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Characterization of pilocarpine-loaded chitosan/Carbopol nanoparticles

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Abstract

Patients using ophthalmic drops are faced with frequent dosing schedules and difficult drop instillation. Therefore, a long-lasting pilocarpine-loaded chitosan (CS)/Carbopol nanoparticle ophthalmic formulation was developed. The physicochemical properties of the prepared nanoparticles were investigated using dynamic light scattering, zeta-potential, transmission electron microscopy, Fourier transform infrared ray spectroscopy (FT-IR) and differential scanning calorimetry (DSC). The sustained-release effects of pilocarpine-loaded nanoparticles were evaluated using in-vitro release and in-vivo miotic tests, and compared with pilocarpine in solution, gel and liposomes. We found that the prepared nanoparticles were about 294 nm in size. DSC and FT-IR studies suggested that an electrostatic interaction between CS and Carbopol contributes at least in part to the stabilization of pilocarpine/CS/Carbopol nanoparticles. When compared with pilocarpine in solution, gel or liposomes, the best slow-release profile of pilocarpine from the prepared nanoparticles occurred in a dissolution test. In the in-vivo miotic study, pilocarpine-loaded CS/Carbopol nanoparticles showed the most significant long-lasting decrease in the pupil diameter of rabbits. The advantages of CS and Carbopol are good biocompatibility, biodegradability and low toxicity. CS is also a mucoadhesive polymer. Thus, pilocarpine/CS/Carbopol nanoparticles may provide an excellent potential alternative ophthalmic sustained-release formulation of pilocarpine for clinical use. CS/Carbopol nanoparticles may also be useful for a variety of other therapeutic delivery systems.

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Introduction

Pilocarpine, a para-sympathomimetic, remains a miotic of choice for open-angle glaucoma because it increases the outflow of aqueous humour. The drug penetrates the eye well, with miosis beginning 15–30 min after topical application and lasting for 4–8 h (Zimmerman 1981). Pilocarpine ophthalmic drops are administered as 1 or 2 drops per dose, with 6 drops per day as the maximum recommended dosage. Patients on pilocarpine ophthalmic drops are faced with frequent dosing schedules and difficult drop instillation. While ointment preparations offer a second option, this dosage form causes poor patient compliance because of the blurred vision and discomfort resulting from the messy and greasy properties of the ointment (Lee 1990). Therefore, new and long-acting ophthalmic pilocarpine formulations are needed (Li & Xu 2002; Aktas et al 2003).

Recently, nanoparticles composed of various polymers have been widely investigated as carriers for drug delivery (Harmia et al 1986; Gref et al 1994; Zimmer et al 1994; Dyer et al 2002; Yoncheva et al 2003). Examples of hydrophobic polymer-based nanoparticles are poly-ε-caprolactone (PCL), polylactide (PLA), and their copolymers. These polymers, however, are not compatible with hydrophilic drugs. On the contrary, poly(ethylene glycol) (PEG) modified polyester nanoparticles are promising carriers for hydrophilic drugs, although possible residuals of solvents and surfactants (e.g., Pluronic F68) that might cause ophthalmic irritation make it necessary to develop polymers that are more biocompatible.

A chitosan/Carbopol nanoparticle, with a well-dispersed and stable system in aqueous solution, is considered an excellent candidate for controlled drug delivery (Ahn et al 2001; Hu et al 2002). Chitosan (CS; (1,4)-[2-amino-2-deoxy-β-D-glucan]), a mixture of glucosamine and *N*-acetyl-glucosamine, is a cationic polysaccharide obtained from the chitin of crustacean shells (Sandri et al 2004). Chitosan is biocompatible, biodegradable, not highly toxic and mucoadhesive (Borchard et al 1996; Mao et al 2004). Carbopol, a

poly(acrylic acid) (PAA) polymer recently introduced in controlled drug delivery, has the disadvantages of a high glass transition temperature and high water solubility, resulting in rapid dissolution and a short duration for drugs to permeate the membrane (Ahn et al 2001). In the CS/Carbopol nanoparticle, however, the dissolution rate of Carbopol is delayed by the partial formation of an electrostatic complex between CS and Carbopol. The small size and positive charges of CS/Carbopol nanoparticles may improve their interaction with negatively charged biological membranes (de la Torre et al 2003; Torrado et al 2004).

In this study, we report an approach for preparing pilocarpine-loaded CS/Carbopol nanoparticles. We evaluated the physicochemical characterization and morphology of pilocarpine-loaded CS/Carbopol nanoparticles using particle size, zeta-potential, pilocarpine encapsulation efficiency and transmission electron microscopy (TEM) measurement. The possible interaction of pilocarpine, CS and Carbopol in the nanoparticle formulation was investigated using the combination of a differential scanning calorimeter (DSC) and Fourier-transform infrared (FT-IR) spectroscopy. In addition, the in-vitro release profile and in-vivo miotic effect of prepared pilocarpine CS/Carbopol nanoparticles were compared with those properties of pilocarpine in the formulations of traditional eye drops, liposomes and gels.

Materials and Methods

Materials

Chitosan (CS) was purchased from Fluka Chemical Co. (Tokyo, Japan), Carbopol from BF Goodrich Chemical Co. (Cleveland, OH, USA) and pilocarpine from Sigma Chemical Co. (St Louis, MO, USA). Most of the other chemical reagents were purchased from either Merck (Darmstadt, Germany) or Sigma Chemical Co. Simulated tear fluid (STF) was composed of 0.67 g of NaCl, 0.20 g of $NaHCO_3$, 0.008 g of $CaCl_2$ $2H_2O$ and distilled de-ionized water to 100 g.

Preparation of CS/Carbopol nanoparticles

CS/Carbopol nanoparticles were prepared by mixing positively charged CS and negatively charged Carbopol using the dropping method (Aktas et al 2003). A 0.02% CS solution was prepared by dissolving 0.02 g of CS (MW 80 kDa) in 100 mL of a 1% (w/v) acetic acid solution. Then 1 mL of the 0.02% CS solution was added dropwise into 5 mL of a 0.02% Carbopol (M_n 100 kDa) dispersion using a homogenizer with magnetic stirring for 10 min to form an opalescent suspension. The pH and temperature of the system were maintained at 4.5 and 45°C, respectively.

Preparation of pilocarpine-loaded CS/Carbopol nanoparticles

The pilocarpine-loaded nanoparticles were prepared by dissolving 50 mg of pilocarpine in 50 mL of CS/Carbopol nanoparticles, resulting in a final pilocarpine concentration of 0.1% (w/v). This formulation was prepared using the dropping method (Aktas et al 2003) and incubated in a 45°C water bath for 90 min. The whole preparation was then maintained under magnetic stirring for 48 h. The nanoparticles were separated from the aqueous phase using ultracentrifugation (50000 rev min^{-1} at 4°C for 50 min). Next, the pilocarpine-loaded CS/Carbopol nanoparticles were lyophilized to obtain dried pilocarpine-loaded CS/Carbopol nanoparticles for further studies.

Pilocarpine encapsulation efficiency in the nanoparticles

After ultracentrifugation, the amount of free pilocarpine in the clear supernatant was measured using a UV spectrophotometer (U-2001; Hitachi, Tokyo, Japan). Pilocarpine encapsulation efficiency (EE) was calculated with the following equation:

$$\mathrm{EE}\,(\%) = \frac{\text{total amount of pilocarpine} - \text{free amount of pilocarpine} \times 100}{\text{total amount of pilocarpine}} \quad (1)$$

Preparation of pilocarpine-loaded liposomes

Mixed lipid of egg yolk and cholesterol (molar ratio 7:3) was dissolved in Tween 80/absolute ethanol to assure a homogeneous mixture. A 0.1% concentration of pilocarpine was dissolved in chitosan/acetate solution. The lipid and pilocarpine solution were then mixed under a vortex in a round-bottom flask and kept in a 45°C water bath. The solvent was then evaporated. The mixture of pilocarpine in liposomes was thoroughly lyophilized using a freeze-drier overnight (Takeuchi et al 2003).

Pilocarpine in gel preparation

Carboxymethylcellulose (CMC; 0.5% concentration) was dispersed with high shear in cold water to swell into sticky gel grains. The solution was then heated with moderate shear to about 60°C to form gel. Pilocarpine (0.1% w/v concentration) was added to the gel solution. The homogenous mixture was maintained in a 45°C water bath and stirred continuously for 1 h.

Transmission electron microscopic (TEM) study

TEM was used to observe the morphology of pilocarpine/CS/Carbopol nanoparticles. Samples were placed onto a cooper grill covered with nitrocellulose. They were dried at room temperature and were then examined using a TEM (JEOL-1200EX, Japan) without being negative stained.

Particle size and zeta-potential of pilocarpine/CS/Carbopol nanoparticles

The mean size and size-distribution of the CS/Carbopol nanoparticles were measured using a dynamic light scattering (DLS) system (Zetasizer 3000 HS; Malvern Instruments Ltd., Malvern, Worcestershire, UK) in buffer solution. All DLS measurements were done using a wavelength of 633 nm at

25°C with an angle detection of 90°. Each sample was measured 3 times and the reported values are the mean diameter ± s.d.

The zeta-potential of the pilocarpine/CS/Carbopol nanoparticles were measured using the same DLS system. The samples were diluted with 10 mM of NaCl solution (pH 4.5) to maintain a constant ionic strength. Each sample was measured three times, and the values reported are the mean ± s.d.

Thermal analysis using differential scanning calorimetry (DSC)

The enthalpy change (ΔH) and melting temperature (T_m) of CS, Carbopol, pilocarpine, CS/Carbopol polymer complexes and pilocarpine-loaded CS/Carbopol nanoparticles were measured using a thermal analysis system (DSC 7; Perkin Elmer, Shelton, CT, USA). Approximately 5 mg of sample was packed into an aluminium DSC pan that was hermetically sealed using a sample-encapsulation press. Helium was used as a purge gas at the rate of 30 $cm^3 min^{-1}$. The sample was heated from 20 to 250°C at a scan rate of 3°C min^{-1}.

Fourier Transform Infrared (FT-IR) spectrum analysis

FT-IR spectra were measured using an optical bench (Magna-IR 560; Nicolet Instrument Corporation, Madison, WI, USA) to determine the possible interactions between pilocarpine, CS and Carbopol. The pilocarpine-loaded CS/Carbopol nanoparticles were lyophilized using a freeze dryer to obtain dried nanoparticles. These dried CS/Carbopol nanoparticles were mixed with potassium bromide (KBr) and pressed to form a plate for measurement. Two hundred and fifty-six scans at 4 cm^{-1} resolution were used to obtain each spectrum.

Dissolution test (in-vitro drug release from the nanoparticles)

Ten grams of pilocarpine-loaded CS/Carbopol nanoparticles were put into a dissolution apparatus (DT6; Hsiangtai Machinery Industry Co. Ltd., Japan) at a rotation of 75 rev min^{-1}. Samples were immersed in 900 mL of simulated tear fluid (STF: NaCl 0.67 g, $NaHCO_3$ 0.20 g, $CaCl_2$ $2H_2O$ 0.008 g and distilled, de-ionized water to 100 g) in triplicate at 37°C. At predetermined time points of 0, 15, 30, 45, 60, 90, 120, 240, 360, 480, 600, 720 and 1440 min, 3 mL of sample was withdrawn and centrifuged, and the supernatant was assayed for pilocarpine using a spectrophotometer (U2001; Hitachi Ltd). The intra-day and inter-day validation tests of pilocarpine spectrophotometric measurements at 220 nm were verified. The release percentage of the pilocarpine/CS/Carbopol complex was plotted as a function of time.

In-vivo miotic study

New Zealand albino rabbits, 1.5–2.0 kg, were used in the in-vivo experiments. The rabbits were kept in restraining boxes throughout each experiment. All tests were performed in the same room under standard lighting. After 30 min of acclimatization, the difference in pupil diameter between the left and right eyes was measured four times, and the mean value of those measurements was used as a reference (A) for calibration in further experiments. Rabbits were dosed with 100 μL of pilocarpine preparation, always in the right eye, and simulated tear fluid (STF), always in the left eye, as the control. The difference (B) between the left and right eyes was measured at the desired time point. The value obtained from (B) minus (A) is the decrease in pupil diameter at the specific time point. Each preparation was tested in groups of six different rabbits. The rabbits were released from their restraining boxes between the sampling time intervals. They were all treated under the appropriate national and university guidelines for the care and use of laboratory animals in research and teaching (National Science Council, Republic of China, Taiwan). The research project was approved by the university's Institutional Animal Care and Use Committee.

Statistical analysis

Results are given as mean ± s.d. of at least three measurements. Statistical significance was set at $P < 0.05$. For both the in-vitro dissolution tests and the in-vivo miotic study, all experiments were run at several time points, each with four pilocarpine formulations handled in a paralleled pattern. Statistical analysis was done using repeated-measure analysis of variance and the Tukey multiple comparison post test. For the in-vivo miotic study, the area under the miosis–time curve (AUC) was calculated using four different pilocarpine formulations. These different AUC values were compared using the non-parametric Kruskal–Wallis test. Dunn's post test was used to compare all pairs of AUC values. All statistical analyses were performed using Prism 4.0 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results and Discussion

Structure of CS/Carbopol nanoparticles

When CS was dropped into Carbopol solution, CS/Carbopol nanoparticles with a CS core and a CS/Carbopol membrane were formed. The pK_a values of Carbopol and CS were 5.5 and 6.5, respectively (Davies et al 1991; Ahn et al 2001). At pH 4.5, Carbopol and CS are partially ionized, which means that Carbopol may bear negative charges and CS may bear positive charges. Carbopol and CS thus may partially form a complex through ionic interaction. Because CS does not swell in the acid solution, there are no cavities formed in the nanoparticles when they are dried for TEM characterization (Figure 1). Instead, one should see a structure with a dark solid and spherical shape, which is, in fact, what we saw. Similar results were found in other studies (Gaserod et al 1999; Hu et al 2002). Pilocarpine was entrapped between the core and the membrane. The nanoparticles shown in Figure 1 were evenly distributed, and particle sizes ranged between 50 and 200 nm. Such well-dispersed nanoparticles are useful as drug carriers because they offer excellent absorption and distribution.

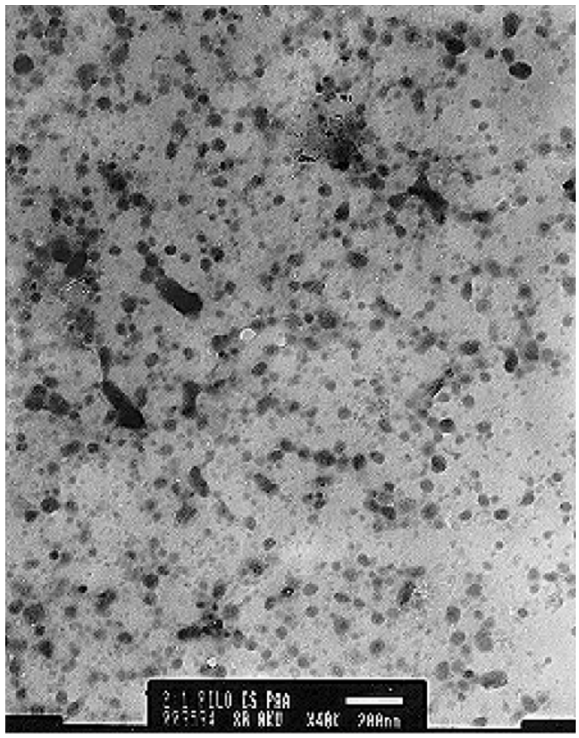

Figure 1 Transmission electron microphotography (TEM) of pilocarpine/CS/carbopol nanoparticles.

Fourier Transform Infrared spectroscopic analysis

To investigate the complex formed with CS, Carbopol, and pilocarpine molecules, we did an FT-IR study. Figure 2 shows the FT-IR spectra of pilocarpine, CS, Carbopol, CS/Carbopol, and pilocarpine/CS/Carbopol nanoparticles. The carbonyl absorption band of pilocarpine was observed at 1776 cm^{-1}. Amine bands of amide I and amide II regions of CS itself were located at 1654 and 1593 cm^{-1}, respectively. In addition, the carbonyl-stretching vibrational band of Carbopol itself appeared at 1705 cm^{-1} (Ahn et al 2002). The carbonyl absorption band of Carbopol in CS/Carbopol was shifted to 1716 cm^{-1}, and the amine bands of the amide I and II regions of CS were shifted to 1623 and 1557 cm^{-1} (Figure 2), suggesting the formation of a polyionic complex between CS and Carbopol molecules. The shift of the amide I and II bands was attributed to the formation of $-NH_3^+$ when the complex was being formed. The carbonyl absorption band of the pilocarpine for pilocarpine/CS/Carbopol was shifted to 1767 cm^{-1}. The amide I band of CS and the carbonyl band of Carbopol in the pilocarpine/CS/Carbopol nanoparticle disappeared, indicating that pilocarpine was stabilized in the CS/Carbopol nanoparticles and that an inclusion complex was formed in them. Moreover, the absorption peak at 1408 cm^{-1} could be assigned to the symmetric stretching vibration of carboxylate anion (COO^-) groups, indicating the dissociation of carboxylic groups of Carbopol into carboxylate anion groups, which form stable complexes with protonated amino groups of CS through electrostatic interaction. Similar electrostatic interaction was observed in other studies (Peniche et al 1999; Ahn et al 2002; Hu et al 2002; Torrado et al 2004). They also suggest that the most impressive characteristic of the CS/Carbopol system is the capability of the carboxylic functional groups in the acrylic monomers to form ionic complexes with the basic amino groups in the chitosan chain, leading to the formation of a highly swollen interpenetrating polymeric network (IPN) (Peniche et al 1999; Ahn et al 2002; Hu et al 2002; Torrado et al 2004).

Particle size and zeta-potential of pilocarpine/CS/Carbopol nanoparticles

The particle-size distribution of CS, CS/Carbopol, and pilocarpine/CS/Carbopol was characterized using dynamic light scattering. The particle size, which increased from 250 nm in CS/Carbopol particles to 294 nm in the pilocarpine/CS/Carbopol nanoparticles (Table 1), indicated that the encapsulation of pilocarpine expands the nanoparticles, possibly because of the partial location of pilocarpine in the interface of the CS core and the CS/Carbopol shell.

Zeta-potential is the difference in electrical potential between a tightly bound layer of ions on particle surfaces and the bulk liquid in which the particles are suspended. It can be quantified by tracking the charged particles when they migrate in a voltage field, as measured in a zeta-potential analyser. The zeta-potential in this study decreased from 73.46 mV in CS to 50.66 mV in the CS/Carbopol mixture. It is possible that Carbopol partially coated the surface of the nanoparticles, thus reducing their zeta-potential. In addition, because partially ionized Carbopol may be negatively charged and partially ionized CS may be positively charged, we suggest that partial neutralization between these opposite charges may decrease the zeta-potential. When pilocarpine was incorporated, the zeta-potential increased slightly from 50.66 to 55.78 mV because pilocarpine has a positive charge. The positive value of zeta-potential in the nanoparticles also suggests that the more positively charged chitosan or pilocarpine molecules, or both, are distributed nearer the surface than those of negatively charged Carbopol molecules. The positive surface charges of nanoparticles make it easier for them to interact with the biological membranes in the eyes.

Thermal analysis using a differential scanning calorimeter

The enthalpy change (ΔH) and melting temperature (T_m) of CS, Carbopol, pilocarpine and pilocarpine-loaded CS/Carbopol nanoparticles were measured using a differential scanning calorimeter. The T_m of the CS/Carbopol system was not significantly different from that of either CS or Carbopol alone (Table 2). The CS/Carbopol mixture showed a single peak at higher temperature. It is possible that this peak was simply a merging or a masking of the two peaks of CS and Carbopol. Another possibility is that the partial formation of an electrostatic complex between CS and Carbopol, as suggested by FT-IR and zeta-potential studies, may have increased the melting temperature. The T_m of

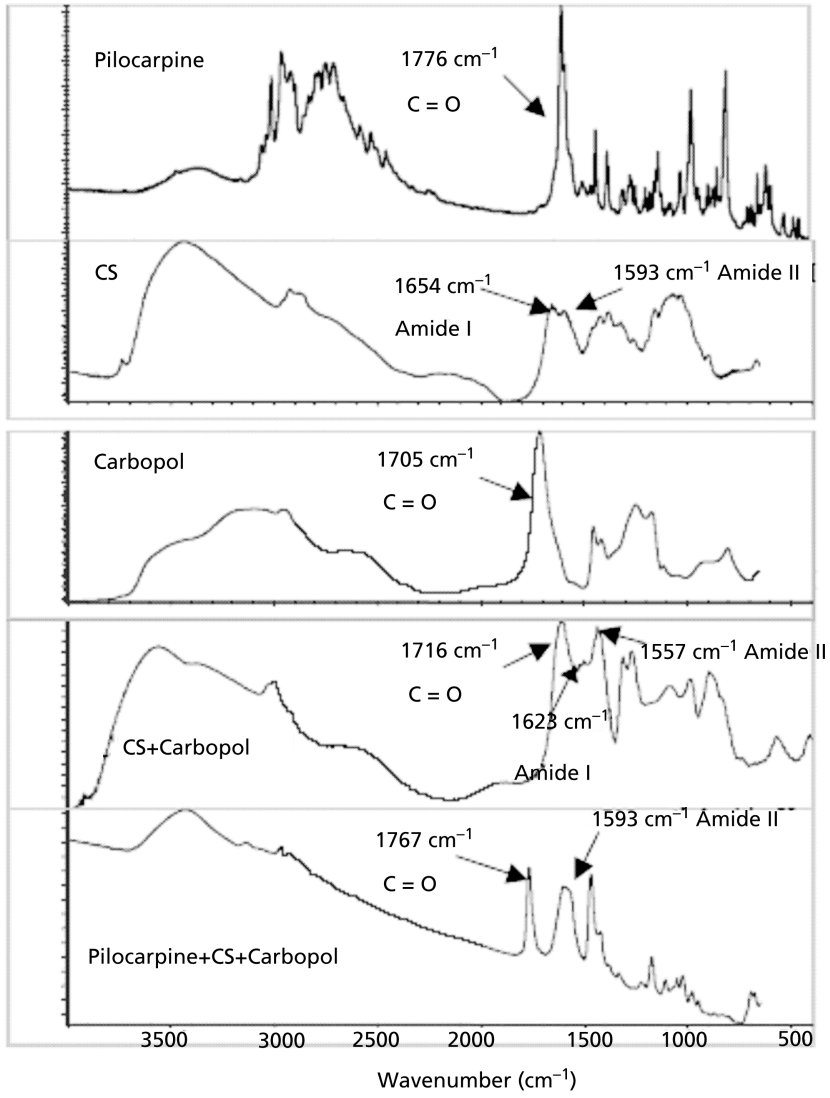

Figure 2 FT-IR spectra of pilocarpine, CS, carbopol, CS/carbopol and pilocarpine/CS/carbopol nanoparticle powders (from top to bottom).

pilocarpine/CS/Carbopol nanoparticles was much higher than that of CS and Carbopol particles alone and of CS/Carbopol nanoparticles. We hypothesize that the disappearance of the melting endotherms of individual CS, Carbopol and pilocarpine in the pilocarpine/CS/Carbopol nanoparticle formulation means that pilocarpine was included in the partial electrostatic CS/Carbopol mixture. And even if this hypothesis proves false, the stabilization of CS, Carbopol and pilocarpine in the formulation by the other two components merges the individual transition peaks to a higher mixed melting temperature in pilocarpine/CS/Carbopol nanoparticles. This inference is also supported by our FT-IR study.

Encapsulation efficiency (EE)

The encapsulation efficiency of pilocarpine in the CS/Carbopol nanoparticles was 77±4%. Thus, biocompatible CS/Carbopol nanoparticles prepared by the dropping method resulted in an acceptable pilocarpine encapsulation.

Table 1 Mean particle size and zeta-potential of chitosan, chitosan/Carbopol and pilocarpine/chitosan/Carbopol nanoparticles

Sample	Chitosan	CS/Carbopol	Pilo/CS/Carbopol
Mean diameter (nm)	146±18	250±23	294±30
Zeta potential (mV)	73.46±10.34	50.66±7.85	55.78±3.41

CS, chitosan; pilo, pilocarpine.

Dissolution test: in-vitro pilocarpine release from nanoparticles

Pilocarpine loaded in eye drops was released very quickly, and more than 90% of the loaded pilocarpine was released and reached a plateau within 4 h (Figure 3). Pilocarpine

Table 2 Melting temperature (T_m) and enthalpy change (ΔH) of chitosan, Carbopol, pilocarpine, chitosan/Carbopol and pilocarpine/chitosan/Carbopol nanoparticles

	CS	Carbopol	Pilo	CS/Carbopol	Pilo/CS/Carbopol
T_m (°C)	187.8 ± 3.6	189.5 ± 12.8	198.7 ± 4.1	195.3 ± 18.2	225.5 ± 6.4
ΔH (J g^{-1})	145.1 ± 4.2	107.8 ± 7.6	88.4 ± 1.8	148.6 ± 6.5	72.3 ± 9.7

CS, chitosan; Pilo, pilocarpine

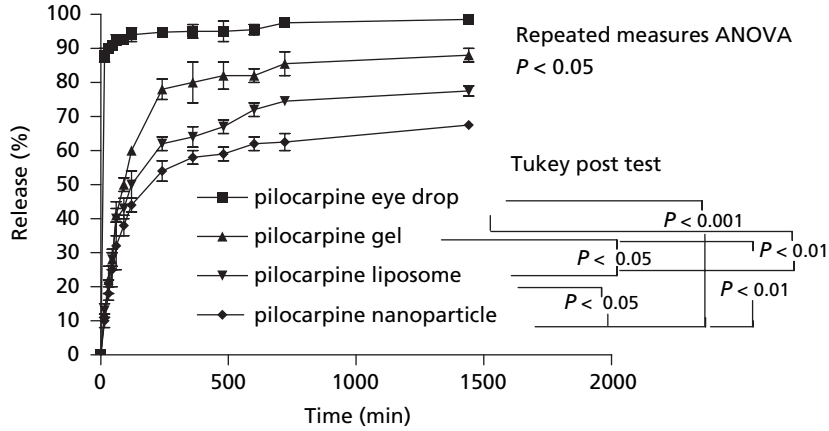

Figure 3 In-vitro release of pilocarpine as eye drops, nanoparticles, liposomes and gel as a function of time in simulated tear fluid (STF) at pH 7.4 and 37°C for 24 h.

loaded in nanoparticles, liposomes and gel showed an initial burst-release followed by a continuous and sustained release for 24 h. Nanoparticles proved to be the best of the four formulations at sustaining the release of pilocarpine for the longest time period. This, as we hypothesized earlier, is probably because pilocarpine was encapsulated and complexed in the polymer, as suggested in the DSC and FT-IR studies.

To provide a more precise understanding of the effect of CS/Carbopol nanoparticle formation on the release of pilocarpine, the release profile was analysed using the following equations:

$$M_t/M_\infty = Kt^n \quad (2)$$

$$\text{Log}(M_t/M_\infty) = \log K + n\log t \quad (3)$$

where M_t/M_∞ is the amount (%) of pilocarpine released at time t (min), n is a diffusional exponent, and K is the apparent release rate (% min^{-1}) (Ahn et al 2002; de la Torre et al 2003). Our data show that pilocarpine/CS/Carbopol nanoparticles presented a non-Fickian release with an n-value of 0.39 and a K value of 5.31. This low value of n is related to a high initial burst-release of pilocarpine from the CS/Carbopol nanoparticles, and these data may suggest that the release of pilocarpine from the CS/Carbopol nanoparticles was controlled by a combination of diffusion and dissolution of the polyelectrolyte complex, which was also a finding in the de la Torre et al (2003) study.

In-vivo miotic study

After pilocarpine in various formulations – eye drop solution, liposomes, gel and nanoparticles – had been instilled in the eyes of rabbits, its mitotic responses were compared. We found that at all time intervals, the decrease in pupil diameter was greatest for the pilocarpine/CS/Carbopol nanoparticles (Figure 4). The decrease in pupil diameter was in the order of pilocarpine nanoparticles > pilocarpine liposomes > pilocarpine gel > pilocarpine eye drops. The miosis efficiency of pilocarpine in liposomes and nanoparticles led to a prolonged effect of up to 24 h, a finding for pilocarpine in liposomes similar to that in the Monem et al (2000) study as well. Pilocarpine in the nanoparticle formulation had the largest AUC_{0-24} of the four formulations tested, a difference that was statistically significant ($P < 0.01$ and $P < 0.001$; Dunn's post test; Figure 4). This indicates that this formulation is the most efficient delivery vehicle for pilocarpine.

The in-vivo study demonstrates the excellent extended miosis effect of pilocarpine in the CS/Carbopol nanoparticles. Some studies that used insulin and pilocarpine also suggest that nanoparticles composed of either natural polymers (e.g. chitosan) or synthetic copolymers (e.g. Pluronic F127 and polybutylcyanoacrylate), are good carriers of protein drugs and ophthalmic drugs (Zimmer et al 1994; Sznitowska et al 2001; Pepic et al 2004).

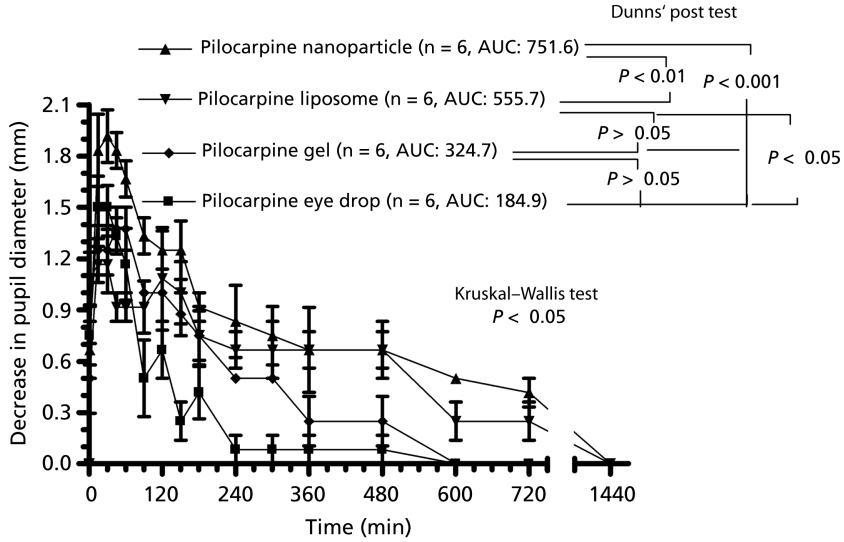

Figure 4 In-vivo decrease in pupil diameter versus time profiles for pilocarpine loaded in eye drops, nanoparticles, liposomes and gel as a function of time at 25 °C for 24 h.

Conclusions

Chitosan (CS) and Carbopol are non-toxic, highly bio-compatible, and bio-degradable materials. CS-Carbopol nanoparticles can be prepared easily using the dropping method (i.e. adding positively charged CS dropwise into negatively charged Carbopol). The expected sustained-release effects of pilocarpine-loaded nanoparticles were verified by in-vitro and in-vivo tests. Thus, pilocarpine/CS/Carbopol nanoparticles may provide an excellent potential alternative ophthalmic sustained-release formulation of pilocarpine for clinical use. CS/Carbopol nanoparticles may also be useful for a variety of other therapeutic delivery systems.

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