Preparation and Characterization of Cross-Linked Guar Gum Microspheres: Optimization Using Factorial Design

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In the present work cross-linked guar gum microspheres were prepared for colon specific delivery of ornidazole. Development and optimization of guar gum microspheres for colonic drug delivery was carried out using a 2^4 factorial design based on four independent variables. Microspheres were prepared by emulsification method using glutaraldehyde as cross-linking agent. Morphology and surface characteristics of the formulations were determined by scanning electron microscopy. Particle size of the guar gum microspheres was determined by particle size analyzer. *In vitro* drug-release studies were performed in conditions simulating stomach-to-colon transit in the presence and absence of rat cecal contents. Only a small fraction of drug was released at acidic pH; however, the release of drug was found to be higher in the presence of rat cecal contents, indicating the susceptibility of guar gum matrix to colonic enzymes released from rat cecal contents. The significance of differences was evaluated by analysis of variance (ANOVA). Differences were considered statistically significant at $p<0.05$.

Key words emulsification method; cross-linked; guar gum microsphere; colonic delivery; optimization; factorial design

Amoebiasis is a very common infection of the large intestine caused by *Entamoeba histolytica*, a single celled protozoan parasite. Ornidazole is the drug of choice used in the treatment of amoebiasis, giardiasis, trichomoniasis and anaerobic infections.[1,2] The drug is to be delivered to the colon for its effective action against *E. histolytica* where in the trophozoites resides in the lumen of the caecum and large intestine and adhere to the colonic mucus and epithelial layers.[3] The pharmacokinetic profile of ornidazole indicates that the drug is rapidly absorbed from stomach and small intestine. Since the administration of ornidazole, in the form of conventional tablet dosage might lead to entry of most of the drug into systemic circulation resulting in unwanted side effects and at the same time it is difficult to maintain the effective drug concentration at the desired site of infection, due to the degradation of drug in stomach and small intestine.[4] The targeting of ornidazole to the colon may provide an effective treatment of intestinal amoebiasis (with lower dose) with minimal or no systemic side effects. An ideal colon targeted drug delivery system should not only protect its drug from being released in the physiological environment of stomach and small intestine but also deliver its drug to the colon.[5—8] Polysaccharides such as chitosan, pectin, inulin, and guar gum have been explored for their potential in colon-specific drug delivery.[9—11]

In the present work guar gum was selected as carrier for the preparation of microspheres. Guar gum does not disintegrate in the stomach and small intestine, but in the colon the presence of bacterial polysaccharidases degrade the polymer, hence the maximum drug release takes place in the colon, at the desired site.[12,13] The microparticulate delivery systems are considered and accepted as a reliable means to deliver the drug to the target site with specificity, if modified, and to maintain the desired concentration at the site of interest without untoward effect(s). The main objective of our research work is formulation and characterization of cross-linking guar gum microspheres for colon specific delivery of ornidazole. Guar gum was selected as a carrier system; it is a natural polymer, and having swelling property and large amount of drug can be loaded into system.[14,15]

Experimental

Ornidazole (100.1% pure) was gift samples from M/s Aristo Pharmaceuticals, Bhopal, India. Tween-80 and Guar gum (viscosity of 1% aqueous dispersion is 125 cps at 258) was obtained from M/s Ranbaxy Lab. Guargaon, India. Liquid paraffin and Glutaraldehyde were purchased from Sigma Aldrich India. All materials used were of USP/NF quality.

Preparation of Cross-Linked Guar Gum Microspheres Different batches of microspheres were prepared by water-in-oil (w/o) emulsification method as reported by Kumarsh and Tejraj[16] with minor modifications. In brief, aqueous dispersion of guar gum (20 ml) containing 20% Ornidazole, was acidified with 0.5 ml of dilute sulphuric acid solution and emulsified into 40 ml of liquid paraffin with 2% tween-80 and in order to cross-link the polymer glutaraldehyde was added to the solution. The mixture was stirred continuously using a mechanical stirrer for 4 h at room temperature. The hardened microspheres were filtered and washed repeatedly with hexane and water to remove liquid paraffin, unreacted glutaraldehyde and any adhered tween-80. The microspheres were then dried at 40 °C over night and kept in desiccators until further use (Table 2).

Experiment Design In this study the orthogonal experimental design was introduced to optimize the formulation of ornidazole loaded cross-linked guar gum microspheres. Objective function for the present study was selected as maximizing encapsulation efficiency and minimizing drug release in simulated gastro intestinal fluid (SGIF).

A design matrix comprising of 16 experimental runs was constructed using Design Expert 6.0 to investigate the effect of three factors (polymer concentration as X_1, cross-linking agent as X_2, swelling time as X_3 and stirring speed as X_4) on the response variable by Y_1 (% drug release) and Y_2 (% encapsulation efficiency). The various levels of experimental parameters were summarized in Table 1.

The statistical design provides a polynomial describing quadratic effect, as well as interactions of each study factors on considered response variables.

The general model corresponds to the following equations:

$$Y = b_0 + b_1X_1 \cdots + b_4X_4 + b_{12}X_1X_2 + \cdots b_{123}X_1X_2X_3 + \cdots b_{1234}X_1X_2X_3X_4$$

Where Y is the majored response associated with each factor level combination; b_0 is an intercept b_1 to b_{234} are the regression coefficients; and X_1, X_2, X_3 and X_4 are the independent variable. The variables selected are shown in Table 1. These high, and low levels were selected from the preliminary experimentation. The amounts of guargum (X_1), glutaraldehyde (X_2), swelling time (X_3) and stirring speed (X_4), used to prepare each of the 16 formulations

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are in Table 2.

***In Vitro* Characterization of Guar Gum Microspheres. Fourier Transform Infra-Red Characterization (FT-IR) of Microspheres** FT-IR (470 Plus, Jasco, Japan) spectra of the guar gum and cross-linked microspheres were determined to check the cross-linking between guar gum and glutaraldehyde.

Size and Surface Morphology Morphology and surface characteristics of the microspheres were determined using scanning electron microscopy (SEM) (AIIMS, New Delhi, India). Particle size of the cross-linked guar gum microspheres was determined by using particle size analyzer (Cilas 1064 L, Marcoussis France).

Encapsulation Efficiency Encapsulation efficiency is the amount of added drug (in percent) that is encapsulated in the formulation of microspheres. Encapsulation efficiency was calculated in terms of the ratio of drug in the final formulation to the amount of added drug. An accurately weighed amount (100 mg) of the formulation of microspheres was dispersed in 100 ml of phosphate buffer saline (PBS) pH 7.4. The sample was ultrasonicated for 3 consecutive periods of 5 min each, with a resting period of 5 min each. It was left to equilibrate for 24 h at room temperature, and the suspension was then centrifuged at 3000 rpm for 15 min. The supernatant was diluted appropriately with PBS (pH 7.4) and analyzed for concentration of ornidazole using UV/visible spectrophotometer (UV-1700 CE by Shimadzu Corporation, Japan).

$$\text{encapsulation efficiency} = \frac{\text{concentration of drug in final formulation}}{\text{concentration of drug initially loaded to microspheres}} \times 100$$

Swelling Study The equilibrium water uptake of the cross-linked empty and drug loaded microspheres were determined by measuring the extent of swelling in water.

To ensure complete equilibrium the samples were allowed to swell for 24 h. The excess liquid drops adhered on the surface were removed by blotting and the swollen microspheres were weighed. The microspheres were dried in oven at 60 °C for 5 h, until there was no further change in the dried mass of samples. The degree of swelling was then calculated from the following formula:

$$\text{swelling index} = \frac{\text{mass of swollen microspheres} - \text{mass of dry microspheres}}{\text{mass of dry microspheres}} \times 100$$

***In Vitro* Drug Release Study. *In Vitro* Drug Release Study in Simulated Gastrointestinal Fluid** Simulated Gastro-Intestinal Fluid (SGIF): It consisted of simulated gastric fluid (SGF) pH 1.2, simulated intestinal fluid (SIF) pH 4.5, and pH 7.5. SGF pH 1.2 consisted of NaCl (2.0 g) and HCl (7 ml) and pH was adjusted to 1.2±0.5. SIF pH 7.5 consisted of KH_2PO_4 (6.8 g) and 0.2 N NaOH (190 ml), pH was adjusted to 7.5±0.1. SIF pH 4.5 was prepared by mixing SGF pH 1.2 and SIF pH 7.5 in a ratio of 39 : 61. The drug release studies were conducted in SGF for the first 2 h and in mixed SGF and SIF for 3 h. The dissolution medium was then replaced with SIF and tested for the next 3 h. The *in vitro* drug release of ornidazole from the guar gum microspheres was carried out in a USP-II rotating paddle apparatus at a rotation speed of 100 rpm at 37±0.1 °C. The release studies were performed in SGIF and in rat cecal content release medium (pH 6.8). The drug release studies in SGIF (900 ml) were carried out for 8 h. The simulation of gastrointestinal conditions was achieved by using different dissolution media, in simulated gastric fluid for 2 h and in SIF pH 4.5 for 3 h. The dissolution medium was then replaced with SIF and tested for further 3 h. The replacement was made by filtering SIF pH 4.5 through 0.2 μ disc filter paper (Pall Life Science, U.S.A.) and the marc (substance retained on filter paper) remaining on the filter paper was dried at 40 °C and placed in 900 ml dissolution flask containing SIF pH 7.5.

At regular interval of time the aliquots (5 ml) were withdrawn and analyzed for ornidazole using UV/visible spectrophotometer. Sink conditions were maintained with the addition of an equal volume of fresh dissolution medium.

***In Vitro* Drug Release Study in Rat Cecal Content** The study was carried out to check the ability of guar gum microspheres to release the ornidazole in presence of rat cecal content release medium resembling the physiological environment of colon.

Preparation of Rat Cecal Content Release Medium The medium was prepared by the method reported by Van and Kinget[17] Krishnaiah *et al*.[18] The cecum bag was opened and its contents were weighed and homogenized, then suspended in Phosphate buffer (pH 6.8) to give the desired concentrations (0%, 2%, 5%) of cecal contents. The suspension was filtered through glass wool and sonicated (50 W) for 20 min at 4 °C to disrupt the bacterial cells. After sonication, the mixture was centrifuged at 2000 rpm for 20 min. Because the cecum's environment is naturally anaerobic, all the operations were performed in a CO_2 atmosphere, since it increases polysaccharidases

Table 1. Experiment Design-Levels of Process Parameters

Factors	Levels used	
	Low	High
X_1 polymer concentration (%w/v)	1	4
X_2 cross-linking agent (%v/v)	0.5	1.5
X_3 swelling time (h)	0	4
X_4 stirring speed (rpm)	500	1500
Response variable		
Y_1 drug release		
Y_2 encapsulation efficiency		

Table 2. Effect of Various Process Parameters on Ornidazole Release from Cross-Linked Guar Gum Microspheres

Formulation	X_1: % polymer concentration (w/v)	X_2: cross-linking agent (ml)	X_3: swelling time (h)	X_4: stirring speed (rpm)	Y_1: % Drug release		Y_2: % Encapsulation efficiency	
					Actual	Predicted	Actual	Predicted
1	1	0.5	0	500	27.80	30.92	76.57	74.20
2	4	0.5	0	500	23.35	25.17	71.20	74.20
3	1	1.5	0	500	32.90	26.93	70.00	74.20
4	4	1.5	0	500	20.00	21.18	73.00	74.20
5	1	0.5	4	500	32.00	30.92	80.20	81.79
6	4	0.5	4	500	28.00	25.17	85.45	81.79
7	1	1.5	4	500	25.20	26.93	78.25	81.79
8	4	1.5	4	500	22.10	21.18	82.80	81.79
9	1	0.5	0	1500	31.60	30.92	68.20	74.20
10	4	0.5	0	1500	26.20	25.17	79.20	74.20
11	1	1.5	0	1500	26.23	26.93	80.23	74.20
12	4	1.5	0	1500	21.30	21.18	75.20	74.20
13	1	0.5	4	1500	31.20	30.92	82.10	81.79
14	4	0.5	4	1500	24.20	25.17	83.40	81.79
15	1	1.5	4	1500	24.48	26.93	78.10	81.79
16	4	1.5	4	1500	20.23	21.18	84.00	81.79

Table 3. *In Vitro* (%) Cumulative Drug Release in Simulated Gastrointestinal Fluid

Formulation	Time (h)							
	SGF (pH 1.2)		SIF (pH 4.5)			SIF (pH 7.5)		
	1	2	3	4	5	6	7	8
1	2.45±1.23	5.47±1.76	7.86±1.34	11.56±1.22	12.26±1.22	15.75±1.10	21.43±1.22	27.80±1.24
2	2.78±1.34	4.87±1.43	6.87±1.32	10.34±1.48	13.45±1.34	15.56±1.24	19.64±1.28	23.35±1.45
3	3.54±1.54	6.76±1.34	8.22±1.16	12.34±1.18	14.26±1.45	19.65±1.33	25.46±1.37	32.90±1.64
4	2.35±1.2	6.12±1.30	8.72±1.18	10.64±1.26	13.7±0.8	15.23±1.20	18.84±1.34	20.00±1.78
5	3.4±1.43	5.34±0.9	7.92±1.22	10.81±1.32	14.24±0.74	19.66±1.14	25.12±1.32	32.00±1.42
6	4.2±1.18	7.51±1.24	9.66±0.93	11.22±1.22	13.46±1.26	19.24±1.17	22.76±1.31	28.00±1.56
7	5.42±1.24	8.24±1.04	10.24±1.03	12.3±1.04	15.11±1.33	18.75±1.12	20.12±1.04	25.20±1.86
8	4.85±1.34	9.13±0.8	10.46±1.02	13.24±1.16	14.8±1.17	17.04±1.26	19.25±1.25	22.10±1.33
9	3.56±1.46	8.73±0.6	10.73±1.11	12.27±1.32	13.52±0.7	19.07±0.92	23.23±1.14	31.60±1.56
10	2.58±1.56	7.14±1.34	9.78±0.94	11.33±1.11	12.88±1.48	19.6±0.83	22.41±1.27	26.20±1.48
11	8.48±1.53	14.84±1.23	17.13±0.87	19.8±1.02	22.62±1.23	25.33±1.34	27.33±1.25	26.23±2.34
12	4.76±1.45	9.43±1.13	11.70±1.03	14.3±1.26	16.77±1.18	18.85±1.37	20.08±1.28	21.30±2.68
13	6.34±1.43	13.14±0.9	16.66±0.76	18.77±1.22	21.51±1.34	24.78±1.36	25.14±1.23	31.20±2.55
14	3.64±1.46	9.01±1.32	10.81±1.35	13.78±1.24	15.34±0.85	16.63±1.27	18.24±1.03	24.20±2.58
15	2.46±1.72	8.24±1.24	11.76±1.24	12.16±71.23	14.01±0.93	15.65±1.36	17.12±1.23	24.48±2.48
16	2.82±1.45	7.06±1.38	9.16±1.12	10.36±1.12	12.23±1.22	13.82±1.34	15.96±1.16	20.23±2.44

activity.[19—21] The dissolution media used was 100 ml of pH 6.8 phosphate buffered saline (PBS) containing (0%, 2%, 5%) w/v of rat cecal contents (contained 150-ml beaker). The previously weighed amount of microspheres (an amount equivalent to 100 mg of ornidazole) was placed in the dissolution media.

The samples (2 ml) were withdrawn after a fixed time interval of 1 h for analysis, and the volume of dissolution media was replaced with fresh dissolution media. The studies were performed for 24 h; samples were diluted appropriately with PBS (pH 6.8) and centrifuged at 2000 rpm for 10 min. The supernatant was filtered through Whatman filter paper and the filtrate was analyzed for ornidazole content using UV–visible spectrophotometer.

Statistical Analysis Experimental data have been represented as the mean with standard deviation (S.D.) of different independent determinations. The significance of differences was evaluated by analysis of variance (ANOVA). Differences were considered statistically significant at $p<0.05$.

Results and Discussion

FT-IR Characterization of Microspheres During cross-linking, glutaraldehyde might have reacted with the –OH groups of the guar gum through the formation of ether linkages. Hence, the appearance of a sharp peak at, *ca.* 1246 cm^{-1} in the spectra of the cross-linked microspheres confirms the formation of more ether linkages (Fig. 1).

Particle Size and Surface Morphology The particle size and surface morphology was characterized and determined by using particle size analyzer (Cilas 1064 L, Marcoussis France). The average particle size was observed within range of 8.2 to 29.2 mm. Spherical shaped and smooth surface particles were observed in electron microscope (Fig. 2).

Optimization of Formulation To identify the key process variables for experimental design that influence dissolution profiles of the guar gum microspheres independently, effect of the three parameters (polymer concentration, stirring speed and amount of cross-linking agent) was studied by conducting the experimental runs at randomly selected different levels of the three parameters. Data were collected for drug release from microspheres with respect to time in each run. These data were fitted into Design Expert 6.0 and correlation coefficients along with other statistical parameters were estimated. The mathematical relationship in the form of polynomial equation for the measured response, % of drug release (Y_1) released from guar gum microspheres and % encapsulation efficiency (Y_2).

$$Y_1 = 26.05 - 2.88X_1 - 1.99X_2$$

$$Y_2 = 77.99 + 3.79X_3$$

(a)

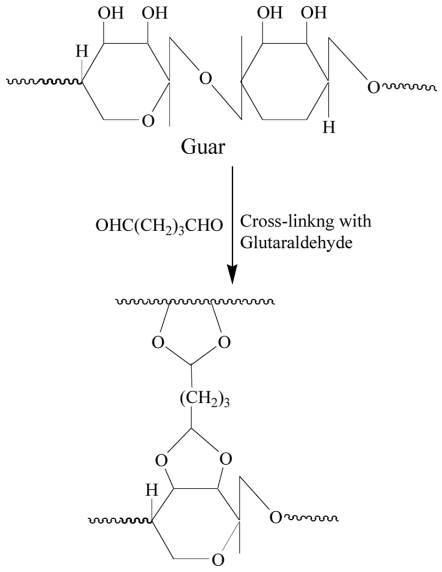

Fig. 1a. Formation of Cross-Linked Guar Gum Microspheres

(b)

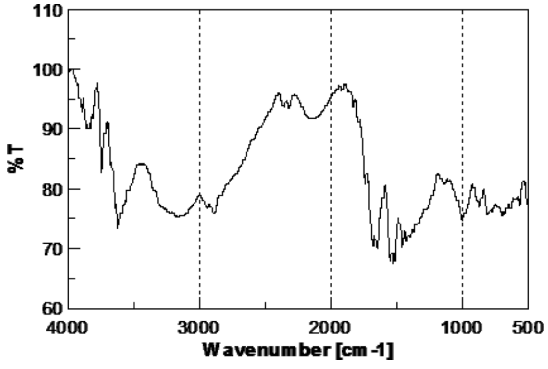

Fig. 1b. FT-IR Spectra of Guar Gum

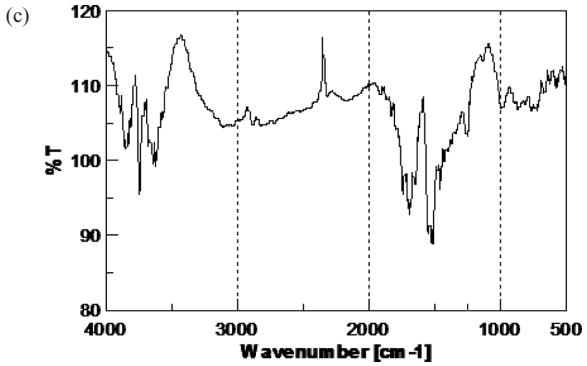

Fig. 1c. FT-IR Spectra of Cross-Linked Guar Gum Microspheres

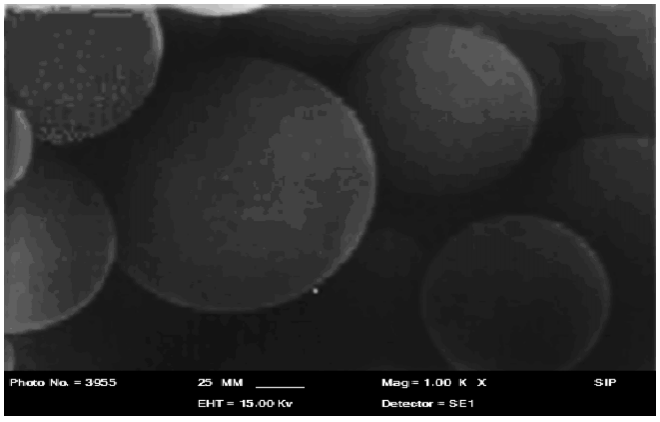

Fig. 2. SEM Micrograph of Cross-Linking Guar Gum Microspheres

Where X_1, X_2 and X_3 represened the polymer concentration, cross-linking agent and swelling time. A positive value represents an effect that favors the optimization, while a negative value indicates an antagonistic effect. The value of X_1, X_2 and X_3 were substituted in the equation to obtain the theoretical values of Y_1 and Y_2. The predicted value and observed value were found to be in good agreement. The effect of pair wise interaction of the prameteres is depicted in the three dimensional graphs (Figs. 3a—d) when the third parameter is kept constant. The optimum condition for the preparation of guar gum microspheres as evident from Table 2 and Fig. 3 are 2.5% w/v polymer concentration, stirring speed at 1000 rpm, amount of cross-linking agent 1% v/v and swelling time 2 h. Therefore the optimum procedure was determined to be the owing; aqueous dispersion of guar gum (20 ml) containing 20% ornidazole, 2.5% w/v of guar gum (accurately weighed amount of polymer was dispersed in cold distilled water and allowed to swell for 2 h) was acidified with 0.5 ml of dilute sulphuric acid. Above solution was emulsified into 40 ml of liquid paraffin with 2% tween-80 and in order to cross-link the polymer, 1 ml of 25% v/v glutaraldehyde was added to the solution formulation OM was prepared using this composition.

The response of ornidazole release from microspheres at 1000 rpm stirring with the polymer concentration 2.5% w/v gives the highest release. When the polymer concentration increased, the release percentage increases initially followed by decrease. The low release of ornidazole from the microspheres at higher polymer concentration may be due to de-

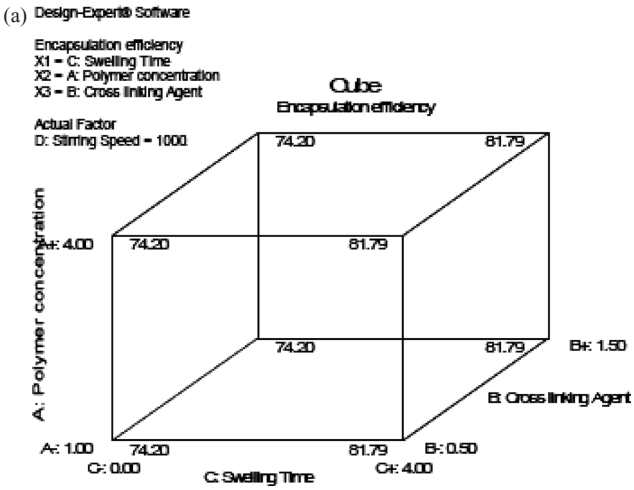

Fig. 3a. Effect of Polymer Concentration, Swelling Time and Cross-Linking Agent on Encapsulation Efficiency

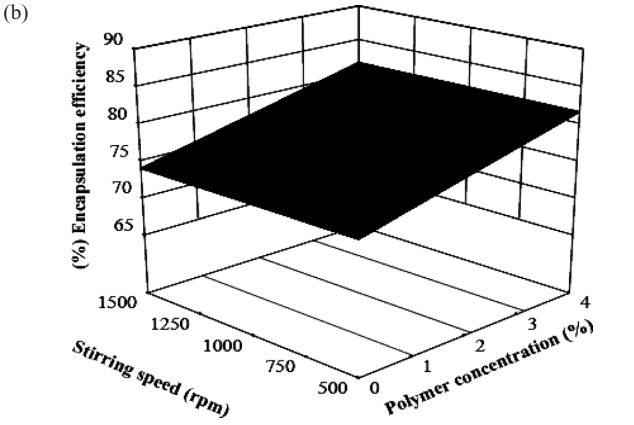

Fig. 3b. Effect of Stirring Speed and Swelling Time on Encapsulation Efficiency

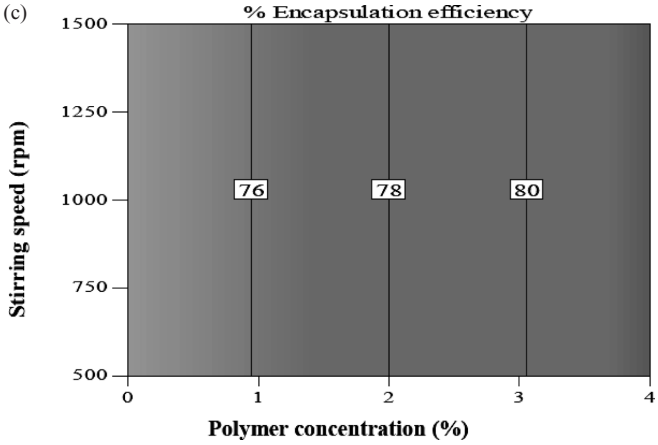

Fig. 3c. Effect of Stirring Speed and Swelling Time on Encapsulation Efficiency

layed swelling of the microspheres with large particle size formation. It is well known fact that at the increasing stirring speed, the loading capacity of drug is very low which may be due to small particle size.[22,23] With the increased amount of cross-linking agent, the ornidazole release % decreases. Loading of ornidazole decreases with increasing cross-linking of the microspheres, because the polymer matrices

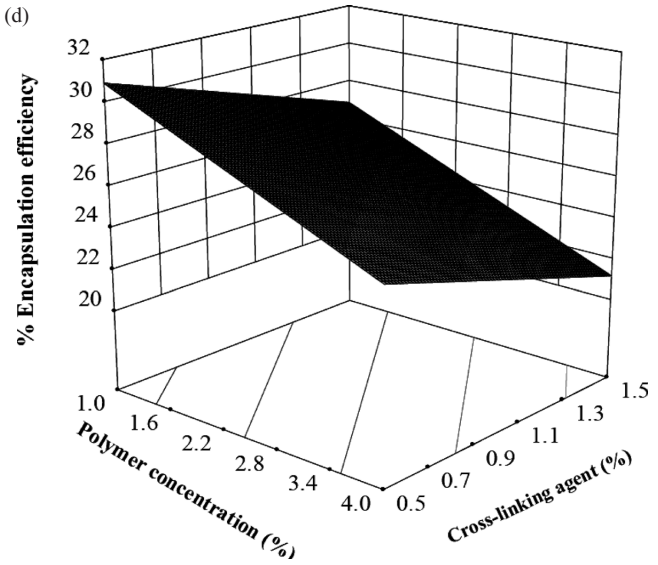

Fig. 3d. Effect of Polymer Concentration and Cross-Linking Agent on Drug Release

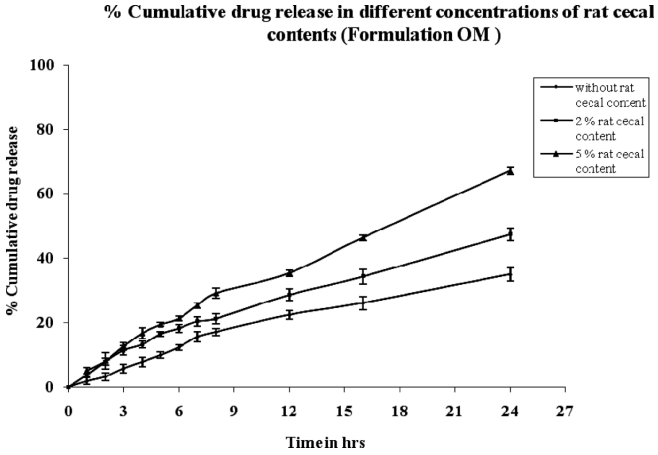

Fig. 4 Mean (±S.D.) % Cumulative Drug Release in Different Concentrations of Rat Cecal Contents (Formulation OM) ($n=3$)

OM: contains ornidazole (20%), polymer (2.5%), swelling time 2 h glutaraldehyde (1 ml), stirring speed (1000 rpm).

formed at higher cross-linking show lesser swelling.

An increase in drug release was observed with increase in swelling time due to rapid hydration/high swelling, governed by dissolution and diffusion of drug through the hydrated path in the polymer matrix formed by swelling. The release of water soluble drug from the swellable matrix occurs only after the penetration of water into the polymeric matrix that allows swelling of the polymer and drug dissolution, following diffusion along the pathway to the surface of microspheres.[24,25]

Increasing the swelling time of polymer the drug loading capacity and encapsulation efficiency also increased up to a maximum further increase in swelling time did not increase the drug loading capacity. An optimum swelling time for preparation of ornidazole microspheres was found 2 h. This was so because swelling allows more drugs loading into the microspheres.

The interaction between cross-linking agent and stirring speed are directly influencing the ornidazole release from microspheres. It may be noted that low amount of cross-linking agent caused with high initial burst effect and higher amount of cross-linking agent retards the ornidazole release from the microspheres. Preparation of guar gum microspheres with 1% v/v of cross-linking agent produced good effect on ornidazole release.

As illustrated in Table 2 a p value of <0.05 for any factor in analysis of variance (ANOVA) indicates significant effect of the corresponding factors on the response, *i.e.* % of ornidazole release (Y_1) and encapsulation efficiency (Y_2).

***In Vitro* Drug Release in Rat Cecal Content Release Medium pH 6.8** *In vitro* drug release in rat cecal content release medium was performed for the formulation OM in different concentration of rat cecal content. As the concentration of cecal content increased (0%, 2%, 5%), the drug release from the microspheres also increased. This could have been due to the presence of bacterial polysaccharidases in rat cecal content which caused the degradation of guar gum.[26,27] So it facilitated the release of drug from the microspheres. The maximum drug release was observed 65% (Fig. 4).

Summary In the present work, colon specific drug delivery systems have been selected, to deliver the drug locally to colon for longer period of time. The guar gum microspheres retarded the release of drug in stomach but it facilitated the release of large amount of drug in the colon in the presence of rat cecal contents, as these have polysaccharides enzymes that degrade the guar gum in the colon. The *in vitro* drug release showed that the formulation retarded the release of drug in the stomach and intestine and it is capable of delivering its maximum load to the colon, which was justified by the release study in the presence of rat cecal content. The release data shows that the a maximum of 32.90% of drug was released in presence of simulated gastrointestinal fluid, (SGIF) and 65% in the presence of rat cecal content release medium (formulation OM, Fig. 4).

Conclusion

Colon targeting of ornidazole using guar gum as carrier system prevents the drug release in stomach and intestine and delivers its maximum drug load to the colon, at the site of action where the action is needed. Our approach minimises side effect associated with conventional dosage form and improves bioavailability if drug also reduces the dose and dosing frequency.

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