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Evaluation of the Factors Influencing Stomach-specific Delivery of Antibacterial Agents for *Helicobacter pylori* Infection

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Abstract

Because *Helicobacter pylori* infection is localized in the gastric mucus layer and at the mucus layer–epithelial cell interface, we have developed amoxycillin- and metronidazole-containing chitosan microspheres for stomach-specific drug delivery.

Drug-loaded porous chitosan microspheres were prepared by simultaneous crosslinking and precipitation with sodium tripolyphosphate. The release of antibacterial agents into simulated gastric fluid (SGF, pH 1·2), and the stability and permeability through gastric mucin, were examined at 37°C. Because of the high porosity of drug-loaded chitosan microspheres, all the amoxycillin and metronidazole were released in 2 h. High-performance liquid chromatography assays of the antibacterial agents in SGF at 37°C indicated 40% degradation of amoxycillin after 10 h. Metronidazole was completely stable for up to 24 h in SGF. Amoxycillin and metronidazole were highly permeable through the gastric mucin gel layer.

The results of this study show that acid-stable antibacterial agents, such as metronidazole, that rapidly permeate the gastric mucus layer would be very effective for the complete eradication of *H. pylori* infection when delivered specifically at the site of infection in the stomach.

In 1982 Warren and Marshall provided the first insight into the role of a bacterial infection as a possible aetiological factor in the development of chronic gastritis that could ultimately lead to peptic ulcer disease and gastric cancer (Marshall 1983). They isolated a Gram-negative, spiral, urease-secreting organism from 84% of patients with stomach ulcers and 100% of patients with duodenal ulcers (Marshall & Warren 1984). The organism, now known as *Helicobacter pylori*, has profoundly altered the therapeutic management of peptic ulcer disease. *H. pylori* contains one to six flagella which facilitate adhesion to the gastric mucus. The corkscrew shape of the organism enables it to imbibe and penetrate its viscous physiological niche (Tytgat et al 1991). *H. pylori* resides exclusively in the gastric mucus layer and the intracellular junctions of the mucus-secreting cells with significant colonization of the gastric antral region (Peterson 1991).

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Although *H. pylori* is very sensitive to acidic pH, the organism's urease-secreting ability enables it to survive in the stomach (Eaton et al 1989).

According to the guidelines presented by the United States National Institutes of Health's Consensus Development Conference Report on *H. pylori* in peptic ulcer disease (1994), ulcer patients who are positive to *H. pylori* infection require treatment with antimicrobial agents, in addition to antisecretory drugs, whether on the first presentation with the illness or its recurrence. Although many antibacterial agents have very low minimum inhibitory concentrations (MIC) against *H. pylori* in culture (Ateshkadi et al 1993), no single agent is effective in the eradication of the infection in-vivo when administered alone (Murray 1993). The MIC50 values (concentrations resulting in 50% inhibition) of amoxycillin and metronidazole, for instance, are as low as 0·008 μg mL^{-1} and 2·0 μg mL^{-1}, respectively (Ateshkadi et al 1993). In addition, single antibiotic therapy is strongly discouraged to prevent the development of resistant strains (Hunt 1995).

There could be one or several reasons for the failure of single-antibiotic therapy against *H.*

pylori. Firstly, the organism resides in the mucus gel layer close to the acidic environment of the gastric fluid. Many antibacterial agents, such as penicillin and erythromycin, degrade rapidly in acid. Secondly, the drug must diffuse into the mucus layer and the bacterial glycocalyx to furnish concentrations sufficient for antibacterial activity. For eradication of *H. pylori* in the stomach the concentrations of antibacterial agents reaching the site of infection from tablets or capsules might not be bactericidal against organisms located in the mucus layer and protected by the glycocalyx. Lastly, the contact time of antibacterial agents with the organism needs to be sufficiently long. Yokel et al (1995) suggested that by increasing the in-vivo contact time of the drugs with *H. pylori*, as with tetracycline-sucralfate complex, the eradication efficiency would be significantly improved.

We have previously studied the pH-sensitive swelling and drug-release properties of porous chitosan-poly(ethylene oxide) (PEO) hydrogels at 37°C (Patel & Amiji 1996). Chitosan, a linear random polymer of D-glucosamine, is obtained by alkaline *N*-deacetylation of chitin (Chandy & Sharma 1990). Chitin is the second most abundant polymer in nature, isolated from the exoskeletons of marine crustaceans such as crabs, shrimps and krill (Li et al 1992). The pK_a of the primary amine groups of D-glucosamine residues in chitosan is approximately 6·5 (Claesson & Ninham 1992). Chitosan with 80% deacetylation will, therefore, be positively charged with a high charge density in acidic media, and will revert to the neutral form in alkaline media (Yao et al 1994). In the acidic environment of the gastric fluid (pH 1·2), chitosan-PEO hydrogels swell by at least ten times more by weight than in the almost neutral environment of intestinal fluid (pH 7·2). We and others have also found that chitosan can bind to the gastrointestinal mucus layer, probably by electrostatic interactions between the positively charged D-glucosamine residues and the negatively charged sialic acid residues of mucin (Illum et al 1994; Fieberg et al 1995; Qaqish & Amiji 1999). The mucoadhesive property of chitosan would be important for increasing the residence time of the delivery system in the stomach (Lehr 1994).

To develop a stomach-specific delivery system, in this study we have examined the factors that must be optimized for complete eradication of *H. pylori* with amoxycillin or metronidazole. As shown in Figure 1, we propose that drug-loaded chitosan microspheres, when formulated to adhere to the gastric mucus layer, will release the contents locally at the site of infection. Mergaud et al (1991) showed that the inhibitory concentration of amoxycillin for 99·9% of *H. pylori* isolates was 1·0 to 10 $\mu g\,mL^{-1}$ for sessile organisms and only 0·001 $\mu g\,mL^{-1}$ for planktonic organisms. Localized delivery of antibacterial agent can achieve concentrations as high as 10 $\mu g\,mL^{-1}$ in the gastric mucus gel layer by adjusting the dose of amoxycillin-containing chitosan microspheres. Drug-containing porous chitosan microspheres were prepared by crosslinking and precipitation. The loading efficiencies and the release profiles of amoxycillin and metronidazole in simulated gastric fluid (SGF, pH 1·2) were studied. We have also examined the stability of antibacterial agents in SGF at 37°C for up to 24 h and their permeability through the gastric mucin gel layer.

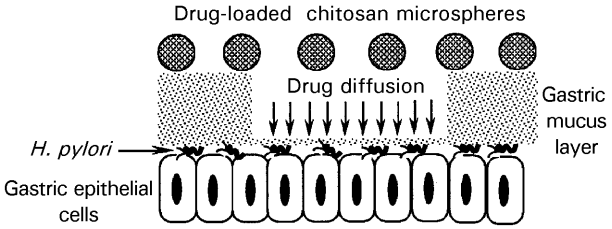

Figure 1. Schematic diagram of the rationale for stomach-specific delivery of antibacterial agents for *Helicobacter pylori* infection using mucoadhesive chitosan microspheres.

Materials and Methods

Materials

Chitosan with a viscosity–average molecular weight of 750 000 Da and 87% deacetylation was obtained from Pronova Biopolymers (Raymond, WA). Pluronic F-68, a poly(ethylene oxide)-poly(-propylene oxide)-poly(ethylene oxide) triblock copolymer, containing 76 ethylene oxide residues and 30 propylene oxide residues was obtained from BASF (Parsipanny, NJ). Amoxycillin, metronidazole, and mucin from porcine gastric mucosa (Type II) were purchased from Sigma (St Louis, MO). Deionized distilled water (Nanopure II, Barnsted/Thermolyne, Dubuque, IO) was used to prepare all aqueous solutions. Other chemicals and reagents were of analytical-grade or better.

Preparation of porous chitosan microspheres

Chitosan was purified from proteins and inorganic impurities by dissolving in 0·10 M acetic acid then precipitating in 0·10 M sodium hydroxide and extensive washing of the precipitate with double-distilled water. The dissolution–precipitation–washing process was repeated twice and the polymer was freeze-dried. Purified chitosan (500 mg) was dissolved in acetic acid (0·10 M, 200 mL)

containing Pluronic F-68 (1·0% w/v) as surfactant. The microspheres were prepared by a modification of the procedure of Berthold et al (1996). Briefly, chitosan solution was vigorously stirred and sodium tripolyphosphate (10% w/v) was added dropwise. A cloudy suspension was formed as a result of simultaneous crosslinking and precipitation of the chitosan microspheres. The microspheres were centrifuged at 3000 *g* for 10 min and the pellet was washed twice with deionized distilled water. The hydrated microspheres were rapidly frozen in liquid nitrogen and freeze-dried to obtain the porous morphology (Patel & Amiji 1996).

Loading and release studies

Amoxycillin- and metronidazole-containing chitosan microspheres were prepared as described above, except that each drug (10 mg) was added to the 200-mL chitosan solution before crosslinking and precipitation. The solution was stirred for 1 h (approx.) to ensure complete dissolution of the drug. After the addition of sodium tripolyphosphate the amount (%) of bound amoxycillin and metronidazole was determined from the total amounts added and the amounts remaining in the washing medium. The porous antibiotic-containing chitosan microspheres were formed by rapid freezing in liquid nitrogen then freeze-drying as described above.

Chitosan microspheres (100 mg) loaded with antibacterial agents were incubated with simulated gastric fluid (SGF, pH 1·2; 10 mL) in a shaking water-bath at 37°C. The SGF was prepared according to the protocol described in the United States Pharmacopeia (1990). At predetermined intervals a sample of the medium was removed and assayed for amoxycillin at 276 nm and for metronidazole at 285 nm with a Shimadzu (Columbia, MD) 160U UV-Vis spectrophotometer, as described elsewhere (Patel & Amiji 1996). The cumulative amounts of amoxycillin or metronidazole released from the microspheres were determined from calibration curves.

Stability studies

To determine the degradation kinetics of amoxycillin and metronidazole in SGF at 37°C, the concentrations of parent antibacterial drug remaining were analysed by reversed-phase high-performance liquid chromatography (HPLC) (Concannon et al 1986; Mathew et al 1994). Amoxycillin and metronidazole solutions (1·0 mg mL^{-1}) were prepared in SGF. At different times a sample of the drug solution was removed and mixed with internal standards (phenoxyacetic acid and salicylic acid, for amoxycillin and metronidazole, respectively) and 20 μL of the antibacterial agent–internal standard mixture was injected on to a C-18 HPLC column (Waters, Milford, MA). The mobile phase for the separation of amoxycillin from its degradation products was 0·05 M phosphate buffer (pH 5·5) containing 6·0% (v/v) methanol. For metronidazole, the mobile phase was 20% (v/v) methanol in 0·05 M phosphate buffer (pH 3·0). The ratio of the areas under the absorbance peaks was calculated for the parent drug and the internal standard. The amount (%) of parent drug remaining as a function of time was determined from the calibration curves. The results were treated according to apparent first-order degradation kinetics described by equation 1:

$$C_t = C_0 \exp^{-kt} \quad (1)$$

where C_t is the concentration of drug remaining at any time t, C_0 is the initial concentration (1·0 mg mL^{-1}), and k is the pseudo-first-order rate constant. The half-lives ($t_{\frac{1}{2}}$) and shelf-lives (t90, the time at which only 90% of the active drug remains) of amoxycillin and metronidazole were calculated from the apparent first-order rate constant (Martin 1993).

Permeability studies

Gastric mucin was purified by equilibrium dialysis against repeated changes of double-distilled water and ultra-centrifugation, according to the procedure described by Bhat et al (1996), and freeze-dried. Just before the permeability studies, the high molecular weight fraction of mucin (500 mg), obtained after purification, was reconstituted with SGF (10 mL) and mixed vigorously with a mortar and pestle to obtain a viscous gel. The 50-mg mL^{-1} concentration of gastric mucin is necessary for the interactions that produce gel elasticity (Gu et al 1988). The experiment to measure the permeation of antibacterial agents through the mucin gel layer was performed with a side-by-side glass diffusion cell separated by two Spectrapor dialysis membranes (Spectrum Industries, Los Angeles, CA) with a molecular weight cut-off of 6000–8000 Da (Bhat et al 1995). A custom-made liquid holder containing either gastric fluid or reconstituted mucin (1·25 mL) was mounted between the dialysis membranes. The donor compartment contained antibacterial drug solution in SGF (1·0 mg mL^{-1}), and the receptor compartment contained sodium chloride (0·15 M). The solutions in both compartments were continuously stirred with magnetic stirrers to ensure uniform mixing and maintained at 37°C with a circulating water-bath. At different times a sample from the receptor compartment was

removed and assayed for the drug spectrophotometrically as described above. The apparent permeability coefficient ($cm\,s^{-1}$) of the antibacterial agents through the mucin gel layer was calculated according to Fick's first law of diffusion (Martin 1993).

Results and Discussion

Drug-containing chitosan microspheres

Several independent studies, including ours, have indicated that chitosan can bind to gastrointestinal mucus and improve the permeability of drugs by reducing the diffusional distance at the absorption site (Artursson et al 1994; Illum et al 1994; Leußen et al 1994; Fieberg et al 1995; Qaqish and Amiji 1999). On the basis of the mucoadhesive properties of chitosan, we have developed drug-loaded chitosan microspheres for stomach-specific delivery in the eradication of *H. pylori* infection. Porous chitosan microspheres (approx. 50 μm in diameter) were prepared by simultaneous crosslinking and precipitation with sodium tripolyphosphate. Surface and cross-sectional views of the microsphere sample, as analysed by scanning electron microscopy, showed porous morphology that extended into the matrix. Even after several washing steps, as outlined above, 81·2 ± 4·5% (mean ± s.d., n = 4) of the added amoxycillin and 99·4 ± 6·9% (mean ± s.d., n = 4) of the added metronidazole were incorporated in the chitosan microspheres.

With drug-containing chitosan microspheres in SGF at 37°C in a shaking water-bath, almost 48% of the incorporated amoxycillin and 77% of the incorporated metronidazole were released in 30 min (Figure 2). Metronidazole diffused faster than amoxycillin from the chitosan, possibly because of its lower molecular weight. In 2 h all the incorporated amoxycillin and metronidazole was released from the microspheres. Because mucoadhesion prolongs the gastric residence time, we expect the drug-containing microspheres to stay in the stomach for longer than 2 h.

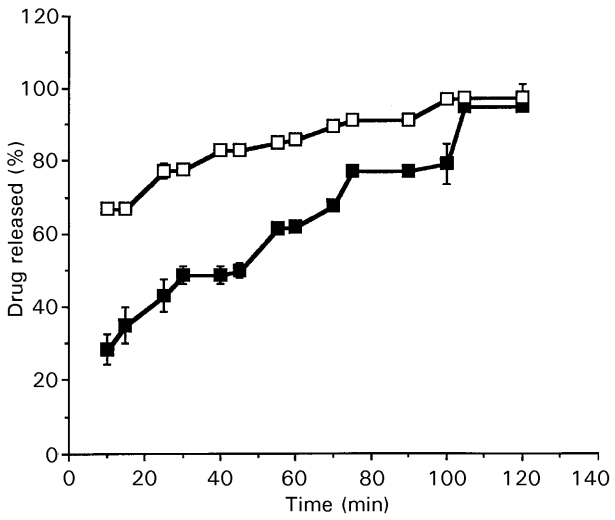

Figure 2. Percentage release of amoxycillin (■) and metronidazole (□) as a function of time from drug-loaded porous chitosan microspheres in simulated gastric fluid (pH 1·2) at 37°C. The error bars indicate standard deviation (n = 4).

Stability of antibacterial agents in gastric fluid

Because the antibacterial agents released from chitosan microspheres must reach the site of infection in their active forms, we examined the stability of amoxycillin and metronidazole at 37°C in SGF for up to 24 h. High-performance liquid chromatography (HPLC) assays were used to separate and quantify the amounts of parent drug remaining and the degradation products formed as a function of time. As shown in Figure 3, the chromatographic separation of amoxycillin revealed the presence of distinct peaks for the parent drug, internal standard and degradation products after 24 h in SGF.

Although relatively acid-stable, amoxycillin did degrade by 40% (approx.) after 10 h in SGF (Figure 4). The acid-catalysed degradation products of amoxycillin were identified from their absorption maxima at 228 nm and 274 nm as penillic acid and piperazine-2,5-dione, respectively (Tsuji et al 1978; Haginaka & Wakai 1986). The pseudo-first-order rate constant, half-life ($t_{\frac{1}{2}}$), and shelf-life (t90) of amoxycillin, as calculated from the slope of the profile of the amount (%) of drug remaining against time (Figure 4), are shown in Table 1. The rate constant of amoxycillin degradation in SGF at 37°C was found to be $4{\cdot}80 \times 10^{-2}\,h^{-1}$. On the basis of this value the half-life and shelf-life of amoxycillin were determined to be 14·3 h and 2·2 h, respectively. Because all the entrapped antibacterial agents were released from chitosan microspheres in

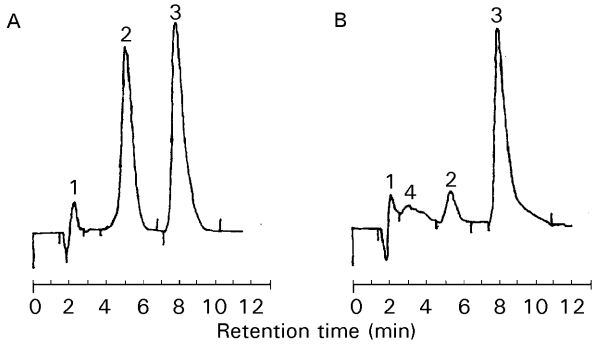

Figure 3. High-performance liquid chromatographic separation of amoxycillin at time zero (A) and after 24 h (B) in simulated gastric fluid (pH 1·2) at 37°C. The assigned peaks correspond to the solvent front (1), amoxycillin (2), the internal standard (3), and degradation product (4).

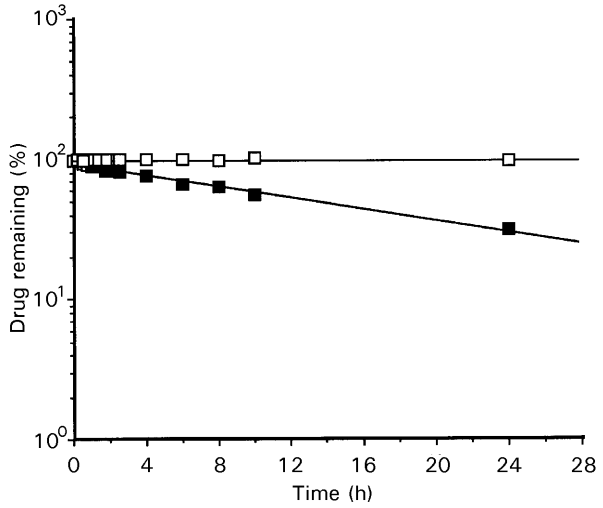

Figure 4. Apparent first-order degradation kinetics of amoxycillin (■) and metronidazole (□) in simulated gastric fluid (pH 1·2) at 37°C.

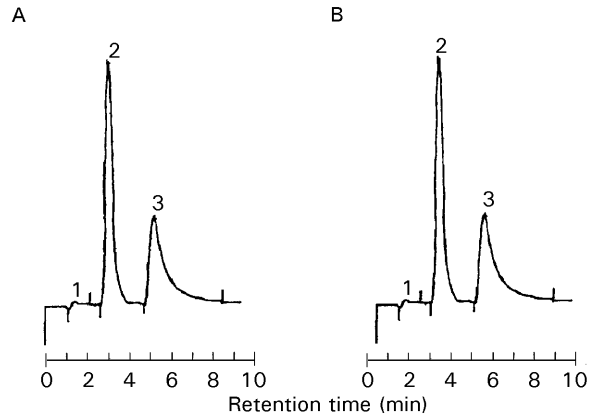

Figure 5. High-performance liquid chromatographic separation of metronidazole at time zero (A) and after 24 h (B) in simulated gastric fluid (pH 1·2) at 37°C. The assigned peaks correspond to the solvent front (1), metronidazole (2), and the internal standard (3).

2 h (approx.; Figure 2), much of the administered amoxycillin will remain therapeutically active for several hours.

Metronidazole, on the other hand, did not degrade in SGF even after 24 h. Figure 5 shows the chromatographic separation of metronidazole and the internal standard at time zero (A) and after 24 h (B) in SGF at 37°C. The absence of a degradation product peak, and no change in the area of the metronidazole peak, are indicative of the complete stability of metronidazole in gastric fluid. The pseudo-first-order rate constant, half-life, and shelf-life of metronidazole are shown in Table 1. The rate constant of metronidazole degradation in SGF at 37°C was found to be $6{\cdot}50 \times 10^{-5}\,h^{-1}$. On the basis of this value, the half-life and shelf-life of metronidazole were determined to be $1{\cdot}07 \times 10^{4}$ h (approx. 1·22 years) and $1{\cdot}62 \times 10^{3}$ h (approx. 2·25 months), respectively. For all practical purposes, the shelf-life of metronidazole in SGF at 37°C would be regarded as infinite.

The stability studies indicate that amoxycillin, when delivered locally in the stomach, would be effective in eradicating *H. pylori* infection if the drug reaches the organisms at bactericidal concentrations in less than 2·2 h. Metronidazole, on the other hand, does not degrade in the gastric fluid and could be used for stomach-specific delivery in *H. pylori* infection irrespective of the time taken to reach the organisms.

Permeability of antibacterial agents through gastric mucin

Because the organisms reside in the gastric mucus layer and at the mucus–epithelial cell interface, an acid-stable drug would need to permeate the gastric mucus layer to reach the site of infection in bactericidal concentrations. The thickness of the antral mucus is 200 μm (approx.; Gu et al 1988). The permeability coefficient of amoxycillin was $5{\cdot}5 \times 10^{-6}\,cm\,s^{-1}$ through gastric fluid and $2{\cdot}3 \times 10^{-6}\,cm\,s^{-1}$ through gastric mucin (Table 2). Assuming that the permeability coefficient of amoxycillin is approximately the same in gastric mucus, the drug would penetrate a 200-μm layer in 2·4 h (approx.). The permeability coefficient of metronidazole was $2{\cdot}75 \times 10^{-5}\,cm\,s^{-1}$ in gastric fluid and $2{\cdot}06 \times 10^{-5}\,cm\,s^{-1}$ in gastric mucin, so

Table 1. Apparent first-order rate constants, half-lives, and shelf-lives of the antibacterial agents in simulated gastric fluid (pH 1·2) at 37°C.

Drug	Rate constant (h^{-1})	Half-life ($t_{\frac{1}{2}}$) (h)	Shelf-life (t90) (h)
Amoxycillin	$4{\cdot}80 \times 10^{-2}$	14·3	2·20
Metronidazole	$6{\cdot}50 \times 10^{-5}$	$1{\cdot}07 \times 10^{4}$	$1{\cdot}62 \times 10^{3}$

The apparent first-order rate constant was calculated from the slope of the plot of the amount (%) of drug remaining as a function of time. The half-life ($t_{\frac{1}{2}}$) and shelf-life (t90) are the times at which 50% and 90% of the active drug remain.

Table 2. Permeability coefficients of the antibacterial agents through gastric mucin at 37°C.

Drug	Molecular weight ($g\,mol^{-1}$)	Permeability coefficient ($cm\,s^{-1} \times 10^{5}$)	
		Gastric fluid	Mucin
Amoxycillin	365·4	0·55	0·23
Metronidazole	171·2	2·75	2·06

The drugs were left to permeate through gastric mucin in side-by-side diffusion cells at 37°C.

metronidazole would permeate a 200-μm mucus layer in 16 min (approx.).

Conclusions

Because *H. pylori* resides in the gastric mucus layer, we have examined the possibility of using stomach-specific delivery of antibacterial agents for eradication of the infection. Chitosan microspheres loaded with amoxycillin and metronidazole might prove very beneficial for increasing the local absorption of the antibacterial agents and eradicating *H. pylori* infection.

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