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International Journal of Pharmaceutics 235 (2002) 87–94

international journal of pharmaceutics

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Stomach-specific anti-*H. pylori* therapy. I: preparation and characterization of tetracyline-loaded chitosan microspheres

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Received 28 August 2001; received in revised form 3 December 2001; accepted 4 December 2001

Abstract

The main objective of the study was to develop a stomach-specific drug delivery system to increase the efficacy of tetracycline against *Helicobacter pylori*. Chitosan microspheres were prepared by ionic cross-linking and precipitation with sodium sulfate. Two different methods were used for drug loading. In method I, tetracycline was mixed with chitosan solution before the simultaneous cross-linking and precipitation. In method II, the drug was incubated with pre-formed microspheres for 48 h. The cumulative amount of tetracycline that was released from chitosan microspheres and the stability of the drug was examined in different pH medium at 37 °C. Microspheres with a spherical shape and an average diameter of 2.0–3.0 μm were formed. When the drug was added to the polymer solution before cross-linking and precipitation only 8% (w/w) was optimally incorporated in the final microsphere formulation. When the drug was incubated with the pre-formed microspheres, on the other hand, a maximum of 69% (w/w) could be loaded. Thirty percent of tetracycline either in solution or when released from microspheres was found to degrade at pH 1.2 in 12 h. The preliminary results from this study suggest that chitosan microspheres can be used to incorporate antibiotic drugs and may be effective when administered locally in the stomach against *H. pylori*.

Keywords: Chitosan microspheres; Tetracycline; Stomach-specific delivery; *Helicobacter pylori* infection

1. Introduction

In 1982, Warren and Marshall isolated a Gram-negative, flagellated, spiral, urease producing microorganism from patients with peptic ulcer. The microorganism, now known as *Helicobacter pylori*, is the main etiologic factor in the development of gastritis, gastric ulcer, and gastric carcinoma (Marshall, 1983; Marshall and Warren, 1984). *H. pylori* reside mainly in the gastric mucosa or at the interface between the mucous layer and the epithelial cells of the antral region of the stomach (Peterson, 1991). The discovery of this microorganism has revolutionized the diagnosis and treatment of peptic ulcer disease. Most antibacterial agents have low minimum inhibitory concentrations (MIC) against *H. pylori* in culture. The MIC_{50} and MIC_{90} of tetracycline, for in-

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0378-5173/02/$ - see front matter
PII: S0378-5173(01)00985-1

stance, are 0.094 and 12 μg ml^{-1}, respectively (Ani et al., 1999). However, single antibiotic therapy is not effective for the eradication of *H. pylori* infection in vivo. This is because of the low concentration of the antibiotic reaching the bacteria under the mucosa, instability of the drug in the low pH of gastric fluid and short residence time of the antibiotic in the stomach (Shah et al., 1999). Combination of more than one antibiotic and anti-secretory agent, therefore, are required for the complete eradication of *H. pylori*.

Other than the multi-antibiotic therapy, different therapeutic strategies have been examined to completely eradicate *H. pylori* from the stomach. One of these approaches is the use of an oligosaccharide, sodium salt of 3′-sialyllactose, to inhibit the adhesion of *H. pylori* to the human epithelial cells (Mysore et al., 1999). However, the anti-adherent compound suppressed the infection in some animal models but not in others. A sustained release liquid formulation of ampicillin was prepared using sodium alginate for eradication of *H. pylori* (Katayama et al., 1999). The alginate formulation gelled in the stomach and released ampicillin locally. However, the authors did not test the efficacy of locally-administered ampicillin in *H. pylori* infection model.

Satoh (1996) examined a combination of amoxycillin, metronidazole, and bismuth subnitrate for eradication of *H. pylori* in humans after instilling into the stomach with a nasogastric tube. *H. pylori* infection was completely eradicated in 96% of patients treated with this topical approach. The non-antibiotic therapies for eradication of *H. pylori* have also gained a lot of attention lately. The activity of tea catechins (Mabe et al., 1999), vitamin C (Zhang et al., 1997) and poly(ethylene glycol)-containing non-ionic emulsifiers (Kane and Plaut, 1996) was tested against *H. pylori*. These non-antibiotic therapies were able to inhibit the growth of *H. pylori* in vitro, as well as, in vivo.

One way to improve the efficacy in eradicating the infection is to deliver the antibiotic locally in the stomach (Yokel et al., 1995; Shah et al., 1999). Better stability and longer residence time will allow more of the antibiotic to penetrate through the gastric mucus layer to act on *H. pylori*. Topical delivery of non-antibiotics has been suggested as an approach to overcome the resistance problems with antibiotics.

In order to develop stomach-specific formulation of tetracycline for eradication of *H. pylori* infection, we have prepared drug-containing chitosan microspheres. Chitosan is a linear polymer of D-glucosamine that is obtained by the alkaline deacetylation of chitin. Chitin, an abundant biopolymer, is harvested from the exoskeleton of marine crustaceans (Kurita, 1986). In the cationic form, the charged amino group of D-glucosamine residues in chitosan will interact with sialic acid (*N*-acetyl nuraminic acid) in the gastric mucus by electrostatic interaction. Thus, chitosan microspheres will improve the gastric residence time of a drug that is loaded into the polymer microsphere (Lehr et al., 1992). Chitosan microspheres can also provide pH-responsive release profile by swelling in acidic environment of the gastric fluid (Patel and Amiji, 1996).

2. Materials and methods

2.1. Materials

Chitosan with an average viscosity molecular weight of 750 000 Da and with 87% degree of deacetylation was obtained from Pronova Biopolymers (Raymond, WA). Tetracycline HCl was purchased from ICN (Aurora, OH). All aqueous solutions were prepared using deionized distilled water (Nanopure II, Barnstead/Thermolyne, Dubuque, IO). The other reagents and chemicals were of analytical grade or better.

2.2. pH stability study of tetracycline solution

The stability of tetracycline in solution (100 μg ml^{-1}) was investigated at the pH values of 1.2, 2.0, 3.5, and 5.0 at 37 °C. The concentrations of the parent drug remaining were analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) assay (Hasan et al., 1985). Periodically, 10 μl of the tetracycline solution was injected into Nova Pak C-18 column in LC Module I HPLC system (Waters, Milford, MA). The

mobile phase consisted of an aqueous solution of oxalic acid (75 mM) and ethylenediamine tetraacetic acid (20 mM) brought to pH 6.4 with triethylamine and dimethylformamide mixed in a volume ratio of 82:18. The flow rate was maintained at 1.4 ml min^{-1} and the detector was set at 277 nm. The percentage of the parent drug remaining was calculated by dividing the peak area of the drug at time t over the peak area of the drug at time zero multiplied by 100%.

The degradation of tetracycline was assumed to follow pseudo-first order kinetics, which is described by the following equation:

$$C = C_o e^{-kt}$$

in which C is the concentration of tetracycline remaining at time t, C_o is the initial concentration of tetracycline, and k is the pseudo-first order degradation rate constant. The half-life ($t_{1/2}$) and the shelf-life (t_{90}) of tetracycline under various conditions were obtained from the pseudo-first order degradation rate constant.

2.3. Preparation of chitosan microspheres

Chitosan solution was prepared by dissolving 500 mg of chitosan in 500 ml of 0.1-M acetic acid. Chitosan microspheres were prepared by ionic cross-linking according to the method of (Berthold et al., 1996) with minor modification. To the stirring solution of chitosan and Tween 80 [1% (w/v)], sodium sulfate [20% (w/v)] was added drop-wise until uniform turbidity was detected. The suspension was further stirred for an additional hour to stabilize the microspheres. The microsphere suspension was centrifuged at $2000 \times g$ for 30 min and the pellet was washed twice with deionized distilled water. Subsequently, the microspheres were rapidly frozen in liquid nitrogen and lyophilized.

2.4. Characterization of microspheres

2.4.1. Particle size analysis

A sample of the microsphere suspension, formed after cross-linking and precipitation, was used for particle size analysis using Beckman/Coulter N4 plus (Fullerton, CA) instrument. The size of the microspheres was measured at a 90° scattering angle and at 25 °C.

2.4.2. Differential interference contrast (DIC) microscopy

A few drops of chitosan microsphere suspension were placed on a clean glass slide. After placing a cover-slip on the sample, it was observed with Zeiss Axioplan-2® confocal microscope (Thornwood, NY). DIC image of the microsphere sample was digitized and processed with ADOBE PHOTOSHOP® software.

2.4.3. Transmission electron microscopy (TEM)

Freeze-dried chitosan microspheres sample was added on a formvar coated grid. The size and the shape of the microspheres were observed using Zeiss EM10® transmission electron microscope (Thornwood, NY) at an accelerating voltage of 60.0 kV. The TEM image was digitized and processed with ADOBE PHOTOSHOP® software.

2.4.4. Surface charge measurements

The surface charge on control and tetracycline-containing chitosan microspheres was measured from zeta potential values in deionized distilled water with a Brookhaven Instrument's Zeta-PALS® (Phase Analysis Light Scattering) Ultra-Sensitive Zeta Potential Analyzer (Holtsville, NY).

2.5. Tetracycline loading studies

Tetracycline was loaded into chitosan microspheres by two different methods. In method I, the drug was mixed with chitosan solution prior to cross-linking and precipitation. In method II, the drug was incubated with the formed microspheres for up to 48 h. The amount of tetracycline loaded in the microspheres was determined by subtracting the amount of the drug in the supernatant from the total amount added. For both methods, the amount of tetracycline loaded was assayed using Shimadzu UV160U spectrophotometer (Columbia, MD) at 350 nm and the actual values were calculated based on a calibration curve.

2.6. In vitro drug release studies

Drug loaded chitosan microspheres (3 mg) were incubated with 1.5 ml of hydrochloric acid solutions adjusted to pH 1.2 and 2.0 and 0.1 M acetate buffers adjusted to 3.5 and 5.0 in an Eppendorf vial in a shaking water-bath at 37 °C. The ionic strength of the release medium was adjusted to 0.16 using sodium chloride. At each time point, the vial was centrifuged at $10\,000 \times g$ for 5 min and 1 ml of supernatant was withdrawn. To maintain sink condition, 1 ml of fresh release medium was added to the vial. The sample was diluted 20 times and assayed for the released tetracycline at 350 nm using Shimadzu UV160U spectrophotometer. The cumulative amount of tetracycline was obtained from the calibration curves of tetracycline in each of the release medium.

2.7. pH stability study of tetracycline loaded into chitosan microspheres

The stability of tetracycline released from chitosan microspheres (100 μg ml^{-1}) was investigated using hydrochloric acid solution adjusted to pH 1.2 at 37 °C. Since the microspheres dissolved in the acidic medium, the procedure for assaying the stability of the drug was the same as that for the drug solution.

3. Results and discussion

3.1. pH stability study of tetracycline solution

To act effectively against *H. pylori* in the stomach, the released tetracycline has to remain stable in the harsh acidic environment of the gastric lumen. Thus, the stability of tetracycline was evaluated at four different pH values (1.2, 2.0, 3.5, and 5.0) at 37 °C for 12 h using RP-HPLC.

Almost 30% of the drug degraded after 12 h at pH 1.2 (Fig. 1). The pseudo-first-order rate constants (k), half-lives (t_{50}) and shelf-lives (t_{90}) were calculated from the slope of log (percent remaining) versus time and are shown in Table 1. The half-life and the shelf-life of tetracycline at pH 1.2 were 23.49 and 3.56 h, respectively.

In a previous study (Shah et al., 1999), the half-life and the shelf-life of amoxycillin at pH 1.2 were 14.30 and 2.20 h, respectively. Since tetracycline is more stable than amoxycillin in the acidic media, it might act more effectively against *H. pylori* in the gastric mucosa.

Fig. 2 shows the effect of buffer type on the stability of tetracycline in solution. It is obvious that the drug is more stable in hydrochloric acid solution than in acetate buffer at the same pH. For example, using hydrochloric acid solution adjusted to pH 3.5, the k value was 0.0041 h^{-1}. However, using acetate buffer adjusted to the same pH, the k value was 0.0265 h^{-1}. In acidic medium (pH 1–6), tetracycline is degraded by the process of epimerization (Remmers et al., 1963; Hussar et al., 1968), where the drug is converted into 4-epitetracycline with the concurrent loss of antibacterial activity. Other than pH, the epimerization process is also dependent on temperature and the buffer system used. It has been reported that citric acid and ascorbate anion significantly enhance tetracycline degradation. Additionally, tetracycline-hydrochloride was more stable than tetracycline-phosphate (Walton et al., 1970). In this study, it has been proven that acetate buffer enhances the degradation of tetracycline more than hydrochloric acid solution. Further studies

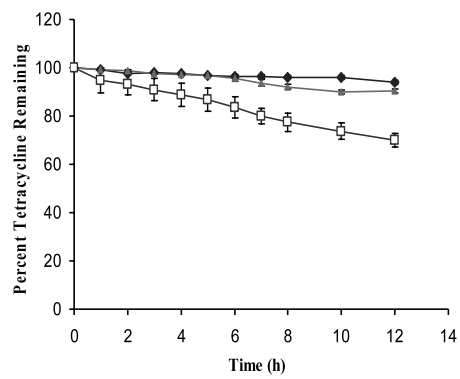

Fig. 1. Stability of tetracycline in the solution represented as percentage of the drug remaining as function of time at 37 °C at different pH medium having ionic strength of 0.16. The symbols represent hydrochloric acid solution pH 1.2 (□), 2.0 (▲), and 3.5 (◆) ($n = 3$; mean ± S.D.).

Table 1
Pseudo-first-order rate constants, half-lives and the shelf-lives of tetracycline at 37 °C and at different pH medium

pH	Pseudo first order degradation rate constant (h^{-1})[a]	Half-life, $t_{1/2}$ (h)	Shelf-life, t_{90} (h)
1.2	0.0295	23.49	3.56
2	0.0097	71.66	10.82
3.5	0.0041	167.17	25.61

[a] The pseudo-first-order rate constants were calculated from the slopes of the logarithmic scale of percentage of the drug remaining as function of time.

are required to know the exact mechanism for tetracycline degradation and why certain buffer systems like the acetate buffer could enhance the degradation.

3.2. Characterization of microspheres

The results of particle size analysis, DIC microscopy, and TEM are shown in Fig. 3. Particle size analysis by Coulter shows that chitosan microspheres had a mean diameter of 2.0–3.0 μm. The DIC image shows distinct spherical particles in suspension. TEM image shows the spherical shape of the freeze-dried chitosan microspheres.

3.3. Surface charge measurements

The average zeta potential of unloaded chitosan microspheres was +7.45 mV. The positive zeta potential values could be explained by the fact that most of the amine groups of chitosan having a pK_a of 6.3 (Claesson and Ninham, 1992) are positively charged at pH 3.7, which was the pH of chitosan microspheres in deionized distilled water. The average zeta potential of tetracycline-loaded chitosan microspheres was +26.68 mV. The higher positive zeta potential value was due to the fact that most of the amine groups of chitosan and the tertiary amine group of tetracycline, having a pK_a of 9.69, are positively charged at pH 3.24, which was the pH of tetracycline-loaded chitosan microspheres in deionized distilled water. The positively charged microspheres are expected to interact with the negatively charged sialic acid residues of mucin in the stomach by electrostatic interactions and prolong the residence time.

3.4. Tetracycline loading studies

The maximum tetracycline loading capacity was found to be only 8% (w/w) when the drug was added to the polymer solution prior to cross-linking. On the other hand, the loading capacity increased to a maximum of 69% (w/w) when the conditions were optimized for loading of preformed microspheres with the drug (Fig. 4). The low-loading capacity for method I could be explained by the fact that some of the hydrophilic drug was extracted from the microspheres during the washing steps. Also, in method I some of the drug could be degraded because of low pH of the 0.1 M acetic acid during the preparation stage. For method II, the amount of the loaded drug into chitosan microspheres increased (almost in a linear fashion) as the total amount of the drug in

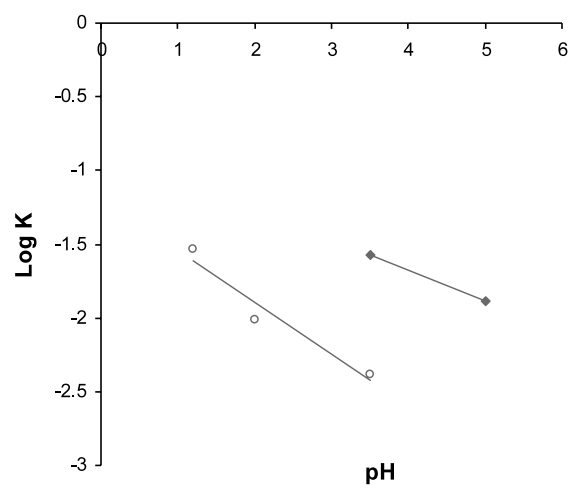

Fig. 2. Degradation rate constant as a function of pH profile of tetracycline in hydrochloric acid (○) and acetate buffer (◆) solutions at 37 °C.

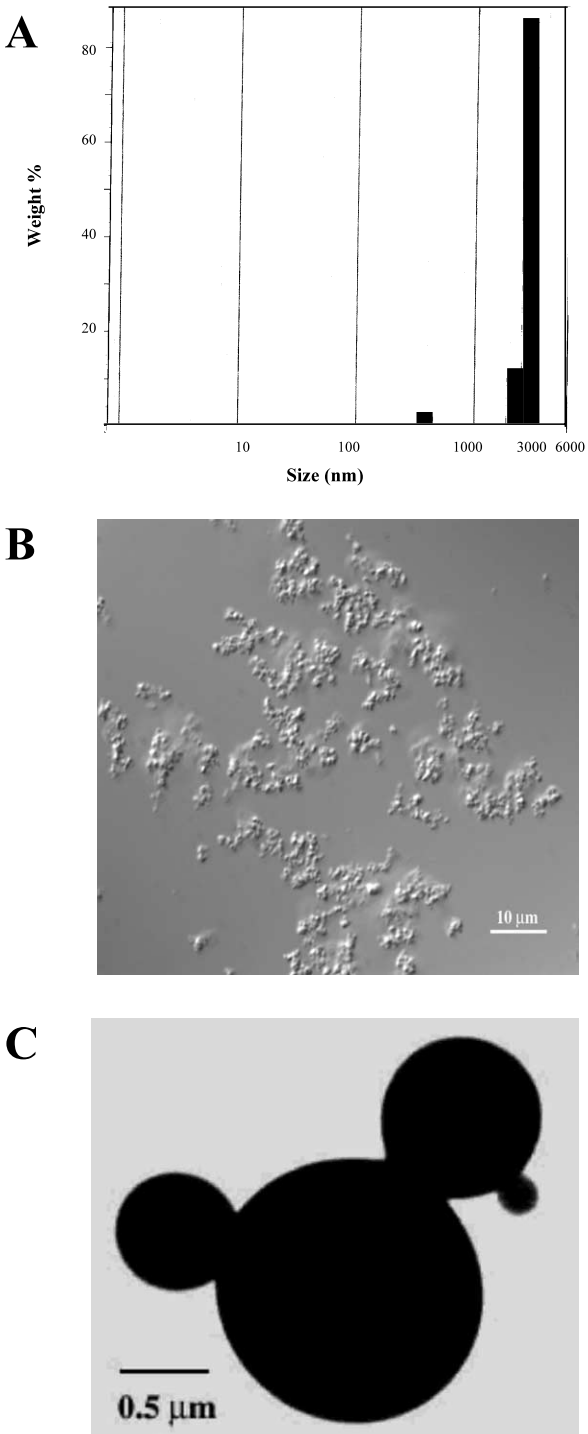

Fig. 3. Results of particle size analysis by Coulter counter (A), differential interference contrast microscopy (B), and transmission electron microscopy (C) images of chitosan microspheres.

the formulation increased. Table 2 shows the loading capacity of tetracycline in the final chitosan microspheres formulations.

3.5. In vitro release studies

For site-specific delivery of tetracycline in the stomach, a pH triggered release system is preferred to allow the drug in solution to diffuse into the mucus layer. The release of the drug from the chitosan microspheres is strongly affected by the pH of medium that can affect the ionization of the glucosamine residues of chitosan. At lower pH, the D-glucosamine residues are ionized resulting in extensive swelling and faster release of the drug. In addition, the pH of the gastric content can vary from a low of 1.2 in the fasted state to a high of 5.0 under fed state or upon concurrent use of antacids. For this reason, tetracycline release kinetics from chitosan microspheres was examined at different pH values. At pH 1.2 and 2.0, the microspheres dissolved and thus all of the entrapped drug was released instantaneously (Fig. 5). The release profile of the drug at pH 3.5 and 5.0 was almost the same. This may be due to the fact that the degree of ionization of the glucosamine groups of chitosan was almost the same at these pH values. Almost 70% of the drug was released after 3 h, while 90% of the drug was released after 8 h at pH 3.0 and 5.0. Assuming that the drug release profile in vivo does match that seen in vitro, 70% of the entrapped tetracycline will be released from the microsphere formulation in 3 h. The amount of the drug released will be readily available for absorption and action against *H. pylori*.

The release profile of tetracycline in this study is different from the release profile of amoxycillin from chitosan microspheres in a previous study (Shah et al., 1999). For example, in this study, tetracycline was released instantaneously at pH 1.2 and 2.0. However, in the previous study (Shah et al., 1999), 48% of amoxycillin was released in 30 min and all of the drug was released after 2 h. For both studies, the microspheres were prepared the same way. However, for the amoxycillin study, the crosslinking agent, tripolyphosphate was able to crosslink multiple sites in chitosan

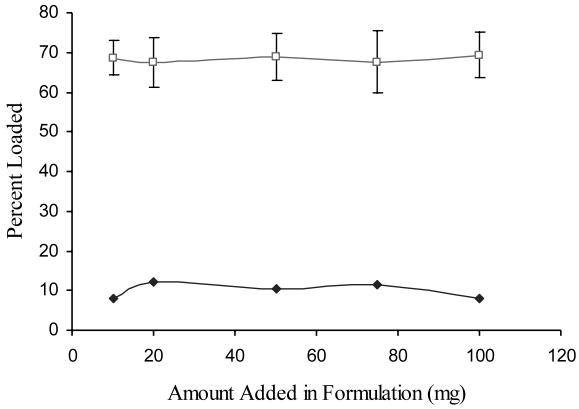

Fig. 4. Percentage of tetracycline loaded in chitosan microspheres as function of the amount of drug added to the formulation. Tetracycline was loaded into the microspheres by two different methods. In method I (◆) the drug was mixed with chitosan solution prior to cross-linking and precipitation. In method II (□) the drug was incubated with the formed microspheres for up to 48 h ($n = 3$; mean ± S.D.).

chains to provide a more stable network system. As a result, the chitosan microspheres did not dissolve in the amoxycillin study (Shah et al., 1999) but dissolved in the current study.

3.6. pH stability study of tetracycline loaded into chitosan microspheres

Almost 30% of the drug degraded after 12 h at pH 1.2. The pseudo-first-order rate constant (k), half-life (t_{50}), and shelf-life (t_{90}) were calculated from the slope of log (percent remaining) versus time. The k value, half-life, and shelf-life of tetracycline loaded into chitosan microspheres at pH 1.2 were 0.0267 h^{-1}, 25.94 and 3.932 h, respectively. The stability of tetracycline released from microspheres was the same as that in solution (Table 1) because the microspheres completely dissolved at pH 1.2. It is important to note from this study that the method of tetracycline loading into the microspheres did not contribute to any degradation of the drug.

Table 2
The amount of tetracycline that is loaded into chitosan microspheres

Loading method	Loading capacity[a]
Method I	8.04 ± 0.00[b]
Method II	69.37 ± 5.84

[a] The amount of the tetracycline loaded in mg per 100 mg chitosan microspheres from 100 mg of the drug added.
[b] Mean ± S.D. ($n = 3$).

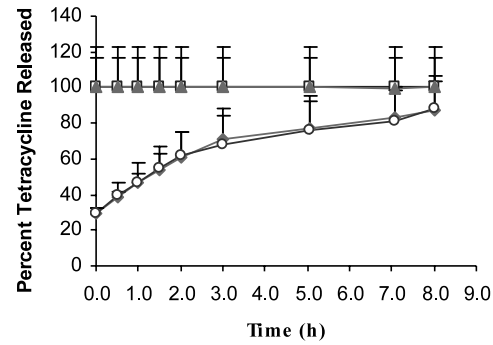

Fig. 5. Percentage of tetracycline released from the microspheres as function of time at 37 °C in different pH medium having ionic strength of 0.16. The symbols represent hydrochloric acid solution, pH 1.2 (□); hydrochloric acid solution, pH 2.0 (▲); acetate buffer, pH 3.5 (◆); and acetate buffer, pH 5.0 (○) ($n = 3$; mean ± S.D.).

4. Conclusions

H. pylori colonize the gastric mucosa leading to gastritis, gastric ulcer, and gastric carcinoma. To increase the efficacy of eradicating the infection, a localized delivery system of anti-*H. pylori* agents in the stomach is required. Tetracycline-loaded chitosan microsphere formulation was prepared to increase the local concentration of the antibiotic in the stomach and, thus eradicate *H. pylori* infection. Sixty nine percent of the tetracycline was loaded into the chitosan microspheres, and the drug was stable for up to 12 h even under acidic condition. The entire drug content was released instantaneously in acidic pH. Further studies are planned to examine the gastric residence time of the microsphere formulation and the efficacy in eradicating *H. pylori* infection in gerbil model.

Acknowledgements

This study was supported by EOS Pharmaceutical Corporation through an NIH SBIR Phase 1 grant and the Northeastern University Research and Development Funds. DIC and TEM images were acquired with the assistance of William Fowle at the Electron Microscopy Center, Northeastern University. Particle size analysis by Coulter was performed in Dr Vladimir Torchilin's laboratory at Northeastern University and zeta potential measurements were performed in Dr Robert Langer's laboratory at the MIT.

References

Ani, A.E., Malu, A.O., Onah, J.A., Queiroz, D.M., Kirschner, G., Rocha, G.A., 1999. Antimicrobial susceptibility test of *Helicobacter pylori* isolated from Jos, Nigeria. Trans. R. Soc. Trop. Med. Hyg. 93, 659–661.

Berthold, A., Cremer, K., Kreuter, J., 1996. Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. J. Control. Rel. 39, 17–25.

Claesson, P.M., Ninham, B.W., 1992. pH-dependent interactions between adsorbed chitosan layers. Langmuir 8, 1406–1412.

Hasan, T., Allen, M., Cooperman, B.S., 1985. Anhydrotetracycline is a major product of tetracycline photolysis. J. Org. Chem. 50, 1755–1757.

Hussar, D.A., Niebergall, P.J., Sugita, E.T., Doluisio, J.T., 1968. Aspects of the epimerization of certain tetracycline derivatives. J. Pharm. Pharmacol. 20, 539–546.

Kane, A.V., Plaut, A.G., 1996. Unique susceptibility of *Helicobacter pylori* to simethicone emulsifiers in alimentary therapeutic agents. Antimicrob. Agents Chemother. 40, 500–502.

Katayama, H., Nishimura, T., Ochi, S., Tsuruta, Y., Yamazaki, Y., Shibata, K., Yoshitomi, H., 1999. Sustained release liquid preparation using sodium alginate for eradication of *Helicobacter pylori*. Biol. Pharm. Bull. 22, 55–60.

Kurita, K., 1986. Chemical modifications of chitin and chitosan. In: Muzzarelli, R.A.A., Jeuniaux, C., Gooday, G.W. (Eds.), Chitin in Nature and Technology. Plenum Press, New York, pp. 287–293.

Lehr, C.M., Bouwstra, J.A., Schacht, E., Junginger, H.E., 1992. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int. J. Pharm. 78, 43–48.

Mabe, K., Yamada, M., Oguni, I., Takahashi, T., 1999. In vitro and in vivo activities of tea catechins against *Helicobacter pylori*. Antimicrob. Agents Chemother. 43, 1788–1791.

Marshall, B.J., 1983. Unidentified curved bacilli in gastric epithelium in active chronic gastritis. Lancet 1, 1273–1275.

Marshall, B.J., Warren, J.R., 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1, 1311–1315.

Mysore, J.V., Wigginton, T., Simon, P.M., Zopf, D., Heman-Ackah, L.M., Dubois, A., 1999. Treatment of *Helicobacter pylori* infection in rhesus monkeys using a novel antiadhesion compound. Gastroenterology 117, 1316–1325.

Patel, V.R., Amiji, M.M., 1996. Preparation and characterization of freeze-dried chitosan-poly (ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach. Pharm. Res. 13, 588–593.

Peterson, W.L., 1991. *Helicobacter pylori* and peptic ulcer disease. N. Engl. J. Med. 324, 1043–1048.

Remmers, E.G., Sieger, G.M., Doerschuk, A.P., 1963. Some observations on the kinetics of the c.4 epimerization of tetracycline. J. Pharm. Sci. 52, 752–756.

Satoh, K., 1996. Treatment of *Helicobacter pylori* infection by topical administration of antimicrobial agents. Scand. J. Gastroenterol. 31 (Suppl 214), 56.

Shah, S., Qaqish, R., Patel, V., Amiji, M., 1999. Evaluation of the factors influencing stomach-specific delivery of antibacterial agents for *Helicobacter pylori* infection. J. Pharm. Pharmacol. 51, 667–672.

Walton, V.C., Howlett, M.R., Selzer, G.B., 1970. Anhydrotetracycline and 4-epianhydrotetracycline in market tetracyclines and aged tetracycline products. J. Pharm. Sci. 59, 1160–1164.

Yokel, R.A., Dickey, K.M., Goldberg, A.H., 1995. Selective adherence of a sucralfate-tetracycline complex to gastric ulcers: implications for the treatment of *Helicobacter pylori*. Biopharm. Drug. Dispos. 16, 475–479.

Zhang, H.M., Wakisaka, N., Maeda, O., Yamamoto, T., 1997. Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: *Helicobacter pylori*. Cancer 80, 1897–1903.