RESEARCH ARTICLE

Formulation and *in vitro* characterization of alginate microspheres loaded with diloxanide furoate for *colon*- specific drug delivery

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The aim of the research work was to develop cyst–targeted alginates microspheres of diloxanide furoate (DF) for the effective treatment of amoebiasis. Calcium alginates microspheres of DF were prepared using emulsification method using calcium chloride as a cross-linking agent. Alginate is a natural polysaccharide found in brown algae. Alginates are widely used in the food and pharmaceutical industries and have been employed as a matrix for the entrapment of drugs, macromolecules and biological cells. Alginate microspheres produced by the emulsification method using calcium chloride. Formulations were characterized for particle size and shape, surface morphology, entrapment efficiency, and *in vitro* drug release in simulated gastrointestinal fluids. XRD and differential scanning calorimetery were used to confirm successful entrapment of DF into the alginates microspheres. All the microsphere formulations showed good % drug entrapment (73.82±1.99). Calcium alginate retarded the release of DF at low pH (1.2 and 4.5) and released microspheres slowly at pH 7.4 in the colon without colonic enzymes.

Key words: *Amoebiasis, colon targeting, diloxanide furoate, microspheres, sodium alginates*

INTRODUCTION

The oral route is considered to be the most convenient for the administration of drugs to patients. On oral administration of conventional dosage form, drug normally dissolves in the gastrointestinal (GI) fluids and is absorbed from the GI tract. It has serious drawback in conditions where localized delivery of the drugs in the colon is required or in conditions where a drug needs to be protected from the hostile environment of upper GIT. Targeting of drugs to colon is advantageous in the treatment of diseases associated with the colon such as amoebiasis, ulcerative colitis, Crohn's disease, or colorectal cancer. The colon is attracting interest as a site where poorly absorbed drug molecules may have improved bioavailability. This region of the colon has a somewhat less hostile environment with less diversity and intensity of activity than the stomach and small intestine.[1] Various strategies are available for targeting drug release selectively to the colon.[2] The simplest method for targeting of drugs to the colon is to obtain slower release rates or longer release periods by the application of thicker layers of conventional enteric coating or extremely slow releasing matrices.[3]

Amoebiasis is an infection of large intestine caused by the protozoal organism *Entamoeba histolytica*, an organism that feeds on cells in the human colon. It is the cause of amebic colitis and liver abscess. Amoebiasis probably is second next to malaria as a protozoal cause of death. *E. histolytica* infection results in 50 million cases of invasive amoebiasis and 100,000 deaths annually.[4,5] DF, tinidazole and metronidazole are drugs of choice in the treatment of amoebiasis, also effective against anaerobic microorganisms and are to be delivered to the colon for their effective action against trophozoites of *E. histolytica* that reside in lumen of the caecum, large intestine and adhere to colonic mucus and epithelial layers.

Targeted drug delivery implies a selective and

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effective localization of pharmacologically active moiety at pre-identified (pre-selected) target(s) in therapeutic concentration, while restricting its access to non-target normal cellular linings, thus minimizing toxic effects and maximizing therapeutic index. [6,7]

Polysaccharides are widely used in oral drug delivery systems because of the simplicity to obtain the desired drug delivery system and drug release profile, by the control of cross-linking, insolubility of crosslinked beads in gastric environment and broad regulatory acceptance. They include sodium alginate, pectin, chitosan, xantan gum, guar gum, starch, dextran and gellan. [8-12]Alginate, a natural polysaccharide found in brown algae, is a linear 1, 4 linked copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G). These monomers are arranged as blocks in a chain. The homopolymeric regions of M blocks and G blocks are interdispersed with regions of alternating structure (MG blocks).[13,14]

Alginates have many advantages as colonic drug carriers, including nontoxicity, biocompatibility, biodegradability by colonic flora, availability, cheapness and also have a protective effect on the mucous membranes of the upper GIT.[15] The dried alginate beads have the property of reswelling and thus they can act as controlled release system. Their reswelling property is susceptible to pH, which protect the acid sensitive drug from gastric juice.[16]

The aim of the present work was to develop cyst-targeted microspheres of DF for the effective treatment of amoebiasis. Calcium alginates used as targeting material, because cyst wall of *E. histolytica* contains glycoproteins having specific affinity toward carbohydrates receptors (polysaccharide). DF is a safer and more effective, direct luminal amoebicidal drug, than other luminal amoebicidal drugs.[17] In the light of the above facts, it was proposed that calcium alginates microspheres could be effective in the treatment of amoebiasis. This system could plug and seal the carbohydrate receptor on the surface of cyst of *E. histolytica* and target the drug at the site of infection.[2,18]

MATERIALS AND METHODS

The drug (DF) was found as a gift sample from M/s Umedica Laboratories Pvt. Ltd. Mumbai,India. All the other reagents used during experiment were of analytical grade. Double distilled water was used during the whole experiment.

Method of preparation of alginate microspheres

The emulsification method was utilized for the preparation of microspheres followed by cross-linking with calcium chloride using the method of Calis *et al., 2002* with some modifications. Ten milliliters of a 5% (w/v) aqueous sodium alginate solution containing 100 mg drug was dispersed in 50 ml light liquid paraffin oil containing span 80 (1.0%) at 70°C. The biphasic system was stirred at 1000 rpm (Remi, India) to form a stable w/o emulsion. After formation of emulsion added drop wise 1 ml $CaCl_2$ (2M) and maintained stirring at 1000 rpm for 10 min at 70°C. Solution was rapidly cooled at 15°C and then, 50 ml of acetone was added in order to dehydrate the alginate droplets. The alginate particles were isolated from the suspension by filtration. The removal of residual oil was performed by washing the microspheres with 3 × 50-ml aliquots of isopropyl alcohol. Microspheres were allowed to dry at room temperature (25°C). Upon drying, free flowing, fine powder was obtained.

Optimization

Various formulation variables e.g. drug concentration, polymer concentration, emulsifier concentration, concentration of cross-linking agent and process variable viz. stirring speed, which would affect the preparation and properties of microspheres were identified and studied. The composition of formulation code of designed formulae of alginate microspheres are given in Table 1.

Particle size measurement

Particle size and size distribution of different microsphere formulations were measured using an optical microscope, and the mean particle size was calculated by measuring 200 particles with the help of a calibrated ocular micrometer. The average particle size was expressed as the volume mean diameter in micrometers.[19-23] The results are given in Table 1.

Table 1:- Effect of various parameters on particle size. % yield and drug entrapment efficiency of microspheres

Formulation	Variables	Particle size	% Yield	Entrapment efficiency
Polymer concentration (mg)				
A–1	200	–	–	–
A–2	300	417.8±3.33	86.66±6.29	48.58±1.07
A–3	400	424.57±3.37	87.33±5.03	47.55±0.62
A–4*	500	453.92±3.2	89.99±3.33	63.98±0.51
Emulsifier concentration (%)				
B–1	.75%	415.14±3.15	86.1±0.95	60.52±0.95
B–2*	1.0%	409.61±2.28	87.49±0.83	63.71±0.95
B–3	1.25%	401.41±5.38	81.66±1.66	57.54±0.98
B–4	1.5%	395.80±3.22	79.16±0.83	47.62±0.98
Cross-linking agent (ml)				
C–1	1M (1ml)	373.71±1.58	86.83±0.67	53.58±1.12
C–2	1M (2ml)	377.44±3.95	87.83±0.86	50.96±1.00
C–3*	2M (1ml)	381.72±1.53	88.88±1.27	71.81±1.05
C–4	2M (2ml)	382.41±2.86	85.27±0.47	60.2±1.04
Stirring speed (rpm)				
D–1	750	455.92±2.2	85.94±0.67	54.82±1.21
D–2*	1000	397.58±4.49	86.66±0.83	73.08±0.48
D–3	1250	382.91±3.38	83.88±0.96	70.89±1.45
D–4	1500	348.24±3.50	82.49±0.83	64.77±2.05
Drug concentration (mg)				
E–1	50	366.57±3.24	82.78±0.81	63.58±2.72
E–2*	100	364.57±2.95	87.49±0.83	73.82±1.99
E–3	150	361.67±1.33	82.3±0.77	59.76±1.55
E–4	200	359.77±3.87	77.09±0.71	51.37±1.96

Shape and surface Morphology

Microspheres were suspended in water; a drop was placed on a glass slide, covered with a cover slip and viewed under the optical microscope to examine their shape.The surface morphology of microspheres was observed scanning electron microscopy (SEM). The samples for SEM were prepared by lightly sprinkling the microspheres powder on a double adhesive tape which stuck to an aluminum stub. The stubs were then coated with gold to a thickness of about 300°A using a sputter coater. These samples were than randomly scanned and photomicrographs were taken, [24] which are shown in Figures 1 and 2.

Drug entrapment efficiency

About 100 mg of microspheres was taken and triturated with of ethanol and distilled water and transferred to a 50-ml standard flask. The volume was made up to 10 ml and mixed well. The solution was then kept aside for 12 hrs. It was then filtered through membrane filter (0.45μm) and estimated for drug content by measuring the absorbance at 260 nm. The drug entrapment efficiency was calculated using the following formula.[19]

$$\text{Entrapment Efficiency} = \frac{\text{Estimated \% Drug Content}}{\text{Theoretical \% Drug Content}} \times 100$$

The entrapment efficiency are reported in the Table 1

Percentage yield

Weight the prepared microsphere and find out percentage yield according to following formula[19]. % Yield = [Weight of microspheres / Weight of drug + polymer] × 100.

Differential scanning calorimeter.

In order to determine the physical state of drug, i.e. amorphous or crystalline, before and after final formulation and to evaluate any possible drug–polymer, drug–other components interaction[25]. differential scanning calorimetery (DSC) of DF, alginate, drug polymer mixture (1:1), blank and drug-loaded alginate microspheres were carried out by heating the sample from 30°C to 350°C at the heating rate of 10°C/min. in a nitrogen environment (nitrogen gas flow rate of 20ml/ min). Thermograms obtained are shown in Figure 3.

The DSC studies were conducted on Jade DSC Instrument, Type Pyris 6 DSC with Software Version: 9.0.1.0174.

XRD

X-ray diffraction (XRD) patterns of DF, alginate, physical mixture (1:1) and optimized blank and loaded formulations were measured by X–ray diffrectometer at 5–50°. Crystallinity of powder, vehicle and the formulations were determined at 2θ degree min^{-1}. Scanning rate, 40 KV and 30 mA with Rigaku generator.XRD pattern of drug, polymer, physical mixture, blank and loaded preparation are shown in Figure 4.

***In vitro* drug release studies**

The *in vitro* release studies of drug-loaded microspheres were carried out in simulated gastric fluids. Microspheres (100 mg) were weighed accurately and gently spread over the surface of 900 ml of dissolution medium (SGF). The content was rotated at 100 rpm at 37±0.5°C. Perfect sink condition was maintained during drug dissolution study period. The simulation of GI transit condition was achieved by altering the pH of dissolution medium at different time intervals. The pH of dissolution medium was maintained at 1.2 for 2 hrs. using 0.1N Hcl. Then KH_2PO_4 (1.7 g) and Na_2HPO_4 (2.2 g) were added to the dissolution medium, adjusting the pH to 4.5 with 0.1M NaOH and the release study was continued for further 2 h. After 4 hrs. the pH of dissolution medium was adjusted to 7.4 with 0.1N NaoH and maintained up to 24 hrs [26]. The medium was filtered through membrane filter (0.45μm), after 2 and 4 hours and the residue on filter paper was added to the next medium immediately.[25.27]

A 5-ml sample was withdrawn from the dissolution medium at various time intervals using a pipette and analyzed drug release using a UV–Vis–spectrophotometer at 261 nm. The receptor volume was maintained constant by replacing with equivalent volume of SGF after each withdrawal. The concentration of DF in the samples was corrected and calculated using regression equation of the calibration curve. The drug release pattern are shown in Figures 5-9.

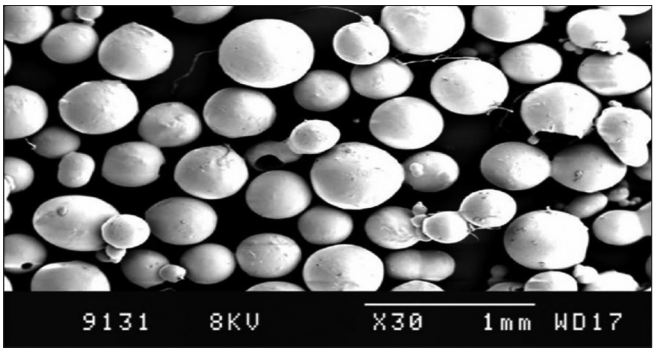

Figure 1: SEM photomicrograph of alginate microspheres without drug

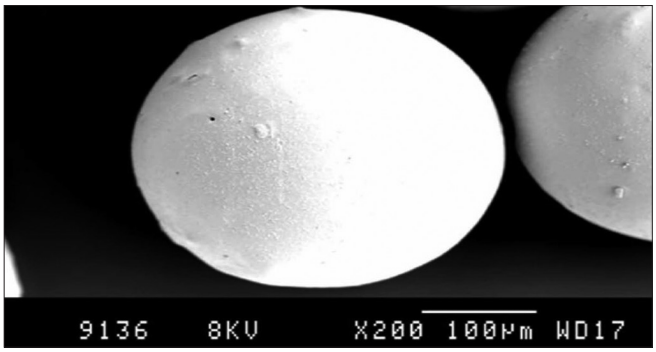

Figure 2: SEM photomicrograph of diloxanide furoate-loaded alginate microspheres

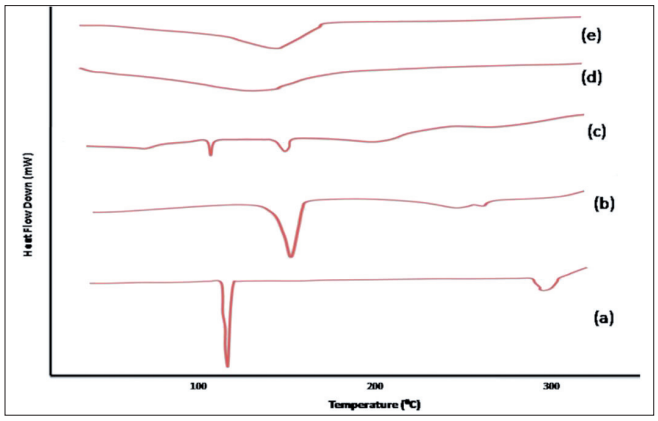

Figure 3: DSC thermogram of (a) diloxanide furoate (DF; Drug) (b) alginate (Polymer) (c) mixture of DF and alginate (d) alginate microspheres without drug (e) alginate microspheres loaded with drug

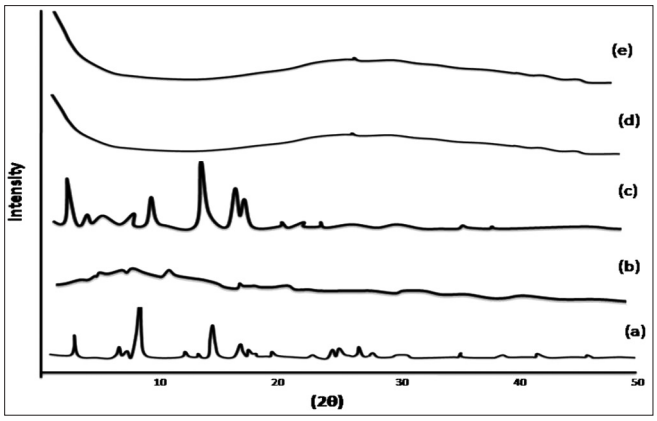

Figure 4: XRD of (a) DF, (b) alginate, (c) mixture of DF and alginate, (d) alginate microspheres without drug, (e) alginate microspheres loaded with drug

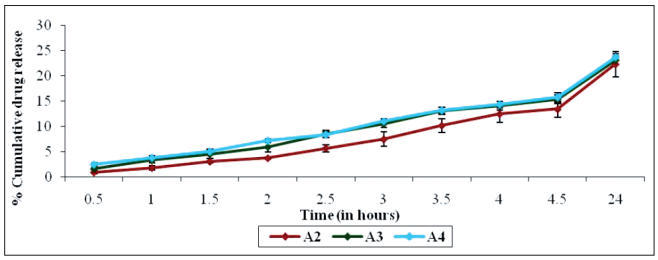

Figure 5: Effect of amount of alginate on dissolution rate of DF-loaded alginate microspheres

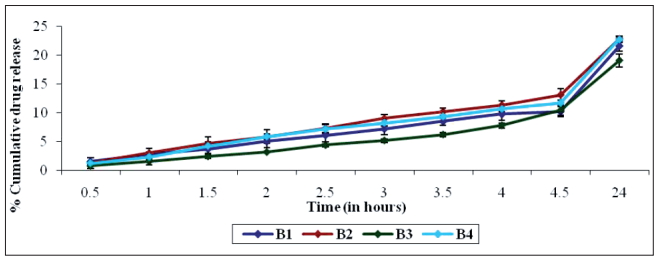

Figure 6: Effect of concentration of span 80 on dissolution rate of DF-loaded alginate microspheres

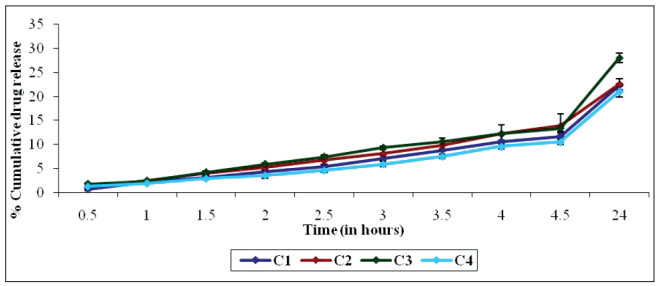

Figure 7: Effect of concentration of CaCl2 of microspheres on dissolution rate of DF-loaded alginate microspheres

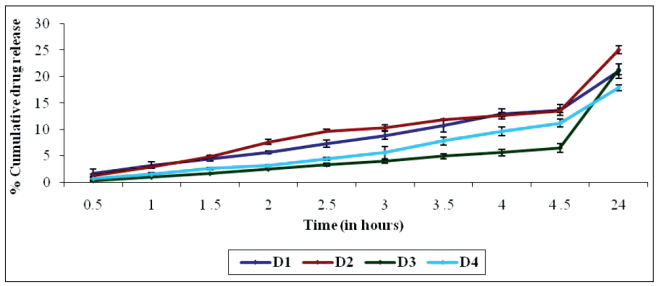

Figure 8: Effect of stirring rate of microspheres on dissolution rate of DF-loaded alginate microspheres

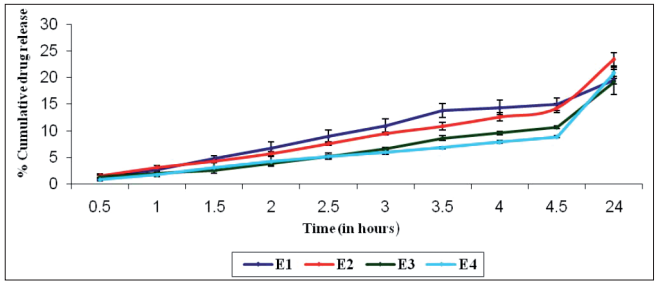

Figure 9: Effect of amount of drug on dissolution rate of DF-loaded alginate microspheres

RESULTS AND DISCUSSION

Alginate microspheres have the potential to be an efficient, viable, safe and cost–effective system for administration of DF on account of their biodegradability, biocompatibility, and suitability for oral applications. DF-loaded alginate microspheres were prepared by emulsion dehydration method.

Particle size of the alginate microspheres increased from 417.8±3.33 to 453.92±3.2 μm with increasing amount of alginate from 200 to 500 mg. The average particle size of microspheres increased with increasing amount of polymer solution, which got dispersed into larger droplets. A higher concentration of polymer produced a more viscous dispersion, which formed larger droplets and consequently larger microspheres as reported by Pongpaibul *et al.* (1984). The drug entrapment efficiency varied from 47.55±0.62% to 63.98±0.51 (Table 1). The particle size of alginate microspheres decreased from 415.14±3.15 to 395.80±3.22 µm with increasing amount of span 80 from 0.75 to 1.50%. Increased emulsifier concentration led to the

formation of particles with a lower mean geometric diameter. Increasing span 80 concentration from 0.75 to 1.50% wt/vol led to stabilization of the emulsion droplets avoiding their coalescence, resulting in smaller microspheres as reported by Maia *et al.,* 2004.The drug entrapment efficiency varied from 47.62±0.98% to 63.71±0.95 (Table 1). The particle size of alginate microspheres showed little difference from 373.71±1.58 to 382.91±3.38 μm when increasing concentration of $Cacl_2$ from 1 to 2ml (1M) to 1 to 2ml (2M) and entrapment efficiency varied from 50.96±1.00 to 71.81±1.05 (Table 1). And similar result was found as reported by Esposito *et al*., 2001. The particle size of alginate microspheres decreased from 455.92±2.2 to 348.24±3.50 µm with increasing stirring rate from 750 to 1500 rpm. Results showed that the particle size of microspheres was controlled by stirring rate. These results show that a high stirring speed produced smaller microspheres due to the smaller emulsion droplets produced by a higher stirring speed, which provided more energy to disperse the oil phase in water, but higher agitation speeds resulted in irregularly shaped microspheres as reported by Jain *et al.,* 2004. The particle size of alginate microspheres decreased from 366.57±3.24 to 359.77±3.87 µm with increasing the amount of DF from 50 to 200 mg. and entrapment efficiency varied from 51.37±1.96 to 73.82±1.99 (Table 1). The effect of drug concentration on microsphere size and percent drug entrapment efficiency was studied and observed that there was very little change in the size of microsphere as on increasing the drug concentration but the percent drug entrapment efficiency enhanced upto 10% drug concentration then it declined because of saturation of the polymer matrix with the drug resulting in no further entrapment of the drug as reported by Vaidya *et al.,*2009. The highest entrapment efficiency and % yield were found with E–2 and the size of microspheres was also sufficiently low therefore this formulation was selected as optimum.

SEM was used to investigate the morphology as well as particle size of microspheres. As showed in Figures.1 and 2, microspheres displayed a spherical shape with a smooth surface and no aggregation was observed. No difference was observed in the morphological properties of microspheres due to presence of the drug as reported by Bigucci f, *et al.*

DSC studies were performed to investigate the physical state of the drug in the microspheres, because this aspect could influence the *in vitro* and *in vivo* release of the drug from the systems. Different combinations of drug/polymer may co-exist in the polymeric carriers, such as: (i) amorphous drug in either an amorphous or a crystalline polymer and (ii) crystalline drug in either an amorphous or a crystalline polymer. Moreover, a drug may be present either as a solid solution or solid dispersion in an amorphous or crystalline polymer. Figure 3 shows the DSC thermograms of pure DF, alginate, combination of drug and polymer, blank alginate microspheres and DF-loaded alginate microspheres. Pure DF showed a endothermic melting peak at 115.1°C. alginate showed an endothermic peak at 158°C. The endothermic peak of the physical mixture of alginate and DF was observed it 158°C and 115.1°C and the peaks were similar to the melting points of alginate and DF. DF melting peak totally disappeared in the thermogram of loaded microspheres, evidencing the absence of crystalline drug in the microsphere samples, at least at the particle surface level. Therefore, it could be concluded that DF in the microspheres was in amorphous phase of a molecular dispersion or a solid solution state in the polymer matrix after the production as previously reported by Anande *et al*., 2008.

The formulation of microspheres was verified by characterization of 2θ (degree) using XRD. Figure. 4 shows the XRD pattern for the DF, alginate, physical mixture, blank and loaded preparation. As shown in this figure, the diffraction peaks of drug which showed its crystalline nature. Pure alginate did not showed any peak; indicating its amorphous nature. Similarly the microspheres prepared with alginate with and without drug also did not showed any peak confirming that polymer shield drug and show good entrapment. Figure.4 also represents the XRD pattern for blank and loaded preparation indicating the stability of the preparation in the presence of alginate.

In vitro drug release study of all microspheres was performed in simulated GI fluid medium of different pH. The *in vitro* release profile of various alginate microspheres showed the cumulative percent drug release of DF from alginate microspheres in SGF and SIF after 4 hrs were varied from 5.62±0.66% to 14.41±1.35%. As desired the drug release in simulated colonic fluid having pH 7.4 after 4 hrs study, was varied from 17.87±0.51% to 28.00±0.97% for alginate microspheres.

The present studies revealed that the alginate microspheres bear DF maintain its integrity in the hostile environment of stomach and small intestine and this system were not show any pH- dependent drug release in GIT due to presence of dextranase. While presence of dextranase microspheres may release drug selectively in colon as well as biodegradable polymeric colloidal systems made up of alginate can entrap DF providing a targeted drug delivery system. Hence, it can be concluded that alginate microspheres can be utilized and are having potential for the site- specific delivery of the drug to the colon.

CONCLUSIONS

The results of our study clearly indicate that there is great potential in delivery of DF to the colonic region as an alternative to the conventional dosage form. Various formulation as well as process variables affect the size, shape and drug release pattern of microspheres. Sodium alginate is a biocompatible polymer; we expect it to cause no harmful effects if used for prolonged periods. Alginate microspheres

show good entrapment, % yield and spherical particle. While in upper part of GIT alginate microspheres did not show drug release due to absence of colonic flora. We can conclude that sodium alginate microspheres of DF showed extended drug release profile in pH progression medium. Therefore, designed drug delivery system can be effectively used for the treatment of luminal amoebiasis.

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