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Lectin Conjugated Gastroretentive Multiparticulate Delivery System of Clarithromycin for the Effective Treatment of *Helicobacter pylori*

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Abstract: The aim of the research work was to develop and characterize a concanavalin-A (Con-A) conjugated gastroretentive multiparticulate delivery system of clarithromycin (CM) for the effective treatment of colonization of *Helicobacter pylori* (*H. pylori*). Ethylcellulose (EC) microspheres containing CM were prepared using emulsification/ evaporation method. Formulations were characterized for micromeritic properties, % drug entrapment, % yield, surface morphology, buoyancy behavior and *in vitro* drug release in simulated gastric fluid. EC microspheres of CM were conjugated with Con-A. IR spectroscopy and differential scanning calorimetry were used to confirm conjugation of Con-A to EC microspheres while Con-A conjugated microspheres were further characterized using the parameters of zeta potential, mucoadhesiveness to gastric mucosa and Con-A conjugation efficiency with microspheres. The gamma scintigraphy of the formulations was carried out in albino rabbits (New Zeeland) to monitor the transit of Con-A conjugated EC microspheres and marketed formulation in gastrointestinal (GI) tract. The microparticles were found to be regular and spherical in shape. The particle size of microspheres was found to vary from 112.45 $\pm$ 3.39 to 124.23 $\pm$ 2.31 μm with polymer concentration from 1% w/v to 3% w/v. IR and DSC studies confirmed the attachment of Con-A with EC microspheres. All the microsphere formulations showed good % drug entrapment (70 $\pm$ 3%). Zeta potential of EC microspheres and Con-A conjugated EC microspheres was found to be -8.77 ± 0.5 mV and 7.56 ± 0.7 mV, respectively. Maximum mucoadhesion (85 $\pm$ 2.6%) was shown by Con-A conjugated EC microspheres as compared with nonconjugated EC microspheres (12.0 $\pm$ 3.2%). Performance of developed formulation in GI tract was visualized successfully by gamma scintigraphy in rabbits. Prolonged gastric residence time (GRT) of over 6 h was achieved in all rabbits for Con-A conjugated microspheres of CM. It is concluded that designed targeted delivery system could possibly treat the colonization of *H. pylori*.

Keywords: Clarithromycin; concanavalin-A; mucoadhesive drug delivery system; microspheres; gamma scintigraphy

Introduction

Helicobactor pylori (*H. pylori*) is a small, spiral, microaerophilic, Gram-negative bacteria with a 4−6 bulbous tipped sheathed flagella at one end, which helps it to penetrate the gastric mucosa and colonize on the gastric antrum.[1,2] The organism exclusively remains on the luminal surface of the gastric mucosa under the mucus gel layer, and access

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of antimicrobial drug(s) to the site is restricted both from the stomach and from the gastric blood supply. Also, the antibiotics are not delivered to the site of infection in effective concentration and in fully active form from conventional drug delivery systems.

The treatment of *H. pylori* remains a challenging proposition; although *H. pylori* is highly sensitive to most antibiotics. Eradication of infection (*H. pylori*) from patients is difficult, even with the current best therapies.[3,4] Conventional tablets or capsules are, in general, used for eradication therapy, but these preparations do not remain in the stomach for long. Therefore, it is difficult to reach minimum inhibitory concentrations in the gastric mucus where *H. pylori* colonizes. To overcome the constraints in *H. pylori* treatment, a novel drug delivery system that localizes the antibiotic at the site of infection to achieve bactericidal concentration is highly desirable. The extended release of the drug can maintain a higher antibiotic concentration in the gastric region where *H. pylori* exists and thereby improve the therapeutic efficacy. Gastroretentive drug delivery systems like floating and bioadhesive drug delivery systems would improve the therapeutic effects of antimicrobial drugs. The bioadhesive drug delivery system can plug and seal the infected and inflammed mucosal cell lines.[5,6] Mucoadhesive microspheres have gained considerable attention due to their ability to adhere to the mucus layer, as well as to release the drug in a sustained manner. Ramteke et al.[7] prepared and evaluated the oral mucoadhesive sustained release nanoparticles of clarithromycin in order to improve patient compliance by simplifying its administration, improving its therapeutic effect and reducing its dose related side effects. In this study, it was proved that clarithromycin resided in the stomach for a longer period of time when it was administered in the form of the mucoadhesive nanoparticles than when administered as a suspension or conventional system. Umamaheswari and Jain[5] reported the receptor mediated targeting of lectin conjugated gliadin nanoparticles bearing acetohydroxamic acid prepared by a desolvation method. These lectin-conjugated gliadin nanoparticles were found to be a potential candidate for targeted drug delivery and are anticipated to be useful in the treatment of *H. pylori*. Nagahara et al.[8] reported the *in vivo* clearance of *H. pylori* following oral administration of the mucoadhesive microspheres and the 0.5% methylcellulose suspension to infected Mongolian gerbils under fed conditions.

Clarithromycin (CM) is a broad spectrum macrolide antibacterial agent that is effective both *in vitro* and *in vivo* against major pathogens responsible for peptic ulcer disease by *H. pylori*.[9] Concanavalin-A (Con-A) is a lectin isolated from the jack bean, *Canavalia ensiformis*. It binds specifically to mono,oligo- and polysaccharides with terminal nonreducing α-D-mannopyranosyl-, α-D-glucopyranosyl- or β-D-fructofuranosyl residues. Lectins bind to a variety of cells, presumably via the carbohydrate portions of glycoproteins and glycolipids on the cell surface.[10] The aim of the present work was to develop lectin a conjugated gastroretentive multiparticulate delivery system of CM for controlled and site specific delivery of drug to treat colonization of *H. pylori*. To achieve this objective an ethylcellulose (EC) microspheres of CM was prepared and conjugated with lectin i.e. Con-A.

Materials and Methods

Materials. Clarithromycin was supplied as a gift sample by M/s Zydus Cadila Health Care Ltd. (Ahmedabad, India). Chitosan was obtained as gift sample from Central Institute of Fisheries Technology (Cochin, India). Concanavalin-A was supplied as a gift sample by Bio-Research Products, Inc. (323 W. Cherry St., North Library, IA 52317). *N*-Hydroxysuccinimide was supplied as gift sample from Shivam Enterprises (Pune, India). 1-Ethyl-3,3-(dimethylaminopropyl)carbodiimide (EDC), hexane, ethylcellulose, stannous chloride and dichloromethane were purchased from HiMedia Laboratory Pvt. Ltd. (Mumbai, India). Polyvinyl alcohol (PVA) was purchased from S. D. Fine Chemical Ltd. (Mumbai, India). Technetium-99m (as pertechnetate) ($^{99m}TcO^{-4}$) was obtained from the Nuclear Medicine Department, Jawaharlal Nehru Cancer Hospital and Research Center (Bhopal, India). All other chemicals were of analytical reagent grade and were used as received.

The *in vivo* and *ex vivo* studies were performed following the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Institutional Animal Ethical Committee

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Table 1. Formulation Codes of Microspheres

no.	drug:polymer ratio	EC microspheres
1	1:1	ECM-1[a]
2	1:2	ECM-2
3	1:3	ECM-3

[a] ECM: ethylcellulose microspheres of clarithromycin.

of Guru Ghasidas University (Bilaspur, India) granted permission for the study.

Preparations of Ethylcellulose Microspheres. Microspheres were prepared by an emulsification/evaporation method.[11] CM (0.5 g) and EC (1.0 g) were dissolved in 25 mL of dichloromethane (DCM), and chitosan (0.15 g) was dispersed in the solution. This suspension was poured into 200 mL of a polyvinyl alcohol (PVA) (1% w/v) solution at 25 °C. The resultant emulsion was continually stirred at 750 rpm with a mechanical stirrer equipped with a 3 blade propeller (Remi, Mumbai, India) for 1 h. Subsequently, the emulsion was heated to 40 °C for 4 h to evaporate the DCM. After evaporation of DCM, the microspheres were collected by filtration. The microspheres were washed with distilled water three times and left to dry in an oven at 40 °C for 8 h. The representative formulations are given in Table 1.

Micromeritic Properties. The microspheres were characterized for their micromeritic properties, such as particle size, true density, tapped density, compressibility index and flow properties. The size was measured using an optical microscope, and the mean particle size was calculated by measuring 600 particles with the help of a calibrated ocular micrometer. The tapping method was used to determine the tapped density and percent compressibility index[12] as follows:

$$\text{tapped density} = \frac{\text{mass of microspheres}}{\text{volume of microspheres after tapping}}$$

$$\%\ \text{compressibility index} = (1 - V/V_0) \times 100$$

Here V and V_0 are the volumes of the sample after and before the standard tappings, respectively.[13] True density was determined using benzene displacement method. Porosity[14] (ε) was calculated using the equation

$$\varepsilon = (1 - P_p/P_t) \times 100$$

where P_t and P_p are the true density and tapped density, respectively. Angle of repose θ of the microspheres, which measures the resistance to particle flow, was determined by a fixed funnel method.[15]

Drug Entrapment and Percent Yield. The % CM entrapment of EC microspheres was determined by dispersing accurately 50 mg formulation in 10 mL of DCM followed by agitation with a magnetic stirrer for 12 h to dissolve the polymer and to extract the drug. After filtration through a 5 μm membrane filter (Millipore, USA), the drug concentration in DCM phase was analyzed spectrophotometrically at 211 nm using UV−visible spectrophotometer (Systronics, Mumbai, India). No interference was found due to other microsphere components at 211 nm. The experiment was performed in triplicate. The percentage drug entrapment and yield were calculated as follows:

$$\%\ \text{drug entrapment} = \frac{\text{calculated drug content}}{\text{theoretical drug content}} \times 100$$

$$\%\ \text{yield} = \frac{\text{total wt of microparticles}}{\text{total wt of drug, polymer and other nonvolatile solids (if added)}} \times 100$$

Floating Behavior. Fifty milligrams of the microparticles were placed in 100 mL of enzyme free simulated gastric fluid (SGF, pH 2.0, KCl/HCl buffer containing 0.02% w/v Tween 20). The mixture was stirred at 100 rpm in a magnetic stirrer. After 8 h, the layer of buoyant microparticles was pipetted and separated by filtration. Particles in the sinking particulate layer were separated by filtration. Particles of both types were dried in a desiccator until constant weight. Both the fractions of microspheres were weighed and buoyancy was determined by the weight ratio of floating particles to the sum of floating and sinking particles.

$$\text{buoyancy}\ (\%) = [W_f/(W_f + W_s)] \times 100$$

where W_f and W_s are the weights of the floating and settled microparticles, respectively. All the determinations were made in triplicate.

***In Vitro* Drug Release.** The release rate of CM from microspheres was determined in a USP XXIII paddle type dissolution apparatus. A weighed amount of microspheres equivalent to 100 mg of drug was filled into a hard gelatin capsule (#5) and placed in the paddle of dissolution rate apparatus. Nine hundred milliliters of the SGF (pH 2.0, KCl/HCl buffer containing 0.02% w/v Tween 20) was used as the dissolution medium. The dissolution fluid was maintained at 37 ± 1 °C at a rotation speed of 100 rpm Perfect sink conditions prevailed during the drug release study. Five-milliliter samples were withdrawn at each 30 min interval and passed through a 0.25 μm membrane filter (Millipore), and the initial volume of the dissolution fluid was maintained by adding 5 mL of fresh dissolution fluid after each withdrawal. Samples were analyzed using a UV−visible spectrophotometer (Systronics, Mumbai, India) at 211 nm. The *in vitro* drug release in SGF from marketed uncoated

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dosage form of CM (Pylokit-50 mg, tablet) was also carried out following the same procedure. All experiments were conducted in triplicate.

Statistics. Differences in *in vitro* drug release of CM from EC microspheres and Pylokit tablets (marketed product of CM) were statistically analyzed by one way analysis of variance (ANOVA) with post test (Dunnett's multiple comparison test). Statistically significant differences between *in vitro* drug releases of formulations were defined as $p < 0.01$. Calculations were performed with the GraphPad-Instat Software Program (GraphPad-Instat Software Inc., San Diego).

Conjugation of Con-A with Microspheres. Activation of carboxyl group of EC microspheres (50 mg) was carried out by addition of 1 mL of 0.1 M 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide (EDC) and 1 mL of 0.11 M of *N*-hydroxysuccinimide (NHS) in phosphate buffer (pH 5.8).[16] After 3 h incubation at room temperature, excess coupling agent was removed by washing of microspheres with phosphate buffer (pH 5.8). The latter were suspended in 1 mL of Con-A solution in phosphate buffer (pH 5.8) and after incubation overnight Con-A conjugated microspheres were obtained by centrifugation (CFC-FREE, C-24, Cooling centrifuge, REMI, India) at 8000 rpm, followed by three to four washings with distilled water. The conjugated microspheres thus obtained were dried at room temperature.

IR Study. IR spectroscopy was carried out to confirm the conjugation of Con-A with EC microspheres. The KBr discs of EC microspheres (without drug) and Con-A conjugated EC microspheres (without drug) were prepared and scanned in an IR spectrophotometer (Perkin-Elmer - Spectrum RX-I, Lambda, USA).

Differential Scanning Calorimetry. DSC examination was conducted for the optimized formulation, pure drug, the polymer, Con-A, EDC and NHS (EDC and NHS are coupling agents) using a DSC instrument (Mettler 305, Switzerland). Samples of 2−6 mg were placed in aluminum pans (Al-Crucibles, 40 Al) and sealed. The probes were heated from 25 to 400 °C at a rate of 10 °C/min under nitrogen atmosphere.[17]

Morphology. The shape and surface morphology of EC microspheres and Con-A conjugated EC microspheres were investigated using scanning electron microscopy (SEM). The samples for SEM study were prepared by lightly sprinkling the formulation on a double adhesive tape stuck to an aluminum stub. The stubs were then coated with gold to a thickness of about 300 Å under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The coated samples were then randomly scanned and photomicrographs were taken with a scanning electron microscope (Jeol JSM-1600, Tokyo, Japan).

Con-A Conjugation Efficiency. The amount of Con-A bound to the EC microspheres containing CM was calculated as the difference between the Con-A added initially and the Con-A recovered after incubation with the microspheres.[18] The amount of lectin in the supernatant was estimated by using Folin−Ciocalteu phenol reagent. Reagent C (10 mL) was added in suspension containing 100 mg of conjugated microspheres in 100 mL of distilled water, mixed thoroughly and allowed to stand for 20 min. Then 1 mL of reagent D was added rapidly with immediate mixing to the above solution. After 30 min, the solution was filtered with Whatman filter paper (#41), volume was made up to 10 mL and absorbance was measured against blank using a UV−visible spectrophotometer (Systronics, Mumbai, India) at 750 nm.[19] The experiment was performed in triplicate.

Reagent A and reagent B were prepared by dissolving Na_2CO_3 (2 g) in 0.1 N NaOH and $CuSO_4 \cdot 5H_2O$ (0.5 g) in 1% sodium/potassium tartrate to make 100 mL solutions each, respectively. Reagent C was prepared by mixing 50 mL of reagent A with 1 mL of reagent B. Reagent D was prepared by diluting Folin−Ciocalteu phenol reagent (2.0 N) with distilled water to make it 1.0 N.

Percent Mucoadhesion. Albino rats (450−500 g, male) were fasted overnight and dissected immediately after being sacrificed. The stomachs of the rats were removed, cut into pieces 2 cm long and 1 cm wide and rinsed with 2 mL of physiological saline. One hundred milligram microspheres of each were scattered uniformly on the surface of the stomach mucosa. Then, the mucosa with the microspheres was placed in a chamber maintained at 93% relative humidity and room temperature. After 20 min, the tissues were taken out and fixed on a polyethylene support at an angle of 45°. The stomach was rinsed with physiological saline solution (pH 1.3) for 5 min at a rate of 22 mL/min. The microspheres remaining at the surface of gastric mucosa were then counted, and the percentage of the remaining microspheres was calculated. The experiment was performed in triplicate. The following formula was used for the determination of % binding:

$$\% \text{ binding} = \frac{\text{initial wt of microspheres} - \text{wt of unbound microspheres}}{\text{initial wt of microspheres}} \times 100$$

Zeta Potential Measurement. Zeta potential was measured by electrophoresis, which was performed with a Malvern Zetasizer instrument (U.K.). The microspheres were suspended in distilled water by ultrasonication for 30 min. The concentration of the suspension was 2% w/v. The cell

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was filled with a measured amount of sample and inserted with its integral gold electrodes close to the lid.

Uptake of Sodium Pertechnetate (^{99m}Tc). Weighed quantity (100 mg) of the microspheres loaded with $SnCl_2$ (3% w/v) was placed in a test tube and soaked in 10 mL of normal saline (0.9% NaCl) for 15 min. A small amount of ^{99m}Tc solution equivalent to radioactivity of 40 mBq in a sterile vial obtained from a technetium generator (column generator, Monrol, Mon-tek, $M_o{}^{99}T_c{}^{99}$, Turkey) was added to the test tube. The suspension was mixed intermittently for 15 min using a vortex shaker (Superfit, India) and microspheres were allowed to equilibrate. The supernatant was removed and the labeled microspheres were recovered by filtration through a Whatman filter paper (#41) followed by washing thoroughly with deionized water. The formulation was then allowed to dry in air for 15 min. The radioactivity in the supernatant and filtrate was counted in an auto gamma counter (Cobra II, Germany) to determine the remaining unbound pertechnetate. The radioactivity of the microspheres was also counted for bound pertechnetate.[20]

Marketed uncoated dosage form of CM (Pylokit, 50 mg, tablet) was powdered; about 100 mg of powder was mixed with 3 mg of $SnCl_2$ and compressed. The compressed tablet was then placed in a test tube. A small amount of ^{99m}Tc solution equivalent to radioactivity of 40 mBq in a sterile vial obtained from a technetium generator was added to the test tube and mixed for 15 min, followed by drying in air for 15 min. The radioactivity of the formulation was counted in an auto gamma counter (Cobra II, Germany) for bound pertechnetate.

Organ Distribution Study. Organ distribution study was performed in three adult male albino rats. ^{99m}Tc-labeled microspheres in gelatin capsule (#5) were orally administered with the help of feeding tube to rats followed by 20 mL of drinking water. The anterior position of the rat was scanned under a gamma camera 2 h post dosing. The rats were sacrificed and thyroid gland, stomach, and small intestine were isolated. These organs were placed separately in petriplates with 10 mL of normal saline.[21] Gamma images and radioactive counts of organs were recorded using E-Cam Single Head gamma camera (Siemen's, Germany).

Gamma Scintigraphy. Six one-year-old male albino rabbits (New Zealand) were used to monitor the *in vivo* transit behavior of Pylokit (tablet), and Con-A conjugated EC microspheres.[21] None of them had symptoms or a past history of GI disease. Animals were divided into two groups of 3 animals each. The animals were fasted for 12 h prior to the commencement of each experiment in order to standardize the condition of GI motility. One capsule of Con-A conjugated EC microspheres was orally administered to animals of the first group, and Pylokit (tablet) to the second group with the help of feeding tube, followed by a sufficient volume of drinking water. All four legs of the rabbit were tied over a piece of plywood (20 × 20 in.), and the location of the formulation in GI tract was monitored every 1 h by keeping the subject in front of gamma camera. The gamma camera had a field view of 40 cm and was fitted with a medium energy parallel hole collimator. The 140 keV γ rays emitted by ^{99m}Tc were imaged. Specific GI tract sites (anterior) were imaged by E-Cam Single Head gamma camera (Siemen's, Germany) after definite time intervals. The gamma images were recorded for a 6 h study period using an online computer system and stored on magnetic disk and analyzed to determine the distribution of activity in the GI tract. During the gamma scanning, the animals were freed and allowed to move and carry out normal activities.

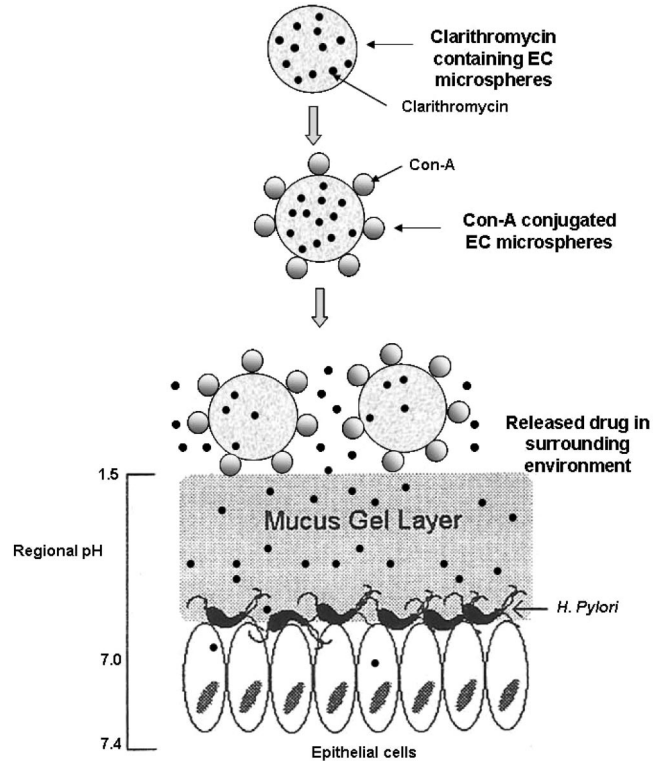

Figure 1. Schematic showing working of Con-A conjugated mucoadhesive drug delivery system on *H. pylori* infection.

Results and Discussion

A combination approach, i.e. mucoadhesive and floating multiparticulate delivery system, is explored for the effective and improved treatment of *H. pylori* infection. The concept of preparing delivery system for treatment of *H. pylori* is explained in Figure 1. Chitnis et al.[22] proposed mucoadhesive-floating systems with uniform gastric distributions to target *H. pylori*-induced infected sites. EC microspheres were prepared by an emulsification/evaporation method. Chitosan

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Table 2. Micromeritic Properties of Ethylcellulose Microsphere Formulations[a]

no.	formulation code	tapped density (g/cm^3)	true density (g/cm^3)	average particle size (μm)	compressibility index (%)	angle of repose (θ)
1	ECM-1	0.590 ± 0.05	0.754 ± 0.08	112.45 ± 3.39	17.37 ± 2.11	29.13 ± 1.44
2	ECM-2	0.627 ± 0.12	0.887 ± 0.18	117.67 ± 4.36	16.02 ± 0.82	24.45 ± 1.22
3	ECM-3	0.718 ± 0.14	0.988 ± 0.30	124.23 ± 2.31	18.21 ± 2.20	25.47 ± 1.81

[a] Values are average of three readings ± standard deviation.

Table 3. Percent Yield, Percent Drug Entrapment and Percent Buoyancy of Different Formulation Batches[a]

no.	formulation code	yield (%)	drug entrapment (%)	buoyancy (%)
1	ECM-1	84.83 ± 1.5	71.45 ± 1.2	75.21 ± 5.2
2	ECM-2	80.56 ± 4.2	68.47 ± 1.8	79.37 ± 2.0
3	ECM-3	85.22 ± 2.2	72.13 ± 1.4	80.20 ± 3.5

[a] Values are average of three readings ± standard deviation.

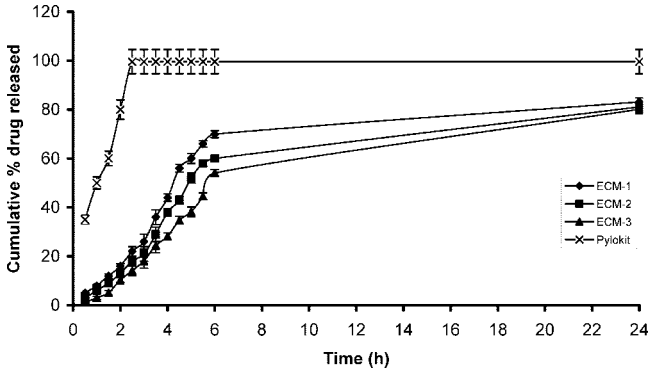

Figure 2. *In vitro* drug release profile of ethylcellulose microspheres of CM and marketed product of CM (Pylokit tablet) in pH 2.0. The values are mean ± SD (n = 3).

Table 4. One Way ANOVA (Dunnett's Multiple Comparison) Test for *in Vitro* Drug Release of CM in SGF (pH 2.0)[a]

comparison	mean difference	q	p value
Pylokit vs ECM-1	47.484	5.017**	<0.01
Pylokit vs ECM-2	53.097	5.610**	<0.01
Pylokit vs ECM-3	58.862	6.219**	<0.01

[a] ANOVA indicates analysis of variance; q, parameter obtained with p when performing ANOVA; ECM indicates ethylcellulose microspheres of clarithromycin; control, Pylokit tablet (marketed product of CM); **, significant.

was included in the preparation as a bioadhesive polymer to act with alginate by ion gelation and further improve the mucoadhesion of Con-A conjugated EC microspheres to the gastric mucosa.[11] Microspheres were prepared by using different drug:polymer ratio.

Micromeritic Properties. The average particle size of microspheres was found to be 112.45 ± 3.39 μm, 117.67 ± 4.36 μm and 124.23 ± 2.31 μm with 1:1, 1:2 and 1:3 drug, polymer ratio, respectively. The average particle size of EC microspheres increased as the amount of polymer was increased, which was due to the increased polymer concentration, and solution viscosity also increased, resulting in large particles. Thus, average particle size also increased. These results are in agreement with the results of Umamaheswari et al.,[2] who demonstrated that an increase in concentration of polymer shows increase in the mean particle size of floating microspheres. The tapped density of microspheres was found to range from 0.532 ± 0.05 g/cm^3 to 0.718 ± 0.14 g/cm^3, and their true density ranged from 0.754 ± 0.08 g/cm^3 to 0.988 ± 0.30 g/cm^3. The compressibility index ranged between 17.37 ± 2.11 and 18.21 ± 2.20. The compressibility index values were all less than 20, suggesting also excellent flowability of microspheres.[14] The angle of repose of floating microspheres was found to be 24.08 ± 0.9 to 29.13 ± 1.4. The lower is the angle of repose (>40°), the better the flow property. The micromeritic properties of EC microspheres are shown in Table 2.

Drug Entrapment, Percent Yield and Buoyancy Behavior. The yield was found to be in the range of 80.56 ± 4.2 to 85.22 ± 2.2%, drug entrapment was found to be in the range of 68.47 ± 1.8 to 72.13 ± 1.4% and buoyancy was found to be in the range of 75.21 ± 5.2% to 80.20 ± 3.5% with varying polymer concentration from 1% w/v to 3% w/v (Table 3). 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.[2]

***In Vitro* Drug Release.** Release of CM from EC microspheres was evaluated in SGF (pH 2.0). Tween 20 (0.02 wt %/vol) was incorporated in the dissolution medium. It would increase wetting and therefore hydration of the permeable EC and Chitosan.[23] Drug release from EC microspheres in SGF took place only through diffusion. Insoluble EC might have played an important role in controlling the CM release from EC microspheres. There was no burst effect from any of these formulations. Soppimath et al.[13] studied the *in vitro* release of verapamil hydrochloride (VRP) and dipyridamole (DIP) in 0.1 N HCl. They reported that cellulose acetate microspheres initially released the VRP and DIP relatively fast, showing a large burst release (about 80%) and subsequent release continued up to more than 15 h. Guiziou et al.[24] have reported significant burst release of the drug from poly (lactide) microspheres prepared by solvent evaporation

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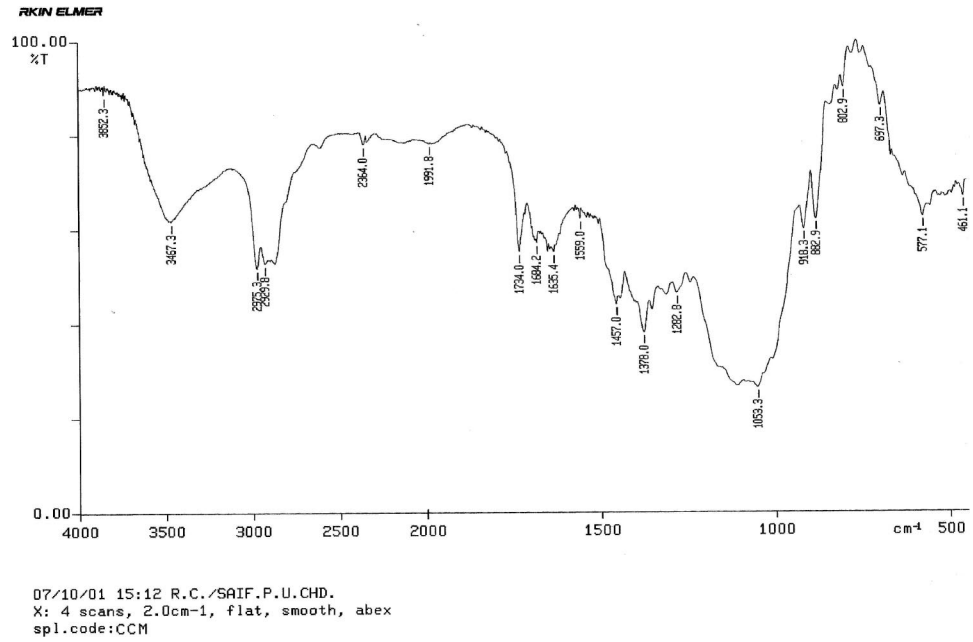

Figure 3. IR of Con- A conjugated ethylcellulose microspheres.

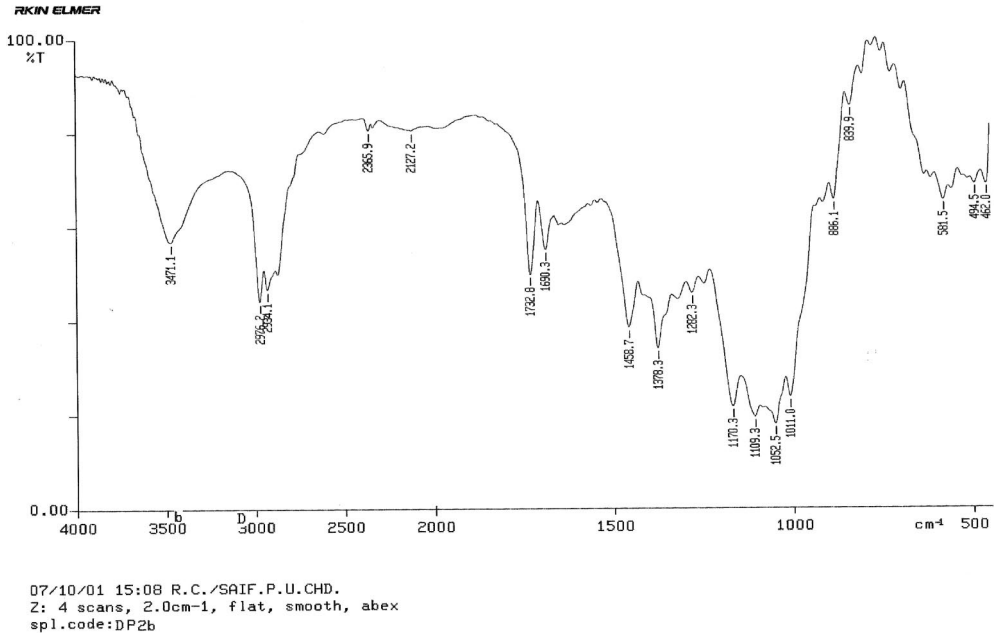

Figure 4. IR of ethylcellulose microspheres.

method. Figure 2 clearly shows that formulation with more polymer concentration released the drug in a more controlled manner as compared with formulation contains lesser polymer concentration. The drug release from different formulations in pH 2.0 followed the order: ECM-1 > ECM-2 > ECM-3. This change in CM release may be due to the increase EC ratio which had increased the density of polymer matrix and also an increase in the diffusional path length that the drug molecules have transverse.[25] When the *in vitro* release data of marketed product of CM (Pylokit tablets) is compared with EC microspheres containing CM by one-way ANOVA (Dunnett's multiple comparison test) post test, the *in vitro* release in SGF (pH 2.0) from ECM-1, ECM-2, and ECM-3 was found to be significant ($p < 0.01$) (Table 4).

Formulation ECM-3 (drug polymer ratio 1:3) showed highest % yield (85.22 ± 2.2), % drug entrapment (72.13 ± 1.4) and better buoyancy behavior (80.20 ± 3.5) as well as regular and spherical in shape. Formulation ECM-3 also showed better controlled release behavior. Hence it was selected as optimized formulation for coupling with Con-A and further studies.

IR and DSC Study. Con-A coupling to the selected EC microspheres formulations was done by carbodiimide tech-

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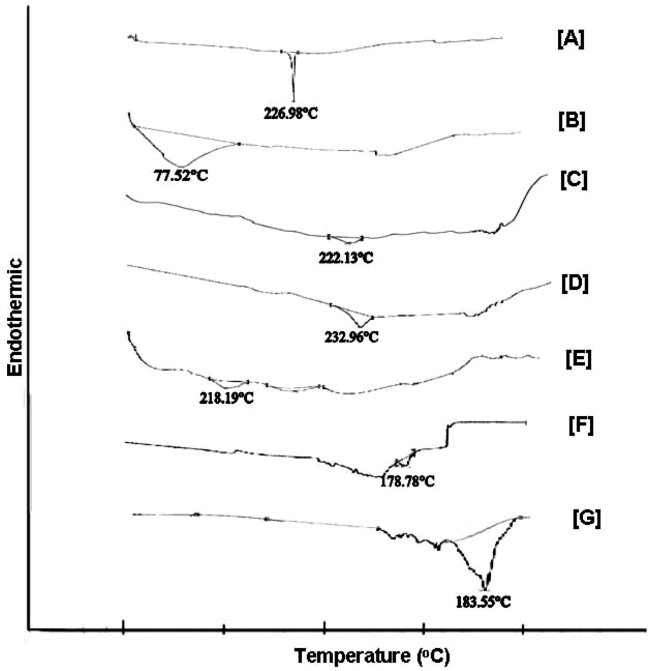

Figure 5. DSC thermogram of (A) clarithromycin, (B) chitosan, (C) ethylcellulose, (D) Con-A, (E) Con-A conjugated microspheres, (F) *N*-hydroxysuccimide, (G) 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide.

nique. 1-Ethyl-3,3-(dimethylaminopropyl)carbodiimide (EDC) is a water-soluble derivative of carbodiimide. *N*-Hydroxysuccinimide (NHS) is often used to assist the carbodiimide coupling in the presence of EDC. The reaction includes formation of the intermediate active ester (the product of condensation of the carboxylic group and *N*-hydroxysuccinimide) that further reacts with the amine function to yield finally the amide bond. IR studies confirmed the attachment of Con-A with microspheres, because coupling of Con-A and EC microspheres was depends on the amide bond between NH_2 group of Con-A and COOH group of EC. IR spectra of Con-A conjugated microspheres (CECM-3) and EC microspheres were taken and compared (Figures 3 and 4). The IR spectrum of Con-A conjugated EC microspheres clearly showed peaks (3467.3, 1735, 1684.2, 800−600, etc.) for amide groups suggesting the presence of amide group in the formulation, but peaks for amide groups were absent in the IR spectrum of EC microspheres.

A sharp melting transition of pure drug CM was observed at 226.98 °C. EC and chitosan showed an endothermic peak at 77.52 and 222.13 °C, respectively. Con-A, EDC and NHS showed an endothermic peak at 232.96, 183.55 and 178.78 °C, respectively (Figure 5). A DSC thermogram of CECM-3 showed the CM peak at 218.19 °C, suggesting that there was some interaction between drug and other components of optimized formulation.

Morphology. Surface morphology and shape of developed formulation was determined using scanning electron microscopy. Photomicrograph shows that EC microspheres were regular and spherical in shape (Figure 6A). The size of Con-A conjugated formulation was found to be increased as compared to nonconjugated microspheres, which may be due to the presence of lectin coating on formulation. The average particle size of Con-A conjugated EC microspheres was 144.35 ± 22 μm. A SEM study in which the Con-A conjugated EC microspheres were found to be regular and spherical in shape indicates Con-A attachment did not affect the microspheres' structural integrity (Figure 6B).

Con-A Conjugation Efficiency, Percent Mucoadhesion and Zeta Potential Measurement. The amount of Con-A bound to the EC microspheres containing CM was determined. The amount of lectin was estimated by using Folin−Ciocalteu reagent. The percent conjugation efficiency of CECM containing CM was calculated as 85.98 ± 1.4%.

Mucoadhesion studies were carried out to ensure the adhesion of the formulation to the mucosa for a prolonged period of time. Maximum mucoadhesion (85 ± 2.6%) was shown by Con-A conjugated EC microspheres as compared with nonconjugated EC microspheres (12.0 ± 3.2%). This significant difference ($p < 0.05$) in the mucoadhesion may be due to affinity of the Con-A toward glycoproteins of mucus membrane of stomach.

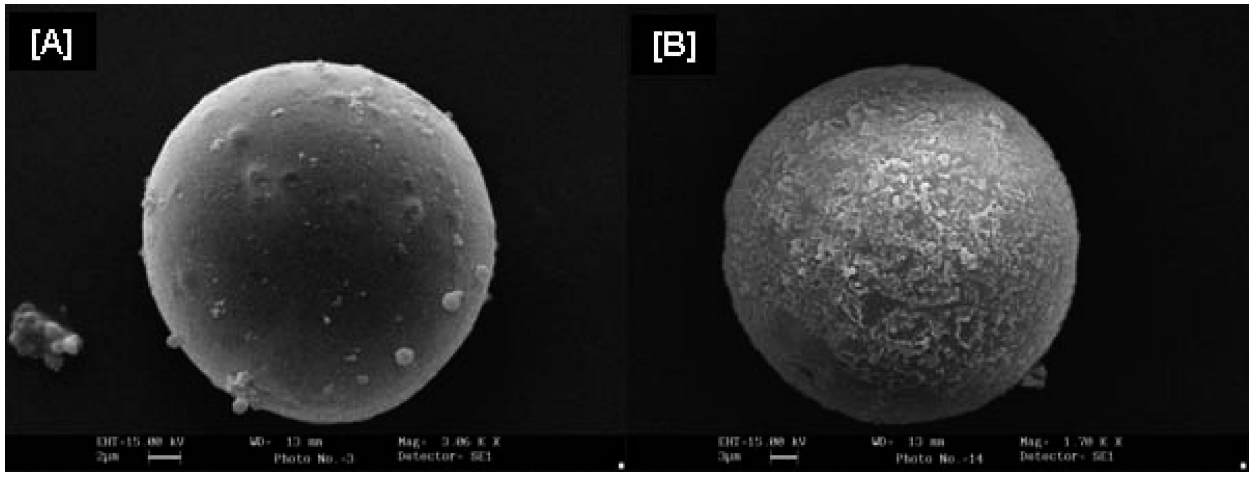

Figure 6. SEM of (A) ethylcellulose microspheres of CM, and (B) Con-A conjugated ethylcellulose microspheres of CM.

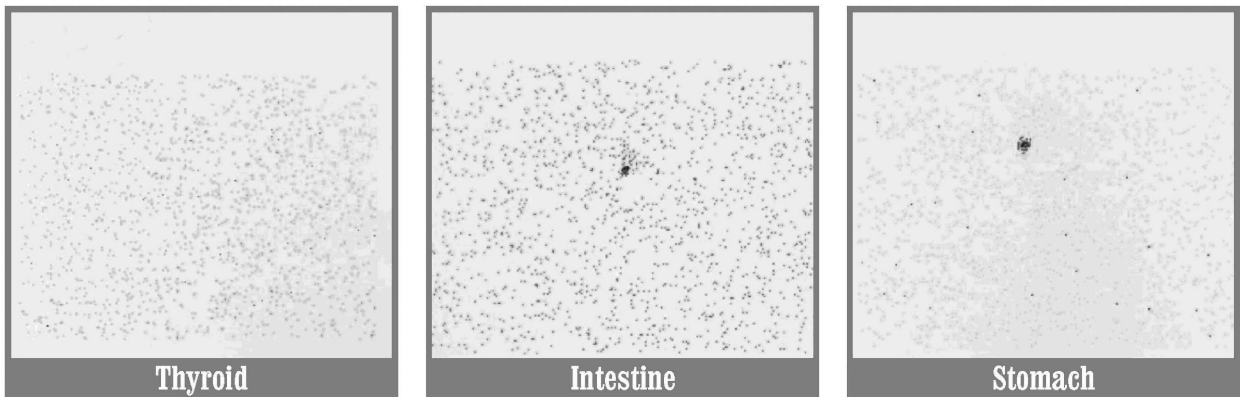

Figure 7. Organ distribution study in albino rats.

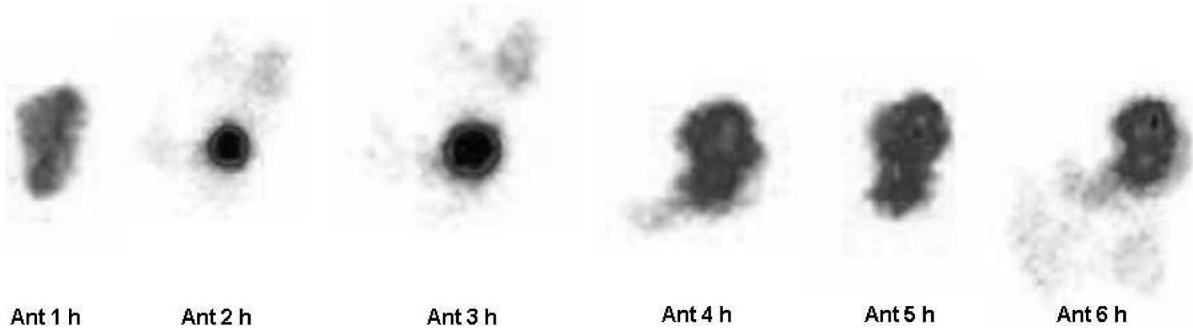

Figure 8. Gamma scintigraphic images of Con-A conjugated EC microspheres in rabbit at different time intervals.

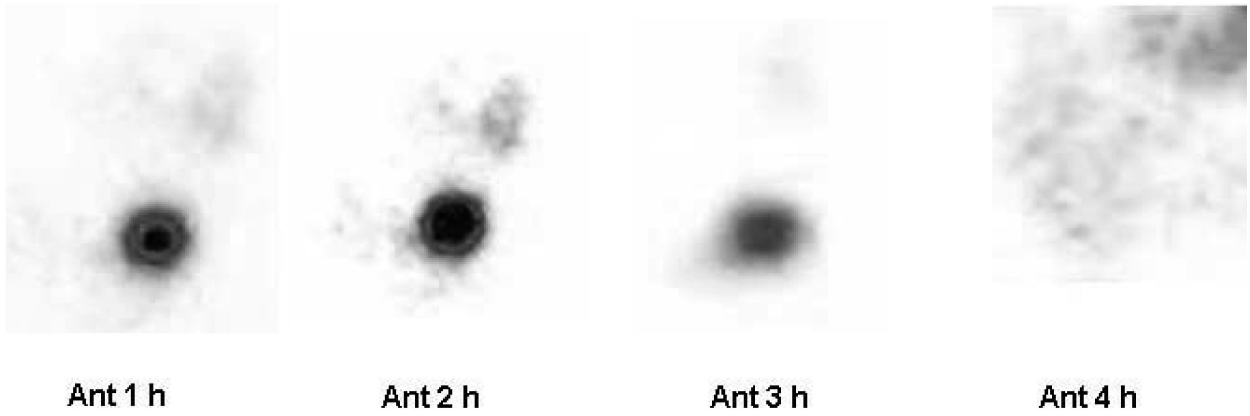

Figure 9. Gamma scintigraphic images of Pylokit (tablet) in rabbit at different time intervals.

Zeta potential of both the formulations was measured. The zeta potential of microspheres (ECM-3) and Con-A conjugated EC microspheres (CECM-3) was found to be -8.77 ± 0.5 mV and 7.56 ± 0.7 mV, respectively. Due to lectin coating the zeta potential of formulation was positively increased. This property is beneficial for interaction of formulation with the gastric mucosa. The positively charged drug loaded microspheres are expected to interact with the negatively charged mucus layer on mucosa in the stomach by electrostatic interactions and prolonged the gastric residence time. These results are in agreement with the results of Umamaheswari and Jain,[5] who demonstrated that due to lectin coating zeta potential of gliadin nanoparticles was increased positively.

Gamma Scintigraphy. The uptake of pertechnetate anions by Con-A conjugated EC microspheres and Pylokit (tablet) was determined using an auto gamma counter. The uptake values were $94 \pm 1.0\%$ and $89 \pm 1.6\%$ for Con-A conjugated EC microspheres and Pylokit (tablet), respectively.

The organ distribution study was performed in albino rats in order to measure the labeling efficiency of the formulation with ^{99m}Tc. Measurable radioactive counts in stomach and intestinal region but zero counts in thyroid gland were observed. The counts per minute (CPM) values were 137.9 ± 5.4 and 112.6 ± 4.2 in stomach and intestinal region, respectively (Figure 7). The thyroid gland is the target organ of free ^{99m}Tc, and if the formulation is not properly tagged with ^{99m}Tc, it will go to the thyroid gland. Therefore zero count in thyroid gland confirmed the proper tagging of ^{99m}Tc with the formulation.[21]

The gamma scintigraphy of the Con-A conjugated EC microspheres and Pylokit (tablet) formulation was performed

in rabbits (New Zealand) in order to establish their gastroretentive behavior.[26] Marketed product of CM, i.e. Pylokit (tablet), was used for comparison with optimized formulation. Gamma images of the ^{99m}Tc-labeled Con-A conjugated EC microspheres and Pylokit (tablet) formulations are shown in Figures 8 and 9. Examination of the sequential gamma scintigraphic images during the study clearly indicated that the Con-A conjugated EC microspheres remained in the stomach for the study period of 6 h. This might be due to the presence of Con-A coating in EC microspheres which produces mucoadhesion in the epithelial mucous surface of the stomach. Measurable number of counts of $^{99}mT_C$ tagged Con-A conjugated EC microspheres during 6 h study period suggested very good gastroretentive property. In contrast, Pylokit (tablet) showed gastroretention of less than 3 h.

Conclusion

Optimized ethylcellulose microspheres of clarithromycin were successfully prepared using emulsification/evaporation method, and Con-A was successfully attached to the microspheres using carbodiimide method. Attachment of lectin to the ethylcellulose microspheres significantly increased the mucoadhesiveness and also controls the release of clarithromycin in simulated gastric fluids. It is concluded that designed targeted delivery system could possibly treat the colonization of *H. pylori* in an effective manner.

Abbreviations Used

H. pylori, *Helicobactor pylori*; GI, gastrointestinal; GRT, gastric residence time; Con-A, concanavalin-A; EC, ethylcellulose; DCM, dichloromethane; CM, clarithromycin; EDC, 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; PVA, polyvinyl alcohol; UV, ultraviolet; IR, infrared; SEM, scanning electron microscopy; SGF, simulated gastric fluid; ECM, ethylcellulose microspheres; CECM, concanavalin-A conjugated ethylcellulose microspheres.

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