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Cross-Linked Cationic Polymer Microparticles: Effect of *N*-Trimethyl Chitosan Chloride on the Release and Permeation of Ibuprofen

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ABSTRACT Microparticles made by cross-linking hydrophilic polymers, such as chitosan, have been used to modify the release rate of a loaded drug. In this study a polymer with fixed positive charges, *N*-trimethyl chitosan chloride (TMC), was used in combination with chitosan to formulate microparticles to investigate its effects on drug release rate and transport across intestinal epithelial cells. The microparticles were prepared by cross-linking these cationic polymer(s) using sodium citrate as the ionic cross-linker. This process was done under homogenization and ultrasonication to control the size of the particles. The addition of TMC to the chitosan microparticles resulted in an increase in particle size of the microparticles and an increase in ibuprofen release rate as compared to the microparticles containing chitosan alone. Permeation of ibuprofen across Caco-2 cell monolayers, after administration of a suspension of the microparticles to the apical side, was not significantly different for the microparticles containing TMC as compared to those consisting of chitosan alone. It was concluded that release of TMC molecules from the microparticles was probably not sufficient to interact with the intestinal epithelial cells in order to change the permeation of the released drug.

KEYWORDS Chitosan, *N*-trimethyl chitosan chloride, Microparticles, Drug release, Drug transport

INTRODUCTION

Multiparticulate dosage forms have several advantages over single unit preparations such as a more uniform dispersion and absorption in the gastrointestinal tract, reduced local irritation, less inter- and intra-individual variability, lower variability in gastrointestinal transit times, and the possibility

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of mixing particles with different release properties together into a single dose (Bodmeier et al., 1989; Silva & Ferreira, 1999; Zhang et al., 2002).

Chitosan is a unique cationic polymer with excellent gel and film-forming properties. This polymer has been investigated extensively for several uses in the pharmaceutical field such as its potential in the development of drug delivery systems (Dodane & Vilivalam, 1998; Felt et al., 1998; Illum, 1998; Paul & Sharma, 2000). This is due to its appealing intrinsic characteristics, which include biodegradability, biocompatibility, bioadhesiveness, absorption enhancing properties, wound healing abilities, and bacteriostatic effects (Berger et al., 2004). N-trimethyl chitosan chloride (TMC) is a partially quaternized derivative of chitosan with fixed positive charges on the amino groups. It has improved solubility as compared to chitosan and showed potential as an absorption enhancer across intestinal epithelial cells even in neutral environments (Kotzé et al., 1997, 1998a; Hamman et al., 2000, 2002, 2003). The mechanism of drug absorption enhancement by chitosan, and most probably also TMC, is opening of tight junctions between adjacent epithelial cells by means of an interaction between the positive charges on the polymer molecules and the anionic components on the surface of epithelial cells (Artursson et al., 1994; Schipper et al., 1997).

Reaction of chitosan with multivalent anions allows the formation of bridges between the polymeric chains, which results in cross-linking between the chitosan molecules. This process of ionotropic gelation has been used for the preparation of chitosan microspheres (Sinha et al., 2004). Chitosan microspheres with a good spherical geometry and a smooth surface were prepared by cross-linking an aqueous acid dispersion of chitosan in paraffin oil with glutaraldehyde (Chithambara Thanoo et al., 1992). The main drawback to the use of covalent cross-linkers (e.g., glutaraldehyde, formaldehyde, hexamethylene diisocyanate, and dichloro- and trichlorotriazine) in chitosan microparticulate drug delivery systems is represented by their toxicity (Genta et al., 2002). A method to overcome this problem and to avoid purification before administration is to prepare systems using reversible physical cross-linking by electrostatic interaction (i.e., using anion cross-linkers like tripolyphosphate, sulfate, and citrate). Water-soluble polymers with positively or negatively charged groups interact with molecules of opposite charges to form three-dimensional networks (Bodmeier et al., 1989). The complexation process between oppositely charged macromolecules and ions to prepare chitosan beads and microspheres has attracted much attention because it is a very simple and mild method (Dumitriu & Chornet, 1998; Shu & Zhu, 2000).

Cross-links between hydrophilic polymer functional groups lead to decreased hydrophilicity, which slows down the diffusion of biological fluids and consequently the diffusion of the drug throughout the microsphere matrix. This modifies the release rate of a loaded drug in cross-linked microparticles (Genta et al., 2002).

The purpose of this study was to investigate the effects of TMC on the release rate and transport properties of cross-linked chitosan microparticles. The release profiles of ibuprofen from two different types of microparticles, which consist of chitosan alone and chitosan in combination with TMC respectively, were determined at a neutral pH value. The transport of ibuprofen released from these microparticles across Caco-2 cells was evaluated after administration of suspensions of these microparticles to the apical side of the cell monolayers. The Caco-2 cell model was used because it represents a well-characterized in vitro transport model for drug absorption across intestinal epithelial cells (Artursson et al., 2001; Hidalgo et al., 1989).

METHODS AND MATERIALS

Materials

The chitosan (91.48% deactylated) was obtained from Fujuan Shipbuilding Industry Group Company (China) and the ibuprofen was obtained from Knoll Pharma Chemicals (Nottingham, England). All other chemicals used were of analytical (AR) grade. The components of the growth medium for the Caco-2 cells were obtained from Bio Whittaker (Walkersville, MD) except for fetal bovine serum that was obtained from Delta Bioproducts (Johannesburg, South Africa) and the 6-well filter plates were from Corning Costar Corporation (USA).

Synthesis of TMC

The TMC was synthesized with reductive methylation of chitosan by a reaction with methyl iodide in

the presence of NaOH as described before (Domard et al., 1986; Sieval et al., 1998). The TMC was precipitated from solution with ethanol and isolated by centrifugation. The degree of quaternization of TMC was calculated from an ^{1}H-NMR spectrum (500 MHz) obtained with a BRUKER DAX500 spectrometer (Karlsruhe, Germany) in D_2O at 80°C with suppression of the water peak as described before (Hamman & Kotzé, 2001; Sieval et al., 1998).

Preparation of Microparticles

Ibuprofen was used as a model drug compound in this study to investigate the effects of TMC on its release and transport behavior from chitosan microparticles. Chitosan (3% w/v) was dissolved in an acetic acid (2% v/v) solution while stirring at 1000 rpm with an overhead mechanical stirrer (Heidolph RZR 2021, Germany) for 20 minutes and ibuprofen (3% w/v) was dispersed in this chitosan solution while stirring with the overhead mechanical stirrer at a speed of 1200 rpm for 20 minutes. A solution of sodium citrate (10% w/v) was added slowly into this chitosan and ibuprofen mixture under homogenization at a speed of 10,000 rpm (Vortex mixers, England) and ultrasonication at a power of 440 W (Bransonic 220, USA) for 10 minutes. The formed microparticles were separated by centrifugation (Minette, England) at a speed of 2000 rpm for 5 minutes, washed twice with ether (Arica et al., 2002), and freeze-dried (Leybold-Heraeus, Germany).

This method was repeated, but with addition of TMC to the chitosan solution in order to prepare microparticles consisting of chitosan in combination with TMC in ratios of chitosan:TMC=75:25 and 50:50.

Determination of Particle Size

Particle size was determined using a Malvern Mastersizer X (Malvern Instruments, Spring Lane South, Worcestershire, United Kingdom) fitted with a MSX1 small volume sample unit and a 300-mm lens. A volume of 15 mL ethanol was used as the dispersant, which was stirred at a constant rate.

The dispersant was added to the sample cell, the optics were aligned, and a background measurement was taken. A sample of the microparticles was subsequently added to the dispersant after ultrasonication for 2 minutes to ensure that all agglomerates were broken down. Enough sample was added to obtain an obscuration between 10% and 30% after which a measurement was taken. The alignment of the optics as well as background measurements were taken before each particle size measurement. Particle size measurements were done in duplicate consisting of 4000 sweeps each. The size of the microparticles was measured based on the volume of the particles, and the average particle size was calculated.

Determination of Microparticle Drug Content

Firstly, the moisture content of the prepared microparticles was determined by means of a loss on drying analyzer (Sartorius, MA30, Germany).

Approximately 100-mg samples of the microparticles were weighed accurately, which were transferred into 100-mL volumetric flasks and made up to volume with a 0.1 M NaOH solution. These were stirred for 24 hours to allow total breakdown of the microparticles or total release of the drug. After filtration (0.45 μm filter membrane), the solutions were assayed using a UV spectrophotometer (Secomam S750, France) at a wavelength of 264 nm. The percentage drug content of the particles was then calculated by means of the following equation:

$$\text{Content \%} = \frac{\text{ABS}_{\text{sample}} \times \frac{\text{W}_{\text{standard}}}{100}}{\text{ABS}_{\text{standard}} \times \frac{\text{W}_{\text{sample}} \times (1 - \text{Moi \%})}{100}} \times 100\%$$

Where $\text{ABS}_{\text{sample}}$=absorbance of the sample at 264 nm, $\text{ABS}_{\text{standard}}$=absorbance of ibuprofen standard at 264 nm, W_{sample}=weight of the microparticle sample, $\text{W}_{\text{standard}}$=weight of the ibuprofen standard, Moi %=percentage moisture in the sample.

Dissolution Studies

For the experimental groups, aliquots of microparticles containing 200 mg ibuprofen (calculated according to the drug content) were weighed accurately and loaded into empty hard gelatin capsules. For the control group, quantities of 200 mg ibuprofen raw material were weighed accurately

and loaded into empty hard gelatin capsules. Dissolution tests on the filled capsules were conducted in triplicate using a six-station dissolution apparatus (Pharma Test, Model PTMS 2, Germany). The dissolution media (900 mL) consisted of a phosphate buffer at pH 7.4 and the rotation speed was 30 rpm at a temperature of 37±0.5°C. Samples (5 mL) were withdrawn at predetermined time intervals, including 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 300, 480, and 600 min. After withdrawal of a sample, it was replaced immediately with 5 mL of preheated dissolution medium.

The samples were filtered (0.45 μm filter membrane) and assayed using a UV spectrophotometer (Secomam S750, France) at a wavelength of 264 nm. The readings were corrected for dilution and the calculated cumulative ibuprofen release values (% of initial value) were plotted as a function of time.

The time where 50% of the initial dose was released (t_{50}) was graphically determined from these dissolution curves and used as an indication of the rate of drug release. The area under the curve (AUC) values were calculated by means of the trapezoidal rule and used as an indication of the extent of drug release.

Permeation Studies

The pH of serum-free Dulbecco's Modified Eagles Medium (DMEM) was adjusted to the appropriate value (i.e., pH 7.4) with 0.1 M HCl and/or 0.1 M NaOH. For the experimental groups, samples of the microparticles containing 50 mg ibuprofen (calculated according to the drug content) were suspended in 10 mL serum-free DMEM and for the control group, 50 mg ibuprofen raw material were suspended in 10 mL serum-free DMEM.

Caco-2 cells (American Type Culture Collection, USA) were seeded on tissue culture treated polycarbonate filters (area=4.70 cm^2) in Costar Transwell six-well plates (Corning Costar Corporation, USA). The volume of growth medium used in these filters was 2.5 mL in each chamber. The cells on the filters were cultured at a temperature of 37°C in an atmosphere of 95% humidified air and 5% CO_2. Permeation studies only commenced when a confluent monolayer was achieved (±21 days after seeding). The growth medium was then removed from the basolateral chambers and replaced with 2.5 mL of permeation medium buffered at pH 7.4 with 25 mM HEPES (n-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]). After 30 min incubation, the growth medium was removed from the apical chambers and replaced with 2.5 mL of the prepared suspensions of microparticles (containing 50 mg ibuprofen) or a suspension of 50 mg ibuprofen raw material for the control group. Samples of 200 μL were drawn from the basolateral chamber at the following time intervals: 20, 40, 60, 90, and 120 minutes, and were replaced with an equal volume of fresh prewarmed transport medium at every time interval.

The samples (200 μL) withdrawn from the basolateral side of the Caco-2 cell monolayers during the transport study were diluted to 3 mL with permeation medium and analyzed with the UV spectrophotometer (Secomam S750, France) at a wavelength of 262 nm. The readings were corrected for dilution and the cumulative ibuprofen permeation values (% of initial value) were plotted as a function of time.

The apparent permeability coefficient (P_{app}) for ibuprofen was calculated by using the following equation (Kotzé et al., 1998b):

$$P_{app} = dQ/dt\{1/(A.60.C_0)\}$$

Where P_{app}=apparent permeability coefficient ($cm.s^{-1}$), dQ/dt=permeability rate (amount permeated per minute), A=diffusion area of the monolayer (cm^2), C_0=initial concentration of the marker molecule.

Data Analysis

Differences between the AUC and t_{50} values of the different dissolution curves of the experimental groups and the control group were statistically evaluated with a one-way repeated analysis of variance (ANOVA). Differences between the apparent permeability coefficient (P_{app}) values for ibuprofen across Caco-2 cell monolayers after it has been released from the different microparticles were statistically evaluated in the same way. Values were considered statistically different if $p<0.05$.

RESULTS AND DISCUSSION

Degree of Quaternization of TMC

The ^{1}H-NMR spectrum of the synthesized TMC is shown in Fig. 1. The degree of quaternization was

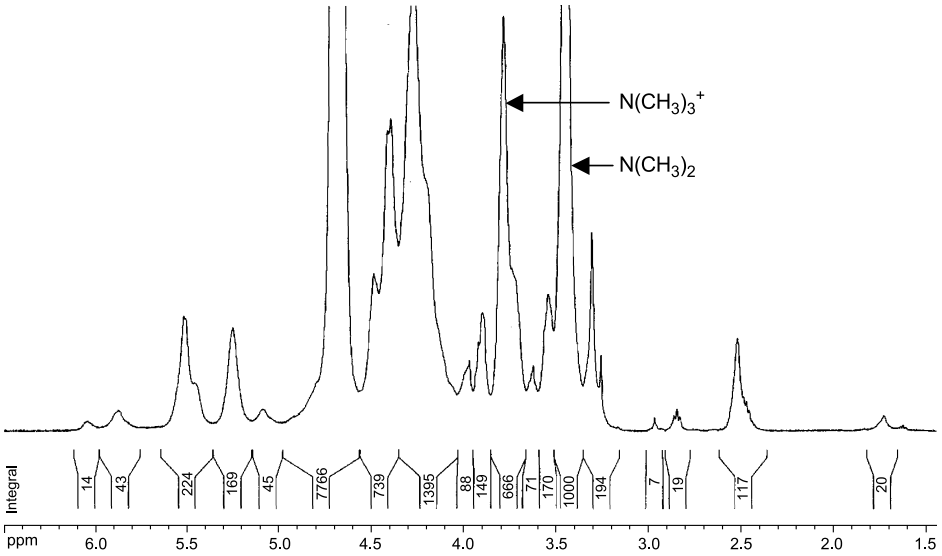

FIGURE 1 **^{1}H-NMR Spectrum of the TMC.**

calculated as 17% from this spectrum by using the integral of the peak for the quaternized amino group according to the published method (Hamman & Kotzé, 2001; Sieval et al., 1998).

Microparticle Size and Drug Content

The measured microparticle size and ibuprofen content are shown in Table 1.

The size of the microparticles increased pronouncedly with addition of TMC to the chitosan microparticles, while addition of TMC only resulted in a marginal increase in the drug content. This may be due to a less compact and more porous structure of the particles containing TMC as a result of steric effects caused by the added methyl groups on the TMC molecules. However, the mechanism of increasing the particle size by addition of TMC was not investigated in this study.

TABLE 1 **Particle Size and Ibuprofen Content of the Microparticles**

Ratio of chitosan:TMC	Particle size (μm)	Drug content (%)
100:0	47.96±0.70	34.08±0.80
75:25	56.31±4.02	36.92±0.78
50:50	109.79±0.34	36.50±0.74

Dissolution Profile at pH 7.4

The cumulative ibuprofen release values (% of initial value) plotted as a function of time is shown in Fig. 2 for the different microparticle preparations and the control group at pH 7.4.

The calculated AUC and t_{50} values for the dissolution profiles of the different microparticle preparations and the control group at pH 7.4 are depicted in Table 2.

There was a significant decrease in ibuprofen release rate and extent from the microparticles as compared to that of the control group. These results indicate therefore that cross-linked chitosan alone or in combination with TMC in microparticulate systems are useful to control ibuprofen release in neutral environments.

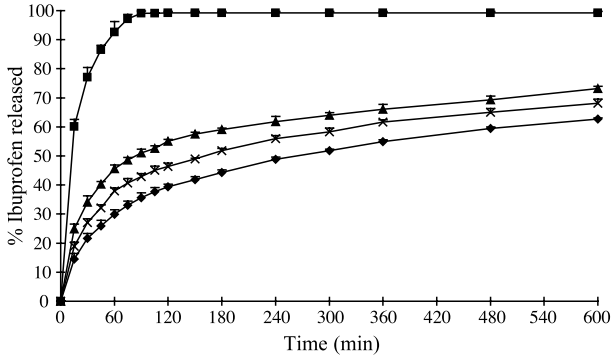

FIGURE 2 **Cumulative Ibuprofen Released (% of Initial Value) as a Function of Time at pH 7.4. Key: Control (■), Chitosan Alone (◆), Chitosan:TMC=75:25 (x), Chitosan:TMC=50:50 (▲).**

The addition of TMC to the microparticles resulted in a statistically, significantly higher ibuprofen release rate and extent as compared to that of the microparticles containing chitosan alone, which was directly proportional to the quantity of TMC added. This increasing effect of TMC on the ibuprofen release rate from the microparticles may be explained by steric effects caused by the added methyl groups on the TMC molecules, thereby probably influencing the cross-linking of polymer molecules in the microparticles. The physicochemical properties of TMC, such as better wettability and solubility, can possibly also contribute to the higher release rate of the drug from the microparticles. In contrast to TMC, chitosan is not soluble in a neutral environment and lack of dissolution of the chitosan molecules (and thereby also reverse of the cross-links) inside the microparticles probably explains why the release of ibuprofen from the microparticles consisting of chitosan alone is slower than that of the control group.

Ibuprofen Permeation at pH 7.4

The cumulative ibuprofen permeation values (% of initial dose) across Caco-2 cell monolayers plotted as a function of time is shown in Fig. 3 for the different microparticle preparations and the control group at pH 7.4.

The apparent permeability coefficient (P_{app}) values calculated for the permeation of ibuprofen across Caco-2 cell monolayers released from the different microparticles and control group are in the following order: control $(4.76\times10^{-6}\pm0.57\times10^{-6})^{A}>$ chitosan: TMC=50:50 $(3.84\times10^{-6}\pm0.14\times10^{-6})^{A,B}>$ chitosan alone $(3.57\times10^{-6}\pm0.24\times10^{-6})^{B,C}>$chitochitosan: TMC=75:25 $(3.38\times10^{-6}\pm0.11\times10^{-6})^{C}$.

TABLE 2 AUC and t_{50} Values of the Dissolution Curves at pH 7.4

Group	AUC[a]	t_{50} (min)[a]
Control	12.77 ± 0.04^{A}	13 ± 0.87^{A}
Chitosan alone	6.44 ± 0.09^{B}	261 ± 15.87^{B}
Chitosan:TMC=75:25	7.29 ± 0.14^{C}	161 ± 4.90^{C}
Chitosan:TMC=50:50	8.06 ± 0.13^{D}	84 ± 6.70^{D}

[a]Values with different letters are statistically significantly different ($p<0.05$).

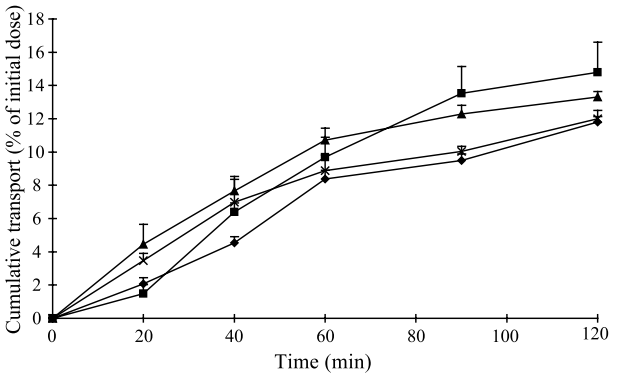

FIGURE 3 Cumulative Ibuprofen Permeated (% of Initial Dose) as a Function of Time at pH 7.4. Key: Control (■), Chitosan Alone (◆), Chitosan:TMC=75:25, (x), Chitosan:TMC=50:50 (▲).

P_{app} values labeled with different letters are statistically significantly different.

In general, all the microparticles have lower P_{app} values as compared to the control group. This can be explained by the slow release of ibuprofen from the microparticles as compared to the control group as indicated by the dissolution profiles. The permeation results at this neutral pH value indicate no significant increase in the P_{app} value of the ibuprofen released from the microparticles containing TMC in combination with chitosan as compared to that of the microparticles containing chitosan alone. This indicates that the release of TMC molecules from the microparticles was not sufficient to affect the permeation of ibuprofen across the intestinal epithelial cells. TMC is soluble and capable of absorption enhancement in a neutral environment (Hamman et al., 2003), while chitosan is only soluble in acidic environments as mentioned earlier. The degradation (or reverse of the cross-links) of the microparticles may therefore be slow due to this insolubility of the chitosan molecules at pH 7.4, thereby preventing the release of TMC molecules from the microparticles up to the time that this transport study was conducted.

CONCLUSION

Use of TMC in combination with chitosan in cross-linked microparticles resulted in an increase in particle size and decrease in drug release compared to the control group. However, an increase in the drug release rate compared to that of microparticles containing chitosan alone was found for the microparticles

containing TMC. This was explained by possible steric effects of the added methyl groups on the amino groups in the C-2 position of the TMC molecules inside the microparticles. These steric effects may adversely affect the physical cross-links between the polymer molecules, which have an effect on the degradation of the microparticles and consequently, drug release. The physicochemical properties of TMC may also contribute to the drug release behavior of the microparticles, as TMC is better soluble compared to chitosan in a neutral environment.

The addition of TMC to the chitosan microparticles did not show a significant increase in the permeation of the drug across intestinal epithelial cells compared to that of microparticles containing chitosan alone. This suggests that the release of TMC molecules from these microparticles was insufficient to affect drug permeation as compared to that of the microparticles consisting of chitosan alone.

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