Chapter 7
Research papers presented and published
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7 Research papers presented and published

7.1 Publications


7.2 Presentation

Formulation and evaluation of stomach-specific amoxicillin-loaded carbopol-934P mucoadhesive microspheres for anti-*Helicobacter pylori* therapy

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Abstract

The purpose of this research was to formulate and systemically evaluate *in vitro* and *in vivo* performances of mucoadhesive amoxicillin microspheres for the potential use in the treatment of gastric and duodenal ulcers, which were associated with *Helicobacter pylori*. Amoxicillin mucoadhesive microspheres containing carbopol-934P as mucoadhesive polymer and ethyl cellulose as carrier polymer were prepared by an emulsion-solvent evaporation technique. Results of preliminary trials indicate that quantity of emulsifying agent, time for stirring, drug-to-polymers ratio and speed of rotation affected the characteristics of microspheres. Microspheres were discrete, spherical, free flowing and showed a good percentage of drug entrapment efficiency. An *in vitro* mucoadhesive test showed that amoxicillin mucoadhesive microspheres adhered more strongly to the gastric mucous layer and could retain in the gastrointestinal tract for an extended period of time. A 3² full factorial design was employed to study the effect of independent variables, drug-to-polymer-to-polymer ratio (amoxicillin-ethyl cellulose-carbopol-934P) \((X_1)\) and stirring speed \((X_2)\) on dependent variables, i.e. percentage mucoadhesion, drug entrapment efficiency, particle size and \(t_{80}\). The best batch exhibited a high drug entrapment efficiency of 56%; mucoadhesion percentage after 1 h was 80% and the particle size was 109μm. A sustained drug release was obtained for more than 12 h. The drug-to-polymer-to-polymer ratio had a more significant effect on the dependent variables. The morphological characteristics of the mucoadhesive microspheres were studied under a scanning electron microscope. *In vitro* release test showed that amoxicillin released slightly faster in pH 1.2 hydrochloric acid than in pH 7.8 phosphate buffer. *In vivo* *H. pylori* clearance tests were also carried out by administering amoxicillin powder and mucoadhesive microspheres 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 a contribution to *H. pylori* complete eradication.

**Key words:** Mucoadhesive; amoxicillin; microspheres; factorial design; *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. They have varied applications and are prepared using assorted polymers. 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 in chronic active gastritis, gastric and duodenal ulcers.

This can be achieved by coupling mucoadhesion characteristics to microspheres and developing mucoadhesive microspheres. Mucoadhesive 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. Carbopol-934P (acrylic acid homopolymer) is an anionic polymer that has been used in mucoadhesive systems by several researchers. Carbopol-934P was selected as a polymer in the preparation of mucoadhesive microspheres because of its good mucoadhesive and biodegradable properties and ethyl cellulose as carrier polymer for microspheres.

In a relatively short time span, Helicobacter pylori (H. pylori) have become recognized as a major gastric pathogen with worldwide distribution. H. pylori are a 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 active gastritis, gastric and duodenal ulcers, and gastric adenocarcinoma, 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. 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.

Treatment regimens for H. pylori infection have been evolving since the early 1990s, when monotherapy was first 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. Tetracycline is used in the rescue therapy. 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 and clarithromycin have been well documented, while resistance to amoxicillin and tetracycline was mainly reported in Asia. 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 semi-synthetic, orally absorbed, broad-spectrum antibiotic. It is now widely used in the 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. These tripe therapies have proved to be effective in clinical application. However, some other reports and clinical trials indicate that the therapies cannot bring out compete eradication of H. pylori and suggest that the therapeutic effect needs more investigation. One reason for the incomplete eradication of H. pylori is probably due to the 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. The other may be the degradation of amoxicillin in gastric acid. Therefore, some researchers had prepared and reported new amoxicillin formulations such as float tablets, mucoadhesive tablets, pH-sensitive excipients 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.

A previous investigation on H. pylori clearance effect showed that there was a tendency for a more effective H. pylori activity of mucoadhesive amoxicillin microspheres prepared using chitosan as mucoadhesive microspheres.

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 carbopol-934P as a mucoadhesive polymer and ethyl cellulose as a carrier polymer and prepare mucoadhesive amoxicillin microspheres. The microspheres were characterized by in-vitro and iv-vivo tests and factorial design was used to optimize the variables.

Materials

Amoxicillin (powder) was obtained as a gift sample from Zydus Cadila (Ahmedabad, India). Carbopol-934P (CP, molecular weight [MW] 3 × 10⁶ Da) was obtained as a gift sample from Noveon (Mumbai, India). Ethyl cellulose and petroleum ether 80:20 were procured from Willson Lab (Mumbai, India) and S. D. Fine Chemicals Ltd (Mumbai, India), respectively. Liquid paraffin and span 80 were purchased from Loba Chemie Pvt Ltd (Mumbai, India). Wistar rats (300 ± 50 g) were obtained as a gift sample from Zydus Cadila (Ahmedabad, India). Skirrow’s medium was purchased from Himedia Ltd.
(Mumbai, India). All other ingredients were of analytical grade.

**Methods**

**Preparation of mucoadhesive amoxicillin microspheres**

Mucoadhesive microspheres of amoxicillin were prepared containing carbopol-934P as a mucoadhesive polymer and ethyl cellulose as a carrier polymer by emulsion-solvent evaporation technique. Briefly, ethyl cellulose (1500 mg) was dissolved in 200 mL of ethanol. Each 500 mg of amoxicillin and carbopol-934P were dispersed in the polymer solution of ethyl cellulose under stirring. In preliminary trail batches the drug-to-polymer-to-polymer (amoxicillin-ethyl cellulose-carbopol-934P) ratio was kept constant at 1:3:1. The resultant mixture was extruded through a syringe (gauge No. 20) in 500 mL of liquid paraffin (mixture of heavy and light, 1:1 ratio) containing 2.0% v/v Span 80 and stirring was carried out using a propeller stirrer (Remi, Mumbai, India) at 1000 rpm. Stirring was continued for 3 h. The amount of emulsifying agent and time for stirring were varied in preliminary trial batches from 1–3% v/v and 1–3 h, respectively. In factorial design batches J1–J9, 2.0% v/v Span 80 was used as an emulsifying agent and time for stirring was kept to 3 h. The drug-to-polymer-to-polymer ratio and stirring speed were varied in batches J1–J9, as shown in Table 1. All other variables were similar as in preliminary trial batches. Microspheres thus obtained were filtered and washed several times with petroleum ether (80:20) to remove traces of oil. The microspheres were then dried at room temperature (25°C and 60% RH) for 24 h. The effect of formulation variables on characteristics of the microspheres of factorial design batches is summarized in Table 1.

### Optimization of microspheres formulation using $3^2$ full factorial design

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

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

(1)

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 $3^2$ full factorial design was employed to study the effect of independent variables, i.e. drug-to-polymer-to-polymer ($X_1$) and the stirring speed ($X_2$) on dependent variables percentage mucoadhesion, drug entrapment efficiency, particle size and the time required for 80% drug dissolution ($t_{80}$).

### Determination of amoxicillin

Amoxicillin was estimated by a UV/Vis spectrophotometric method (Shimadzu UV-1700 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.

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**Table 1.** Amoxicillin mucoadhesive microspheres batches using $3^2$ full factorial design layout.

<table>
<thead>
<tr>
<th>Batch code</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>In-vitro wash-off test (% mucoadhesion after)</th>
<th>Drug entrapment efficiency (%)</th>
<th>Particle size (μm)</th>
<th>$t_{80}$ (min)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1 h</td>
<td>5 h</td>
<td>10 h</td>
<td></td>
</tr>
<tr>
<td>J1</td>
<td>-1</td>
<td>-1</td>
<td>57</td>
<td>50</td>
<td>45</td>
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<td>J2</td>
<td>-1</td>
<td>0</td>
<td>55</td>
<td>49</td>
<td>44</td>
<td>21</td>
</tr>
<tr>
<td>J3</td>
<td>-1</td>
<td>1</td>
<td>40</td>
<td>35</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
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<td>72</td>
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<td>56</td>
</tr>
<tr>
<td>J5</td>
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<td>0</td>
<td>77</td>
<td>70</td>
<td>55</td>
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<td>J6</td>
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<td>1</td>
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<td>65</td>
<td>50</td>
<td>41</td>
</tr>
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<td>80</td>
<td>72</td>
<td>47</td>
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<tr>
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<td>81</td>
<td>75</td>
<td>64</td>
<td>41</td>
</tr>
<tr>
<td>J9</td>
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<td>1</td>
<td>73</td>
<td>64</td>
<td>50</td>
<td>34</td>
</tr>
</tbody>
</table>

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<td>1 h</td>
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<td></td>
</tr>
<tr>
<td>J1</td>
<td>-1</td>
<td>-1</td>
<td>57</td>
<td>50</td>
<td>45</td>
<td>26</td>
</tr>
<tr>
<td>J2</td>
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<td>0</td>
<td>55</td>
<td>49</td>
<td>44</td>
<td>21</td>
</tr>
<tr>
<td>J3</td>
<td>-1</td>
<td>1</td>
<td>40</td>
<td>35</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>J4</td>
<td>0</td>
<td>-1</td>
<td>80</td>
<td>72</td>
<td>60</td>
<td>56</td>
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<tr>
<td>J5</td>
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<td>77</td>
<td>70</td>
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<td>-1</td>
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<tr>
<td>J8</td>
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<td>0</td>
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<td>75</td>
<td>64</td>
<td>41</td>
</tr>
<tr>
<td>J9</td>
<td>1</td>
<td>1</td>
<td>73</td>
<td>64</td>
<td>50</td>
<td>34</td>
</tr>
</tbody>
</table>

Translation of coded levels in actual units.

Variables level: drug-to-polymer-to-polymer ratio ($X_1$) (amoxicillin-ethyl cellulose-carbopol-934P), Low (−1) 1:3:0.5, Medium (0) 1:3:1, High (+1) 1:3:1.5; Stirring speed ($X_2$), rpm, Low 800, Medium 1000, High 1200.

All the batches were prepared using 2% v/v Span 80 and stirring time of 3 h.
spectrophotometer at 272 nm. The method was validated for linearity, accuracy and precision.

**Drug entrapment efficiency**

Two-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 hs 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 J1–J9 is reported in Table 1.

**Particle size of microspheres**

The particle size of the microspheres was determined by using an optical microscopy method. Approximately 300 microspheres were counted for particle size using a calibrated optical microscope (Labomed CX RIII, Ambala, India). The particle size of microspheres of batches J1–J9 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. A 1 × 1 cm piece of rat stomach mucosa was tied onto a glass slide (3 inch-by-1 inch) using thread. Microspheres were spread (≈50) onto the wet rinsed tissue specimen and the prepared slide was hung onto one of the groves of a USP tablet disintegrating test apparatus with continuous oxygen supply. 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 up to 12 h, the number of microspheres still adhering onto the tissue was counted. The results of in vitro wash-off test after 1, 5 and 10 h of batches J1–J9 are shown in Table 1. Also, results of in vitro wash-off test of amoxicillin-loaded carbopol-934P mucoadhesive microspheres of batch J4 is shown in Figure 1.

**Scanning electron microscopy**

Scanning electron photomicrographs of drug-loaded carbopol-934P mucoadhesive microspheres were taken. A small amount of microspheres was spread on a 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. A 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 J4 is depicted in Figure 2.

The photomicrographs of in-vitro wash-off test results after 2 h and 8 h are depicted in Figures 3 and 4, 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.
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 millilitres of sample solution was withdrawn at pre-determined time intervals, filtered through a 0.45 μm membrane filter, diluted suitably and analysed 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 J1–J9 are mentioned in Table 1. The percentage drug release of batch J4 in pH 1.2 and pH 7.8 is shown in Figure 5.

**Data fitting**

An attempt was made to fit the dissolution data into the Hixon-Crowell\(^{43}\) model represented:

\[
m = \left( 100 \times \left( \frac{1}{3} \right) - k \times t \right)^3
\]

(2)

where \(k\) is Hixon-Crowell constant [mass/(time)]\(^{1/3}\). In this model the percentage drug unreleased vs. cube root of time is linear.

The data was treated with the Korsmeyer-Peppas model\(^{44}\) to characterize the mechanism of drug release:

\[
\frac{M_t}{M_{\infty}} = Kptn
\]

(3)

where \(M_t/M_{\infty}\) represents the fraction of drug released at time \(t\) and \(Kp\) is the kinetic constant characterizing the polymeric system and \(n\) stands for the diffusion exponent.

The dissolution data was also analysed using the Weibull equation\(^{43}\) to determine the kinetics of drug release from different batches of mucoadhesive microspheres:

\[
m = 1 - \exp \left[ -\left( t - t_i \right) \frac{b}{a} \right]
\]

(4)
where \(a\) is the scale parameter which defines the time scale of the process, \(t_i\) is the location parameter which represents the lag period before the actual onset of dissolution process (in most cases \(t_i = 0\)) and \(b\) is the shape parameter. In this model the plot of log of time vs. In \(1/C_0\) is linear.

The results of \(F\)-statistics were used for the selection of the most appropriate model. Results of \(F\)-statistics and summary of results of regression analysis are shown in Tables 2 and 3, respectively.

The curve fitting, simulation and plotting was performed in Excel (Microsoft Software Inc., USA) and Sigma plot\textsuperscript{8} version 10.0 (Sigma plot software, Jangel Scientific Software, San Rafael, CA). The effects of independent variables on the response parameters were visualized from the contour plots. Numerical optimization using the desirability approach was employed to locate the optimal settings of the formulation variables so as to obtain the desired response\textsuperscript{45}. An optimized formulation was developed by setting constraints on the dependent and independent variables. The formulation developed was evaluated for the responses and the experimental values obtained were compared with those predicted by the mathematical models generated. Counter plots showing the effect of drug-polymer-polymer ratio (\(X_1\)) and stirring speed (\(X_2\)) on percentage mucoadhesion, particle size, drug entrapment efficiency and \(t_{80}\) appear in Figure 6.

In vivo clearance of \textit{H. pylori}

The \textit{H. pylori} infected animal model was established according to Qian’s method (China Patent, CN 1304729A). Briefly, 0.3 mL of broth containing \(10^9\) CFU ml\(^{-1}\) of \textit{H. pylori}, 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. \textit{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

### Table 2. Results of models fitting of batch J4.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Hixon-Crowell</th>
<th>Korsemeyer and Peppas</th>
<th>Weibull</th>
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<tbody>
<tr>
<td></td>
<td>Calculated cumulative percentage release</td>
<td>Calculated cumulative percentage release</td>
<td>Calculated cumulative percentage release</td>
</tr>
<tr>
<td></td>
<td>Residual square</td>
<td>Residual square</td>
<td>Residual square</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>60</td>
<td>11.0</td>
<td>14.01</td>
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<td>90.2</td>
<td>101.77</td>
<td>90.36</td>
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### Table 3. Summary of results of regression analysis.

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<th>Coefficient</th>
<th>(b_0)</th>
<th>(b_1)</th>
<th>(b_2)</th>
<th>(b_{11})</th>
<th>(b_{22})</th>
<th>(b_{12})</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Mucoadhesion</td>
<td>77.66</td>
<td>15.33</td>
<td>-7.16</td>
<td>0</td>
<td>-10.0</td>
<td>-2.5</td>
<td>0.9803</td>
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<tr>
<td>Drug entrapment efficiency</td>
<td>50.11</td>
<td>9.16</td>
<td>-5.66</td>
<td>-1.75</td>
<td>-18.16</td>
<td>-0.66</td>
<td>0.9954</td>
</tr>
<tr>
<td>Particle size</td>
<td>102.33</td>
<td>7.16</td>
<td>-6.83</td>
<td>0</td>
<td>-1.5</td>
<td>-1.5</td>
<td>0.9824</td>
</tr>
<tr>
<td>(t_{80})</td>
<td>536.22</td>
<td>-169.5</td>
<td>42.66</td>
<td>-23.5</td>
<td>-56.83</td>
<td>8.66</td>
<td>0.9994</td>
</tr>
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</table>
(2% w/v) and phenol red (0.03% v/v). If the solution colour turned into red in several minutes, the urease test was regarded to be positive, which indicated the presence of H. pylori. While if the solution colour did not turn red in several minutes, the urease test was regarded to be negative, this indicated the absence of H. pylori.

Single-dosage administration

To determine the dose for H. pylori clearance, mucoadhesive amoxicillin microspheres and amoxicillin powder were orally administered to the H. pylori infected rats at the dosages of 4.0, 7.5 and 15 mg kg\(^{-1}\) (\(n = 2\)). Physiological saline was given to rats as control (\(n = 2\)). One day after administration of drug to the H. pylori infected rats, they 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 colour turned into red in several minutes, the urease test was regarded to be positive, which indicated H. pylori detection. H. pylori clearance effects of amoxicillin at different doses in different formulations (\(n = 2\)) are shown in Tables 4 and 5.

Multidose administration

To determine whether the mucoadhesive amoxicillin microspheres could completely eradicate H. pylori, a multidose administration therapy was carried out. Briefly, amoxicillin was orally administrated twice a day for 3 days at a dose of 3.5 mg kg\(^{-1}\) in the form of either amoxicillin mucoadhesive microspheres or powder (\(n = 2\)). Physiological saline was given to 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 H. pylori clearance effect was studied using the same method as described for single dose administration. H. pylori clearance effect of amoxicillin at different doses in different formulations is shown in Figure 7.
Results and discussion

Preliminary trials

The mucoadhesive microspheres of amoxicillin using carbopol-934P and ethyl cellulose were prepared by emulsion-solvent evaporation technique. Carbopol-934P was selected as a polymer for the preparation of mucoadhesive microspheres owing to its biodegradable and mucoadhesive properties. Ethyl cellulose was used as carrier polymer. Different concentrations of span 80 from 1–3% v/v were used as emulsifying agent.

Significant effect of concentration of span 80 was observed on percentage mucoadhesion, particles size and drug entrapment efficiency. Results showed that increase in the concentration of span 80 increase the particles size and percentage mucoadhesion but decrease the drug entrapment efficiency. At 1% v/v, percentage mucoadhesion, particles size and drug entrapment efficiency were 66%, 80 μm and 65%, respectively, but irregular shape of microspheres was observed. While at 3% v/v, percentage mucoadhesion, particles size and drug entrapment efficiency were 82%, 210 μm and 38%, respectively; spherical shape of microspheres was observed but particles were coalesced. Therefore, 2% v/v of concentrations of span 80 was used for further study.

One of the important factors related to microspheres as reported by Lee et al.46 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 ethyl cellulose and carbopol-934P concentration was used at a level of 0.5% w/v, whereas maximum sphericity was observed at the 1% w/v level. Non-spherical microspheres were found when polymer concentration was used at the 2% w/v level. Therefore, 1% w/v of ethyl cellulose and carbopol-934P each in ethanol 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 a dispersion medium.

Preliminary trials batches were prepared to study the effect of the time for stirring and stirring speed on the

Table 4. *H. pylori* clearance effect of amoxicillin at different doses in different formulations (n = 2).

<table>
<thead>
<tr>
<th>Colony counts</th>
<th>Doses (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Amoxicillin mucoadhesive microspheres</td>
<td>20 (23 ± 4.24)*</td>
</tr>
<tr>
<td>Amoxicillin powder</td>
<td>72 (78 ± 8.48)*</td>
</tr>
<tr>
<td>Physiological saline</td>
<td>98 (94 ± 5.65)*</td>
</tr>
</tbody>
</table>

*Figure showed mean ± SD.

Table 5. *In vivo* clearance of *H. pylori* after the administration of amoxicillin powder, mucoadhesive amoxicillin microspheres and physiological saline (n = 2).

<table>
<thead>
<tr>
<th><em>H. pylori</em> Condition</th>
<th>Mucoadhesive amoxicillin microspheres</th>
<th>Amoxicillin powder</th>
<th>Physiological saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Condition:</td>
<td>−/−</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
</tbody>
</table>

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.

Figure 7. *H. pylori* clearance effect of amoxicillin at different doses in different formulations.
percentage mucoadhesion, drug entrapment efficiency and characteristics of the microspheres. Increase in the time for stirring from 1 to 3 h showed an increase in percentages of mucoadhesion, but a decrease in drug entrapment efficiency and particles size. Thus, 3 h of stirring time was selected for further study. Since stirring speed has a significant effect on percentage mucoadhesion, drug entrapment efficiency and particles size, it was selected as an important factor for further study.

On the basis of the preliminary trials a $3^2$ full factorial design was employed to study the effect of independent variables (i.e. drug-to-polymer-to-polymer ratio [$X_1$] and the stirring speed [$X_2$]) on dependent variables percentage mucoadhesion, drug entrapment efficiency, particle size and $t_{50}$. 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 nine batches (J1–J9). The fitted equations (full models) relating the responses (i.e. percentage mucoadhesion, drug entrapment efficiency, particle size and $t_{50}$) to the transformed factor are shown in Table 3. 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 3) 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 percentage mucoadhesion**

The *in vitro* mucoadhesiveness test showed that the percentage of mucoadhesive microspheres remaining on the stomach mucosa (Table 1). Figure 1 showed that even after 12 h, 52% microspheres were adhered to the gastric mucous layer. The mucoadhesive microspheres of all the batches of the factorial design were spherical (Figure 2, batch J4) and free flowing.

The linear model generated for percentage mucoadhesion was found to be significant with an $F$-value of 29.96 ($p < 0.0001$) and $R^2$ value of 0.9803:

\[
\text{% Mucoadhesion} = 77.66 + 15.33X_1 - 7.16X_2 - 2.5X_1X_2 - 10X_2^2
\]

The counter plot (Figure 6(a)) shows that the *in vitro* wash-off test for percentage mucoadhesion of microspheres increased from 40 to 57 and 73 to 90 at lower and higher levels of drug-to-polymer-to-polymer ratio, respectively, as stirring speed decreased. Results of equation indicate that the effect of $X_1$ (drug-to-polymer-to-polymer) 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 drug-to-polymer-to-polymer ratio increases, the percentage mucoadhesion also increases; because more amounts of polymer results in higher amount of free $\text{–COOH}$ groups\textsuperscript{13}, 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 gastrointestinal tract for an extended period of time (Figures 3 and 4.). Figure 4 showed that even after 8 h, some of the microspheres were adhered to the gastric mucous layer. All factorial batches showed more than 50 mucoadhesion even after 10 h.

**Factorial equation for particle size**

The linear model generated for particle size was found to be significant with an $F$-value of 33.6 ($p < 0.0001$) and $R^2$ value of 0.9824:

\[
\text{Particle size} = 102.33 + 7.16X_1 - 6.83X_2 - 1.5X_1X_2 - 1.5X_2^2
\]

The counter plot (Figure 6(b)) shows that the particle size of microspheres increased from 86.0 to 99.0 $\mu$m and 99.0 to 112 $\mu$m at lower and higher levels of drug-to-polymer-to-polymer ratio, respectively, as stirring speed decreased. Results indicate that the effect of $X_1$ (drug-to-polymer-to-polymer) is more significant than $X_2$ (stirring speed). This means, as the stirring speed increased, the particle sizes decreased, which directly affected the percentage mucoadhesion.

Thus, one can conclude that the amount of polymer (carbopol-934P) and stirring speed directly affects the percentage mucoadhesion and particles size.

**Factorial equation for drug entrapment efficiency**

The drug entrapment efficiency and $t_{50}$ 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, physico-chemical properties of drug and formulation variables. The linear model generated for drug entrapment efficiency
was found to be significant with an F-value of 40.70 (p < 0.0001) and R² value of 0.9954:

Drug entrapment efficiency = 50.11 + 9.16X₁
− 5.66X₂ − 0.66X₁X₂
− 1.75X₁² − 18.16X₂² (7)

The counter plot (Figure 6(c)) shows that the percentage drug entrapment efficiency of microspheres increased from 20.0 to 26.0 and 34.0 to 47.0 at lower and higher levels of drug-to-polymer-to-polymer ratio, respectively, as stirring speed decreased. However, at medium level of drug-to-polymer-to-polymer ratio, as stirring speed decreased, percentage drug entrapment efficiency of microspheres showed an increase from 41.0 to 56.0. The results of this equation indicate that the effect of X₁ (drug-to-polymer-to-polymer) is more significant than X₂ (stirring speed). Moreover, the stirring speed had a negative effect on the percentage drug entrapment efficiency (i.e. as the stirring speed increased, the particle size decreased and thus drug entrapment efficiency decreased).

**Factorial equation for t₈₀**

The linear model generated for t₈₀ was found to be significant with an F-value of 115.91 (p < 0.0001) and R² value of 0.9994:

\[ t₈₀ = 536.22 - 169.5X₁ + 42.66X₂ \\
- 23.5X₁X₂ - 56.83X₁² - 8.66X₂² \] (8)

The counter plot (Figure 6(d)) shows that the percentage drug released in vitro from microspheres decreased from 727 to 592 min and 340 to 299 min at lower and higher levels of drug-to-polymer-to-polymer ratio, respectively, as stirring speed decreased. The results depicted in Table 3 indicate that the percentage drug released in vitro is highly dependent on the drug-to-polymer-to-polymer ratio and stirring speed. The drug-to-polymer-to-polymer ratio has a negative effect on t₈₀, while stirring speed has a positive effect on t₈₀ because as the particle size decreases the drug release decreases.

A numerical optimization technique using the desirability approach was employed to develop a new formulation with the desired responses. Constraints like maximizing percentage mucoadhesion, drug entrapment efficiency, particle size and release at the end of 12 h in addition to minimizing the t₈₀ were set as goals to locate the optimum settings of independent variables in the new formulation. The optimized microsphere formulation (110) was developed using a 1:3:1.25 drug-to-polymer-to-polymer ratio and 950 rpm stirring speed. The optimized formulation was evaluated for percentage mucoadhesion, drug entrapment efficiency, particle size and drug release. The results of experimentally observed responses and those predicted by mathematical models along with the percentage prediction errors were compared. The prediction error for the response parameters ranged between 0.50–2.15%, with the value of absolute error of 1.36 ± 0.70%. The low values of error indicate the high prognostic ability of factorial equation and counter plot methodology. The drug release from the optimized formulation was found to be low t₈₀ (410 min), thus, batch J₄ was selected for further study, which exhibited a high t₈₀ of 502 min and seemed to be a promising candidate for achieving drug release of up to 12 h. The drug release profile of batch J₄ is shown in Figure 5. The figure reveals that drug release rate slowed after 4 h. The study focus was the preparation of mucoadhesive microspheres, thus the microspheres of batch J₄ were also evaluated in simulated gastric fluid USP (pH 1.2). In vitro release test showed that amoxicillin released faster in pH 1.2 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 1.2 as the f₂ (similarity factor) value was 63.48.

The results of curve fitting of best batch into different mathematical models are given in Table 2. The mechanism of drug release from the microspheres was found to be diffusion controlled because plots of percentage cumulative drug release vs square root of time were found to be linear with the regression coefficient (R²) values ranging from 0.9780–0.9871 for the best batch. Results of F-statistics are shown in Table 2. The release profile fitted to Weibull equation F-value was found to be 6.92. The value of correlation coefficient was found to be 0.9931. The values of slope and intercept were found to be 1.27 and −3.17, respectively. The release profile fitted to Korsmeyer-Peppas equation, F-value was found to be 20.36. The value of correlation coefficient was found to be 0.9935. The values of slope and intercept were found to be 0.8611 and −2.38, respectively; and release profile fitted to Hixon-Crowell equation, F-value was found to be 10.49. The value of correlation coefficient was found to be 0.9889. The values of slope and intercept were found to be 0.0046 and −0.1038, respectively. The results of F-statistics were used for the selection of the most appropriate model, thus it was concluded that the release profile fitted best to Weibull equation (F = 6.92).

**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 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 2.48–30. 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, which might cover up its shortcoming of being unstable in acidic medium. However, for the mucoadhesive microspheres, which would stay in the stomach for a much longer time, the stability of amoxicillin should be seriously considered. This study found that amoxicillin microspheres were more stable in pH 1.2 HCl than amoxicillin powder.

From the result of the in vivo *H. pylori* clearance test, it was observed 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. Means, 4 mg kg⁻¹ dose of amoxicillin mucoadhesive microspheres administrated colony counts were 23 ± 4.24 and on increase in the doses to 7.5 and 15 mg kg⁻¹ colony counts were 7.0 ± 1.41 and 2 ± 0, respectively. On administrating Amoxicillin powder 4 mg kg⁻¹ dose colony counts were 78 ± 8.48 and on increase in the doses to 7.5 and 15 mg kg⁻¹ colony counts were 29 ± 5.65 and 18.0 ± 16.97, respectively. While in the case of physiological saline 4 mg kg⁻¹ dose administrated colony counts were 94 ± 5.65 and then increases in the doses to 7.5 and 15 mg kg⁻¹ colony counts were 92 ± 9.89 and 92.5 ± 17.67, respectively. Physiological saline did not show any decrease in colony count. However, the ratio of colony counts between amoxicillin powder and mucoadhesive microspheres increased rapidly from 3.39 at 4 mg kg⁻¹ to 9.0 at 15 mg kg⁻¹ (Table 4, Figure 7). This phenomenon indicated that, with the increase in dose, mucoadhesive amoxicillin microspheres showed more effective clearance of *H. pylori* than that in the case of amoxicillin powder. It is inferred that this might be due to the lack of repetition of drug administration. Therefore, another multidose administration regimen was tried. The results showed that, at the dose of 3.5 mg kg⁻¹, when amoxicillin microspheres, powder or physiological saline was administrated, respectively, to the *H. pylori* infectious rat twice a day for three consecutive days (Table 5). Neither *H. pylori* colony was found nor was urease test positive in rats whom mucoadhesive amoxicillin microspheres were administrated. It is concluded that the mucoadhesive amoxicillin microspheres showed a more complete *H. pylori* clearance effect.

### Conclusion

The Amoxicillin mucoadhesive microspheres developed employing a 3² full factorial design showed high percentage mucoadhesion, drug entrapment efficiency and exhibited a sustained release property for per oral use in the form of capsules. Drug-to-polymer-to-polymer (amoxicillin-ethyl cellulose-carbopol-934P) and stirring speed significant influence on percentage mucoadhesion, drug entrapment efficiency, particle size and fₚ₀. The optimized formulation, developed using the desirability approach, showed more effective *H. pylori* activity of mucoadhesive amoxicillin microspheres compared to amoxicillin powder and physiological saline, which might indicate a potential use of mucoadhesive amoxicillin microspheres in treating *H. pylori* infection.

#### Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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