ORIGINAL ARTICLE

Effect of cross-linking on physicochemical properties of chitosan mucoadhesive microspheres: A factorial approach

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The objective of the present study was to develop chitosan-based mucoadhesive microspheres of Clarithromycin, to provide a prolonged contact time for drug delivery of antibiotics, to treat stomach ulcers. Chitosan mucoadhesive microspheres with small particle size and good sphericity were prepared by an emulsification technique using glutaraldehyde as a cross linking agent. Glutaraldehyde, the aldehyde most frequently employed as a chemical cross linker agent for proteins, was also used as a control. The prepared microspheres were optimized by 3^2 factorial design, using the concentration of the cross linking agent (X_1) and time of cross linking (X_2) with respect to their morphological aspects, percentage entrapment, *in-vitro* drug release and percentage mucoadhesion. Microspheres were discrete, spherical and free flowing. The microspheres exhibited a good mucoadhesive property in the *in vitro* wash-off test and also showed a high percentage of drug entrapment efficiency. The best batch exhibited a high drug entrapment efficiency of 45% and percentage mucoadhesion after 5 hours was 15%. The drug release from microspheres was characterized by the initial burst effect followed by sustained release for more than 12 hours. It was concluded that drug entrapment efficiency was increased with an increase in glutaraldehyde concentration and an increase in cross linking time. As the glutaraldehyde concentration and cross linking time increased, the percentage of mucoadhesion decreased. Thus chitosan microspheres appeared to be, technically, promising mucoadhesive drug delivery systems for delivering Clarithromycin, to treat stomach ulcers.

Key words: Chitosan, clarithromycin, cross linking, factorial design, glutaraldehyde, mucoadhesive microspheres

INTRODUCTION

Helicobacter pylori, is a micro-organism that is believed to be the main cause of gastric or peptic ulcer. To eradicate *H. pylori,* a large quantity of antibiotics have been administered orally, which may lead to toxicity, patient discomfort, patient noncompliances and drug resistance.[1,2] The antibiotic with the highest eradication rate in monotherapy *in vivo* is Clarithromycin.[3] One probable reason for the incomplete eradication of *H. pylori* is the short residence time of the dosage form in the stomach, and as a result, effective antimicrobial concentration cannot be achieved in the gastric mucosal layer or epithelial cell surface where *H. pylori* exists.[4,5] Thus, for enhancing the eradication rate of *H. pylori* the residence time of the antibiotic in the stomach can be extended, which will maintain a higher antibiotic concentration in the gastric region where *H. pylori* exists, thereby improving the therapeutic efficacy.[6]

Hence, in the present investigation, mucoadhesive drug delivery has been employed with chitosan, which possesses mucoadhesive properties owing to the molecular attractive forces formed by the electrostatic interaction between the positively charged chitosan and negatively charged mucosal surfaces, which become adhesive on hydration and provide an intimate contact between a dosage form and the absorbing tissue. Hence, this can be used for targeting a drug to a particular region of the body for an extended period of time.[7] The objectives of this study are to prepare Clarithromycin-loaded chitosan mucoadhesive microspheres that provide intimate contact of the drug with the mucosa for an extended period of time and to enhance the bioavailability of drugs to treat stomach ulcers and to observe the effect of processed independent variables, namely, concentration of the cross linking agent and cross linking time on the physicochemical properties of mucoadhesive microspheres by applying the 3^2 factorial design.

MATERIALS AND METHODS

Clarithromycin was obtained as a gift sample from Wockhardt Pvt. Ltd. (Ankleshwar, India). Chitosan (degree of deacetylation of 85%; intrinsic viscosity of 400 mPa/s for 1% solution in 1% aqueous acetic acid at 20°C) was obtained as a gift sample from the Central Institute of Fisheries Technology (Cochin, India). Liquid

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paraffin and glutaraldehyde were purchased from Loba Chemie Pvt Ltd (Mumbai, India). All other chemicals were of analytical grade.

Preparation of Microspheres[8-11]

Mucoadhesive microspheres of chitosan were prepared using a simple emulsification phase-separation technique. The drug (100 mg) was added to the disperse phase of 40 mL of 1% vol/vol aqueous acetic acid containing 1% wt/vol chitosan solution. The resultant mixture was extruded through a syringe (No. 20) in 100 mL of liquid paraffin (heavy and light, 1 : 1 ratio) containing 0.5% Span 85, and it was stirred using a propeller stirrer (Remi, Mumbai, India) at 1000 rpm. After 15 minutes, glutaraldehyde (25% vol/vol aqueous solution) was added and stirring was continued. The amount of cross-linking agent (X_1) and cross-linking time (X_2) were varied in batches CH1 to CH9 from 1 to 3 mL and 1 to 3 hours, respectively, as shown in Table 1. 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 glutaraldehyde. The microspheres were then dried at room temperature (at 25°C and 60% relative humidity [RH]) for 24 hours. The effect of formulation variables on the characteristics of the microspheres is summarized in Table 1.

Factorial Design

A statistical model incorporating interactive and polynomial terms was used to evaluate the responses:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_{11} + b_{22}X_{22} \quad (1)$$

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 factor X_1. 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_{11} and X_{22}) are included to investigate nonlinearity.

Study Design for Optimization of Process Parameters (3^2 Factorial Design)

Batches were prepared to optimize the process parameters for preparation of microspheres, according to a 3^2 factorial design as follows: Two independent variables (Cross linking agent volume and Cross linking time) and three levels of study, as indicated in Table 1.

Scanning Electron Microscopy

A Philips FE1 Company scanning electron microscope (SEM) was used (TGA-7DSC-PYRIS-1DTA-7, Gaseous secondary electron detector, acceleration voltage of 20 kV, chamber pressure of 0.6 mm Hg).

Drug Entrapment Efficiency

Microspheres (50 mg) were crushed in a glass mortar and pestle, and the powdered microspheres were suspended in 10 mL of phosphate buffer (pH 7.4) to extract the drug from the microspheres. After 24 hours, the solution was filtered and the filtrate was analysed for the drug content. The drug entrapment efficiency was calculated using the following formula: (wt of drug in microspheres/wt of drug added) ×100.

***In-vitro* Drug Release Study**

The drug release study was performed using USP Type-II dissolution apparatus at 37°C ± 0.5°C using 500 mL of 0.1 N HCl (pH 1.2) as a dissolution medium. Microspheres (150 mg) were placed in the basket and rotated at a speed of 100 rpm; 5 mL of aliquots of dissolution medium were withdrawn at different time intervals, filtered, diluted suitably, and assayed at 397 nm using the UV visible spectrophotometer.

***In-vitro* Wash-Off Test for Microspheres[12,13]**

Rat stomach mucosa was collected and cleaned with deionized water (3-4 times). A 1 × 1 cm piece of rat stomach mucosa was tied onto a glass slide horizontally (3 × 1 inch). Approximately 100 microspheres were spread onto the wet, rinsed, tissue specimen, and the prepared slide was hung on one of the

Table 1: Effect of glutaraldehyde concentration and cross linking time on dependent variables

Batch no.	Glutaral dehyde (ml)	Cross linking time (hour)	Drug entrapment efficiency (%) Mean ± SD	*In vitro* drug release up to 8 hours Mean ± SD	Percentage mucoadhesion (At 5 hours) Mean ± SD
CH1	2	1	15.05 ± 0.59	68.25 ± 0.44	18 ± 0.56
CH2	2	2	21.42 ± 0.17	58.04 ± 0.37	17 ± 0.16
CH3	2	3	22.94 ± 0.53	56.55 ± 0.58	19 ± 0.46
CH4	3	1	28.99 ± 0.76	75.36 ± 0.67	17 ± 0.34
CH5	3	2	29.12 ± 0.87	77.48 ± 0.49	17 ± 0.64
CH6	3	3	30.37 ± 0.57	62.38 ± 0.79	16 ± 0.67
CH7	4	1	43.02 ± 0.23	54.57 ± 0.49	16 ± 0.76
CH8	4	2	43.21 ± 0.14	54.11 ± 0.56	15 ± 0.88
CH9	4	3	45.21 ± 1.69	55.78 ± 0.34	15 ± 0.94
Translation of coded levels in actual units					
Variables level			Low (-1)	Medium (0)	High (+1)
Glutaraldehyde (ml)			2	3	4
Cross linking time (hours)			1	2	3

groves of a USP tablet disintegrating test apparatus. The disintegrating test apparatus was operated such that the tissue specimen was given regular up and down movements in a beaker containing the simulated gastric fluid USP (pH 1.2). At the end of 30 minutes, 1 hour, and at hourly intervals, for up to 5 hours, the number of microspheres still adhering onto the tissue was counted.

RESULTS AND DISCUSSION

Chitosan mucoadhesive microspheres with small particle size and good sphericity were prepared by an emulsification technique using glutaraldehyde as a cross linking agent where chitosan could be covalently cross-linked with glutaraldehyde through its amino groups. The aldehyde groups of the glutaraldehyde formed covalent imine bonds with the amino groups of chitosan, due to the resonance established with the adjacent double ethylenic bonds via a Schiff reaction [Figure 1]. Various batches of microspheres were prepared by employing the factorial design. A total of nine batches were prepared by employing the 3^2 factorial design. All the batches were evaluated and different equations were derived for percentage encapsulation efficiency, cumulative percentage release (for 8 hours) and for percentage mucoadhesion (after 5 hours).

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 (CH1 to CH9). The fitted equations (full models) relating the responses (Drug entrapment efficiency, percentage drug release up to 8 hours and for percentage mucoadhesion for 5 hours) to the transformed factor are shown in Table 1. The polynomial equations can be used to draw conclusions, after considering the magnitude of coefficient and the mathematical sign it carries, namely, positive or negative. The high values of the correlation coefficient [Table 2], for the dependent variables, indicate a good fit. The equations may be used to obtain estimates of the response since a small error of variance was noticed in the replicates and the derived equation was checked for its validity by preparing a check point batch.

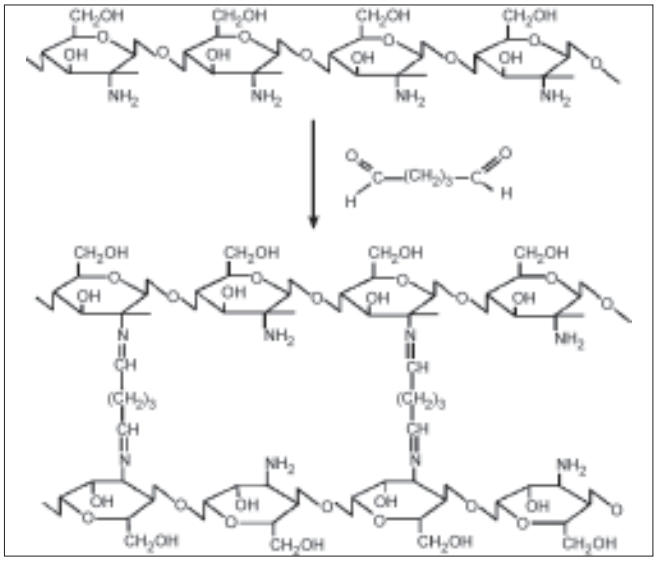

Figure 1: Cross linking process of chitosan treated with glutaraldehyde

Drug Entrapment Efficiency

The following polynomial equation was derived by multiple regression analyses of the data.

$$Y = 29.33 + 12.23X_1 + 1.576X_2 - 1.89X_1X_2 + 2.95X_2^2 - 0.14X_1^2 \quad (2)$$

Where Y = Drug entrapment efficiency (%)
X_1 = Amount of glutaraldehyde (mL)
X_2 = Cross linking time (hours)

The R^2 value found was 0.9940, indicating a good fit. It was found that X_2, X_1X_2, X_1^2 and X_2^2 are nonsignificant terms ($P > 0.05$). Hence these were omitted from the full model equation. Multiple regressions of the remaining term (X_1) gave the following reduced model equation.

$$Y = 31.20 + 12.236X_1 \quad (3)$$

The coefficient of X_1 is positive. Hence the value X_1 (concentration of glutaraldehyde) increases and the drug entrapment efficiency of microspheres also increases. The coefficient of X_1 is 12.23. Hence, it can be concluded that X_1 is a major contributing factor for the drug entrapment efficiency of microspheres.

Percentage Release (Up to 8 hours)

$$Y = 72.44 - 3.063X_1 - 3.911X_2 + 3.227X_1X_2 - 1.0616X_2^2 - 13.85X_1^2 \quad (4)$$

Where Y = *In vitro* drug release up to 8 hours (%)
X_1 = Amount of glutaraldehyde (ml)
X_2 = Cross linking time (hour)

The R^2 value was 0.8773, indicating a good fit. It was evident that X_1, X_2, X_1X_2, and X_2^2 were nonsignificant terms ($P > 0.05$). Hence they were omitted from the full model equation. Multiple regressions of the remaining term X_1^2 gave the following reduced model equation.

$$Y = 63.21 - 13.85\ X_1^2 \quad (5)$$

Table 2: Summary results of regression analysis

Coefficient	b_0	b_1	b_2	b_{11}	b_{22}	b_{12}	R^2
Drug entrapment efficiency	29.33	12.23	1.576	−1.89	2.95	−0.14	0.9940
In vitro drug release up to 8 hours	72.44	−3.063	−3.911	3.22	−1.0616	−13.85	0.8773
Percentage mucoadhesion (At 5 hours)	16.33	−1.833	−0.166	−0.5	0.5	−0.5	0.9305

From the data it was found that the amount of glutaraldehyde had a strong effect on *in vitro* drug release. The negative coefficient suggested that as the concentration of glutaraldehyde increased the drug release decreased, which was well supported by the drug release profiles. Higher levels of glutaraldehyde and more cross linking time, favor the cross-linking reaction and thus slower drug release. Batch CH8 exhibited a drug release of 54.11 ± 0.56% for 8 hours and seemed to be a promising candidate for achieving drug release up to 12 hours. The comparative drug release profiles of all batches are shown in Figure 2.

Chitosan microspheres are known to swell in aqueous environments, due to hydration. As a new polymeric structure is formed by introducing bridges between polymeric chains during the cross-linking procedure, the extent of the swelling process depends on the degree of cross-linking. Therefore, the denser the cross-linking bridges between the chitosan molecules, the more packed is the structure. Such a structure can be characterized by lower and slower penetration of the solvent through the chain structure of the polymer, suggesting that the swelling ratio and hence the release characteristics of the microsphere can be controlled by varying the content of the cross-linking agent used during the manufacturing process.[14] Swelling of microspheres may result in the mobility of chitosan chains, facilitating rapid release rates of the drug, by diffusion, through the polymer. Since glutaraldehyde is responsible for the formation of cross-links, increasing the amount of glutaraldehyde and the cross-linking time will increase the polymer density, resulting in reduction of the macromolecular chain mobility, and the formation of more stable and rigid spheres that show a lower tendency to swell. The finding of this investigation is in agreement with an earlier study performed by a group of researchers. [15-19] As shown in this study, this effect may be attributed to the fact that the cross-linking process usually hardens the chitosan matrix and can also increase the resistance for the penetration of the release medium.

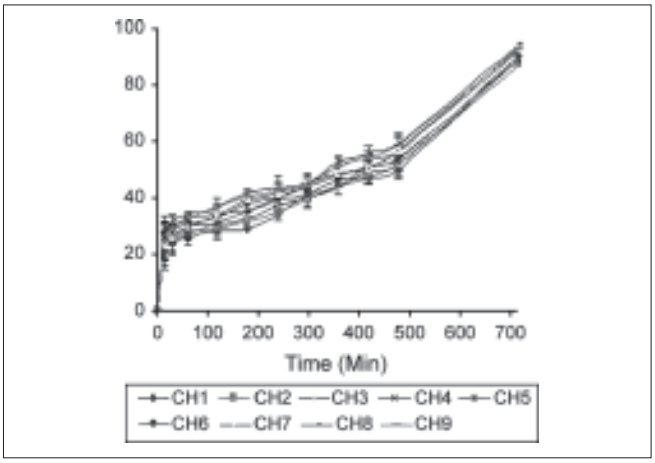

Figure 2: Cumulative percentage drug release (CH1 to CH9)

The dissolution data of batch CH8 were further analysed, to ascertain the mechanism of drug release. The release profile fitted the Weibull equation (F = 9. 05) best. The value of the correlation coefficient was found to be 0.978. The values of slope and intercept were found to be 1.42 and –2.06, respectively.

Percentage Mucoadhesion (At 5 hours)

$$Y = 16.33 - 1.833X_1 - 0.166X_2 - 0.5X_1X_2 + 0.5X_2^2 - 0.5X_1^2 \quad (6)$$

Where Y = Percentage Mucoadhesion (At 5 hours)
X_1 = Amount of glutaraldehyde (ml)
X_2 = Cross linking time (hour)

The R^2 value was 0.9305, indicating a good fit. It was found that X_2, X_1X_2, X_1^2 and X_2^2 were nonsignificant terms ($P > 0.05$). Hence, they were omitted from the full model equation. Multiple regressions of the remaining term X_1 gave the following reduced model equation.

$$Y = 16.33 - 1.83\ X_1 \quad (7)$$

The concentration of glutaraldehyde had a negative effect on the percentage of mucoadhesion, that is, as the concentration increased, the percentage of mucoadhesion decreased. This resulted from the increased glutaraldehyde concentration that made the microspheres harder, thus affecting the swelling index, and thereby decreasing the percentage of mucoadhesion.

Validation of Polynomial Equations

The response of dependent variables, namely, drug entrapment efficiency, *in vitro* drug release up to 8 hours and percentage of mucoadhesion (at 5 hours) of the check point batch were experimentally determined [Table 3]. The experimentally determined values of the dependent variables were compared with the predicted values obtained from the equations. There were no significant differences between the experimentally determined values and the values derived from the equations. Thus, validity of equations 3, 5 and 7 were established, and it was concluded that they had good predicting power.

Surface Morphology

The shape and surface morphology of the produced microspheres have been studied with the aid of a scanning electron microscope and an optical microscope. The w/o emulsion system yields regular shaped microspheres with apparently homogeneous surfaces. The microspheres were spherical in shape and had smooth surfaces [Figure 3].

Table 3: Comparison of check point batch for dependent variables

Batch	Glutaral-dehyde (ml)	Cross linking time	% EE		*In vitro* drug release up to 8 hrs		Percentage mucoadhesion (at 5 hrs)	
			EV	PV	EV	PV	EV	PV
CH10	1.8 ml	1.8 hour	13.06	14.08	61.78	63.893	16	18.01

EE- Drug entrapment efficiency, EV- Experimental value and PV- Predicted value

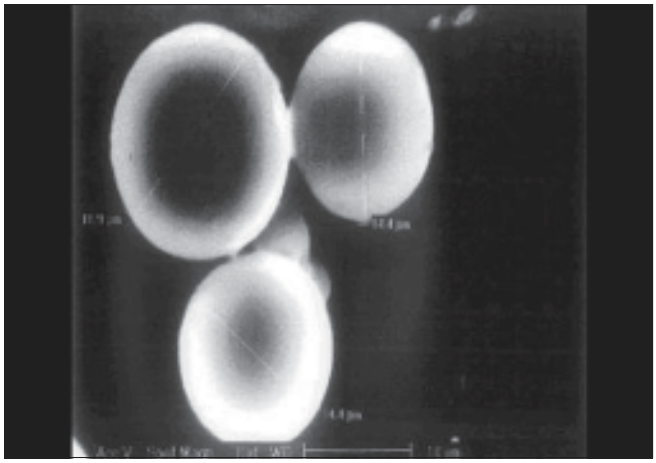

Figure 3: Photomicrographs of prepared microspheres (a) Batch CH8

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