Chapter 6
Experimental –Nitrendipine loaded Chitosan Microspheres

6.1 MATERIALS AND METHODS

6.1.1 Materials

- Nitrendipine (NTD) was a gift sample from USV Limited, Mumbai, 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.

6.1.2 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 6390 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)

6.2 Preparation of Chitosan Microspheres

Chitosan microspheres were prepared by simple W/O emulsification-cross linking process using method described in **Section 4.2.**

6.3 Optimization of Formulation Parameters

Optimization of formulation parameters was carried out similar to **Section 4.3**

6.4 Preparation of NTD 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. Then nitrendipine (0.1 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.

6.5 Characterization of Microspheres

Characterization of the microspheres was carried out as per the methods described in **Section 4.5** for following studies:

6.5.1 Particle Size Measurements

6.5.2 Morphological Characterization

6.5.3 Flow Properties

6.5.4 Determination of Entrapment Efficiency (EE)

The NTD-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 355 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 355 nm. The percentage entrapment efficiency was calculated using following equation:

$$\% \text{ Entrapment efficiency} = \frac{\text{Actual loading}}{\text{Theoretical loading}} \times 100 \quad \ldots\ldots\ldots \quad \textbf{6.1}$$

6.5.5 Mucus Glycoprotein Assay

6.5.6 Adsorption of Mucin to Chitosan Microspheres

6.5.7 Measurement of In Vitro Mucoadhesion

6.5.8 In Vitro Drug Release

6.5.9 Differential Scanning Calorimetry (DSC) Analysis

6.5.10 X-ray Diffraction Studies

6.5.11 Histology studies

6.5.12 Stability study

6.6 RESULTS AND DISCUSSION

6.6.1 Optimization of Formulation Parameters

As per the results and discussion in **Section 4.6.1,** 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%

6.6.2 Preparation of NTD loaded microspheres

The formula for the various batches of NTD loaded microspheres is shown in Table 6.1.

Table 6.1 Various formulation parameters used in the preparation of NTD loaded microspheres

Formulation code	Drug: polymer ratio	Volume of GA (mL)	Cross linking time (h)
CHNT1	1:2	1	1
CHNT2	1:1	1	1
CHNT3	1:2	2	1
CHNT4	1:1	2	1
CHNT5	1:2	1	2
CHNT6	1:1	1	2
CHNT7	1:2	2	2
CHNT8	1:1	2	2

4.6.3 Characterization of NTD loaded Microspheres

4.6.3.1 Particle size measurements

The particle size of chitosan microspheres loaded with NTD was in the range of 26.12–48.65µm (Table 6.2), 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 6.2 Characteristics of prepared NTD loaded chitosan microspheres

Formulation Code	Particle size (μm)	Angle of repose (θ)	Compressibility Index (%)	Entrapment Efficiency (%)	In vitro mucoadhesion (%)
CHNT1	28.68±2.42	28.34±1.68	15.68±2.14	50.14±1.68	85.67±1.97
CHNT2	46.72±1.96	30.98±2.12	16.24±1.62	66.94±2.48	83.12±2.53
CHNT3	26.12±2.37	26.12±1.48	19.67±1.98	47.59±1.92	78.61±2.47
CHNT4	42.37±1.86	33.64±1.69	15.24±1.76	60.46±1.38	76.31±1.84
CHNT5	37.56±2.75	25.24±1.38	16.68±1.82	56.86±2.59	87.48±2.13
CHNT6	48.65±2.67	34.56±1.98	19.12±1.67	69.37±2.26	81.45±1.86
CHNT7	39.34±2.12	29.61±2.14	17.98±1.74	46.63±1.75	80.65±2.92
CHNT8	44.34±2.68	34.42±1.53	18.84±1.34	57.62±1.67	75.04±2.42

6.6.3.2 Morphological characterization

The chitosan microspheres loaded with NTD were non aggregated, free flowing powders with spherical shape and smooth surface (Fig. 6.1 and Fig. 6.1). However, one noticeable characteristic of the cross linked chitosan microspheres was their yellow to brownish color (Fig. 6.1). 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.

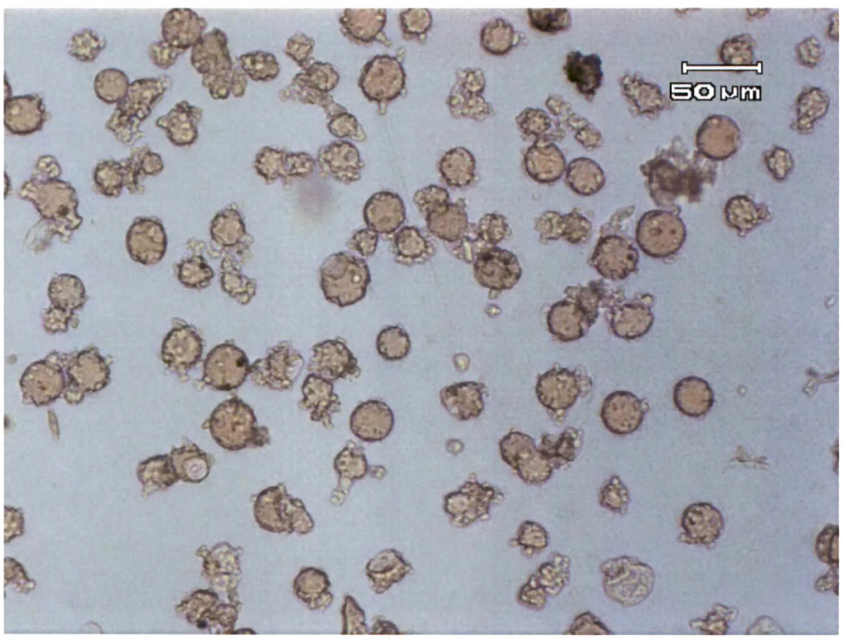

Fig. 6.1 Photomicrograph of NTD loaded chitosan microspheres.

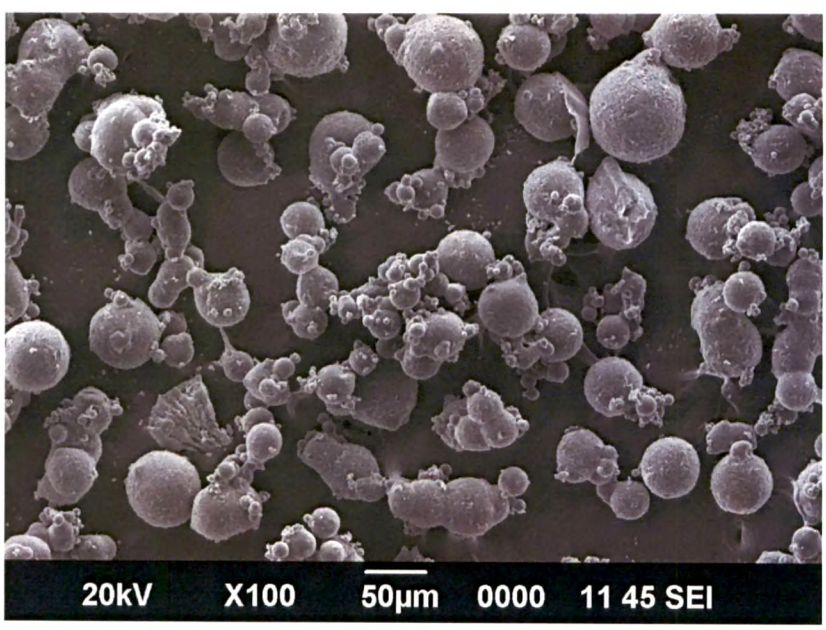

Fig. 6.2 SEM photograph of NTD loaded chitosan microspheres.

The cross linking agent (GA) used here is reported to bind to different reaction sites in the chitosan. The aldehyde groups of glutaraldehyde form covalent imine bonds with the amino groups of chitosan, due to the resonance established with adjacent double ethylenic bonds via a Schiff reaction (Fig. 6.3).

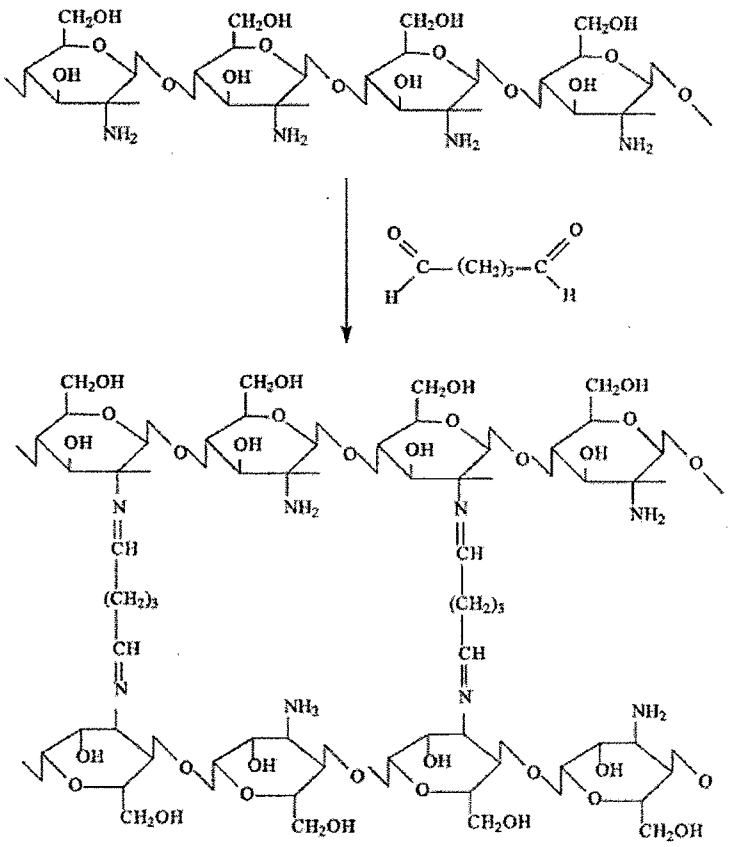

Fig. 6.3 Cross linking process of chitosan treated with glutaraldehyde.

6.6.3.3 Flow Properties

The results of flow properties measurements are shown in Table 6.2. The values of angle of repose were in the range of $25.24^0 \pm 1.38$ to $34.56^0 \pm 1.98$ 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 15.24±1.76 to 19.67±1.98, also indicating good flow characteristics of the microspheres.

6.6.3.4 Entrapment efficiency

The % EE was found to be in the range between 46.63 and 69.37. 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 6.2). 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.

6.6.3.5 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. 6.4). Thus, these studies indicate that mucoadhesion may increase the residence time of the formulation in the nasal cavity.

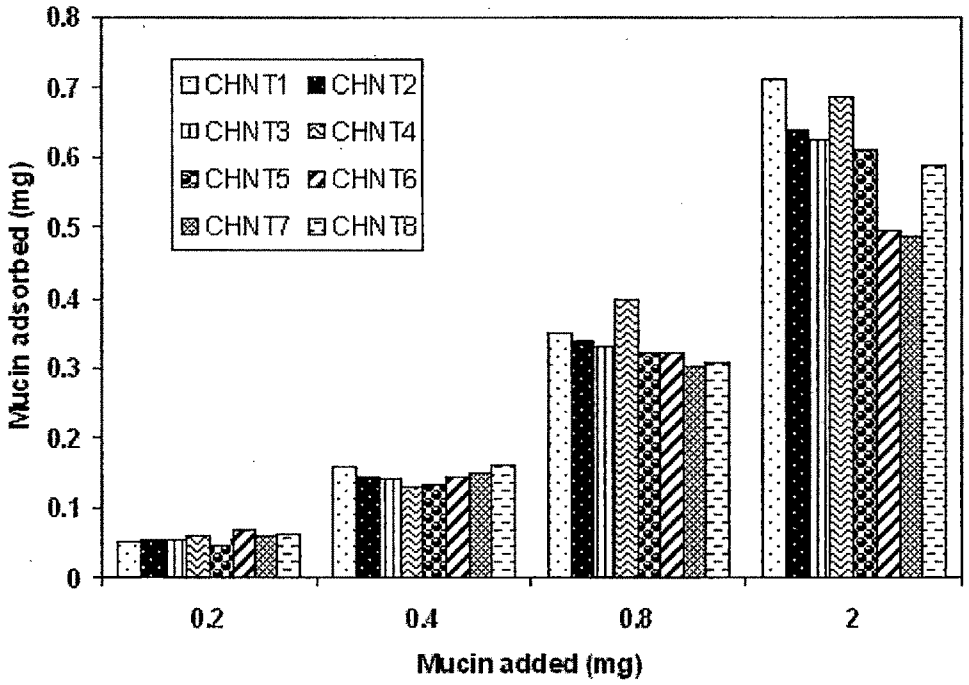

Fig. 6.4 Adsorption of mucin on different microspheres with respect to the amount of mucin added.

6.6.3.6 Adsorption Isotherms

The data obtained from adsorption studies were fitted to Freundlich and Langmuir equations. Straight lines (Fig.6.5 and Fig. 6.6) 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 6.3. 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.

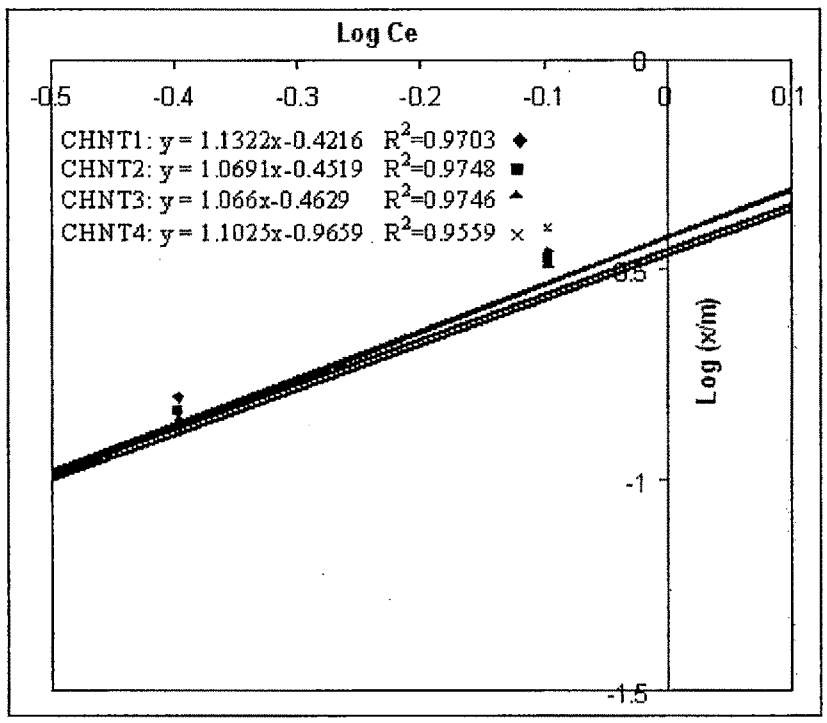

Fig. 6.5A Freundlich adsorption isotherms for mucin adsorbed on chitosan microspheres (Batches CHNT1 to CHNT4)

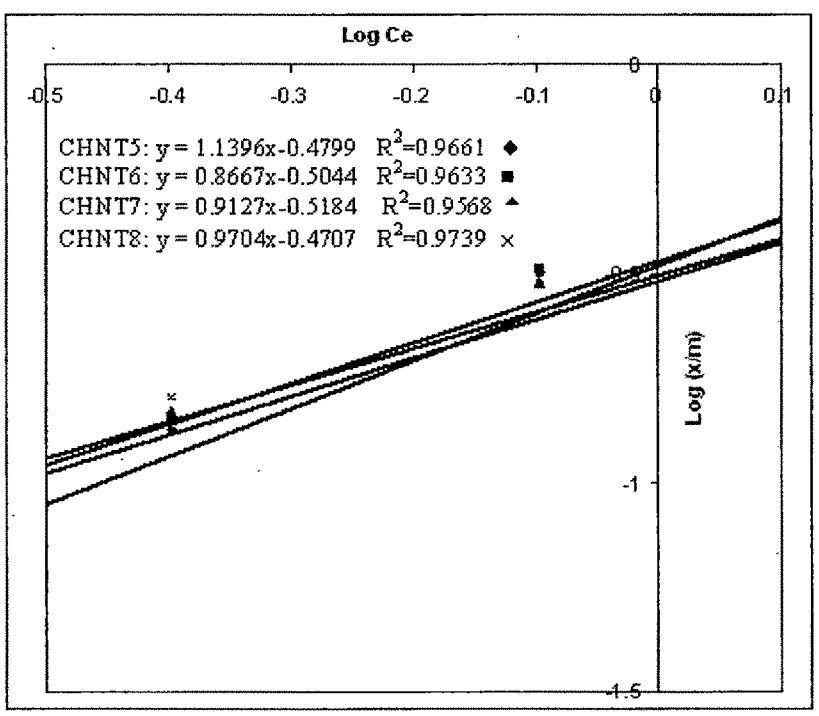

Fig. 6.5B Freundlich adsorption isotherms for mucin adsorbed on chitosan microspheres (Batches CHNT5 to CHNT8)

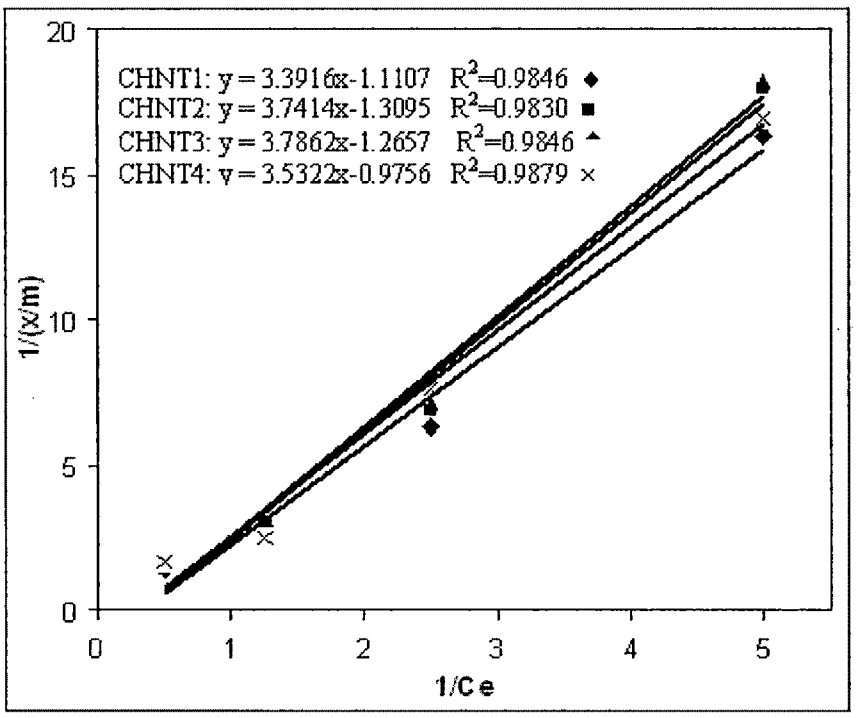

Fig. 6.6A Langmuir adsorption isotherms of mucin adsorbed on chitosan microspheres (Batches CHNT1 to CHNT4)

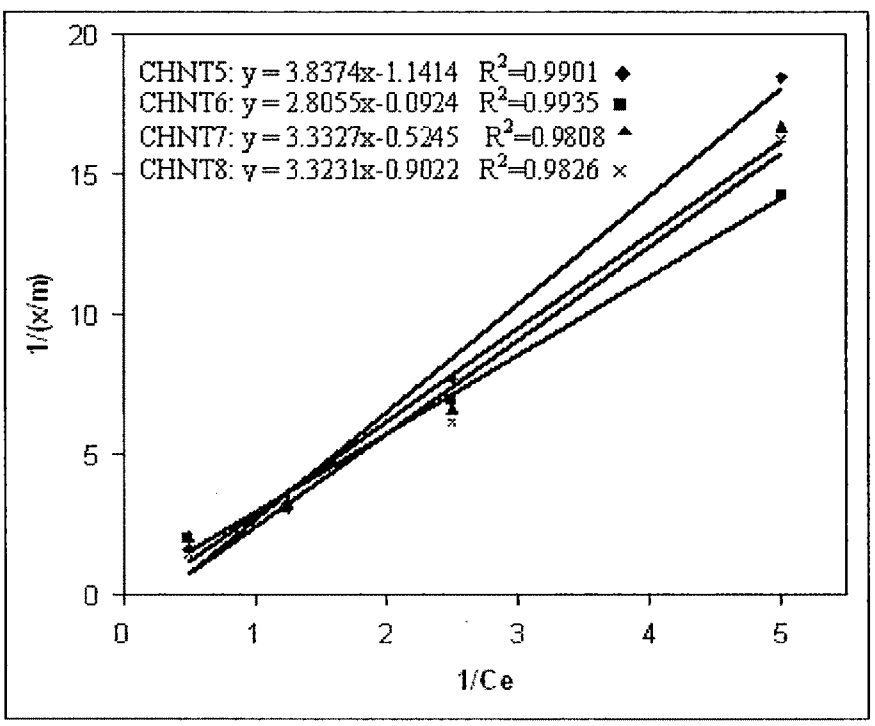

Fig. 6.6B Langmuir adsorption isotherms of mucin adsorbed on chitosan microspheres (Batches CHNT5 to CHNT8)

Table 6.3 Constants for Langmuir and Freundlich Equations

	Freundlich isotherm*			Langmuir isotherm•		
Batch code	**n**	**K**	**R^2**	**a**	**b**	**R^2**
CHNT1	0.883	2.63	0.9703	1.1107	3.3916	0.9846
CHNT2	0.935	2.83	0.9748	1.3095	3.7414	0.9830
CHNT3	0.938	2.90	0.9746	1.2657	3.7862	0.9846
CHNT4	0.907	2.64	0.9659	0.9756	3.5322	0.9879
CHNT5	0.887	3.01	0.9661	1.1414	3.8374	0.9901
CHNT6	1.153	3.19	0.9633	0.0924	2.8055	0.9935
CHNT7	1.095	3.29	0.9568	0.5245	3.3327	0.9808
CHNT8	1.03	2.95	0.9739	0.9022	3.3231	0.9826

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

•$1/(x/m) = a+b \cdot 1/Ce$

6.6.3.7 In vitro mucoadhesion

The results of in vitro mucoadhesion (Table 6.2) showed that all the batches of microspheres had mucoadhesive property ranging from 75.04±2.42 to 87.48±2.13 % (Fig. 6.7) 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 6.2), attributed to increased rigidity and reduced binding sites for mucoadhesion. Most of the studies showed that the prerequisite for a 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, could possibly decrease the mucoadhesion strength of the microspheres (Illum et al., 1987).

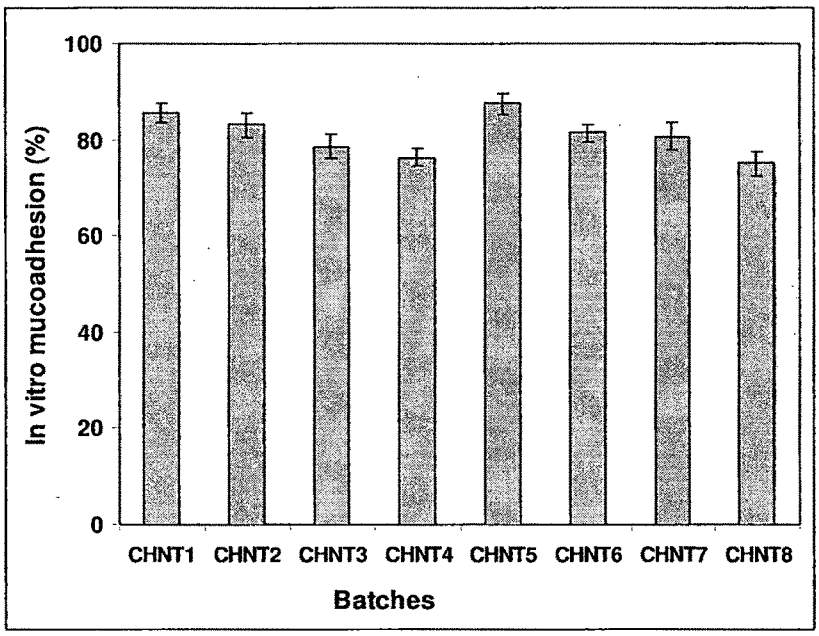

Fig. 6.7 Percentage in vitro mucoadhesion for different batches of microspheres

6.6.3.8 In vitro drug release

The in vitro drug release profile of NTD from the chitosan microspheres is shown in Fig. 6.8. The release pattern showed a moderate and controlled release following near zero order release. When microspheres of hydrophilic polymers such as chitosan immersed in water, they swell and form a gel diffusion layer that hinders the outward transport of the drug within the matrix, hence producing a controlled release effect. The initial slight burst drug release may be attributed to the release of drug molecules held loosely into or just beneath the surface of microspheres. Such a burst effect was reported previously for gelatin microspheres by Ugwoke et al. (Ugwoke et al., 1997). *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.

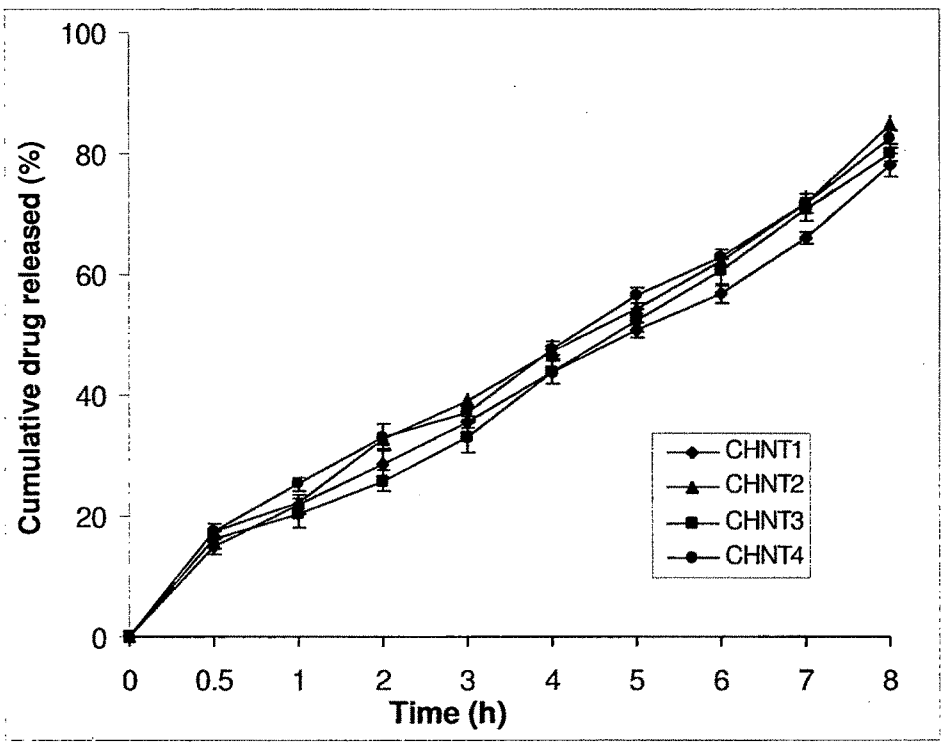

Fig. 6.8A *In vitro* drug release profile of chitosan microspheres of NTD (Batches CHNT1 to CHNT4). The values are mean ± SD (n = 3).

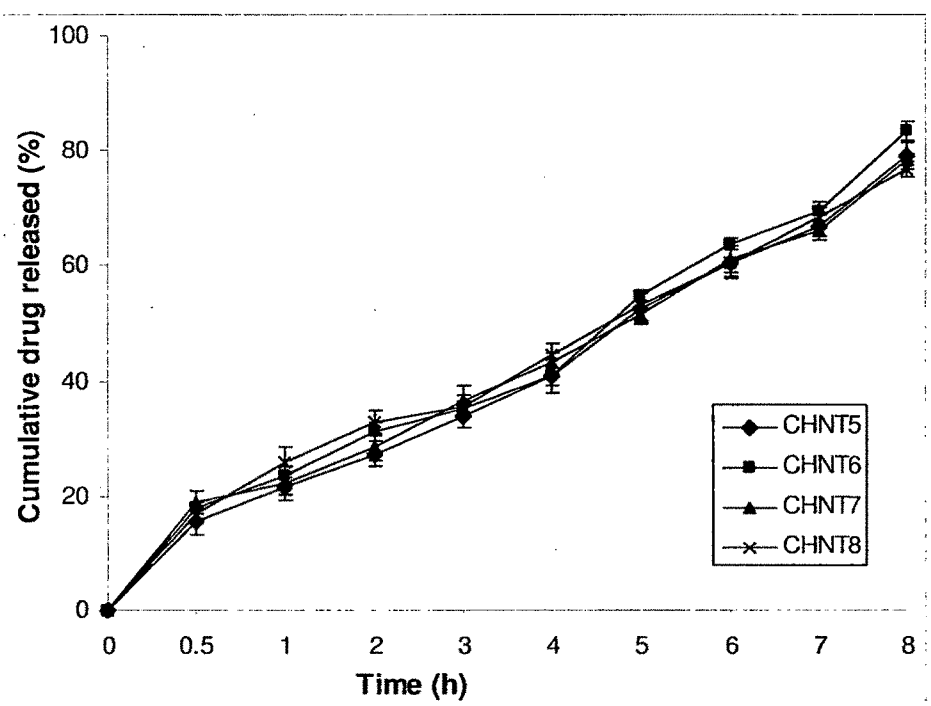

Fig. 6.8B *In vitro* drug release profile of chitosan microspheres of NTD (Batches CHNT5 to CHNT8). The values are mean ± SD (n = 3).

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 6.4. A model with the greatest regression coefficient (r^2) was chosen as the dominant model.

Table 6.4 Kinetic model of Carvedilol release from chitosan microspheres

Formulation code	Regression coefficient (r^2)			Release kinetics
	Zero-order	First-order	Higuchi model	
CHNT1	0.9759	0.9353	0.9624	Zero-order
CHNT2	0.9748	0.9353	0.9757	Higuchi
CHNT3	0.9846	0.9397	0.9486	Zero-order
CHNT4	0.9696	0.9367	0.9768	Higuchi
CHNT5	0.9796	0.9339	0.9500	Zero-order
CHNT6	0.9693	0.9032	0.9337	Zero-order
CHNT7	0.9711	0.9386	0.9518	Zero-order
CHNT8	0.9617	0.9570	0.9625	Higuchi

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

$$\frac{Mt}{M\infty} = kt^n \qquad \text{..... 6.2}$$

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 6.5). 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 0.50 to 0.58 indicating non-Fickian or anomalous type of transport which is a further indication of the diffusion-controlled drug release. 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 6.5 Release mechanisms of different formulations.

Batch code	n	k	Correlation coefficient, r^2	Release mechanism
CHNT1	0.57	0.304	0.9815	Non-Fickian
CHNT2	0.56	0.311	0.9788	Non-Fickian
CHNT3	0.58	0.294	0.9532	Non-Fickian
CHNT4	0.53	0.327	0.9749	Non-Fickian
CHNT5	0.50	0.303	0.9641	Non-Fickian
CHNT6	0.53	0.329	0.9259	Non-Fickian
CHNT7	0.51	0.342	0.9509	Non-Fickian
CHNT8	0.51	0.343	0.9707	Non-Fickian

6.6.3.9 Differential scanning calorimetry

Investigation of the thermal properties of microspheres is done by DSC, which provides both qualitative and quantitative information about the physicochemical state of drug inside the microspheres (Dubernet, 1995). If the drug is present in a molecular dispersion or solid solution state in the polymeric microspheres, then no detectable endotherm of drug observed (Mu and Feng, 2001). DSC thermograms of pure NTD (A), NTD loaded chitosan microspheres (B) and blank chitosan microspheres (C) are displayed in Fig. 6.9. Thermogram of NTD showed a sharp peak at 159 ^{0}C, indicating the melting of the drug. In case of placebo microspheres, a broad peak was observed at 69 ^{0}C which was mainly due to the transitions associated with loss of water corresponding to the hydrophilic nature of the functional groups of the polymer. The second thermal event registered was an exothermic peak occurring at 272 °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 83 ^{0}C and exothermic peak at 272 ^{0}C was observed. However, there was no peak corresponding to NTD, indicating that NTD is molecularly dispersed in the matrix of the chitosan microspheres.

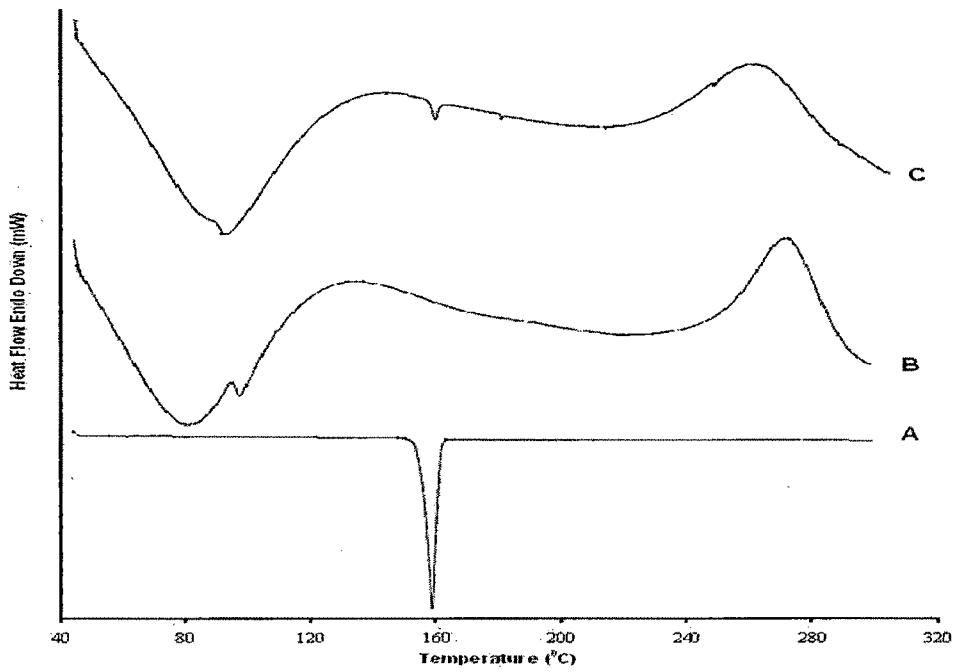

Fig. 6.9 DSC thermograms of (A) pure nitrendipine; (B) placebo microspheres; and (C) NTD loaded microspheres

6.6.3.10 X-ray diffraction studies

XRD patterns recorded for plain NTD (A), placebo microspheres (B) and NTD loaded microspheres (C) are presented in Fig. 6.10. In case of plain NTD, major peaks observed at 2θ of 9.99^0, 11.33^0, 13.09^0, 23.80^0, 24.30^0, 27.45^0 and 28.70^0 are due to the crystalline nature of NTD. These peaks were not observed in the NTD 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.

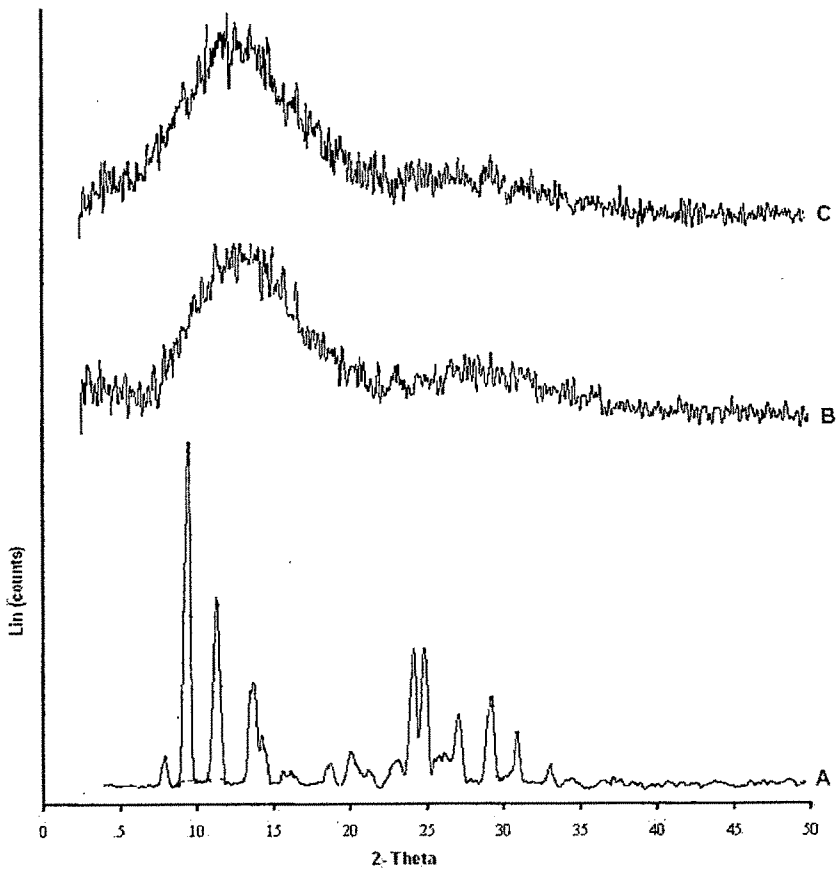

Fig. 6.10 Powder X-ray diffraction patterns of (A) pure nitrendipine; (B) placebo microspheres; and (C) nitrendipine loaded microspheres

6.6.3.11 Histology studies

The histology of control and treated nasal mucosa after 8 hours is shown in Fig. 6.11 (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.

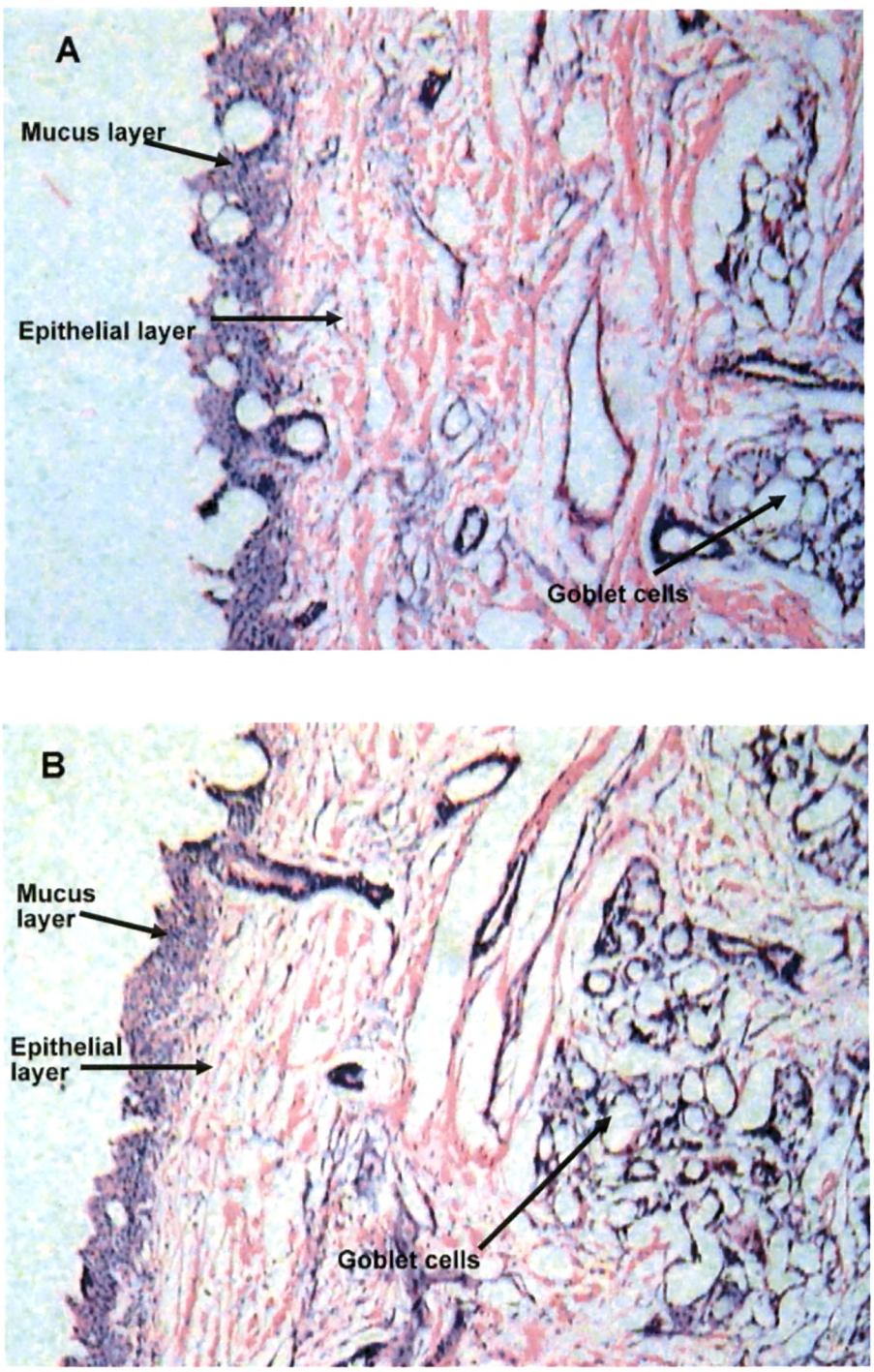

Fig. 6.11 Histology evaluations of sections of sheep nasal mucosa. (A) Control mucosa after incubation with phosphate buffer pH 6.2 in diffusion chamber; (B) Mucosa after incubation in diffusion chamber with microsphere formulation.

6.6.3.12 Stability studies

The stability data of the chitosan microspheres of nitrendipine is shown in Table 6.6. 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 would remain stable under normal storage conditions.

Table 6.6 Stability study results for NTD loaded chitosan microspheres under accelerated condition

Time/months	Appearance	Particle size* (μm)	Drug content* (%)
0	Light brown	48.65±2.67	100.0±2.68
1	Light brown	47.12±2.64	98.86±3.04
2	Light brown	46.96±2.12	98.14±2.37
3	Light brown	46.38±2.49	97.98±2.36

*n = 3; Mean ± SD

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