Lectin conjugated gastro-retentive microspheres of amoxicillin for effective treatment of *Helicobacter pylori*

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Helicobacter pylori **mainly exists deep inside the gastric mucosae and adheres to epithelial cells of the stomach. In the present work, concanavalin-A (Con-A) conjugated gastro-retentive microspheres of amoxicillin trihydrate (AMT) were formulated and thoroughly evaluated. Eudragit S100 microspheres were prepared by emulsion solvent diffusion method and characterized for micromeritic properties, per cent drug entrapment, per cent yield, surface morphology, buoyancy behaviour and *in vitro* drug release in simulated gastric fluid. The microspheres were then conjugated with Con-A and conjugation was verified by IR spectroscopy and differential scanning calorimetry. Moreover, Con-A conjugated microspheres were further characterized for zeta potential, muco-adhesiveness to gastric mucosae and Con-A conjugation efficiency. The microparticles were found to be regular and spherical in shape with a size range 106.4–192.4 μm depending on drug to polymer ratio (1 : 1 to 1 : 3). All the microsphere formulations showed noteworthy drug entrapment from 62.3% to 70.2%. *In vitro* floating test clearly showed that most of the microspheres were floating even after 12 h of testing period. Zeta potential of optimized formulation and Con-A conjugated microspheres was found to be 5.6 and 18.7 mV respectively. Attachment of lectin to the Eudragit microspheres significantly increases the muco-adhesiveness (83.7%) compared to non-conjugated microspheres (16.7%) and also controlled the release of drug in simulated GI fluids. The results suggest that these carriers can be used to incorporate other antibiotic agents and could provide better therapeutic effect against *H. pylori* infection.**

Keywords: Amoxicillin, concanavalin-A, *Helicobacter pylori*, microspheres.

HELICOBACTER PYLORI was the first microaerophilic Gram-negative bacteria isolated from the gastric mucosae of gastritis patients[1]. It is a spiral-shaped, highly motile organism with a unipolar flagellum found within and beneath the mucous layer of the stomach and often attached to the gastric mucosae. *H. pylori* enters into the stomach and attaches itself to the lining of the stomach to establish an environment to grow[2]. The microorganism mainly exists deep within the gastric mucus layer, where it adheres to gastric epithelial cells through a variety of adhesion-like proteins[3–5]. In order to effectively exterminate *H. pylori* infection, the therapeutic agent must be able to go through the gastric mucus layer and maintain minimum effective drug levels at the infected site for a suitable length of time in order to show antibacterial activity. Persistent of infection with *H. pylori* always produces gastritis and increases the risk of developing gastric cancer by about 20-fold[6].

Concanavalin-A (Con-A) is the lectin isolated from jack-bean, *Canavalia ensiformis*. It binds specifically to mono-, oligo- and poly-saccharides with terminal non-reducing R-D-mannopyranosyl, R-D-glucopyranosyl or D-fructofuranosyl residues[7]; hence it can be used as a ligand and can also provide mucoadhesive characteristics to the carriers. The protein consists of 237 amino acids and has two metal-binding sites. The binding site for the sugar is adjacent to the metal atoms. Nitrogen atoms from Asp_{14}, Leu_{99}, Tyr_{100}, Asp_{208} and Arg_{228} are involved in fixing the saccharide[8]. Amoxicillin, a broad-spectrum antibiotic, is effective both *in vitro* and *in vivo* against the most important pathogen responsible for peptic ulcer disease caused by *H. pylori*. It is rapidly absorbed after oral administration, stable in acidic atmosphere of the stomach and has a biological half life of 0.7–1.4 h (ref. 9). Amoxicillin has shown better and complete therapeutic eradication against *H. pylori* compared to clarithromycin, other antimicrobial agents and drug combinations with a highest therapeutic success rate up to 90% (in some cases); hence it has been chosen as a candidate drug for the current study[10,11].

Cross-linked *N*-isopropylacrylamide-acrylic acid-hydroxyethyl methacrylate [P(NIPASM-AA-HEM)] hydrogel nanoparticles (NPs) containing amoxicillin were prepared to be used in the treatment of *H. pylori* infection[12]. Arora *et al.*[13] reported chitosan–alginate polyelectrolyte complex (CS–ALG PEC) nanoparticles of amoxicillin for

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complete eradication of *H. pylori*, colonized deep into the gastric mucosal lining. Even though *H. pylori* is highly sensitive to most of the antibiotics, its elimination is not easy even with the best currently available therapy[14,15]. One main reason for the partial eradication of *H. pylori* is probably due to the short residence time of antimicrobial agents in the stomach[16,17]. In most of the cases, conventional dosage form like tablets or capsules are employed for therapy; however, these agents do not stay in the stomach for prolonged period and hence it is difficult to achieve minimum inhibitory concentrations in the gastric mucus where *H. pylori* colonizes. In view of all these facts, amoxicillin has been chosen as a drug candidate in the present study. To overcome all these constraints in *H. pylori* treatment, an attempt is made to develop lectin conjugated multiparticulate delivery system of amoxicillin trihydrate (AMT) to deliver the antimicrobial agent to the target cells.

Materials and methods

AMT was obtained from M/s Ranbaxy Research Laboratories, Gurgaon. Eudragit S100 was obtained as a gift sample from M/s S. Zhaveri and Company Mumbai. Concanavalin-A (Con-A) was supplied as a gift sample by Bio-Research Products, Inc., Iowa, USA. *N*-Hydroxysuccinimide (NHS) was supplied as a gift sample from Shivam Enterprises, Pune. 1-Ethyl-3, 3-(dimethylaminopropyl) carbodiimide (EDC) was procured from HiMedia Laboratories Pvt Ltd, Mumbai. Polyvinyl alcohol (PVA) was obtained from Qualigens Fine Chemicals, Mumbai. Dichloromethane, ethanol and all other chemicals were of analytical reagent grade and used as received.

The *ex vivo* studies were carried out according to 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. Approval was granted by Institutional Animal Ethical Committee of SLT Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur to carry out the study.

Preparation of floating microspheres

Microspheres were prepared by emulsion solvent diffusion method as reported by Kawashima *et al.*[18] with minor modifications. Eudragit S100 was dissolved in 20 ml mixture of ethanol and dichloromethane (1 : 1 ratio) at room temperature. AMT was dispersed in polymer solution; the dispersion was added dropwise to aqueous solution of PVA (0.5% w/v, 200 ml) through a 22G needle at 30–40°C and the resultant o/w type emulsion was stirred on a mechanical stirrer (Macro Scientific Works, New Delhi) at 300 rpm. The finally dispersed droplets of the polymer solution of the drug were solidified in aqueous phase by diffusion of solvent on heating at 40°C for 1 h till dichloromethane evaporated. Resulting microspheres were filtered, washed thrice with distilled water and dried overnight at room temperature.

Drug entrapment and per cent yield

Entrapment efficiency was determined by a method suggested by Whitehead *et al.*[19], with minor modifications. The AMT content in Eudragit microspheres was determined by dispersing 50 mg of microspheres in 20 ml mixture of ethanol and dichloromethane (1 : 1), followed by agitation on a magnetic stirrer for 12 h to dissolve the polymer and extract out the drug. After filtration through Whatman filter paper (#41), the filtrate was analysed spectrophotometrically at 272 nm using ultra-violet (UV) double-beam spectrophotometer (UV 1800 spectrophotometer, Shimadzu, Japan). The experiment was performed in triplicate. Percentage drug entrapment and percentage yield were calculated using the following formula

$$\text{Drug entrapment (\%)} = \frac{\text{Calculated drug content}}{\text{Theoretical drug content}} \times 100,$$

$$\text{Yield (\%)} = \frac{\left(\begin{array}{c}\text{Total weight of}\\ \text{microparticles}\end{array}\right)}{\left(\begin{array}{c}\text{Total weight of drug, polymer}\\ \text{and other nonvolatile}\\ \text{solids (if added)}\end{array}\right)} \times 100.$$

Micromeritic studies

The microspheres were evaluated for micromeritic properties such as particle size, true density, tapped density, compressibility index and flow properties. All the experiments were performed in triplicate. The size was determined using an optical microscope, and the mean particle size was estimated by measuring 200–300 particles with the help of a calibrated ocular micrometer. Tapped density was determined by tapping method and per cent compressibility index was calculated as follows

$$\text{Tapped density} = \frac{\text{Mass of microspheres}}{\text{Volume of microspheres after tapping}},$$

$$\text{\% Compressibility index} = \left[1 - \frac{V}{V_0}\right] \times 100,$$

where V_0 and V are the volumes of the sample before and after the standard tappings respectively.

True density was determined using a helium densitometer (No. 1305, Shimadzu, Japan). Porosity (ε) was calculated using the equation

$$\varepsilon = \left[1 - \frac{P_p}{P_t}\right] \times 100,$$

where P_t and P_p are the true density and tapped density respectively.

Angle of repose (θ) of the microspheres was determined by the fixed funnel method and calculated as

$$\tan\theta = \frac{2H}{D},$$

where H and D are the free standing height and diameter of the microsphere heap respectively, which is the result of free flow of microspheres through a glass funnel on a graph paper.

Floating behaviour studies

Percentage buoyancy was calculated by a method suggested by Jain *et al.*[20]. Briefly, 50 mg of the floating microspheres was stirred at 100 rpm on a magnetic stirrer in 100 ml of the simulated gastric fluid (SGF; pH 1.2) containing 0.02% w/v Tween 20. After 12 h, the fraction of buoyant microspheres was pipetted out and estranged by filtration, and the shrunken fraction was collected. Both the fractions of microspheres were dried in desiccators until constant weight and weight was measured. Buoyancy was determined by the weight ratio of floating particles to the sum of floating and sinking particles. The experiment was carried out in triplicate and percentage of buoyancy was determined using the following formula

$$\text{Buoyancy (\%)} = \frac{W_f}{\left(W_f + W_S\right)} \times 100,$$

where W_f and W_S are the weights of the floating and shrunken microspheres respectively.

Conjugation of Con-A with microspheres and per cent conjugation efficiency

The conjugation procedure to be used for covalent attachment depends on functional groups present on the carrier surface[21]. Lectin coupling was carried out using carbodiimide technique reported by Olde Damink *et al.*[22]. Briefly, surface activation of carboxyl group was achieved by addition of 4 ml of 0.1 M EDC and 4 ml of NHS to 200 mg of microspheres in phosphate buffer (pH 5.8). After 3 h incubation at room temperature, excess coupling reagent was removed by washing the microspheres with phosphate buffer (pH 5.8). Con-A solution (10 ml) in phosphate buffer (pH 5.8) was added to the suspension and incubated overnight. Con-A conjugated microspheres were obtained by filtration through Whatman filter paper (# 41), followed by 3–4 washing with distilled water. Finally, Con-A conjugated microspheres of amoxicillin trihydrate (CMAMT) were dried overnight at room temperature.

The amount of Con-A bound to the microspheres was calculated by determining the amount of Con-A added initially and that recovered after incubation with the microspheres. The quantity of lectin in the supernatant was estimated using Lowry's method for protein estimation.

Morphology

The external and internal morphology of the microspheres (AMT3b and CMAMT) was studied by scanning electron microscopy (SEM; JEOL, JSM-6390, Japan). All microsphere formulations were sprinkled on a double adhesive tape, which was previously stuck to an aluminum stub. The stubs were coated with gold up to a thickness of about 300 Å under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Prepared samples were randomly scanned and photomicrographs were obtained using electron microscope.

FTIR spectroscopy and differential scanning calorimetry

Conjugation of Con-A with Eudragit microspheres was confirmed using Fourier transform infrared (FTIR) spectroscopy. The KBr discs of AMT3b and CMAMT were prepared and scanned on IR spectrophotometer (IR-Prestige 21 FTIR Spectrometer, Shimadzu, Japan). Differential scanning calorimetry (DSC) studies were conducted for the optimized formulation, CMAMT, pure drug, polymer, Con-A, EDC and NHS (EDC and NHS are coupling agents), using a DSC instrument (Zade DSC, Pyris 6 DSC). Samples of 2–6 mg were placed in aluminum pans (Al-Crucibles, 40 Al) and sealed. The probes were heated from 30–350°C at a rate of 10°C/min under nitrogen atmosphere.

In vitro drug release study, statistical treatment and data analysis

The release rate of amoxicillin from AMT3b and CMAMT was determined using a USP type-II paddle-type dissolution apparatus. Hard gelatin capsule (# 3) filled with an accurately weighed quantity of microspheres, equivalent to 100 mg of drug, was placed in the basket-type dissolution test apparatus. SGF (pH 1.2,

900 ml) containing 0.02% w/v Tween 20 was used as the dissolution medium and maintained at 37 ± 0.5°C at 100 rpm. Samples (5 ml) were withdrawn at predetermined time intervals for 24 h and the same volume of fresh medium was replaced. The samples were filtered through Whatman filter paper (# 41) and analysed using a UV double-beam spectrophotometer at 272 nm against blank after suitable dilution. The *in vitro* drug release study of marketed dosage form of AMT (MOX-P-250 mg, tablet) was also carried out following the same procedure. All experiments were conducted in triplicate.

In vitro drug release of AMT from AMT3b, CMAMT and marketed formulation (MF) of amoxicillin trihydrate (MOX-P) were statistically treated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post test using software program (GraphPad-Instat Software Inc., San Diego). A probability level of $P < 0.05$ was considered to be significant.

Five kinetic models, including the zero-order, first-order, Higuchi matrix, Peppas–Korsmeyer and Hixson–Crowell release equations were applied to interpret the *in vitro* release data in order to find out the best fit equation using PCP Disso v3 software[23,24].

Mucoadhesion

Everted sac experiment[25] was carried out in order to determine percentage of mucoadhesion using rat stomach for selected microspheres (AMT3b) and CMAMT. Male albino rats (400 ± 20 g) were fasted overnight and sacrificed the next day to isolate the stomach. The stomach was cut into pieces of 2 cm length and 1 cm width and the pieces were rinsed with 2 ml physiological saline. One hundred microspheres of each formulation were sprinkled evenly on the gastric mucosae. The mucosae with the microspheres were incubated in a humidity chamber maintained at 93% relative humidity and room temperature. After 20 min incubation, the tissues were placed on a polyethylene support at an angle of 45° and rinsed with pH 1.3 physiological saline solution for 5 min at a rate of 22 ml/min. The remaining microspheres on the surface of the gastric mucosae were counted and percentage of binding was calculated using the following formula. The experiment was performed in triplicate.

$$\text{Binding (\%)} = \frac{\left(\begin{array}{c}\text{Initial weight of microspheres} - \\ \text{weight of unbound microspheres}\end{array}\right)}{\text{Initial weight of microspheres}} \times 100.$$

Zeta potential determination

Zeta potential was measured by electrophoresis using Malvern Zetasizer (UK). The microspheres were suspended in distilled water by ultrasonication for 30 min. The cell was filled with the required quantity of microsphere suspension and inserted with its integral gold electrodes close to the lid into the Zetasizer.

Results and discussion

The aim of the drug treatment is complete eradication of *H. pylori* from the foregut. There are two opportunities for improvement of *H. pylori* therapy: site-specific drug delivery carriers and gastro-retentive drug delivery systems. A greater eradication rate of around 90% compared to clarithromycin and other antibiotics prompted us to study the amoxicillin carriers for prolonged GI retention. In the present work, a two-pronged approach of floating carriers (gastro-retentive formulations) and bioadhesive (plugging and sealing effect) systems has been studied.

Preparation of conjugated microspheres

Floating microspheres were prepared by modified solvent diffusion method and optimized for various drug–polymer ratios at different stirring rates. To optimize drug–polymer ratio, the amount of drug was kept constant and the polymer ratio was varied, i.e. 1 : 1, 1 : 2 and 1 : 3. All these formulations were prepared at different stirring speeds, viz. 100, 300, 500 rpm (total nine formulations). Chemical reaction between Eudragit S100 and Con-A for conjugation is shown in Figure 1. EDC by itself is not particularly efficient in crosslinking because failure to react rapidly with an amine results in hydrolysis and regeneration of the carboxyl moiety. EDC reacts with a carboxyl group on molecule #1, forming an amine-

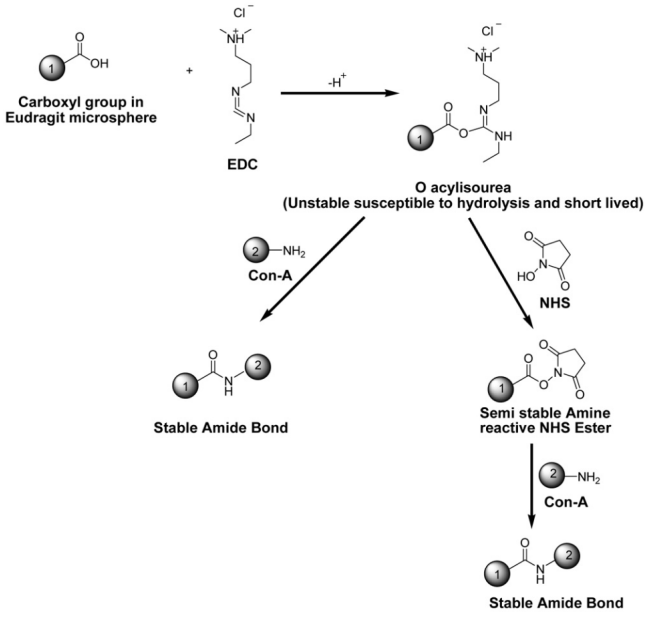

Figure 1. Schematic diagram of chemical reaction for lectin conjugation with Eudragit microspheres.

Table 1. Formulation composition, variables, per cent yield and per cent drug entrapment of various formulations

Formulation code	Formulation variables		Yield (%)	Drug entrapment (%)
	Drug : polymer ratio	Stirring rate (rpm)		
AMT1a	1 :1	100	72.18 ± 2.30	63.29 ± 0.08
AMT1b		300	77.78 ± 2.43	65.81 ± 0.32
AMT1c		500	72.23 ± 2.14	62.31 ± 0.18
AMT2a	1 :2	100	71.82 ± 1.38	67.15 ± 0.24
AMT2b		300	77.56 ± 1.76	69.40 ± 0.27
AMT2c		500	70.64 ± 2.52	66.26 ± 0.15
AMT3a	1 :3	100	72.21 ± 2.83	65.94 ± 0.31
AMT3b		300	80.22 ± 3.13	70.22 ± 0.14
AMT3c		500	69.63 ± 1.77	65.24 ± 0.40

Values are average of readings (mean ± SD; $n = 3$); AMT, Amoxicillin trihydrate.

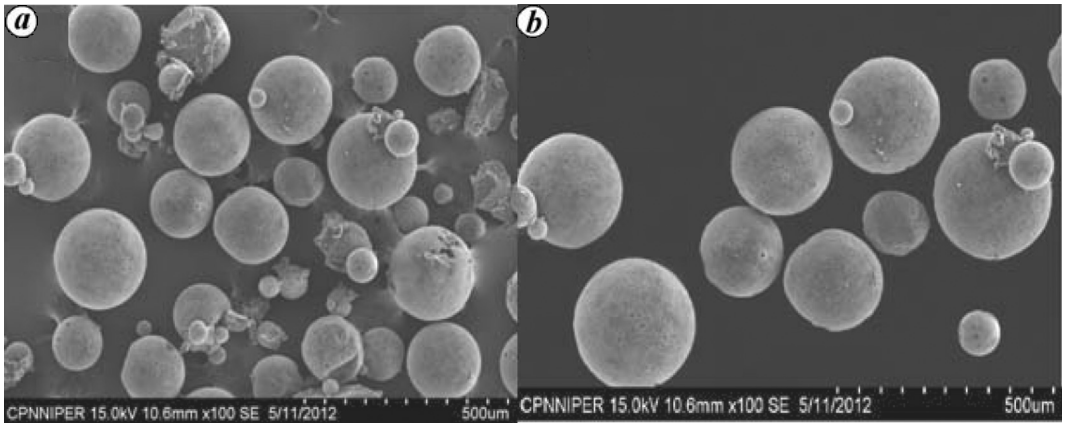

Figure 2. SEM images of (***a***) Eudragit microspheres and (***b***) Lectin conjugated Eudragit microspheres.

reactive *O*-acylisourea intermediate. This intermediate may react with an amine on molecule #2, yielding a conjugate of the two molecules attached through a stable amide bond. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. The addition of NHS stabilizes the amine-reactive intermediate by converting it to an amine-reactive NHS ester, hence increasing the efficiency of EDC-mediated coupling reactions[26,27]. Thus, amine-reactive NHS ester intermediate has sufficient stability to permit two-step crosslinking procedures, which allow the carboxyl groups on protein #2 to remain unaltered.

Morphology

The SEM images of both AMT3b and CMAMT microsphere showed a nearly spherical shape with a smooth and rigid uniform surface (Figure 2). Formulation shows that some of the microspheres have depressions on the surface which might be due to evaporation of dichloromethane. The results were similar to those reported by Gupta and Pathak[28], who had observed numerous depressions on famotidine microballoons prepared by solvent evaporation diffusion method.

Per cent yield, drug entrapment efficiency and buoyancy behaviour

Percentage yield of the prepared formulations was found in the range 69.63 ± 1.8–80.22 ± 3.1, whereas drug entrapment efficiency (DEE) was found to be in the range 62.31 ± 0.18–70.22 ± 0.14% (Table 1). Upon increasing polymer concentration, an increase in encapsulation efficiency was observed, which may be due to highly dense internal structure of polymer matrix, increased bonding and encapsulation of drug particles into the microspheres.

Variable data of buoyancy (%), ranging from 42.34 to 84.76 were found for the developed formulations (Figure 3). The *in vitro* floating test clearly showed that most of the microballoons were floating even after 12 h of testing period because of their low densities. The microballoons with higher concentration of polymer were floating longer than those with lower concentration of polymer. This may be attributed to a decrease in density of microballoons with an increase in polymer concentration.

Micromeritic properties

The average particle size of microspheres was found to be in the size range 106.39 ± 1.74–192.43 ± 2.64 μm which

Table 2. Micromeritic studies of prepared formulations

Formulation code	Particle size (μm)	Tapped density (g cm^{-3})	Apparent density (g cm^{-3})	True density (g cm^{-3})	Porosity (%)	Compressibility index (%)	Angle of repose (θ)
AMT1a	113.48 ± 3.15	0.68 ± 0.01	0.53 ± 0.04	0.73 ± 0.08	34.62 ± 2.11	23.24 ± 0.99	27.25 ± 0.50
AMT1b	110.26 ± 1.45	0.74 ± 0.02	0.55 ± 0.02	0.74 ± 0.02	29.98 ± 0.45	20.43 ± 0.82	28.21 ± 0.84
AMT1c	106.39 ± 1.74	0.76 ± 0.01	0.59 ± 0.01	0.76 ± 0.02	25.94 ± 1.48	18.33 ± 0.69	28.92 ± 0.65
AMT2a	145.90 ± 0.98	0.76 ± 0.02	0.61 ± 0.01	0.78 ± 0.03	33.32 ± 0.73	18.99 ± 1.44	26.90 ± 0.48
AMT2b	141.61 ± 2.31	0.81 ± 0.02	0.63 ± 0.02	0.80 ± 0.08	30.31 ± 2.25	16.86 ± 1.18	26.73 ± 0.48
AMT2c	137.12 ± 4.23	0.85 ± 0.02	0.64 ± 0.01	0.84 ± 0.04	28.38 ± 0.72	16.91 ± 0.87	24.87 ± 0.60
AMT3a	192.43 ± 2.64	0.88 ± 0.01	0.67 ± 0.02	0.90 ± 0.02	28.90 ± 0.51	16.09 ± 0.20	23.73 ± 0.34
AMT3b	188.18 ± 2.46	0.93 ± 0.01	0.68 ± 0.03	0.93 ± 0.02	24.75 ± 0.65	15.30 ± 0.55	24.89 ± 0.40
AMT3c	182.19 ± 3.87	0.96 ± 0.01	0.70 ± 0.04	0.96 ± 0.05	26.19 ± 0.27	17.52 ± 0.76	25.36 ± 0.68

Values are average of readings (mean ± SD; $n = 3$).

Table 3. One-way ANOVA (Dunnett's multiple comparison) test for *in vitro* release of AMT in SGF (pH 1.2)

Comparison	Mean difference	*Q*	*P* value
MF vs AMT3b	30.764	2.264^{ns}	> 0.05
MF vs CMAMT	36.607	2.694*	< 0.05

Q, Parameter obtained with *P* when performing ANOVA. ^{ns}Non significant. *Significant. MF, Marketed formulation of AMT; AMT3b, Microspheres of AMT; CMAMT, Conjugated microspheres of AMT.

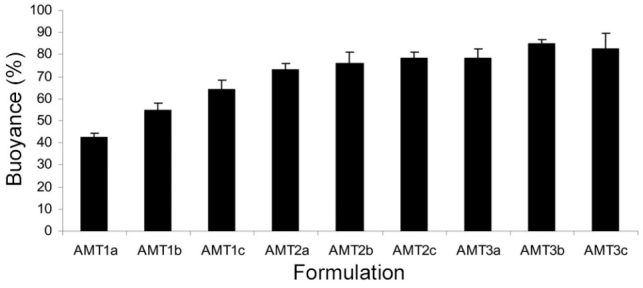

Figure 3. Per cent buoyancy behaviour of various prepared formulations in simulated gastri fluid pH 1.2 after 12 h of testing period. Values are mean ± s.d. ($n = 3$).

increases on increasing polymer concentration from 1 : 1 to 1 : 3 (Table 2). It is obvious that rpm affects the yield and size distribution of the microsphere. As the rpm increased from 100 to 500, the average particle size decreased. The size of Con-A conjugated formulation (237.32 ± 1.2 μm) was higher compared to non-conjugated microspheres (188.18 ± 2.46 μm), which may be due to the presence of lectin coating on the formulation.

Optimum speed for the system was found to be 300 rpm based on the result of particle size and loading efficiency. Below 300 rpm, shear force was insufficient to form stable emulsion droplets; consequently larger droplets were formed and aggregated eventually. Above 500 rpm, consequently smaller droplets were formed (Table 2). Also, the average particle size of floating microspheres increases as the amount of polymer increases, which is due to the fact that increase in polymer concentration increases solution viscosity, resulting in larger particles. Similar findings were reported earlier[29], where the authors demonstrated that an increase in concentration of polymer shows increase in the mean particle size of floating microspheres.

The apparent density is found between 0.53 ± 0.004 and 0.70 ± 0.008 g cm^{-3}, while the tapped density ranged from 0.68 ± 0.007 to 0.96 ± 0.003 g cm^{-3}. Obviously, these values are less than the density of SGF (i.e. 1.004 g cm^{-3}), thereby implying that the microspheres will have the propensity to exhibit an excellent buoyancy effect *in vivo*[30]. Compressibility index of the microspheres ranged between 15.30 ± 0.55% and 23.24 ± 0.99%, suggesting good flow characteristics of the microspheres[31]. The per cent porosity of all prepared formulations was in the 24.75 ± 0.65–34.61 ± 2.10 range. All formulations showed excellent flow ability as expressed in terms of angle of repose ranging from 23.72 ± 0.34 to 28.92 ± 0.65 (Table 2). In a nutshell, results of all these micromeritic properties (tapped density, porosity and compressibility) suggest that these buoyant mucoadhesive microspheres are ideal candidates for direct compression of tablets.

Confirmation of Con-A coupling to Eudragit microspheres by FTIR

Coupling of Con-A and Eudragit microspheres depends on the amide bond formation between NH_2 group of Con-A and COOH group of Eudragit S100. IR spectrum of CMAMT showed peaks corresponding to amide groups (3451.29, 1671.57, 1651.09, 800-600, etc.), suggesting the presence of amide group in the formulation, whereas these peaks were absent in the IR spectrum of Eudragit microspheres AMT3b (Figure 4).

Differential scanning calorimetry

DSC thermogram of pure drug (AMT) showed a sharp endothermic peak at 125.01°C. Eudragit S100 showed a

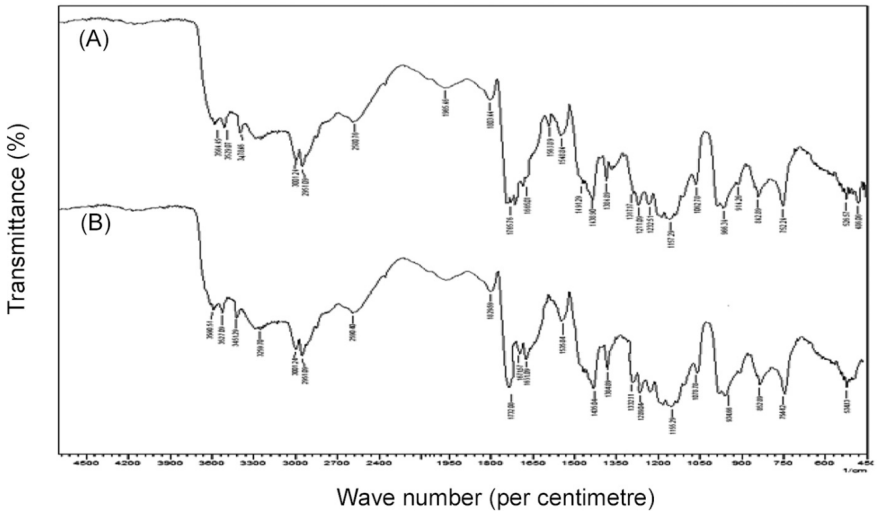

Figure 4. Comparison between FTIR spectra of formulations (A) AMT3b and (B) CMAMT.

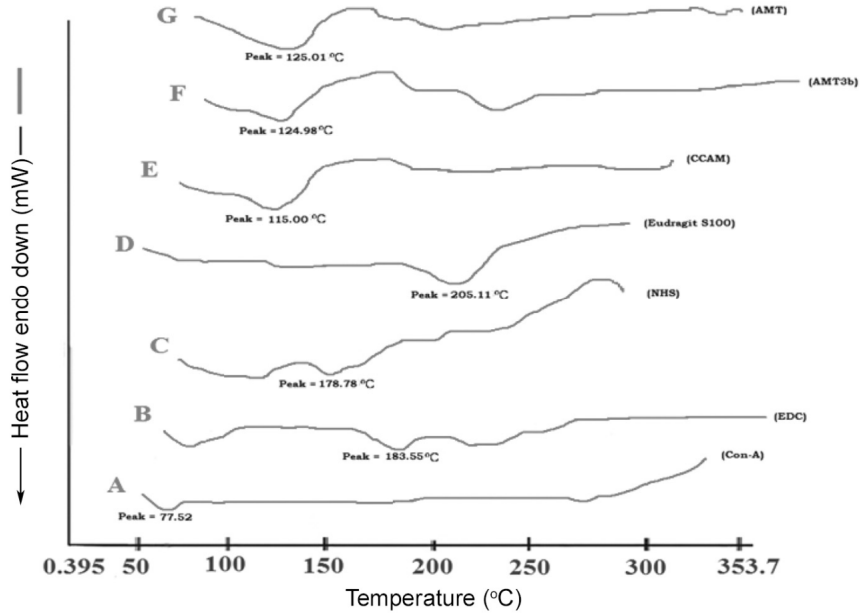

Figure 5. DSC thermogram of (A) Con-A, (B) 1-ethyl-3, 3-(dimethylaminopropyl) carbodiimide (EDC), (C) *N*-hydroxysuccinimide (NHS), (D) Eudragit S 100, (E) Con-A conjugated microspheres (CMAMT), (F) Optimized formulation AMT3b and (G) Amoxicillin trihydrate (AMT).

broad transition from 205.11°C to 232.56°C. Con-A, EDC and NHS showed an endothermic peak at 77.52°C, 183.55°C, 178.78°C respectively (Figure 5). DSC thermogram of non-conjugated microspheres (AMT3b) showed an endothermic peak at 124.98°C, suggesting that there was no interaction between drug and polymer. A slight variation in melting endotherm of CMAMT formulation showed an endothermic peak at 115°C, suggesting little interaction between optimized formulation and Con-A. Absence of EDC and NHS peaks in DSC thermogram of optimized formulation CMAMT confirms the absence of EDC and NHS in the formulation.

In vitro drug release study

The release profile of drug was studied in SGF (pH 1.2) as dissolution medium to simulate the gastric pH conditions. Gradual release of AMT was seen up to 24 h from all the formulations. At the end of the release study, AMT release from all formulations was found to be more than 75%; however, AMT3b formulation, which has the highest concentration of polymer, showed slightly lower release of 74.64 ± 0.73%. The highest cumulative per cent drug release was observed from AMT1a (84.78 ± 0.47%), the formulation with lowest concentration of polymer

(Figure 6). Eudragit S100 is insoluble in acidic medium and also exhibits low permeability[32]. Faster release rate was obtained with smaller devices, which possess larger specific area exposed to dissolution medium resulting in faster drug release (AMT1a) compared to larger particles (AMT3b).

In vitro drug release study of CMAMT was also performed to compare its release profile with optimized formulation (AMT3b) and MF MOX-P. The comparison of dissolution rates revealed that about 50% of drug was released in 4 h from MF, while AMT3b and CMAMT had taken more than 6 h for the same degree of release

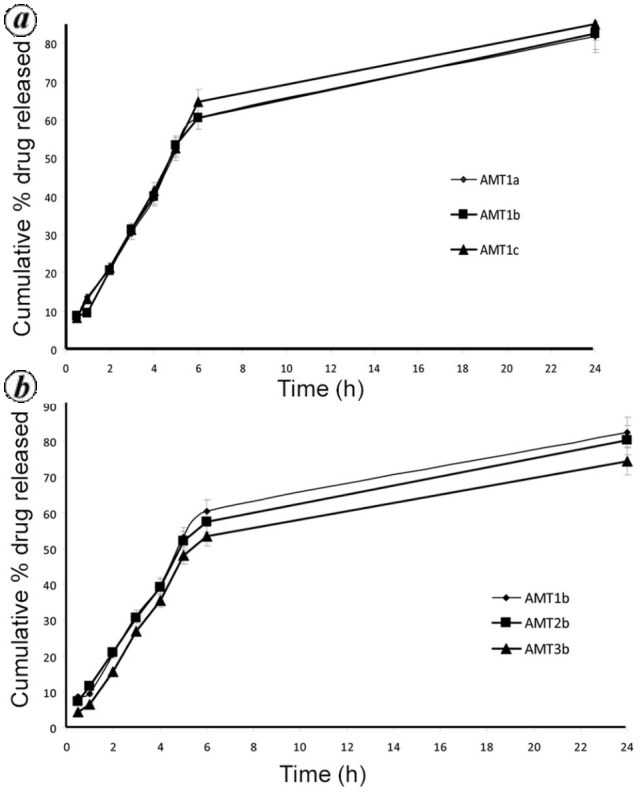

Figure 6. *In vitro* drug release profile of AMT from different formulations in simulated gastric fluid (pH 1.2) at 37°C. ***a***, Effect of stirring speed on *in vitro* drug release profile. ***b***, Effect of polymer concentration on *in vitro* drug release profile. Values are mean ± SD ($n = 3$).

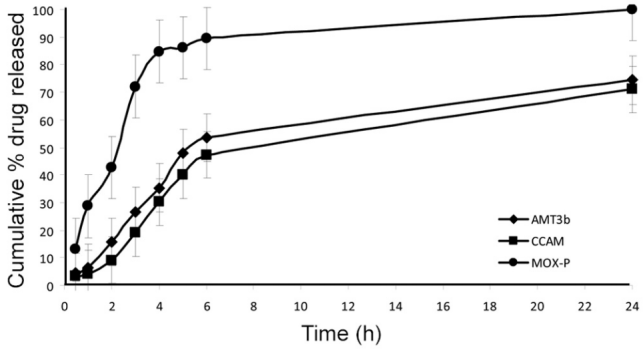

Figure 7. Comparative cumulative per cent drug release profile of AMT3b, CMAMT and MF in simulated gastric fluid (pH 1.2) at 37°C. Values are mean ± SD ($n = 3$).

(Figure 7). Hence, it is clear that Con-A coupling to microspheres sustained drug release compared to non-conjugated microspheres. The one-way ANOVA (Dunnett's multiple comparison) test shows that Con-A coupling with the formulations exhibits significant difference ($P < 0.05$) in drug release compared to MF (Table 3).

The correlation coefficient of all formulations was found to be higher (≥ 0.9) with Higuchi matrix model compared to other models. The value of n was obtained from 0.389 to 0.449, which is > 0.5, suggesting that the mechanism of drug release followed the pathway of diffusion through matrix[33]. It is concluded that all the formulations followed a Fickian release mechanism, i.e. release was governed by Higuchi kinetics indicating diffusion as a predominant mechanism of drug release (Table 4).

Overall, it could be concluded that formulation AMT3b (drug polymer ratio 1 : 3; and stirring rate 300 rpm) showed highest yield 80.22 ± 3.1%, drug entrapment 70.22 ± 0.44%, buoyancy 84.76%, average particle size 188.18 ± 2.46 µm and also controlled *in vitro* release profile compared to other prepared formulations. Hence, it was selected for conjugation with Con-A and later studies.

Con-A conjugation efficiency, percentage mucoadhesive study and zeta potential determination

Folin–Ciocalteu reagent was used to determine the amount of Con-A bound to the Eudragit microspheres containing AMT and per cent conjugation efficiency of CMAMT was found to be 78.26 ± 0.98. Everted sac method was used to test mucoadhesive property of Eudragit microspheres (AMT3b) and Con-A conjugated microspheres (CMAMT; Figure 8). Results indicate that formulation AMT3b showed minimum mucoadhesion (16.66 ± 1.66%), whereas maximum mucoadhesion (83.71 ± 1.039%) was shown by Con-A conjugated microspheres (Figure 8 *a*). The one way ANOVA study showed that Con-A coupling to AMT3b significantly increased per cent mucoadhesion ($P < 0.05$), which may be due to affinity of Con-A towards glycoproteins of the mucus membrane of the stomach.

Zeta potential of AMT3b and CMAMT was 5.57 ± 0.47 and 18.7 ± 0.38 mV respectively. Lectin coating to Eudragit microspheres increased zeta potential towards positivity. The positively charged particles interact strongly with the negatively charged mucus membrane in the stomach because of electrostatic interactions and prolonged the gastric residence time. Also, a previous report demonstrated that zeta potential of gliadin nanoparticles increased due to lectin coating[34]. In accordance with earlier reports[35,36], sustained release of amoxicillin for a longer period of time along with a boost of prolonged

Table 4. Comparison of different dissolution kinetics models for release of AMT from different formulations in SGF (pH 1.2)

	Zero-order	First-order	Higuchi matrix	Hixson–Crowell	Peppas–Korsmeyer	
Formulation code	*r*	*r*	*r*	*r*	*r*	*n*
AMT1a	0.723	0.882	0.904	0.723	0.836	0.389
AMT1b	0.718	0.881	0.893	0.718	0.834	0.418
AMT1c	0.726	0.887	0.898	0.726	0.835	0.435
AMT2a	0.729	0.891	0.900	0.729	0.834	0.415
AMT2b	0.736	0.896	0.905	0.736	0.836	0.422
AMT2c	0.743	0.902	0.907	0.743	0.834	0.438
AMT3a	0.736	0.895	0.898	0.736	0.828	0.425
AMT3b	0.722	0.874	0.886	0.722	0.822	0.428
AMT3c	0.747	0.901	0.902	0.747	0.825	0.449

r, Correlation coefficient; *n*, Diffusional exponent.

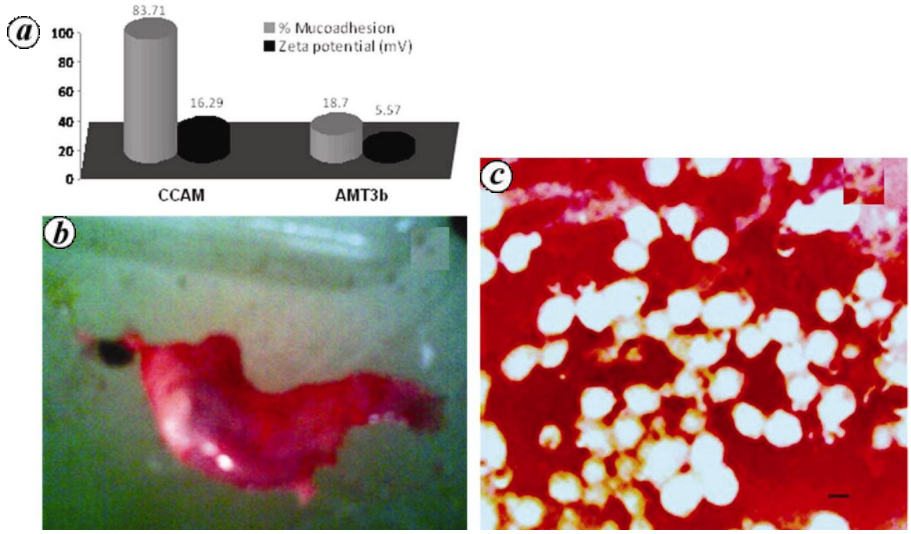

Figure 8. ***a***, Mucoadhesion (%) and zeta potential (mv) of AMT3b and CCAM; ***b***, Photograph of rat stomach. ***c***, Photograph of microspheres spreading on rat stomach (scale bar = 100 μm).

residence time as well as floating behaviour of formulation might help in timely and complete eradication of *H. pylori* infection.

Conclusion and future perspectives

Con-A conjugated Eudragit microspheres of amoxicillin were successfully prepared using modified emulsion solvent diffusion method. Attachment of lectin (Con-A) to the Eudragit microspheres through carbodiimide-mediated conjugation significantly enhanced the mucoadhesiveness and also controlled amoxicillin release. Preliminary results from this study suggest that these Con-A conjugated Eudragit microspheres can be used to incorporate antibiotic drugs and could be effective against infection caused by *H. pylori*. Outcomes of micromeritic studies of developed floating microspheres suggest that the proposed formulation is a suitable candidate for direct compression of fast disintegrating tablets. Such developed formulations could be subjected to *in vivo* studies in future for complete eradication of *H. pylori* infection.

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ACKNOWLEDGEMENTS. The authors gratefully acknowledge M/S Ranbaxy Research Laboratories, Gurgaon, India, for the supply of Amoxicillin trihydrate as a gift sample.

Received 1 April 2013; revised accepted 28 October 2013