Formulation and Evaluation of Clarithromycin Microspheres for Eradication of *Helicobacter pylori*

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The objective of the study was to develop a stomach-specific drug delivery system for controlled release of clarithromycin for eradication of *Helicobacter pylori* (*H. pylori*). Floating-bioadhesive microspheres of clarithromycin (FBMC) were prepared by emulsification-solvent evaporation method using ethylcellulose as matrix polymer and Carbopol 934P as mucoadhesive polymer. The prepared microspheres were subjected to evaluation for particle size, incorporation efficiency, *in vitro* buoyancy, *in vitro* mucoadhesion and *in vitro* drug release characteristics. The prepared microspheres showed a strong mucoadhesive property with good buoyancy. The formulation variables like polymer concentration and drug concentration influenced the *in vitro* drug release significantly in simulated gastric fluid (pH. 2.0). The *in vivo H. pylori* clearance efficiency of prepared FBMC in reference to clarithromycin suspension following repeated oral administration to *H. pylori* infected Mongolian gerbils was examined by polymerase chain reaction (PCR) technique and by a microbial culture method. The FBMC showed a significant anti-*H. pylori* effect in the *in vivo* gerbil model. It was also noted that the required amount of clarithromycin for eradication of *H. pylori* was significantly less in FBMC than from corresponding clarithromycin suspension. The results further substantiated that FBMC improved the gastric stability of clarithromycin (due to entrapment within the microsphere) and eradicated *H. pylori* from the gastrointestinal tract more effectively than clarithromycin suspension because of the prolonged gastrointestinal residence time of the formulation.

Key words clarithromycin; microspheres; *Helicobacter pylori*; carbopol; ethylcellulose

Marshall and Warren discovered *Helicobacter pylori*, which is now recognized as a major gastric pathogen with worldwide distribution. *H. pylori* is a prevalent human-specific pathogen and a causative agent in chronic active gastritis, duodenal ulcers, and gastric adenocarcinoma.[1—3] Although the microorganism is susceptible to many antimicrobial agents *in vitro*, clinical trials with a single antimicrobial agent have resulted in a low eradication rate of *H. pylori*.[3,4] 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 the short residence time of the antibiotic in the stomach.[5] Therefore, eradicating *H. pylori* is a prerequisite for curing a gastric or peptic ulcer and preventing a recurrence.[3] Triple therapy consisting of combined use of antibiotics, such as amoxicillin, clarithromycin/metronidazole, and a proton pump inhibitor gives a high eradication rate, and is now frequently used for clinical treatment of *H. pylori* associated gastroduodenal disease. However, eradication is not always successful and harmful side effects, cost of therapy, and the lack of willingness to take many different drug products are the major drawbacks of the therapy.[5,6]

Other than the multi-antibiotic therapy, other strategies that can completely eradicate *H. pylori* from the stomach have been examined. One way to improve the efficacy in eradicating the infection is to deliver the antibiotic locally in the stomach[5,7] by increasing residence time of antibiotics at infected site. Another way is to improve the stability of antibiotics in gastric environment. The antibiotics with better stability and longer residence time will allow more of the antibiotic to penetrate through the gastric mucus layer to act effectively on *H. pylori*. Gastroretentive drug delivery systems like floating and bioadhesive system would improve the therapeutic effect of antimicrobial drugs.[8] Research into gastroretentive drug delivery systems has resulted in the development of several approaches including the use of tablets-in-capsule,[9] floating systems[10,11] *in situ* gelling systems,[12,13] mucoadhesive tablet, pH-sensitive excipient composition mucoadhesive microspheres[14,15] *etc.*, which were able to reside in the gastrointestinal tract for an extended period of time for a more effective *H. pylori* eradication.

Mucoadhesive drug carriers may prolong the residence time in the gastrointestinal tract (GI) tract because they can adhere to the mucus surface, resulting in an effective localized drug concentration.[10,15] Among several mucoadhesive polymers, Carbopol usually has strong mucoadhesive properties and is known to be biocompatible and nontoxic. Multiple unit gastroretentive dosage forms, like floating microspheres or microcapsules, can be distributed widely throughout the GI tract, providing the possibility of achieving a longer lasting and more reliable release of drugs.[16,17] Clarithromycin is a macrolide, orally absorbed, broad-spectrum antibiotic. It is widely used in a standard eradication treatment of *H. pylori* infection combined with a second antibiotic and an acid-suppressing agent.[3,18] Clarithromycin has highest rate of eradication of *H. pylori* in monotherapy *in vivo* and hence was selected as a model drug in this study. In order to improve the efficacy of anti-*H. pylori* clarithromycin, we propose a new concept based on both floating and bioadhesive system with site-specific drug delivery of clarithromycin using calcium carbonate as gas forming agent. This stomach-specific delivery system would increase the gastric residence time, de-

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crease the diffusional distance, and allow more of the antibiotic to penetrate through the gastric mucus layer and act locally at the infectious site benefits[19,20] and also minimize the resistance problems associated with systemic administration of antibiotics. The main purpose of this study was to develop clarithromycin floating-mucoadhesive microspheres and to evaluate the effectiveness of the microspheres for *H. pylori* eradication therapy in Mongolian gerbil animal model.

Experimental

Clarithromycin was gifted by Ranbaxy Laboratories Ltd. (New Delhi, India) and Carbopol 934P and ehtylcellulose were gifted by Micro Labs Ltd. (Hosur, India). Modified Skirrow's medium, Brucella broth, and fetal calf serum (FCS) were purchased from Himedia (Mumbai, India). Agarose was purchased from FMC BioProducts (Rockland, U.S.A.) and *Taq* DNA polymerase was purchased from Takara Shuzo, Otsu and Shiga, Japan. All other reagents were of analytical grade.

Animals Six-week-old male specific pathogen free Mongolian gerbils (body weight 50—60 g) were purchased from Central Drug Research Institute (Lucknow, India) and were maintained under standard laboratory conditions (room temperature, 23±2 °C; relative humidity, 55±5%; 12/12 h light/dark cycle) with free access to a commercial rodent diet and tap water.

Methods. Preparation Floating-Mucoadhesive Microspheres The Clarithromycin floating-bioadhesive microspheres were prepared by emulsification and solvent evaporation method, reported in the literature[21] with little modification. Briefly, different concentrations of ethylcellulose solution were prepared by dissolving different amount of ethylcellulose in 50 ml of acetone with stirring. Then different concentrations of carbopol 934P, clarithromycin and calcium carbonate were added to the polymer solution under continuous stirring and the mixture was blended for 24 h. Then the suspension was slowly added into 200 ml of light liquid paraffin containing 2.0% Span 80 and stirred at a rate of 1200 rpm using Remi stirrer for 30 min. After 30 min of emulsification, acetone was evaporated gradually with the help of a water-circulating vacuum pump until the microspheres were formed. The formed microspheres were washed with petroleum ether and dried at room temperature.

Morphology and Particle Size Analysis The shape and surface morphology of various batches of microspheres prepared, were determined by scanning electron microscopy (SEM-JEOL Model 8404, Japan at magnification 500×). Particle size was determined using an optical microscope (Olympus, NewDelhi, India) fitted with a stage and an ocular micrometer.

Determination of Drug Encapsulation Efficiency To determine the total drug content of microspheres a known amount of microspheres were ground to fine powder. Accurately weighed (50 mg) grounded powder of microspheres were soaked in 50 ml of distilled water and sonicated using probe sonicator (UP 400 s, Dr. Hielscher GmbH, Germany) for 2 h. The whole solution was centrifuged using a tabletop centrifuge (Jouan, MR 23i, France) to remove the polymeric debris. Then the polymeric debris was washed twice with fresh solvent (water) to extract any adhered drug. The clear supernatant solution was filtrated through a 0.45 μm syringe filter then analyzed for clarithromycin content by reversed-phase high performance liquid chromatography (RP-HPLC) method using a mobile phase consisting of acetonitrile–aqueous 0.05 M phosphate buffer solution of pH 4.0 (40 : 60 v/v). The apparatus used for HPLC analysis was an Agilent 1100 quaternary pump, with a variable wavelength detector, thermostatted autosampler and column thermostat. A Hypersil ODS C18 column (250 mm×4.6 mm ID, 5 μm, Thermo, U.K.) was fitted with a Phenomenex guard column packed with octadecyl C18 (Phenomenex, U.S.A.). The column temperature was maintained at 40 °C and flow rate of 1 ml/min. The detection was done on UV detector at 210 nm.[22] Each study was conducted in triplicate. Incorporation efficiency was calculated using the following formula:

$$\text{incorporation efficiency} = b/a$$

where 'a' is the theoretical drug content and 'b' is the drug entrapped.

Measurement of *in Vitro* Bioadhesion *In vitro* bioadhesion (in triplicate) was determined by following a previously reported method.[23] The stomachs of the over night fasted rats were removed and cut into pieces 2 cm long and 1 cm wide and were rinsed with 2 ml of physiological saline and 50 mg of microspheres were placed uniformly on the surface of the stomach mucosa. The mucosa with microspheres were placed in a humidity temperature control cabinet (Narang Scientific works Pvt. Ltd., NewDelhi, India) at 80% R.H. and temperature of 25±0.5 °C to allow hydration of microspheres for 20 min. The mucosal lumen was rinsed with HCl-physiological saline at angle of 45°. The washings were dried at 60 °C in a hot air oven. The ratio of adhered and applied microspheres were computed as percent bioadhesion.

***In Vitro* Buoyancy Studies** The *in vitro* floating study was determined using USP dissolution apparatus II having 900 ml of simulated gastric fluid (SGF, pH 2.0) .The medium temperature was kept at 37 °C. The floating mucoadhesive microspheres (400 mg) were spread over the surface of the dissolution medium and medium was agitated by paddle at 100 rpm. After agitation the microspheres that floated or coated over the surface of the medium and those that settled down at bottom of the flask were recovered separately and dried. The floating percentage of floating was determined by following equation:

$$\text{buoyancy (\%)} = (\text{weight of microspheres floated on medium}) / (\text{weight of microspheres floated in medium} + \text{weight of microspheres settled at bottom of flask}) \times 100$$

Measurement of *in Vitro* Drug Release Drug release from mucoadhesive microspheres of clarithromycin was determined by reported method[24] using USP dissolution test apparatus I (USP 24) with stirrer at 100 rpm. Nine hundred milliliters of 0.1 N HCl (pH 2.0) was used as the dissolution medium and the temperature was maintained at 37±0.2 °C. Samples were taken at appropriate time intervals and replaced with an equal volume of fresh dissolution medium. The withdrawn samples were filtered through 0.45 μm syringe filter and neutralized with NaOH solution (0.014 M) to adjust the pH of sample to approximately 5.0 in order to prevent the further degradation of drug before analyzed by RP-HPLC as described above. These experiments were conducted in triplicate.

Degradation of Clarithromycin in simulated gastric fluid (SGF) pH 2.0 The degradation rate of the antimicrobial agents at pH 2.0 was examined by reported method[21] with slight modification. A known amount of clarithromycin was added to the medium, which was preheated at 37±0.2 °C, to make a final concentration of 10.0 μg/ml. An aliquot of the medium was withdrawn at predetermined time intervals and neutralized with a NaOH solution before being quantified by HPLC. The concentrations of the parent drug remaining were analyzed by RP-HPLC assay. The degradation of clarithromycin 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 clarithromycin remaining at time t, C_o is the initial concentration of clarithromycin, and k is the pseudo-first order degradation rate constant. The half-life ($t_{1/2}$) of clarithromycin was determined from the pseudo-first order degradation rate constant.

Stability of Clarithromycin in Microspheres in SGF pH 2.0 Fifty milligrams of mucoadhesive microspheres were suspended in 30 ml of SGF pH 2.0 in four graduated centrifuge tube with lid. The tube was then placed in a thermostatic vibrator and vibrated at a speed of 100 rpm at 37±1 °C for 1, 2, 4 and 6 h, respectively. The whole samples were withdrawn at different time interval and neutralized with NaOH solution (0.05 M) to adjust the pH of sample to approximately 5.0 in order to prevent further degradation of drug. The samples were taken out at different time intervals and microspheres were collected separately by filtration. The drug content of the filtrate (amount of clarithromycin released from microspheres) and microspheres (amount clarithromycin entrapped in microspheres) were determined separately by HPLC method as described earlier.

***In Vivo H. pylori* Clearance Study** The bacterial strain used in this study was originally isolated from a human patient (age 50 years) with gastric ulcer in SSG hospital, Banaras Hindu University, Varanasi, India. Six animals in each group were assigned to 9 groups and were inoculated with 1 ml of culture broth *via* intragastric gavage after fasting for 24 h. Each dose contained 10^9 CFU of *H. pylori*. The protocol of the study was approved by Institutional Animals Ethical Committee of the Department. Fourteen days after infection, clarithromycin was orally administered once a day for 3 consecutive days at a dose of 3, 30, 60 and 90 mg/kg in the form of either floating-mucoadhesive microspheres or clarithromycin suspension (clarithromycin was dispersed well in 0.5% w/v of methylcellulose solution). Placebo floating mucoadhesive microspheres used as a control, were administered in the same manner.

Microbial Culture Method One day after administration of the final dose, the Mongolian gerbils were sacrificed and the stomachs were removed. Each stomach was homogenized with Brucella broth (3 ml/stomach), and serial dilutions were plated on modified Skirrow's medium. The agar plates were incubated for 4 d at 37 °C under microaerobic conditions in GasPak (BD Diagnostic Systems, Sparks, MD, U.S.A.). The viable cell counts for

each stomach were calculated by counting the number of colonies on the agar plates. The colonies were identified as *H. pylori* by morphology and urease activity.[25] The number of colonies per plate was counted and expressed as log CFU per gastric wall. The advantage of this evaluation method is that errors caused by sampling site variation can be avoided because the whole stomach is used to determine the bacterial cell count.

Isolation of DNA The DNA of *H. pylori* was isolated as described by Kusuhara *et al.*[26] The gastric tissue was collected by centrifugation from 1 ml of homogenates from each gerbil stomach. The tissue was suspended in 500 μl of Tris ethylene diamine tetra acetate, pH 8.0 (TE) containing 100 mM NaCl and 100 g/ml egg white lysozyme, and incubated at room temperature for 10 min. Sodium dodecyl sulfate was added at a final concentration of 0.1%, and the mixture was incubated at 60 °C for 10 min. DNA was then extracted in an equal volume of phenol : chloroform (1 : 1), and precipitated at 20 °C for 2 h in the presence of 300 mM sodium acetate and two volumes of absolute ethanol. The precipitated DNA was collected by centrifugation and allowed to air dry. The pellet was then dissolved in 50 μl of TE.

Polymerase Chain Reaction (PCR) Amplification of the 16S ribosomal RNA (rRNA) Gene of *H. pylori* The PCR analysis was done as described previously with the *H. pylori*-specific primer.[11—13] The reaction mixture contained 5 μl of 10×PCR buffer (500 mM KCl, 100 mM Tris–HCl, 15 mM $MgCl_2$), 4 μl of deoxynucleotide solution (2.5 mM each dATP, dCTP, dGTP, and dTTP), 0.5 μl of each primer (50 μM), 1.25 U *Taq* DNA polymerase and DNA in a final volume of 50 μl. DNA from the homogenates of each gerbil stomach was serially diluted tenfold in sterile water, and 1 μl of each diluted sample was used in one assay. Each reaction tube was then placed in a thermal cycler (Perkin-Elmer 9600; Perkin-Elmer, Emeryville, CA, U.S.A.). The following conditions were used for the amplification, denaturation at 94 °C for 30 s; anealing at 56 °C for 30 s; and extension at 72 °C for 1 min. A total of 34 cycles were performed, followed by an extension step at 72 °C for 7.0 min. The PCR product (501 bp) was separated by electrophoresis through a 2% gel containing ethidium bromide, and visualized with a UV light source. The PCR product from gel was photographed by alpha imager.

Statistics The difference between the control-treated and the clarithromycin-treated groups in bacterial counts of gastric wall were statistically analyzed by one-way analysis of variance with post-test Dunnett's multiple comparison test. Statistically significant differences between groups were defined as $p<0.05$.

Results and Discussion

Morphological Properties and Size of Microspheres The scanning electron micrograph (SEM) of the microspheres obtained is shown in Fig. 1a. The results of SEM revealed that prepared floating-bioadhesive microspheres were discrete and spherical in shape with a rough outer surface because of the surface-associated drug (Fig. 1b). The surface-associated drug adsorbed on the surface of the microspheres might give a burst release and help enhance the clarithromycin concentration for the effective *H. pylori* clearance shortly after oral administration. The results (Table 2) indicated that as the amount of ethyl cellulose in the microspheres was increased, the particle size also proportionally increased. Decreasing the ethyl cellulose polymer concentration below 2.5% w/v resulted in clumping of microspheres. Whereas high ethyl cellulose concentration (at above 15% w/v) resulted in formation of large discrete microspheres with size above 400 μm. This could be attributed to an increase in the relative viscosity at higher concentration of polymer and formation of larger particles during emulsification. There was a marginal increase in mean diameter of microspheres with increasing concentrations of carbopol and drug loading (Table 2). The mean diameter of the microspheres was found to be in the range of 85.74 to 129.72 μm. All formulations showed good flowability, as represented in terms of angle of repose (from range of 32.10—35.45). The relative density of the microspheres indicates that microspheres have good packability and these micromeritic properties suggest that microspheres could be easily handled.

Table 1. Formulation Composition of Floating-Bioadhesive Microspheres of Clarithromycin

Batch code	Ethyl cellulose (% w/v)	Carbopol 934 P (% w/v)	Clarithromycin (% w/v)
FBMC1	2.50	1.0	2.50
FBMC2	5.0	1.0	2.50
FBMC3	10.0	1.0	2.50
FBMC4	15.0	1.0	2.50
FBMC5	5.0	0.5	2.50
FBMC6	5.0	1.5	2.50
FBMC7	5.0	2.0	2.50
FBMC8	5.0	1.0	1.0
FBMC9	5.0	1.0	5.0
FBMC10	5.0	1.0	10.0

All the formulation containing 1.5% w/v of calcium carbonate as floating agent.

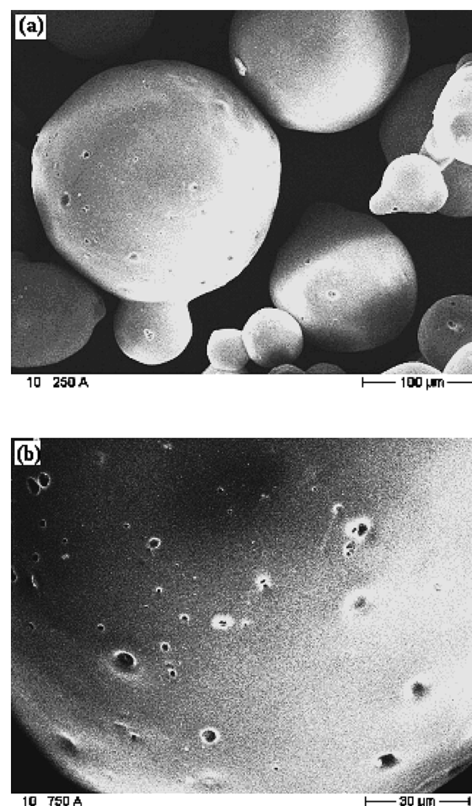

Fig. 1. SEM Photograph of Prepared Floating-Bioadhesive Microspheres
(a) Drug loaded microspheres, (b) morphological surface of microspheres.

Incorporation Efficiency of Microspheres The effect of various formulation parameters on the incorporation efficiency of the prepared microspheres are shown in Table 2. The incorporation efficiency of the prepared microspheres varied from 48.35% for batch FBMC5 to 78.25% for batch FBMC4. The incorporation efficiency increased progressively with increasing both ethyl cellulose and carbopol concentration. Similarly, the incorporation efficiency was increased proportionally with drug concentration. An increase in the concentration of ethyl cellulose from 2.5 to 15% w/v resulted in the formation of larger microspheres thus entrapping more amount of the drug. Similarly, an increase in concentration of carbopol resulted in the formation of larger mi-

Table 2. Physico-Chemical Characteristics of the Floating-Bioadhesive Microspheres of Clarithromycin

Batch code	Buoyancy (%)[a]	Relative density[a] (g/cm^3)	Mean particle size in μm (Mean[a]±S.D.)	Incorporation efficiency (%) (Mean[a]±S.D.)	Bioadhesion (%) (Mean[a]±S.E.M.)
FBMC1	79.25±2.42	0.898±0.22	89.30±7.92	54.30±2.49	82.24±2.174
FBMC2	83.41±2.14	0.892±0.092	101.11±9.82	62.81±3.98	85.27±1.873
FBMC3	89.23±1.89	0.852±0.085	109.57±12.72	69.21±2.52	85.56±1.890
FBMC4	92.23±3.12	0.8013±0.23	129.72±13.87	78.25±2.65	84.24±0.992
FBMC5	87.23±2.12	0.856±0.312	86.89±12.45	48.35±2.41	52.45±1.452
FBMC6	85.82±1.98	0.901±0.190	91.81±8.74	58.11±2.80	89.56±1.876
FBMC7	83.45±3.89	0.854±0.089	96.47±10.78	64.80±4.12	93.24±1.890
FBMC8	88.36±3.45	0.874±0.152	90.57±8.12	68.72±3.56	80.75±1.779
FBMC9	82.78±2.01	0.892±0.124	93.72±9.75	72.80±1.65	79.37±1.658
FBMC10	80.54±2.78	0.911±0.256	92.98±11.47	54.78±4.0	82.30±1.720

a) $n=3$.

crospheres. Increase in clarithromycin loading above 10% w/v caused a marginal decrease in the incorporation efficiency suggesting that quantity of ethyl cellulose becomes insufficient to entrap the drug.

Bioadhesivity The study of *in vitro* bioadhesion revealed that all the batches of prepared microspheres had good bioadhesive property ranging from 79.37 to 93.24%. On increasing the polymer (carbopol) concentration, the bioadhesive property of the microspheres also increased as shown in Table 2. The formulation FBMC7 showed the highest bioadhesive property (93.24%). These studies suggest that the spherical matrix of microspheres can interact with mucosubstrate on the surface of the stomach, and adhere to mucosa more strongly and could stay in stomach for prolong period for more effective *H. pylori* clearance.

***In Vitro* Buoyancy Efficiency** The floating ability of the prepared formulations was evaluated in SGF (pH 2.0). The time the formulation took to emerge on the medium surface (floating lag time) and the percentage of the microspheres that floated on the dissolution medium surface were evaluated and are shown in Table 2. Upon contact with an acidic medium the formulations swells and provides a gel barrier at the surface of the formulation. The calcium carbonate effervesced, releasing carbon dioxide and the released carbon dioxide is entrapped in the gel network producing buoyant formulation for prolonged periods. We observed that all the microsphere ascended to the upper one-third of the dissolution vessel within a short time, and remained floating until the completion of drug release. The buoyancy lag time for this system was in the range of 5—15 min. The *in vitro* floating test clearly showed that most of the microspheres floated for around 20 h.The microspheres with the higher concentration of polymer were more floatable than those with lower concentrations of polymer. This may be attributed to a decrease in density of microspheres with an increase in polymer concentration (Table 2).

***In Vitro* Drug Release** The effect of ethyl cellulose concentration on clarithromycin release from different batches of microspheres is shown in Fig. 2. A significant ($p<0.01$) decrease in the rate and extent of drug release was observed with the increase in polymer concentration in microspheres and could be attributed to increase in the density of the polymer matrix and also increase in the diffusional path length which the drug molecules have to traverse. Similarly, Fig. 3 indicates the effect of carbopol concentration on release

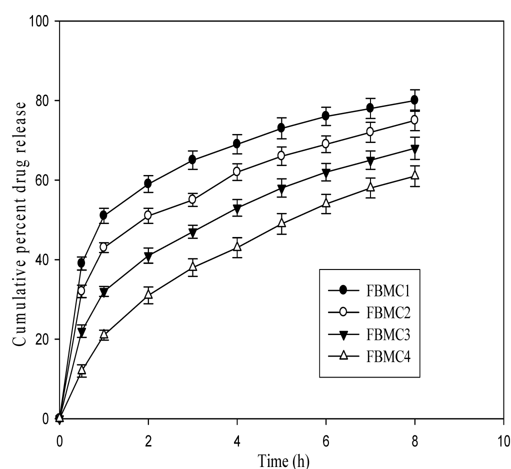

Fig. 2. Effect of Ethyl Cellulose on the *in Vitro* Drug Release Charateristics of Floating-Mucoadhesive Microspheres of Clarithromycin in SGF pH 2.0

Bars represent mean±S.D. ($n=3$).

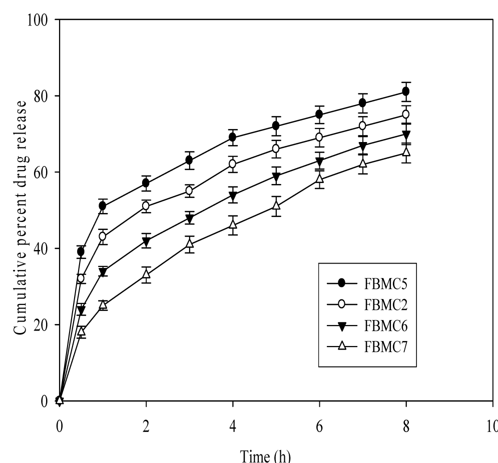

Fig. 3. Effect of Carbopol on the *in Vitro* Drug Release Charateristics of Floating-Mucoadhesive Microspheres of Clarithromycin in SGF pH 2.0

Bars represent mean±S.D. ($n=3$).

properties of clarithromycin from microspheres. An increase in carbopol concentration caused retardation in drug release from the microspheres because of an increase in the viscosity of polymer solution and formation larger size microspheres.

The release of clarithromycin from these batches were

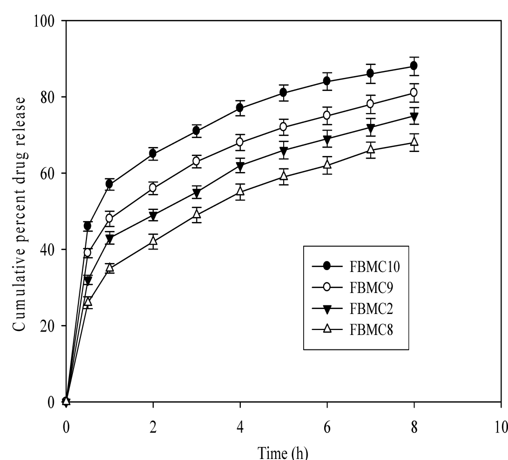

Fig. 4. Effect of Drug Loading on *in Vitro* Calrithromycin Release Characteristics of Floating-Mucoadhesive Microspheres of Clarithromycin in SGF pH 2.0

Bars represent mean±S.D. ($n=3$).

Table 3. *In Vitro* Drug Release Kinetic of Floating-Bioadhesive Microspheres of Clarithromycin

Batch Code	Drug release kinetic, correlation coefficients $(r^2)^{a)}$			Release exponent $(n)^{b)}$
	Zero order	First order	Higuchi	
FBMC1	0.7621	0.9507	0.9865	0.4225
FBMC2	0.8202	0.9715	0.9892	0.4901
FBMC3	0.8770	0.9823	0.9956	0.5612
FBMC4	0.9212	0.9771	0.9986	0.6945
FBMC5	0.7855	0.9651	0.9745	0.4989
FBMC6	0.8669	0.9831	0.9845	0.4892
FBMC7	0.9135	0.9663	0.9994	0.5224
FBMC8	0.8125	0.9683	0.9745	0.5017
FBMC9	0.8369	0.9708	0.9812	0.4985
FBMC10	0.8551	0.9798	0.9913	0.4819

a) By regression analysis. *b)* By following peppas equation.

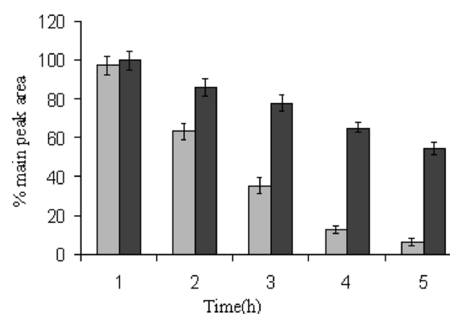

Fig. 5. Stability of Clarithromycin on Floating-Mucoadhesive Microspheeres in SGF pH 2.0

■, stability of clarithromycin released from microspheres; ■, stability of clarithromycin entrapped in microspheres. Bars represent mean±S.D. ($n=3$).

characterized by an initial phase of high release (burst effect) followed by a second phase of moderate release. This bi-phasic pattern of release is a characteristic feature of matrix diffusion kinetics.[11,27] The initial burst effect was considerably reduced with increase in polymer concentration. The fact that an increase in polymer concentration resulted in better incorporation efficiency could be the reason for the observed decrease in burst effect since the amount of surface associated drug decreases with an increase in incorporation efficiency. The results shown in Fig. 4 indicated that rate and extent of drug release was significantly ($p<0.05$) increased from the microspheres with increasing the concentration of clarithromycin. Ethyl cellulose is insoluble at pH 2.0 whereas the solubility of clarithromycin is pH dependent *i.e.*, it dissolves at a lower pH below 3.0.[28] The initial burst effect was always higher for batches (FBMC10 and FBMC9) containing higher concentration of drug. This is probably due to higher solubility of drug in low pH of dissolution medium.

The release data obtained were evaluated kinetically by zero order, first order and Higuchi model.[29] According to the determination coefficients (r^2) release data was best characterized by Higuchi model suggesting a similarity to release from a matrix [Higuchi]. The linear regression analysis is summarized in Table 3. The examination of coefficient of determination (r^2) values indicated that drug release followed the diffusion control mechanism from the microspheres. Further, to understand the drug release mechanism, the data were fitted to Peppas exponential model[30] $M_t/M_\infty=K_t^n$, where M_t/M_∞ is fraction of drug released after time 't' and 'K' is kinetic constant and 'n' is release exponent which characterizes the drug transport mechanism. Values for release exponent 'n' are listed in Table 3. The values of 'n' were in the range of 0.4225—0.6945, which was further indicative of the drug release following a diffusion control mechanism.

Stability of Clarithromycin in Floating-Bioadhesive Microspheres Most of present studies on gastroretentive formulations containing antibiotics for anti-*H. pylori* therapy are focused on prolonging the gastric retarding time. The stability of antibiotics in acidic medium was neglected. In fact, number of antibiotics, such as erythromycin, was reported with strong *in vitro H. pylori* clearance effect but with poor *in vivo* result. The one of the reasons was due to their instability in acidic medium and poor bioavailability at local site of infection. Clarithromycin was also reported to be unstable in mediums with low pH.[31,32] To act effectively against *H. pylori* in the stomach, the released clarithromycin has to remain stable in the harsh acidic environment of the gastric lumen. The degradation rate constant and the degradation half life of the clarithromycin in SGF pH 2.0 were found to be $0.4521\,h^{-1}$ and 1.53 h respectively. The stability of clarithromycin from floating-bioadhesive microspheres was determined in SGF pH 2.0 and shown in Fig. 5. The percentage of main peak area of clarithromycin on HPLC spectrum was calculated for both amount of clarithromycin, which is released from microspheres and amount of clarithromycin, which is retained in the microspheres at 1, 2, 4 and 6 h time interval after suspending the microspheres in SGF pH 2.0. The percentage of main peak of clarithromycin (Clarithromycin released from microspheres) at 1, 2, 4 and 6 h time interval were reduced to 63.12, 35.42, 14.23 and 6.56 respectively. On other hand the percentage of main peak area of clarithromycin (Clarithromycin retained in microspheres) were 85.23, 77.56, 66.45 and 54.45 respectively at 1, 2, 4 and 6 h time intervals after being suspended in SGF pH 2.0 medium. The clarithromycin, which is entrapped in the microspheres, were significantly more stable than that released from microspheres. The result indicated that microspheres

Table 4. Effect of Repetitive Administration of Clarithromycin Floating–Bioadhesive Microspheres and Clarithromycin Suspension against Gastric Infection Caused by *H. pylori* in Mongolian Gerbils

Formulations	Dose (mg/kg)[a]	Clearance rate (no. of. gerbils cleared infection/ total no. (%)	Bacterial recovery (log CFU/ stomach)[b]
Placebo FBMC (Control)	0	0/6 (0)	7.86±0.46
Clarithromycin suspension	3	0/6 (0)	7.55±0.52
	30	1/6 (17)	6.95±0.93
	60	2/6 (33)	4.59±0.87
	90	4/6 (66)	2.72±0.74**
Clarithromycin floating-bioadhesive microspheres (FBMC)	3	2/6 (33)	4.68±0.68
	30	5/6 (83)	2.35±0.57**
	60	6/6 (100)	ND
	90	6/6 (100)	ND

FBMC, Floating-mucoadhesive microspheres of clarithromycin, CFU, colony-forming unit, ND, not detected. *a*) Drugs were administered once daily for 3 d as clarithromycin. *b*) Bacterial cell counts less than $10^{1.45}$ CFU were considered to be $10^{1.45}$ to calculate the mean. Values are means±S.E., $**p<0.01$ (both significant level in reference to control).

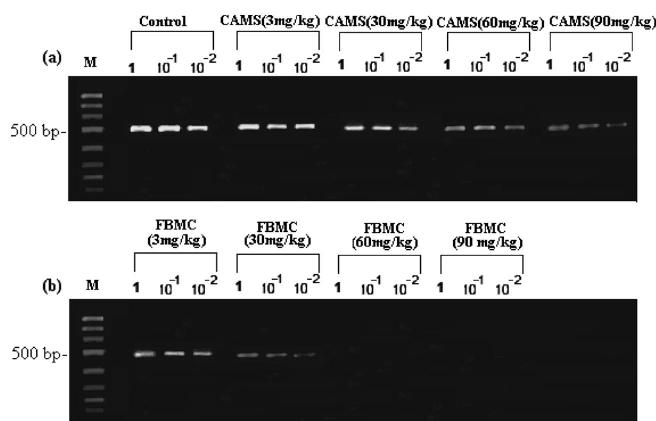

Fig. 6. Polymerase Chain Reaction (PCR) Amplipication of 16S rRNA Gene of *H. pylori* Infected Gerbils Stomchs

DNA from the homogenates of each stomach was serially diluted tenfold (1—10 M^{-2} dilutions) and 1 μl of each samples amplified with 16S rRNA gene-specific primers. (a) PCR detection of *H. pylori* from clarihromycin suspention (CAMS) treated gerbils stomach. (b) PCR detection of *H. pylori* from floating-bioadhesive microspheres of clarithromycin (FBMC) treated gerbils stomach. M, molecular marker.

could help protect clarithromycin from degrading in gastric acid and it may improve the therapeutic efficiency of the clarithromycin in the treatment of *H. pylori* infection.

***In Vivo H. pylori* Clearance Efficiency** *In vivo H. pylori* clearance study of prepared FBMC was carried out with an animal model-mongolian gerbils infected with human *H. pylori*. The mean bacterial count in gerbil's stomach after oral administration of the both clarithromycin suspension and FBMC under fed condition at doses of 3, 30, 60 and 90 mg/kg once daily for three consecutive days in *H. pylori* infected gerbil's stomach are presented in Table 4. In the control group receiving no drug, around $10^{7.86}$ (CFU/stomach) viable bacteria colonized the stomach. As expected the mean bacterial count in gerbil's stomach after oral administration of the clarithromycin suspension decreased as the dose of clarithromycin increased; however, complete clearance of *H. pylori* was not obtained even with the highest dose of 90 mg/kg. The possible reasons for the discrepancy could be the low pH of gastric fluid, which accelerates the degradation of antibiotics, as well as the short residence time of formulation during which antibiotics are present in the stomach before gastric emptying. Moreover, the high concentrations of antibiotics necessary for bactericidal activity are not readily achieved under the layer of gastric mucus. To inhibit *H. pylori*, bactericidal concentrations of the drug should be available around the adherent mucus layer and gastric epithelial cells where *H. pylori* resides.[11]

The residence time of clarithromycin in the stomach after oral administration of the conventional dosage form is expected to be short.[33] Therefore, the resulting insufficient duration of contact with the gastric mucosa by conventional dosage form of clarithromycin may be the reason for the incomplete eradication of *H. pylori*. The mean bacterial count after 3 d of treatment with FBMC with an clarithromycin dose of 3 mg/kg around 33% of *H. pylori* inhibition was obtained which was almost equal to that of clarithromycin suspension at the dose of 30 mg/kg, which is significantly ($p<0.01$) lower than that of clarithromycin suspension. This results clearly indicates that due to stomach specific delivery of clarithromycin from FBMC, it is equally effective at much lower dose than the clarithromycin suspension.

The *H. pylori* therapy with FBMC at clarithromycin doses of 60 and 90 mg/kg, the bacteria were completely cleared in gerbil's stomachs and no bacterial colony was detected in the original dilution from all gastric sample from these groups indicating 100% clearance rate and 83% of inhibition was obtained with FBMC at clarithromycin dose of 30 mg/kg. This is because of the longer residence time of FBMC in the stomach, which enabled high concentration of clarithromycin to reach the bacteria underlying the gastric mucosal layer. The effect of the therapy was further conformed by PCR technique, which has the propensity to detect bacteria more sensitively than microbial culture method. The PCR sample of DNA band was clearly detectable at a concentration of 10^2 dilution of the original DNA extract (Fig. 6a) from the clarithromycin suspension (all dose level) treated group and control group. On the other hand the PCR sample was barely detectable in the original DNA extract at clarithromycin dose of 3 and 30 mg/kg and was undetectable in the original DNA extract at clarithromycin dose of 60 and 90 mg/kg from the FBMC treated group (Fig. 6b). This study clearly demonstrates that, as expected the prepared floating-bioadhesive microspheres of clarithromycin could stay in the gastrointestinal tract for a longer period of time and could keep the entrapped clarithromycin stable in gastric surrounding. This result also indicates clearly that clarithromycin administered in the form of FBMC more effectively clears *H. pylori* from gerbils stomach than clarithromycin administered in the form of a suspension. Further, FBMC may provide therapeutic concentration at a much lower dose, which may significantly reduce the adverse effects.

Conclusion

In vitro buoyancy and *in vitro* mucoadhesive studies clearly indicates that the prepared formulations possess both floating and bioadhesive properties. These properties enable the microspheres to adhere to the gastric mucosal surface and

stay in stomach for prolonged periods and could ensure the stability of clarithromycin in gastric environment, which eventually resulted in better eradication of *H. pylori* than the conventional suspension. FBMC can be used as a stomach site-specific drug delivery system of clarithromycin for treatment of peptic ulcer disease caused by *H. pylori* and FBMC may provide therapeutic concentration at a much lower dose, also may reduce the adverse effects.

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