88.56±1.74
CHCR6	49.26±1.89	32.08±1.58	17.47±1.07	66.86±1.77	80.27±2.49
CHCR7	33.11±2.37	31.33±1.02	15.85±1.48	41.25±1.01	79.60±3.15
CHCR8	46.02±1.67	33.32±1.46	18.89±1.21	54.30±1.23	74.72±1.54

4.6.3.2 Morphological characterization

The microspheres obtained were non aggregated, free flowing powders with spherical shape and smooth surface (Fig. 4.4 and Fig. 4.5). However, one noticeable characteristic of the cross linked chitosan microspheres was their yellow to brownish color (Fig. 4). The intensity of the color of the microspheres increased as the volume of GA and time of cross linking increased. Roberts and Taylor (Roberts and Taylor, 1989) reported that the chitosan/GA gels of yellow-brown color were produced due to inter-chain cross-link formation.

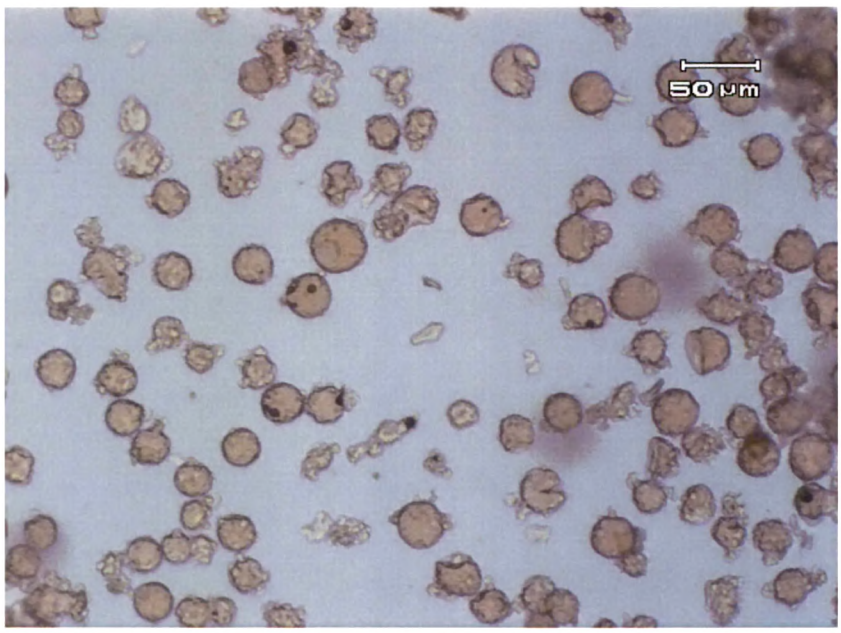

Fig. 4.4 Photomicrograph of CRV loaded chitosan microspheres.

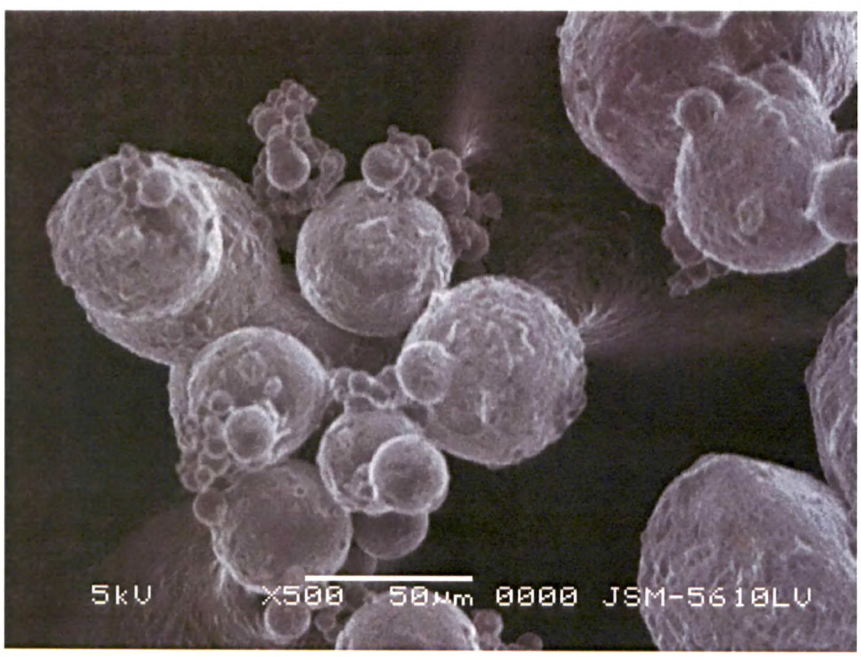

Fig. 4.5 SEM photograph of CRV loaded chitosan microspheres.

4.6.3.3 Flow Properties

The results of flow properties measurements are shown in Table 4.3. The values of angle of repose were in the range of $28.81^0 \pm 1.34$ to $33.32^0 \pm 1.46$ which is within the

normal acceptable range of 20^0 – 40^0 (Wells and Aulton, 1988). The microspheres thus showed reasonably good flow potential. The values of compressibility index which were in the range 15.33±1.15 to 18.89±1.21, also indicating good flow characteristics of the microspheres.

4.6.3.4 Entrapment efficiency

The % EE was found to be in the range between 42.62 and 67.24. The % EE was found to be proportional to drug loading. The formulations loaded with higher amount of drug exhibited higher entrapment efficiencies. The increase in drug loading may cause subsequent increase in viscosity of the polymer dispersion, which would decrease the probability of drug diffusion into the external phase during preparation, resulting in higher drug entrapment efficiency. However, with increasing amount of cross linking agent, entrapment efficiency decreased (Table 4.3). This may be due to an increase in cross – link density that will thereby reduce the free spaces within the polymer matrix and hence lead to reduced entrapment efficiency.

4.6.3.5 Assessment of the Mucoadhesive Behavior of Chitosan Microspheres by Mucus Glycoprotein Assay

Mucoadhesion involves various types of interaction forces between mucoadhesive materials and mucus surface, such as hydrogen bonding, electrostatic attraction, van der Waals forces and mechanical interpenetration and entanglement. Many methods have been employed to evaluate these interactions in vitro and in vivo. Commercial mucin is frequently used as a substitute for fresh mucin because of its reproducible quality and easy availability. As a strong interaction exists between mucin and chitosan, mucin should be spontaneously adsorbed on the surface of the chitosan microspheres. Hence, the mucoadhesive property of chitosan microspheres was assessed by the suspension of chitosan microspheres in different amounts of mucin (Type III) in aqueous solutions at room temperature. The linearity range for mucin at the wavelength of detection of 555 nm was obtained as 0.05 – 0.25 mg/mL. The linear regression equation obtained by least square method was, $y = 1.978x + 0.4493$. As the mucin concentration was increased, the amount of mucin adsorbed increased (Fig. 4.6). Thus, these studies indicate that mucoadhesion may increase the residence time of the formulation in the nasal cavity.

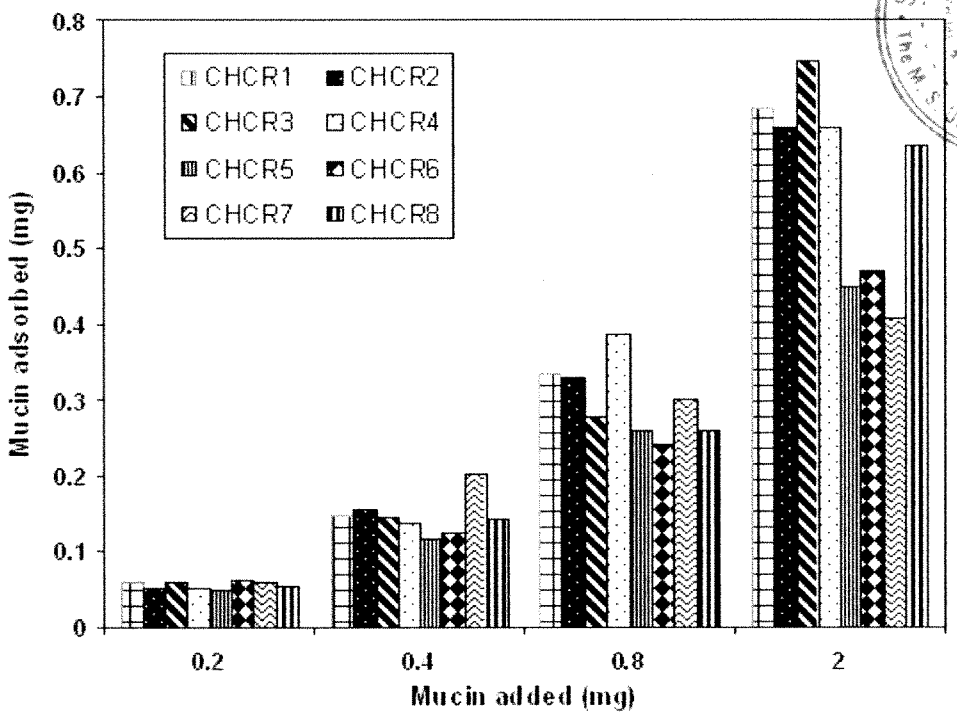

Fig. 4.6 Adsorption of mucin on different microspheres with respect to the amount of mucin added.

4.6.3.6 Adsorption Isotherms

The data obtained from adsorption studies were fitted to Freundlich and Langmuir equations. Straight lines (Fig.4.7 and Fig. 4.8) were obtained, and the constants (n, K in case of Freundlich isotherm and a, b for Langmuir isotherm) from these lines were calculated and are listed in Table 4.4. It was found that the values of R^2 were significantly higher (P <0.05) for the Langmuir equation as compared to the Freundlich equation. This explains a more specific adsorption process where electrostatic interaction is involved. The adsorption of mucin to chitosan is expected to be dominated by the electrostatic attraction between the positively charged chitosan (containing amino groups) and negatively charged mucin (containing ionized sialic acid). As the mucin concentration was increased, the amount of mucin adsorbed increased as the chitosan microspheres have the ability to adsorb mucin.

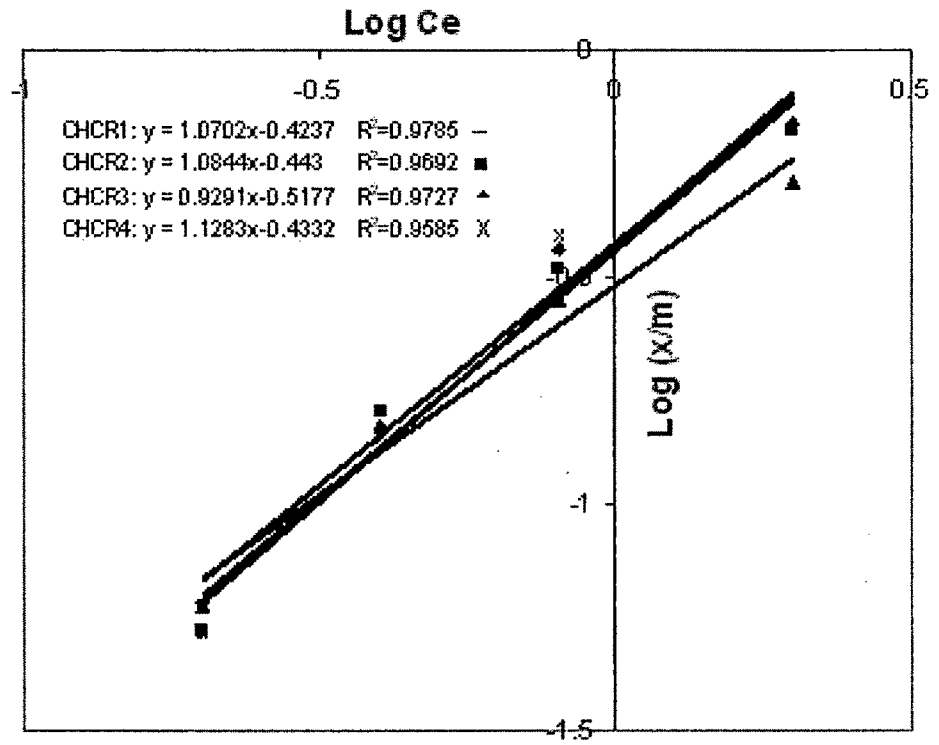

Fig. 4.7A Freundlich adsorption isotherms for mucin adsorbed on chitosan microspheres (Batches CHCR1 to CHCR4)

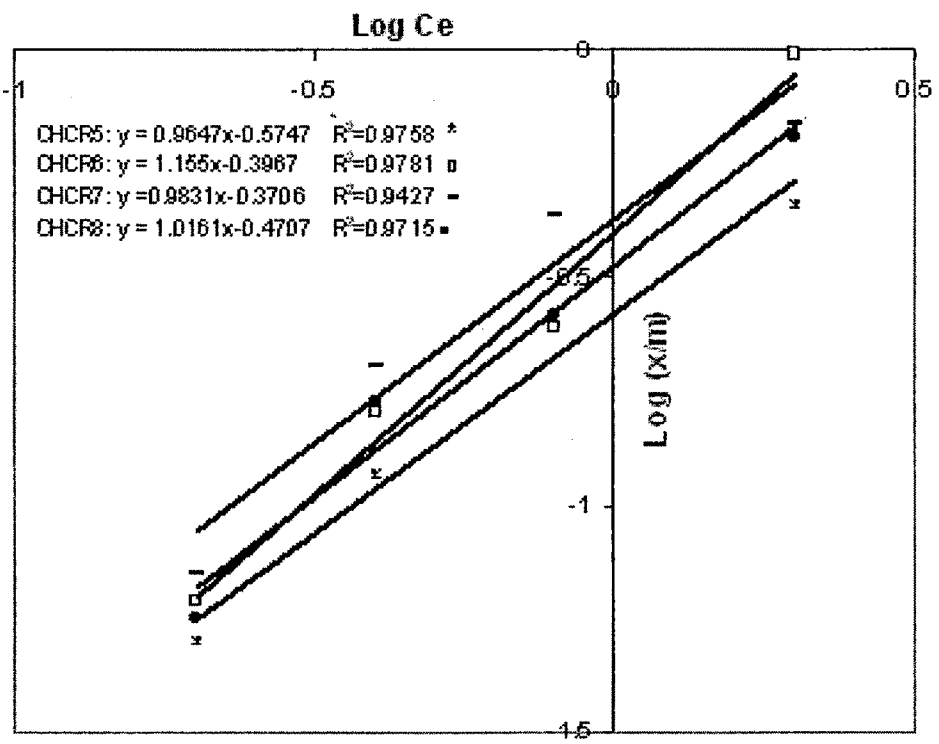

Fig. 4.7B Freundlich adsorption isotherms for mucin adsorbed on chitosan microspheres (Batches CHCR5 to CHCR8)

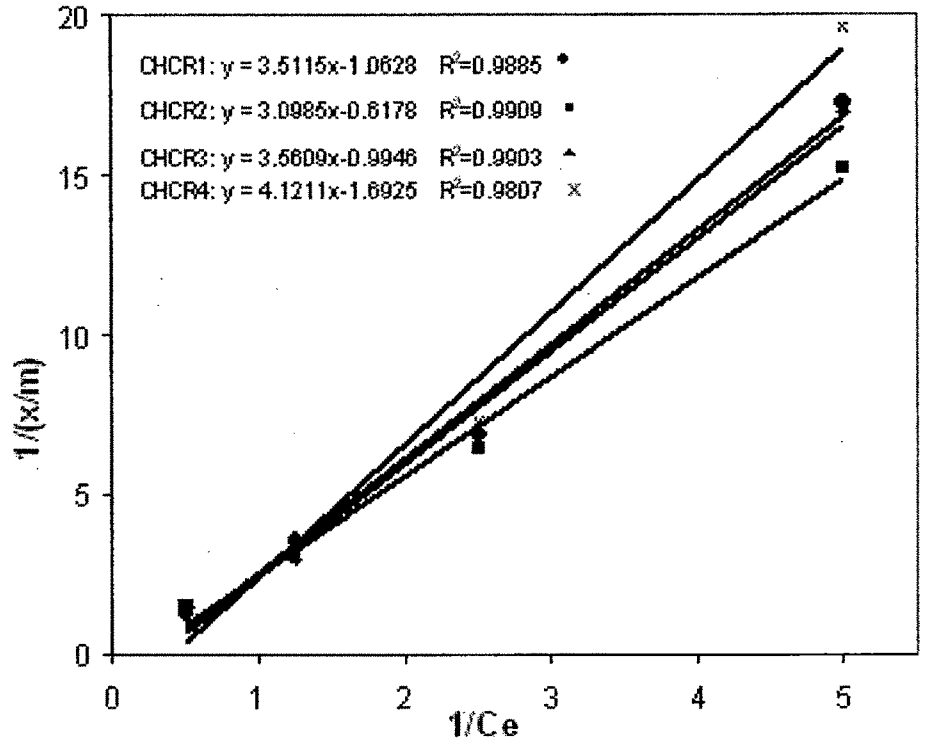

Fig. 4.8A Langmuir adsorption isotherms of mucin adsorbed on chitosan microspheres (Batches CHCR1 to CHCR4)

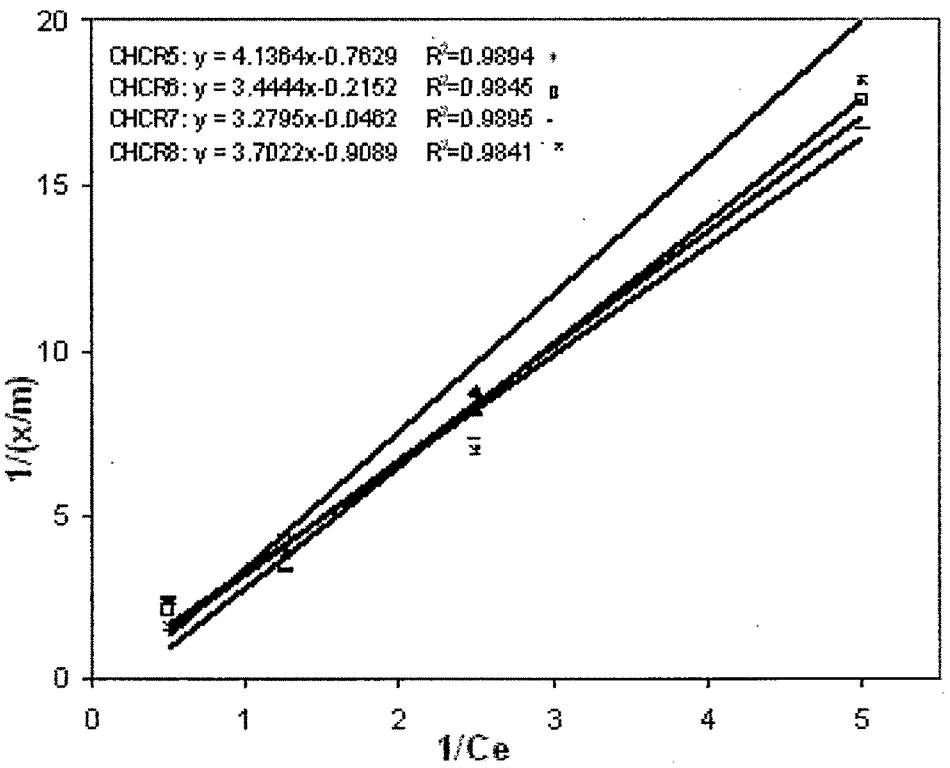

Fig. 4.8B Langmuir adsorption isotherms of mucin adsorbed on chitosan microspheres (Batches CHCR5 to CHCR8)

Table 4.4 Constants for Langmuir and Freundlich Equations

	Freundlich isotherm*			Langmuir isotherm•		
Batch code	**n**	**K**	**R^2**	**a**	**b**	**R^2**
CHCR1	0.934	2.65	0.9785	1.0628	3.5150	0.9885
CHCR2	0.922	2.77	0.9692	0.6178	3.0985	0.9909
CHCR3	1.07	3.29	0.9727	0.9946	3.5609	0.9903
CHCR4	0.88	2.71	0.9585	1.6925	4.1211	0.9807
CHCR5	1.03	3.75	0.9758	0.7629	4.1364	0.9894
CHCR6	0.86	2.49	0.9781	0.2152	3.4444	0.9845
CHCR7	1.01	2.34	0.9427	0.0462	3.2795	0.9895
CHCR8	0.98	2.95	0.9715	0.9089	3.7022	0.9841

*$x/m = K \cdot Ce^{1/n}$

•$1/(x/m) = a+b \cdot 1/Ce$

4.6.3.7 In vitro mucoadhesion

The results of in vitro mucoadhesion (Table 4.3) showed that all the batches of microspheres had mucoadhesive property ranging from 74.72±1.54 to 88.56±1.74 % (Fig. 4.9) and could adequately adhere on nasal mucosa. The results also showed that with increasing polymer ratio, higher mucoadhesion percentages were obtained. This may be due to the fact that, as the amount of polymer increased, the amino groups available for binding with the sialic acid residues in mucus layer also increase, resulting in increased mucoadhesion. The percent in vitro mucoadhesion was found to decrease slightly with increase in the volume of glutaraldehyde (Table 3), attributed to increased rigidity and reduced binding sites for mucoadhesion. Most of the studies showed that the prerequisite for good mucoadhesion is the high flexibility of polymer backbone structure and of its polar functional groups. Such a flexibility of the polymer chains, however, is reduced if the polymer molecules are highly cross linked. Although highly cross-linked microspheres will absorb water, they are insoluble and will not form a liquid gel on the nasal epithelium but rather a more solid gel-like structure. This decrease in flexibility imposed upon polymer chains by the cross-linking agent makes it more difficult for highly cross-linked polymers to penetrate the

mucin network (Berger et al., 2004). Thus, highly cross linked microparticles effectively limits the length of polymer chains that can penetrate the mucus layer and could possibly decrease the mucoadhesion strength of the microspheres (Illum et al., 1987).

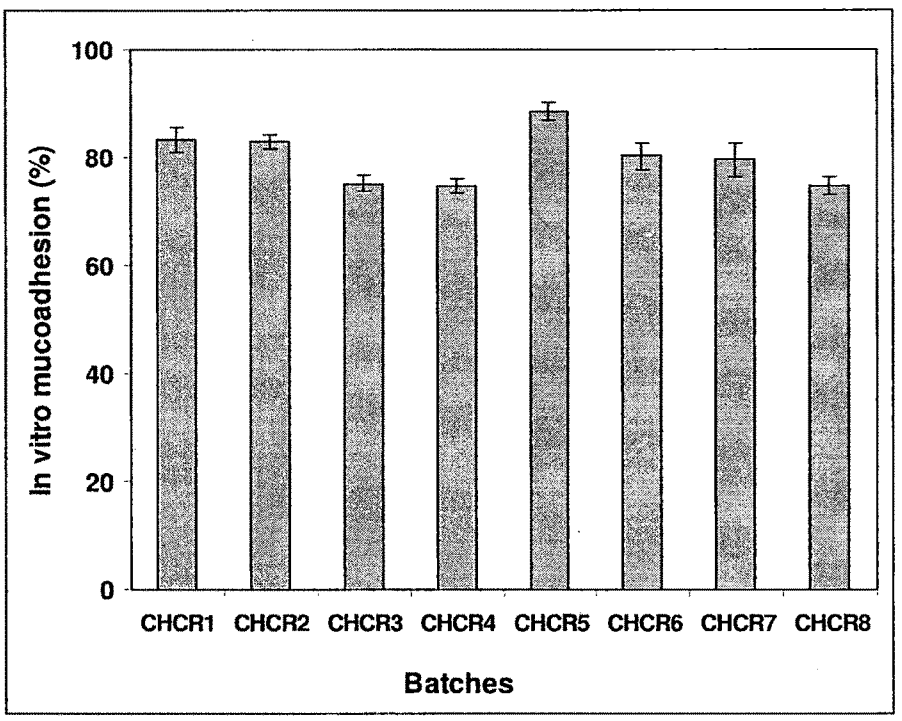

Fig. 4.9 Percentage in vitro mucoadhesion for different batches of microspheres

4.6.3.8 In vitro drug release

The in vitro drug release profile of CRV from the chitosan microspheres is shown in Fig. 4.10. The release pattern showed a moderate and controlled release following near zero order release. *In vitro* drug release proportionally increased with increasing the drug concentration. As expected (Thanoo et al., 1992), with an increase in the crosslinking agent concentration, a respective decrease in the rate and extent of drug release was observed.

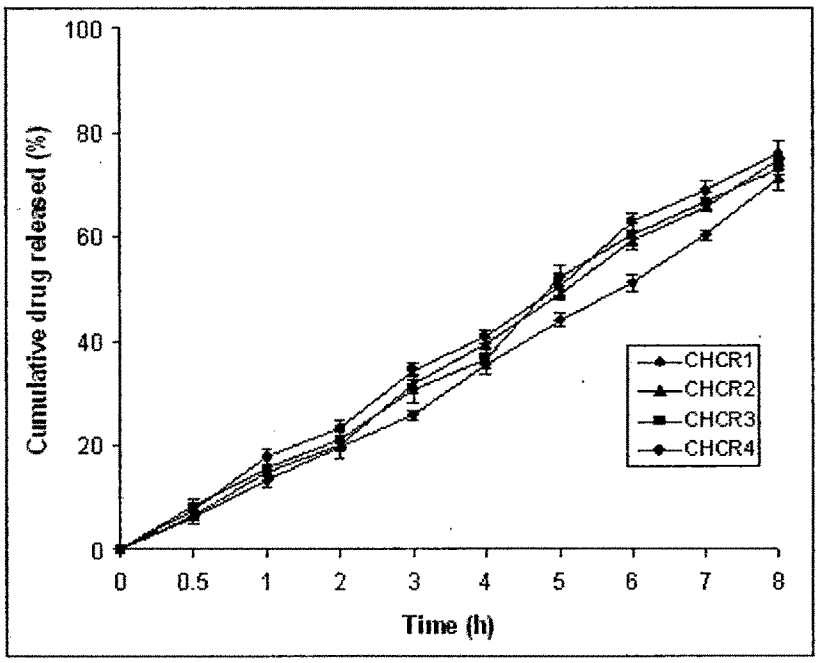

Fig. 4.10A *In vitro* drug release profile of chitosan microspheres of CRV (Batches CHC1 to CHC4). The values are mean ± SD (n = 3).

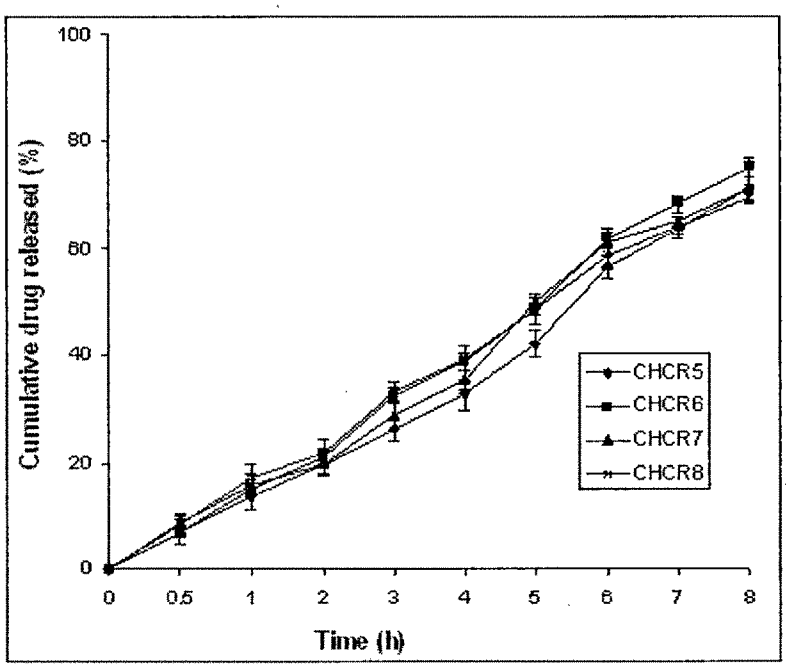

Fig. 4.10B *In vitro* drug release profile of chitosan microspheres of CRV (Batches CHC5 to CHC8). The values are mean ± SD (n = 3).

Mathematical modeling of release kinetics

In order to understand the mechanism and kinetics of drug release, the data was fitted to various kinetic equations. Three kinetic models including the zero-order release equation ($Q_t=K_1t$), first-order equation ($Q_t=Q_0e^{-K_2t}$), and Higuchi equation ($Q_t=K_3t^{1/2}$) were applied to process the in vitro data to find the equation with the best fit (James et al., 1994; Wu et al., 1994; Jones et al, 2004; Costa et al, 2001). Where Q_t is the release percentage at time t. K_1, K_2, and K_3 are the rate constants of zero-order, first-order, and Higuchi respectively. The following plots were plotted: Q_t *vs.* t (zero order kinetic model); log (Q_0-Q_t) *vs.* t (first order kinetic model,) and Q_t *vs.* square root of t (Higuchi model). Where Q_t is the amount of drug released at time t and Q_0 is the initial amount of drug present in microspheres (Korsmeyer et al, 1983). The linear regression analyses are summarized in Table 4.5. A model with the greatest regression coefficient (r^2) was chosen as the dominant model.

Table 4.5 Kinetic model of Carvedilol release from chitosan microspheres

Formulation code	Regression coefficient (r^2)			Release kinetics
	Zero-order	First-order	Higuchi model	
CHCR1	0.9961	0.9739	0.9607	Zero-order
CHCR2	0.9956	0.9739	0.9757	Zero-order
CHCR3	0.99	0.9757	0.9653	Zero-order
CHCR4	0.991	0.9746	0.9768	Zero-order
CHCR5	0.9912	0.9341	0.9455	Zero-order
CHCR6	0.9939	0.9715	0.9712	Zero-order
CHCR7	0.9871	0.9714	0.9559	Zero-order
CHCR8	0.9874	0.9877	0.981	First-order

In order to further investigate the release mechanism, the data were analyzed using the Peppas equation (Peppas, 1985; Ritger and Peppas, 1987),

$$Mt/M\infty = kt^n \quad \text{..... 4.5}$$

Here Mt is the amount of drug released at time t, M∞ is the amount released at time ∞, Mt/M∞ is the fraction of drug released at time t, k is a constant characteristic of the drug-polymer system and n is the diffusional exponent, a measure of the primary mechanism of drug release. Using the least squares procedure, the values of n, k and correlation coefficient (r^2) were estimated (Table 4.6). In spherical matrices, if $n \leq 0.43$, a Fickian diffusion (case-I), $0.43 \leq n < 0.85$, anomalous or non-Fickian transport and $n \geq 0.85$, a case-II transport (zero order) drug release mechanism dominates. The values of n for all the batches ranged from 0.74 to 0.84 with correlation coefficient close to 0.99, indicating non-Fickian or anomalous type of transport. Non- Fickian release is described by two mechanisms: a combination of drug diffusion and polymer relaxation. The release mechanism is known to be influenced by (a) non homogeneous gel microstructure as well as the existence of polymeric domains within the swollen gel, (b) rate of fluid ingress into the matrix, (c) dissociation/ erosion and total disentanglement at the dissolution front and (d) rate of matrix swelling, relaxation as well as molecular diffusion of drug through the swollen gel (Mundargi et al., 2008).

Table 4.6 Release mechanisms of different formulations.

Batch code	n	k	Correlation coefficient, r^2	Release mechanism
CHCR1	0.83	0.175	0.991	Non-Fickian
CHCR2	0.84	0.172	0.9903	Non-Fickian
CHCR3	0.78	0.194	0.9861	Non-Fickian
CHCR4	0.79	0.191	0.9871	Non-Fickian
CHCR5	0.81	0.182	0.9852	Non-Fickian
CHCR6	0.84	0.172	0.9869	Non-Fickian
CHCR7	0.76	0.204	0.9778	Non-Fickian
CHCR8	0.74	0.212	0.9894	Non-Fickian

Formulation CHCR6 showed entrapment efficiency of 66.86±1.77 % with particle size of 49.26±1.89 µm and in vitro mucoadhesion of 80.27±2.49 % with around 75 % release of drug in 8 h. Hence it was selected as optimized formulation for further studies including in vivo evaluation.

4.6.3.9 Differential scanning calorimetry

DSC thermograms of pure CRV (A), CRV loaded chitosan microspheres (B) and blank chitosan microspheres (C) are displayed in Fig. 4.11. Thermogram of CRV showed a sharp peak at 122 ^{0}C, indicating the melting of the drug. In case of blank microspheres, a broad peak was observed at 96 ^{0}C was mainly due to water evaporation. The transitions associated with loss of water correspond to the hydrophilic nature of the functional groups of the polymer. The second thermal event registered was an exothermic peak occurring at 273 °C which can express the overall exothermic effect connected with decomposition (Hekmatara et al., 2006). Similarly in case of drug loaded microspheres, a broad endothermic peak at 76 ^{0}C and exothermic peak at 274 ^{0}C was observed. However, there was no peak corresponding to CRV, indicating that CRV is molecularly dispersed in the matrix.

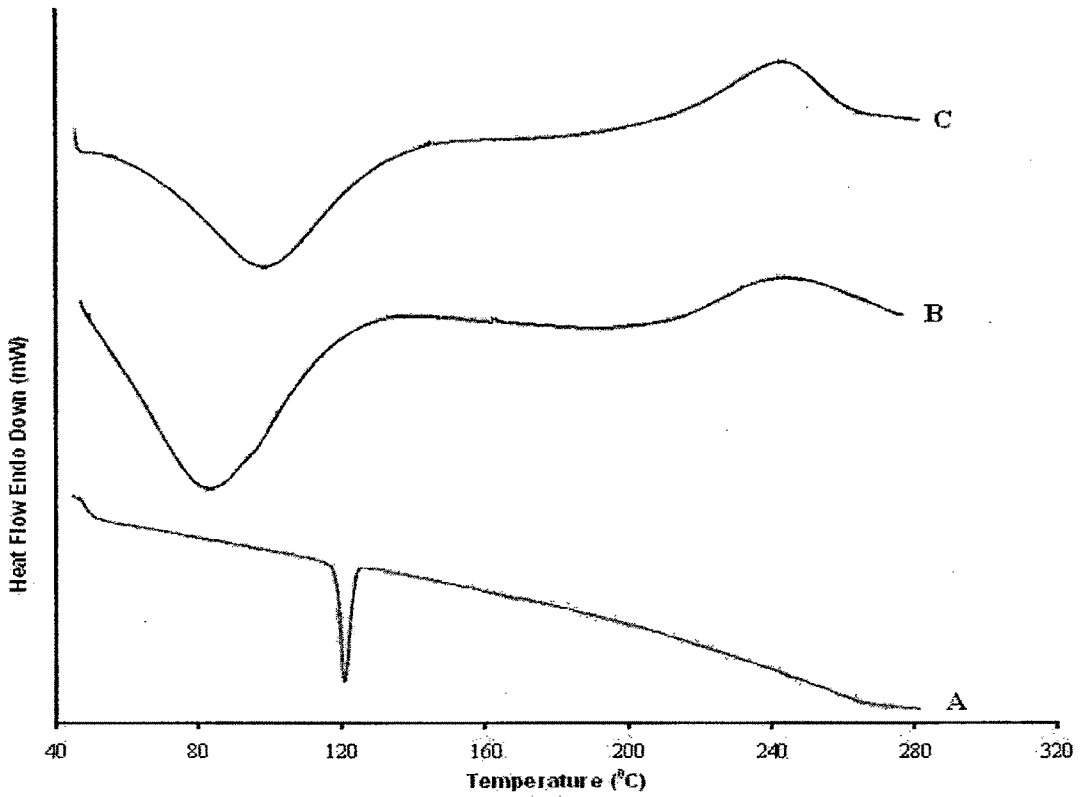

Fig. 4.11 DSC thermograms of (A) pure carvedilol; (B) drug loaded microspheres; and (C) placebo microspheres

4.6.3.10 X-ray diffraction studies

XRD patterns recorded for plain CRV (A), placebo microspheres (B) and CRV loaded microspheres (C) are presented in Fig. 4.12. CRV peaks observed at 2θ of 11.18^0, 12.80^0, 13.48^0, 15.06^0, 17.38^0, 18.29^0, 20.15^0, 24.18^0 and 26.02^0 are due to the crystalline nature of CRV. These peaks were not observed in the CRV loaded microspheres. This indicates that drug particles are dispersed at molecular level in the polymer matrices since no indication about the crystalline nature of the drugs was observed in the drug loaded microspheres.

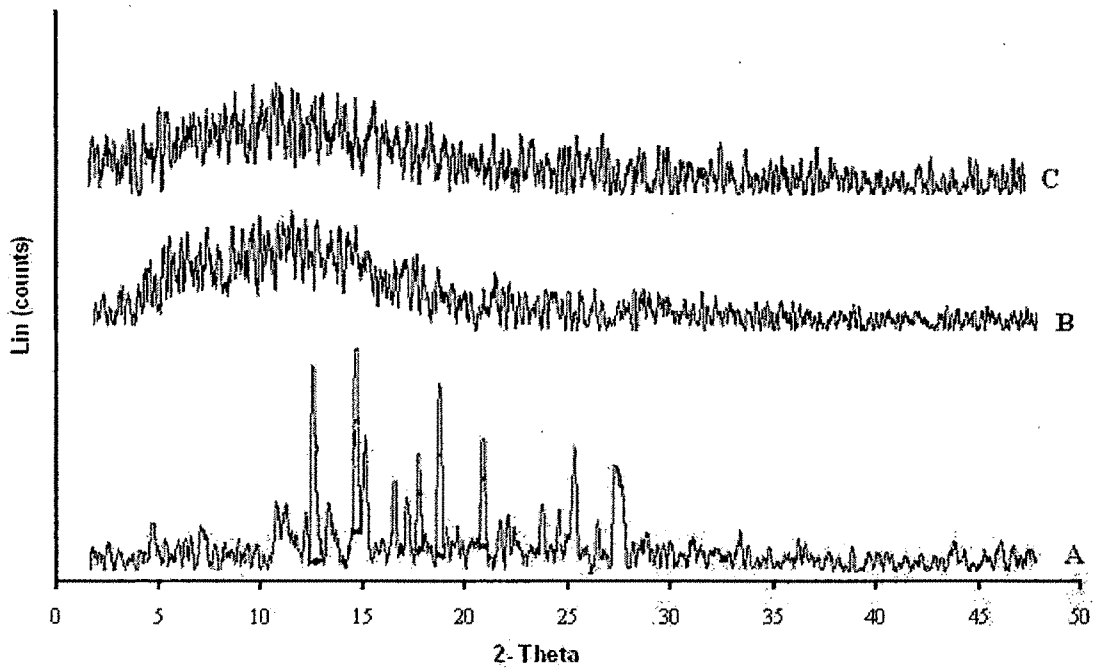

Fig. 4.12 Powder X-ray diffraction patterns of (A) pure carvedilol; (B) drug loaded microspheres; and (C) placebo microspheres

4.6.3.11 Histology studies

The histology of control and treated nasal mucosa after 8 hours is shown in Fig. 4.13 (A and B). The microscopic observations indicated that the optimized formulation has no significant effect on the microscopic structure of sheep nasal mucosa. No major changes in the ultrastructure of mucosa morphology could be seen and the epithelial cells appeared mostly unchanged with slight modification of the epithelial layer because of use of chitosan, as it improves the paracellular transport by opening the tight junctions in the epithelial layer. It can also be assumed that the slight change in epithelial layer was may be due to retention of drug on mucosa because from the data available in the literature, it appears that the effect of chitosan on mucosa may be due to increasing the retention of the drug at the mucosal surface (Nicolazzo et al., 2005). Neither cell necrosis nor removal of the epithelium from the nasal mucosa was observed after diffusion study as compared with control mucosa treated with phosphate buffer pH 6.2 Thus, the microsphere formulation seems to be safe with respect to nasal administration.

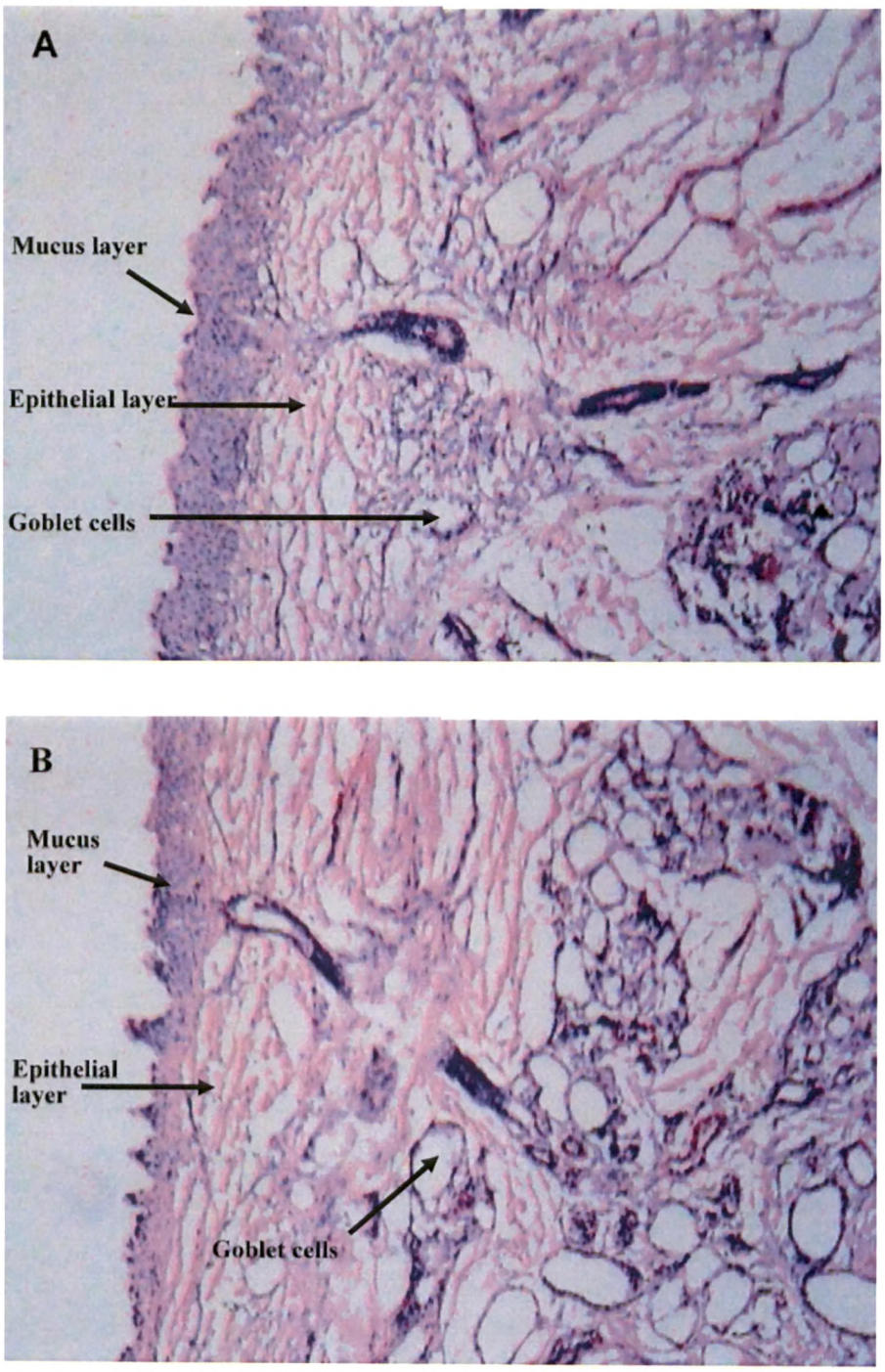

Fig. 4.13 Histology evaluations of sections of sheep nasal mucosa. (A) Control mucosa after incubation with phosphate buffer pH 6.2 in diffusion chamber; (B) Mucosa after incubation in diffusion chamber with microsphere formulation.

4.6.3.12 Stability studies

The stability data of the chitosan microspheres of carvedilol is shown in Table 4.7. No macroscopical physical changes were observed during storage. The results obtained in the stability test showed that the particle size and drug content from the system stored at a temperature of 40 ^{0}C and a relative humidity of 75% was unchanged during a 3-month period of accelerated storage conditions. Particle size and drug content values after 1, 2 and 3 months showed no significant differences ($p>0.05$). This indicated that the microsphere formulation was stable.

Table 4.7 Stability study results for CRV loaded chitosan microspheres under accelerated condition

Time/months	Appearance	Particle size	Drug content (%)
0	Light brown	34.86±1.58	100.0±3.03
1	Light brown	34.56±2.42	100.6±3.12
2	Light brown	33.98±2..28	99.28±2.64
3	Light brown	33.53±1.96	98.86±2.92

*n = 3; Mean ± SD

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