

Stomach Specific Anti-Helicobacter Pylori Therapy: Preparation and Evaluation of Amoxicillin-Loaded Chitosan Mucoadhesive Microspheres

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Abstract: The purpose of this research was to formulate and systematically evaluate *in vitro* and *in vivo* performances of mucoadhesive amoxicillin microspheres for the potential use of treating gastric and duodenal ulcers, which were associated with *Helicobacter pylori*. Amoxicillin mucoadhesive microspheres containing chitosan as mucoadhesive polymer were prepared by simple emulsification phase separation technique using glutaraldehyde as a cross-linking agent. Results of preliminary trials indicate that volume of cross-linking agent, time for cross-linking, polymer-to-drug ratio, and speed of rotation affected characteristics of microspheres. Microspheres were discrete, spherical, free flowing and also showed high percentage drug entrapment efficiency. *In vitro* mucoadhesive test showed that amoxicillin mucoadhesive microspheres adhered more strongly to gastric mucous layer and could retain in gastrointestinal tract for an extended period of time. A 3² full factorial design was employed to study the effect of independent variables, polymer-to-drug ratio (X_1), and stirring speed (X_2) on dependent variables i.e. percentage mucoadhesion, t_{80} , drug entrapment efficiency, particle size and swelling index. The best batch exhibited a high drug entrapment efficiency of 70 % and a swelling index of 1.39; percentage mucoadhesion after 1 h was 79 %. The drug release was also sustained for more than 12 h. The polymer-to-drug ratio had a more significant effect on the dependent variables. The morphological characteristics of the mucoadhesive microspheres were studied using scanning electron microscopy. *In vitro* release test showed that amoxicillin released slightly faster in pH 1.0 hydrochloric acid than in pH 7.8 phosphate buffer. *In vivo* *H. pylori* clearance tests were also carried out by administering amoxicillin mucoadhesive microspheres and powder, to *H. pylori* infectious Wistar rats under fed conditions at single dose or multiple dose(s) in oral administration. The results showed that amoxicillin mucoadhesive microspheres had a better clearance effect than amoxicillin powder. In conclusion, the prolonged gastrointestinal residence time and enhanced amoxicillin stability resulting from the mucoadhesive microspheres of amoxicillin might make contribution complete eradication of *H. pylori*.

INTRODUCTION

Microsphere carrier systems made from the naturally occurring biodegradable polymers have attracted considerable attention for several years in sustained drug delivery. Recently, dosage forms that can precisely control the release rates and target drugs to a specific body site have made an enormous impact in the formulation and development of novel drug delivery systems. Microspheres form an important part of such novel drug delivery systems [1-3]. They have varied applications and are prepared using assorted polymers [4]. However, the success of these microspheres is limited owing to their short residence time at the site of absorption. It would, therefore, be advantageous to have means for providing an intimate contact of the drug delivery system with the absorbing membranes [5-8]. This can be achieved by coupling bioadhesion characteristics to microspheres and developing bioadhesive microspheres. Bioadhesive microspheres have advantages such as efficient absorption and enhanced bioavailability of drugs owing to a high surface-to-volume ratio, a much more intimate contact with the mucus layer, and specific targeting of drugs to the absorption site

[9-12]. Chitosan (obtained by deacetylation of chitin) is a cationic polymer that has been proposed for use in microsphere systems by a number of authors [13-17]. Chitosan was selected as a polymer in the preparation of mucoadhesive microspheres because of its good mucoadhesive and biodegradable properties.

In a relatively short time span, *Helicobacter pylori* (*H. pylori*) have become recognized as a major gastric pathogen with world-wide distribution. *H. pylori* are spiral-shaped bacterium found in the stomach, which (along with acid secretion) damages stomach and duodenal tissue, causing inflammation and peptic ulcers. *H. pylori*, a prevalent human-specific pathogen, is a causative agent in chronic gastritis [18], gastric and duodenal ulcers [19], and gastric adenocarcinoma [20], one of the most common forms of cancer in humans. Epidemiological, laboratory, and interventional human studies strongly suggest that *H. pylori* play a pathogenic role in the development of adenocarcinoma of the distal stomach [21]. The mechanisms by which *H. pylori* may cause gastroduodenal disease and contribute to gastric carcinogenesis are still hypothetical. However, the production of specific virulence factors by the bacterium, the inflammatory response of the host, and the association with environmental contributors may all be responsible [22].

Treatment regimens for *H. pylori* infection have been evolving since the early 1990s, when monotherapy was first

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recommended. Antimicrobial therapy for this infection is a complex issue, and the following drugs are currently used in combination regimens: proton-pump inhibitors and/or bismuth, metronidazole, clarithromycin, and amoxicillin [23]. Tetracycline is used in the rescue therapy [24]. Although optimal first-line treatment is associated with high cure rates, the rising prevalence of resistance to the antibiotic component of current eradication regimens increasingly threatens to compromise the efficacy of these regimens. Strains resistant to metronidazole [25] and clarithromycin [26] have been well documented, while resistance to amoxicillin [27] and tetracycline was mainly reported in Asia [28]. Therapeutic regimens directed against *H. pylori* infection will continue to evolve. What is required is a simpler and more efficacious strategy for the treatment of *H. pylori* infection. *H. pylori* is susceptible to many antibiotics *in vitro* but has proved difficult to eradicate (to root out) *in vivo*.

Amoxicillin (α -amino-hydroxybenzylpenicillin) is a semisynthetic, orally absorbed, broad-spectrum antibiotic. It is now widely used in a standard eradication treatment of gastric and duodenal ulcers, which are associated with *H. pylori* infection combined with a second antibiotic and an acid-suppressing agent [29-31]. These triple therapies are provided to be effective in clinical application. However, some other reports and clinical trials indicate that the therapies cannot bring out complete eradication of *H. pylori* and suggest that the therapeutic effect needs more investigation [32-33]. One reason for the incomplete eradication of *H. pylori* is probably due to short residence time of dosage form in the stomach so that effective antimicrobial concentration cannot be achieved in gastric mucous layer or epithelial cell surfaces where *H. pylori* exist [34-35]. The other may be the degradation of amoxicillin in gastric acid [36-37]. Therefore, some researchers have prepared and reported new amoxicillin formulations such as floating and mucoadhesive tablets, pH-sensitive excipient composition microspheres, etc., which were able to reside in the gastrointestinal tract for an extended period of time for a more effective *H. pylori* eradication [38-39].

In context of the above principles, a strong need was felt to develop a dosage form that delivered amoxicillin in the stomach and would increase the efficiency of the drug, providing sustained action. Thus, an attempt was made in the present investigation to use chitosan as a mucoadhesive polymer and prepare mucoadhesive amoxicillin microspheres. The microspheres were characterized by *in vitro* and *in vivo* tests and factorial design was used to optimize the variables.

MATERIALS AND METHODS

Materials

Amoxicillin (powder) was obtained as gift sample from Zydus Cadila (Ahmedabad, India). Chitosan (degree of deacetylation of 85%; intrinsic viscosity, 1390 mL/g in 0.30 M acetic acid/0.2 M sodium acetate solution; and viscometric molecular weight, 4.08×10^5 Da) was obtained as gift sample from Central Institute of Fisheries Technology (Cochin, India). Dioctyl sodium sulfosuccinate (DOSS) and petroleum ether 80:20 were procured from Willson Lab

(Mumbai, India) and S. D. Fine Chemicals Ltd (Mumbai, India), respectively. Liquid paraffin and glutaraldehyde were purchased from Loba Chemie Pvt Ltd (Mumbai, India). Wistar rats (300±50 g) were obtained as gift sample from Zydus Cadila (Ahmedabad, India). Skirrow's medium was purchased from Himedia Ltd. (Mumbai, India).

METHODS

Preparation of Mucoadhesive Amoxicillin Microspheres

Mucoadhesive microspheres of chitosan containing amoxicillin were prepared by simple emulsification phase separation technique. Chitosan, used as a polymer was cross-linked using glutaraldehyde as per method described by Thanoo *et al.* [13].

Chitosan (900 mg) was dissolved in 90 mL of 1 % v/v aqueous acetic acid solution. Three hundred milligrams of drug was dispersed in the polymer solution. In preliminary trial batches the polymer-to-drug ratio was kept constant at 3:1. The resultant mixture was extruded through a syringe (No. 20) in 900 mL of liquid paraffin (heavy and light, 1:1 ratio) containing 0.2 % w/v DOSS and stirring was carried out using a propeller stirrer (Remi, Mumbai, India) at 1000 rpm. After 15 min, glutaraldehyde (25 % v/v aqueous solution) was added and stirring was continued. The amount of cross-linking agent and cross-linking time were varied in preliminary trial batches from 5 to 50 mL and 1 to 3 h respectively. In factorial design batches B1 to B9, 40 mL glutaraldehyde was used as a cross-linking agent and cross-linking time was kept to 1 h. The polymer-to-drug ratio and stirring speed were varied in batches B1 to B9 as shown in Table 1. All other variables were similar as per preliminary trial batches. Microspheres thus, obtained were filtered and washed several times with petroleum ether (80:20) to remove traces of oil. They were finally washed with water to remove excess of glutaraldehyde. The microspheres were then dried at room temperature (25 °C and 60 % RH) for 24 hours. The effect of formulation variables on characteristics of the microspheres of factorial design batches is summarized in Table 1.

OPTIMIZATION OF MICROSPHERES FORMULATION USING 3² FULL FACTORIAL DESIGN

A statistical model incorporating interactive and polynomial terms was utilized to evaluate the responses.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$$

Where, Y is the dependent variable, b_0 is the arithmetic mean response of the nine runs, and b_i is the estimated coefficient for the factor X_i . The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X_1X_2) show how the response changes when two factors are simultaneously changed. The polynomial terms (X_1^2 and X_2^2) are included to investigate non-linearity. On the basis of the preliminary trials a 3² full factorial design was employed to study the effect of independent variables i.e. polymer-to-drug ratio (X_1) and the stirring speed (X_2) on dependent variables % mucoadhesion, the time required for 80 % drug dissolution (t_{80}), drug entrapment efficiency, particle size and swelling index.

Table 1. Amoxicillin Microspheres Batches using 3² Full Factorial Design Layout

Batch code	Variable levels in coded form		<i>In vitro</i> wash-off test (% mucoadhesion after 1 h)	t ₈₀ (min)	Drug entrapment efficiency (%)	Swelling index	Particle size (μm)
	X ₁	X ₂					
B1	-1	-1	53	225	45	0.859	62.2
B2	-1	0	46	223	42	0.808	58.6
B3	-1	1	43	211	38	0.796	50.4
B4	0	-1	75	196	65	1.164	68.3
B5	0	0	67	228	62	1.109	65.4
B6	0	1	60	241	58	0.971	62.6
B7	1	-1	79	465	70	1.390	98.2
B8	1	0	72	447	68	1.271	90.6
B9	1	1	64	371	64	1.219	73.4
Translation of coded levels in actual units							
Variables level		Low (-1)		Medium (0)		High (+1)	
Polymer-to-drug ratio (X ₁)		1:1		3:1		6:1	
Stirring speed (X ₂) rpm		500		1000		1500	
All the batches were prepared using 40 mL glutaraldehyde and crosslinking time 1h							

DETERMINATION OF AMOXICILLIN

Amoxicillin was estimated by UV/Vis spectrophotometric method (Shimadzu UV-1601 UV/Vis double beam spectrophotometer, Kyoto, Japan). Aqueous solutions of amoxicillin were prepared in phosphate buffer (pH 7.8) and absorbance was measured on a Shimadzu UV/Vis spectrophotometer at 272 nm. The method was validated for linearity, accuracy and precision.

DRUG ENTRAPMENT EFFICIENCY

One hundred milligrams of accurately weighed microspheres were crushed in a glass mortar-pestle and the powdered microspheres were suspended in 10 mL phosphate buffer (pH 7.8). After 24 h the solution was filtered and the filtrate was analysed for the drug content. The drug entrapment efficiency was calculated using the following formula: Practical drug content/Theoretical drug content × 100. The drug entrapment efficiency for batches B1 to B9 is reported in Table 1.

PARTICLE SIZE & SWELLING INDEX OF MICROSPHERES

The particle size of the microspheres was determined by using optical microscopy method [40]. Approximately 300

microspheres were counted for particle size using a calibrated optical microscope (Labomed CX RIII, Ambala, India).

For estimating the swelling index, the microspheres (~100) were suspended in 5 mL of simulated gastric fluid USP (pH 1.2). The particle size was monitored by microscopy technique every 1 h using an optical microscope (Labomed CX RIII). The increase in particle size of the microspheres was noted for up to 8 h, and the swelling index was calculated as per method described by Ibrahim [41]. The swelling index for microspheres of batches B1 to B9 is reported in Table 1.

IN VITRO WASH-OFF TEST FOR MICROSPHERES

The mucoadhesive properties of the microspheres were evaluated by *in vitro* wash-off test as reported by Lehr *et al.* [42]. A 1x1 cm piece of rat stomach mucosa was tied onto a glass slide (-3 inch-by-1inch-) using thread. Microspheres were spread (~50) onto the wet rinsed tissue specimen and the prepared slide was hung onto one of the grooves of a USP tablet disintegrating test apparatus. The disintegrating test apparatus was operated whereby the tissue specimen was given regular up and down movements in the beaker of the disintegration apparatus, which contained the gastric fluid

(pH 1.2). At the end of 30 min, 1 h and at hourly intervals upto 12 h, the number of microspheres still adhering onto the tissue was counted. The results of *in vitro* wash-off test of batches B1 to B9 are shown in Table 1.

SCANNING ELECTRON MICROSCOPY

Scanning electron photomicrograph of drug-loaded chitosan mucoadhesive microspheres were taken. A small amount of microspheres was spread on glass stub. Afterwards, the stub containing the sample was placed in the scanning electron microscope (JSM 5610 LV SEM, JEOL, Datum Ltd, Tokyo, Japan) chamber. Scanning electron photomicrograph was taken at the acceleration voltage of 20 KV, chamber pressure of 0.6 mm Hg, at different magnification. The photomicrograph of batch B7 is depicted in Fig. (1).

The photomicrograph of *in vitro* wash-off test results after 2 h and 12 h are depicted in Figs. (2) and (3), respectively.

DRUG RELEASE STUDY

The drug release study was carried out using USP XXIV basket apparatus (Electrolab, TDT-06T, India) at 37°C ± 0.5°C and at 100 rpm using 900 mL of phosphate buffer (pH 7.8) as a dissolution medium (n=5) as per USP XXVI dissolution test prescribed for amoxicillin tablets. Microspheres equivalent to 100 mg of amoxicillin were used for the test. Five milliliters of sample solution was withdrawn at predetermined time intervals, filtered through a 0.45 µm mem-

brane filter, diluted suitably, and analyzed spectrophotometrically. An equal amount of fresh dissolution medium was replaced immediately after withdrawal of the test sample. Percentage drug dissolved at different time intervals was calculated using the Lambert-Beer's equation. The t_{80} was calculated using the Weibull equation [43]. The average values of t_{80} for batches B1 to B9 are mentioned in Table 1. The percentage drug release of batch B7 in pH 1.0 and pH 7.8 is shown in Fig. (4).

IN VIVO CLEARANCE OF *H. PYLORI*

The *H. pylori* infections animal model was established according to Qian's method (China Patent, CN 1304729A). Briefly, 0.3 mL of broth containing 10⁹ CFU/ml of *H. pylori*, which was isolated from patients with gastritis and gastric ulcer, was inoculated into the stomachs of 6-week-old male Wistar rats. Then, the rats were fed for 4 weeks. *H. pylori* infection in rat was detected using the "golden standard" culture, W-S stain and rapid urease test etc. The rapid urease test was carried out by collecting and transferring the bacterial colonies into small tubes containing 0.5 mL of mixture of phosphate buffer, urea (2% w/v) and phenol red (0.03% v/v). If the solution color turned into red in several minutes, the urease test was regarded to be positive, which indicated presence of *H. pylori* (detection). While if the solution color did not turn red in several minutes, the urease test was regarded to be negative, which indicated absence of *H. pylori* (detection).

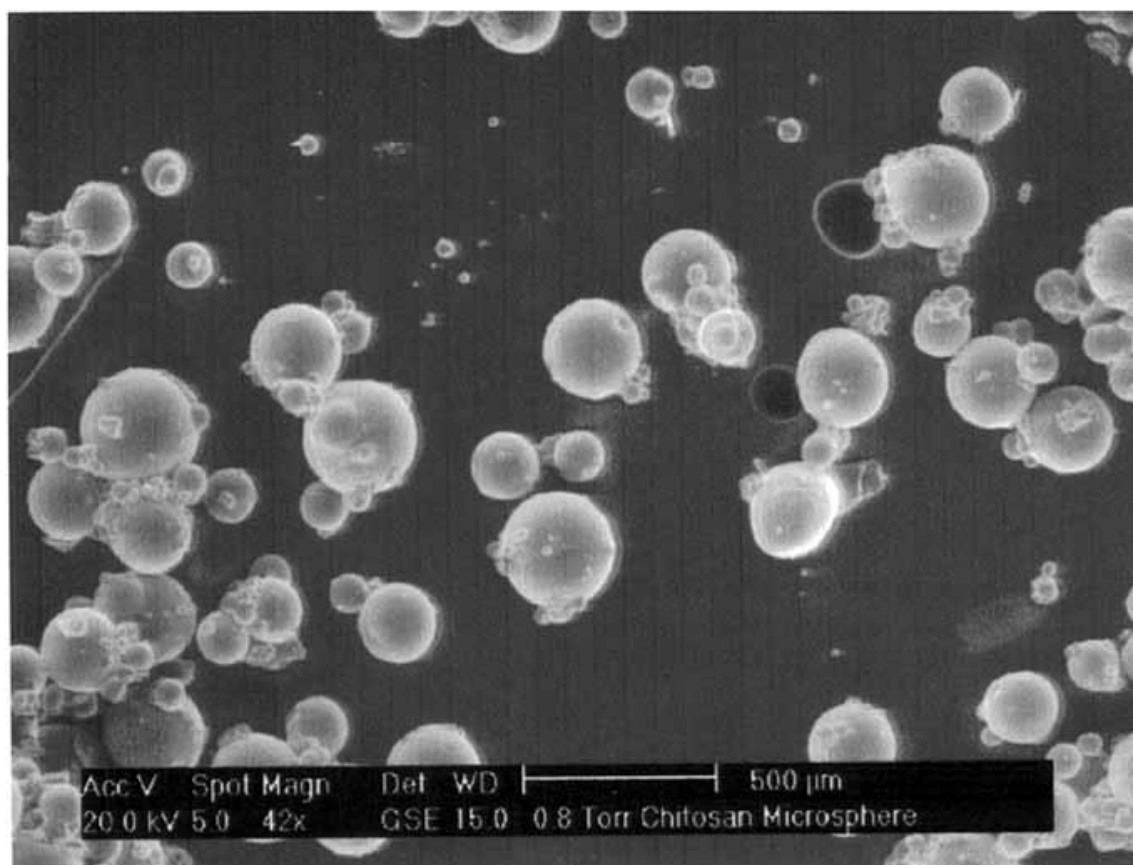


Fig. (1). Scanning electron photomicrograph of amoxicillin loaded chitosan mucoadhesive microspheres (batch B7).



Fig. (2). *In vitro* wash-off test of amoxicillin loaded chitosan mucoadhesive microspheres (batch B7) on rat stomach after 2 h.

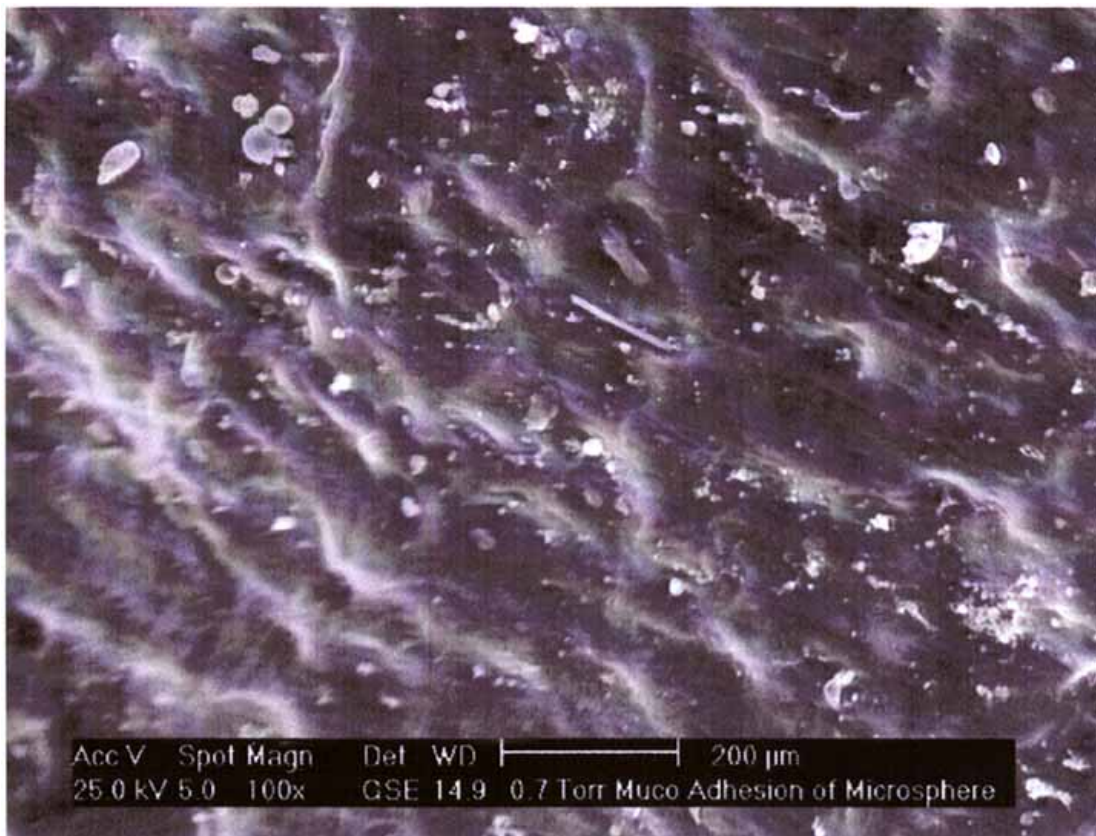


Fig. (3). *In vitro* wash-off test of amoxicillin loaded chitosan mucoadhesive microspheres (batch B7) on rat stomach after 12 h.

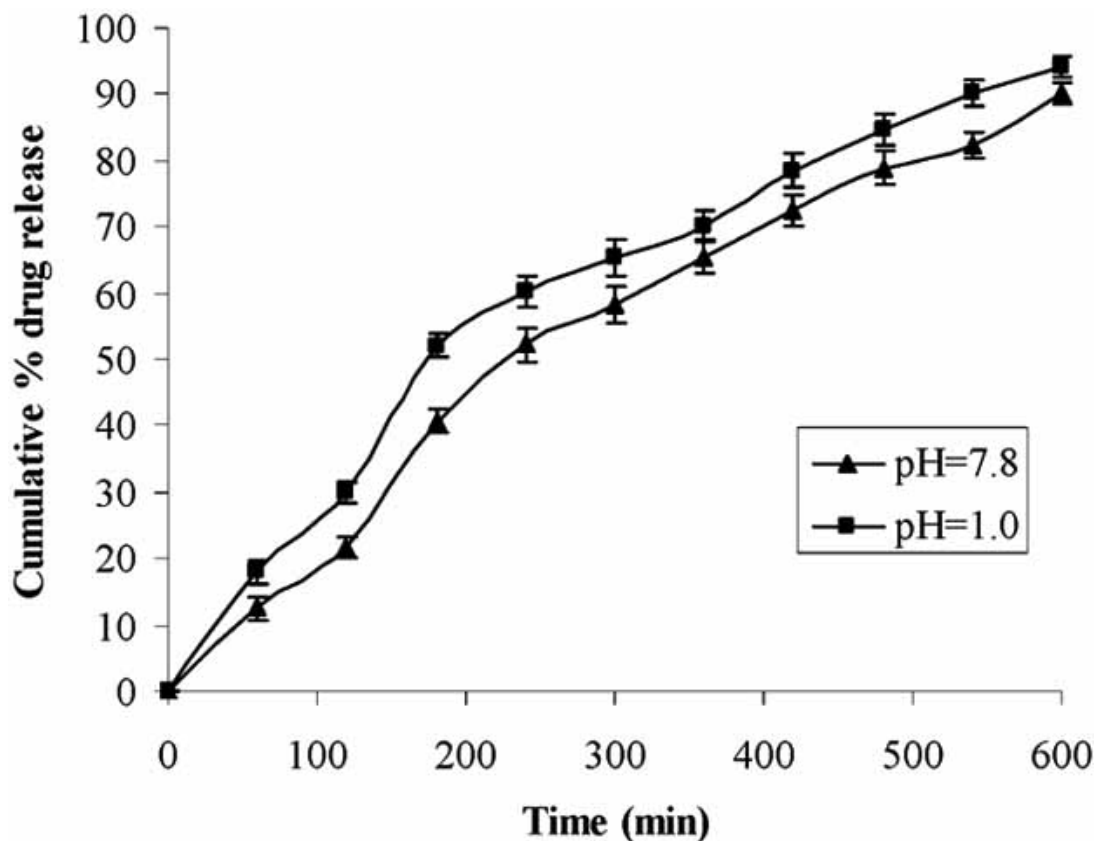


Fig. (4). *In vitro* dissolution of amoxicillin from mucoadhesive microspheres of batch B7.

SINGLE-DOSAGE ADMINISTRATION

To determine the dose for *H. pylori* clearance, mucoadhesive amoxicillin microspheres and amoxicillin powder were orally administered to the *H. pylori* infectious rats at the dosages of 4.0, 7.5 and 15 mg/kg (n=2). Physiological saline was given to the rats as control (n=2). One day after administration, the *H. pylori* infectious rats were killed, and their stomachs were removed and cut. Then, the gastric tissue was daubed on the modified Skirrow's medium. The plates were incubated for 3 days at 37 °C under microaerobic conditions. *H. pylori* clearance effect was judged by both bacterial colony counts and rapid urease test. The rapid urease test was carried out by collecting and transferring the bacterial colonies into small tubes containing 0.5 mL of mixture of phosphate buffer, urea (2% w/v) and phenol red (0.03% v/v). If the solution color turned red in several minutes, the urease test was regarded to be positive, which indicated for *H. pylori* detection.

MULTIDOSAGE ADMINISTRATION

To determine whether the mucoadhesive amoxicillin microspheres could completely eradicate *H. pylori*, a multidosage administration therapy was carried out. Briefly, amoxicillin was orally administered twice a day for three days at a dose of 3.5 mg/kg in the form of either amoxicillin mucoadhesive microspheres or powder (n=2). Physiological saline was given to rats as a control (n=2). One day after administration, the *H. pylori* infectious rats were killed, and their

stomachs were removed and cut. The gastric tissue was then daubed on the modified Skirrow's medium. *H. pylori* clearance effect was studied using the same method as that described for single dose administration.

RESULTS AND DISCUSSION

Preliminary Trials

The mucoadhesive microspheres of amoxicillin using chitosan were prepared by simple emulsification phase separation technique. Chitosan was selected as a polymer for the preparation of mucoadhesive microspheres owing to its biodegradable and mucoadhesive properties. Different concentrations of acetic acid from 1% w/v to 6% w/v were used for preparing the polymer solution, but no significant effect of concentration of acetic acid was observed on percentage mucoadhesion or drug entrapment efficiency, therefore 1% w/v of acetic acid was used. This finding could be owing to good solubility of chitosan in acetic acid.

One of the important factors related to microspheres as reported by Lee *et al.* [44] is the viscosity of the polymer solution. Polymer concentrations of 0.5%, 1%, and 2% w/v were selected for preliminary trials. Flake formation was observed when chitosan concentration was used at a level of 0.5% w/v, whereas maximum sphericity was observed at the 1% w/v level. The chitosan solution was found to be too viscous to pass through the syringe when used at the 2% w/v level. Therefore 1% w/v of chitosan in 1% v/v acetic acid was found to be the optimum concentration for the polymer

solution. A 1:1 mixture of heavy and light liquid paraffin was found to be suitable as the dispersion medium. The addition of 0.2% w/v of DOSS to the dispersion medium was found to be essential to minimize aggregation of microspheres.

Preliminary trial batches were prepared to study the effect of the volume of cross-linking agent (glutaraldehyde), time for cross-linking, and stirring speed on the percentage mucoadhesion, drug entrapment efficiency, and characteristics of the microspheres.

The volume of glutaraldehyde was varied from 5 to 50 mL. Discrete spherical microspheres were obtained using 30, 40, and 50 mL of glutaraldehyde. Batches prepared using 5 and 10 mL of glutaraldehyde yielded irregular microspheres. The higher amount of glutaraldehyde appears to favor the cross-linking reaction, and hence spherical free-flowing microspheres were obtained. Microspheres of batches prepared using 30 mL of glutaraldehyde showed good percentage mucoadhesion, but drug entrapment efficiency was below 60%. Batches prepared using 40 mL of glutaraldehyde also showed good mucoadhesion as well as 70% drug entrapment efficiency. In the microspheres of batches prepared using 50 mL of glutaraldehyde the drug entrapment efficiency was above 72%, but mucoadhesion decreased. The decrease in mucoadhesion could possibly be attributed to the greater amount of cross-linking agent giving a more rigid cross-linked polymer whose adhesion is decreased. Thus, we can conclude that 40 mL of glutaraldehyde was the optimum amount. Increase in the cross-linking time (1 to 3 h) in all preliminary trial batches inversely affected the percentage mucoadhesion. The cross-linking polymer probably becomes more rigid and thus mucoadhesiveness decreases. The cross-linking time did not have a significant effect on the percentage drug entrapment efficiency.

On the basis of the preliminary trials a 3^2 full factorial design was employed to study the effect of independent variables (i.e. polymer-to-drug ratio [X_1] and the stirring speed [X_2]) on dependent variables percentage mucoadhesion, t_{80} , drug entrapment efficiency, particle size and swelling index. The results depicted in Table 1 clearly indicate that all the dependent variables are strongly dependent on the selected independent variables as they show a wide variation among the 9 batches (B1 to B9). The fitted equations (full models) relating the responses (i.e. percentage mucoadhesion, t_{80} , drug entrapment efficiency, particle size and swelling index)

to the transformed factor are shown in Table 2. The polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries (i.e. positive or negative). The high values of correlation coefficient (Table 2) for the dependent variables indicate a good fit. The equations may be used to obtain estimates of the response since small error of variance was noticed in the replicates.

FACTORIAL EQUATION FOR PARTICLE SIZE, % MUCOADHESION AND SWELLING INDEX

The mucoadhesive microspheres of all the batches of the factorial design were spherical (Fig. 1, batch B7) and free flowing. They ranged from a particle size of 50.4 to 98.2 μm and showed good correlation co-efficient (0.9698). Results indicate that the effect of X_1 (polymer-to-drug ratio) is more significant than X_2 (stirring speed). Thus, as the stirring speed increases, the particle size decreases which directly affects the percentage mucoadhesion.

The *in vitro* wash-off test for % mucoadhesion after 1 h varied from 53 to 79 and showed good correlation co-efficient (0.9967). Results of equation indicate that the effect of X_1 (polymer-to-drug ratio) is more significant than X_2 (stirring speed). Moreover, stirring speed had a negative effect on the percentage mucoadhesion (i.e. as the stirring speed increased, the percentage mucoadhesion decreased). This finding may be attributed to the change in particle size that affects mucoadhesion. As the polymer-to-drug ratio increases, the % mucoadhesion also increases because more amount of polymer results in higher amount of free $-\text{NH}_2$ groups, which are responsible for binding with sialic acid groups in mucus membrane and thus results in increase in mucoadhesive properties of microspheres. *In vitro* mucoadhesive test showed that amoxicillin mucoadhesive microspheres adhered more strongly to gastric mucous layer and could retain in the gastrointestinal tract for an extended period of time (Figs. 2 and 3). Fig. (3) showed that even after 12 h, some of microspheres were adhered to gastric mucous layer.

Similar results were also obtained for swelling index. The amount of polymer directly affected the solvent transfer rate and thus as the polymer concentration increased the swelling index also increased. The swelling index varied from 0.796 to 1.39 and showed good correlation coefficient (0.9907). Thus, we can conclude that the amount of polymer and stir-

Table 2. Summary of Results of Regression Analysis

Coefficient	b_0	b_1	b_2	b_{11}	b_{22}	b_{12}	R^2
% Mucoadhesion	66.88	12.16	-6.66	-1.28	-7.83	0.66	0.9967
t_{80}	231.33	104	-10.5	-20.0	102	-14.5	0.9620
Drug entrapment efficiency	62.11	12.83	-3.33	0.25	-7.16	-0.16	0.9998
Swelling index	1.08	0.24	-0.07	-0.03	-0.02	0.01	0.9907
Particle size	66.02	14.93	-6.8	-3.275	5.86	-2.23	0.9698

ring speed directly affects the percentage mucoadhesion and swelling index.

FACTORIAL EQUATION FOR DRUG ENTRAPMENT EFFICIENCY AND T_{80}

The drug entrapment efficiency and t_{80} are important variables for assessing the drug loading capacity of microspheres and their drug release profile, thus suggesting the amount of drug availability at site. These parameters are dependent on the process of preparation, physicochemical properties of drug, and formulation variables. The drug entrapment efficiency varied from 38% to 70% and showed good correlation co-efficient (0.9998). Results of equation indicate that the effect of X_1 (polymer-to-drug ratio) is more significant than X_2 (stirring speed). Moreover, stirring speed had a negative effect on the per drug entrapment efficiency (i.e. as the stirring speed increased, the particle size decreased, and thus drug entrapment efficiency decreased).

Results depicted in Table 2 indicate that the % drug released *in vitro* is highly dependent on the polymer-to-drug ratio and stirring speed. The stirring speed has a negative effect on t_{80} because as the particle size increases the drug releases decreases. Higher levels of polymer-to-drug ratio favour the cross-linking reaction and thus higher t_{80} is obtained.

Batch B7 exhibited a high t_{80} of 465 min and seems to be a promising candidate for achieving drug release upto 10 h. The drug release profile of batch B7 is shown in Fig. (4). The figure reveals that drug release rate was slowed after 4 h. The study focus was the preparation of mucoadhesive microspheres, thus the microspheres of batch B7 were also evaluated in simulated gastric fluid USP (pH 1.2). *In vitro* release test showed that amoxicillin released faster in pH 1.0 hydrochloric acid than in pH 7.8 phosphate buffer but the results indicated that no significant difference was observed between dissolution profiles at pH 7.8 and pH 1.0 as the f_2 (similarity factor) value was 59.77. The dissolution data of batch B7 was further analyzed to certain the mechanism of drug release [45]. The release profile fitted best to Weibull equation ($F=8.15$). The value of correlation coefficient was found to be 0.987. The values of slope and intercept were found to be 1.12 and -2.07 respectively.

IN VIVO STUDY

At present, most studies of mucoadhesive formulations loading amoxicillin for anti-*H. pylori* focused on prolonging the gastric retarding time. The stability of amoxicillin in acidic medium was neglected. In fact, lots of antibiotics, such as erythromycin, clarithromycin, were reported with strong *in vitro* *H. pylori* clearance effect but with poor *in vivo* results. Ogwal and Xide [46] suggested that one of the reasons was due to their instability in acidic medium. Amoxicillin was also reported to be unstable in mediums with pH below 2 [47-49]. Amoxicillin can be quickly absorbed after its conventional dosage forms are orally administered. Therefore, its residence time in the stomach is expected to be short [50], which might cover up its shortcoming of being unstable in acidic medium. But for the mucoadhesive microspheres, which would stay in the stomach for a much longer time, the stability of amoxicillin should be seriously considered. In this study, we found that, amoxicillin microspheres were more stable in pH 1.0 HCL than amoxicillin powder.

From the results of the *in vivo* *H. pylori* clearance test, we conclude that, with the increase of amoxicillin's doses, the *H. pylori* clearance effect was enhanced in mucoadhesive amoxicillin microspheres formulation. In the single dosage administration test, it was found that the total colony counts decreased markedly with the increase of the amoxicillin dose in both groups. Administration of a dose of 4 mg/kg amoxicillin mucoadhesive microspheres the colony counts were 23 ± 7.07 , and as the doses were increased to 7.5 and 15 mg/kg the colony counts were 5.5 ± 0.70 and 2 ± 0 , respectively. Administration of a dose of Amoxicillin powder 4 mg/kg dose the colony counts were 78 ± 8.48 , and as the doses were increased to 7.5 and 15 mg/kg, the colony counts were 29 ± 5.65 and 17.5 ± 17.67 , respectively. While in case of Physiological saline 4 mg/kg dose administrated, the colony counts were 94 ± 5.65 , and on increasing the doses to 7.5 and 15 mg/kg the colony counts were 92 ± 9.89 and 92.5 ± 17.67 , respectively. Physiological saline did not show decrease in the colony count. The ratio of colony counts between amoxicillin powder and mucoadhesive microspheres increased rapidly from 3.39 at 4 mg/kg to 8.75 at 15 mg/kg. (Table 3, Fig. 5). This phenomenon indicated that as the dose increases the mucoadhesive amoxicillin microspheres showed more effective clearance of *H. pylori* than that of amoxicillin

Table 3. *H. pylori* Clearance Effect of Amoxicillin at Different Doses in Different Formulations (n=2)

Colony counts	Doses (mg/kg)					
	4		7.5		15	
Amoxicillin mucoadhesive microspheres	18	28	6	5	2	2
	$(23 \pm 7.07)^*$		$(5.5 \pm 0.70)^*$		$(2 \pm 0)^*$	
Amoxicillin powder	72	84	25	33	5	30
	$(78 \pm 8.48)^*$		$(29 \pm 5.65)^*$		$(17.5 \pm 17.67)^*$	
Physiological saline	98	90	99	85	105	80
	$(94 \pm 5.65)^*$		$(92 \pm 9.89)^*$		$(92.5 \pm 17.67)^*$	

* Figure showed mean \pm S.D.

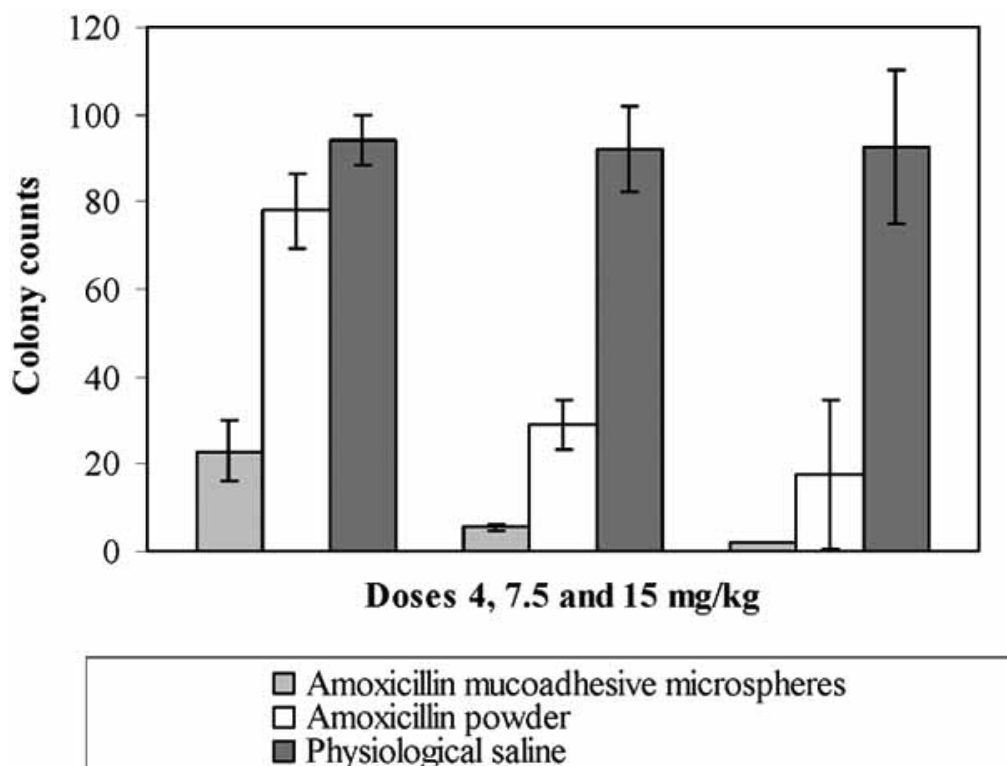


Fig. (5). *H. pylori* clearance effect of amoxicillin at different doses in different formulations.

Table 4. *In Vivo* Clearance of *H. pylori* After the Administration of Amoxicillin Powder, Mucoadhesive Amoxicillin Microspheres and Physiological Saline (n=2)

<i>H. pylori</i> Condition	Mucoadhesive amoxicillin microspheres		Amoxicillin powder		Physiological saline	
	1	2	1	2	1	2
	-/-	-/-	+/+	+/+	+/+	+/+

Negative (-) means neither bacterial colony was found nor rapid urease test was positive (+) means either bacterial colony was found or rapid urease test was positive.

powder. We inferred that this might be due to the lack of repetition of drug administration. Therefore, another multi-dosage administration regimen was tried. The results showed that, at the dose of 3.5 mg/kg, when amoxicillin microspheres, powder or physiological saline was administered, respectively, to the *H. pylori* infectious rat twice a day for three consecutive days (Table 4), neither *H. pylori* colony was found nor was urease test positive in rats that were administered mucoadhesive amoxicillin microspheres. We conclude that the mucoadhesive amoxicillin microspheres showed a more complete *H. pylori* clearance effect.

CONCLUSION

The results of a 3² full factorial design revealed that the polymer-to-drug ratio and stirring speed significantly affected the dependent variables % mucoadhesion, t₈₀, drug entrapment efficiency, particle size and swelling index. The microspheres of the best batch exhibited a high % mucoadhesion of 79 % after 1 h, 70 % drug entrapment efficiency and swelling index of 1.42. The t₈₀ of 465 min indicates that the mucoadhesive microspheres of amoxicillin could sustain

the release of the drug for more than 10 h. The investigation on *H. pylori* clearance effect showed that there was a tendency for a more effective *H. pylori* activity of mucoadhesive amoxicillin microspheres compared to amoxicillin powder and physiological saline, which might indicate the potential use of mucoadhesive amoxicillin microspheres in treating *H. pylori* infection.

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