



Synthesis & design of chitosan derivative pH stimuli sensitive microparticles for colon targeted metronidazol delivery

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ABSTRACT

The objective of the present study was to synthesis of alkyl succinate and phthalate derivative of chitosan by substitution of neucleophilic amino group in order to modify the solubility window of the polymers. The synthesise derivatives were used in preparation of metronidazole (MN) and were investigated the efficacy for pH sensitive colon targeted drug release. The chitosan derivatives microspheres containing MN were prepared using solvent evaporation method. Formulations were characterized for morphology, particle size, % encapsulation efficiency, % drug content and in vitro drug release. The microspheres prepared by N-ethylsuccinate chitosan (batch F₂) was found to be high drug encapsulation efficiency and was showed better stability in simulated gastric fluid. The drug release from this batch was 73±1.4 % (phosphate buffer) whereas in simulated gastric fluid was found 26±1.6% for 2 hr of the study. The mucoadhesion of the batch was found 75±1.5 % whereas, in plain chitosan microparticles was found 56±1.2 %. The result provides evidence that the prepared formulation of batch may be used effectively for pH sensitive colon targeted drug such as metronidazol.

Key words: Metronedazol , emulsion solvent evaporation, erosion, sustained release, microparticle.

INTRODUCTION

Drug delivery systems that can precisely control the release rate of target drug to a specific body site have been gained lots of attention. The targeted delivery of bioactive molecules to specific sites of the gastrointestinal (GIT) has attracted considerable attention for several years. The dosage forms that can precisely control the release rates and target drugs to specific segments of the GIT have made an enormous impact in the development of novel drug delivery systems. In the current scenario numbers of polymers have been use as drug carrier for the design of different system in targeted release of drugs in colon region. The polymers related natural, semi synthetic and synthetic category such as zein, shellac, amylase acetate, phthalate and cellulose derivatives and various ethyl acrylate and methylmethacrylate [1-4]. The most of these carriers may leak active compound at 6.1 to 8.0 pH of small intestine and will not reach the colon targeted segments of GIT [5]. To over come disadvantage of the polymers used for the target drug delivery, newer carriers will be needed. This scarcity was encourage by the synthesise of pH sensitive colon targeted drug carrier.

Metronidazole belongs to nitroimidazole derivative, which is selective toxic for trichomoniasis, amoebiasis and giardiasis etc. The anaerobic protozoan parasites posses an enzyme, pyruvate-ferrodoxin oxido-reductase, which participate in metabolic electron removal reactions. The nitro group in metronedazol serve as an electron acceptor. The cytotoxicity of metronedazol is due to biding of host protein to disrupt replication, transcription and repair process of DNA that result in cell death [6].

Chitosan has gained lots of attention toward using it as an excipients and drug carrier in the development of conventional and novel drug delivery system. Mura et al, 2011[7] prepared 5-Aminosalicylic acids loaded N-

succinyl-chitosan microparticle and was reported that the formulation showed excellent mucoadhesive to the inflamed colonic mucosa. Rekha and Sharma, 2008[8] prepared derivative of chitosan and evaluate its efficacy for targeted delivery for the colon. Rassu *et al*, 2009[9] prepared rokitamycin loaded chitosan derivative microspheres and was reported to improves and prolongs the *in vitro* antiameobic activity.

The objective of present study was to develop alkyl succinate and phthalate derivative as microparticle carrier of the drug. The drug release from the microparticle was modifying in order to release the drug in colon site by the trigger of stimulus of pH 5.0 or above whereas; drug release was inhibited at pH 4.0. The design microparticles have been characterized in terms of morphology, drug loading efficiency dissolution study in order to investigation of the synthesis polymer for colon specific delivery of the drug.

EXPERIMENTAL SECTION

2.1. Materials

Metronidazol (MN) was obtained as a gift sample from the Aarti drugs Ltd. (India) .Chitosan was purchased from Aldrich Ltd. Succinate and phthalate anhydride were purchase from Sigma Aldrich (Steinheim, Germany). All of the other chemicals were of analytical grade and were used without additional purification. Ultra pure water (Billerica, MA) was used throughout the study.

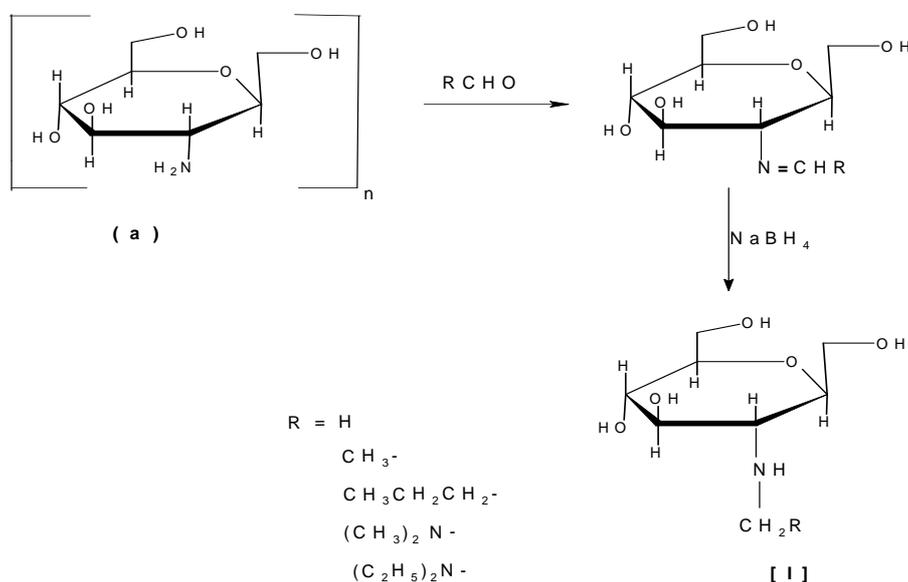


Figure 1 Synthesis of N-alkyl chitosan derivatives

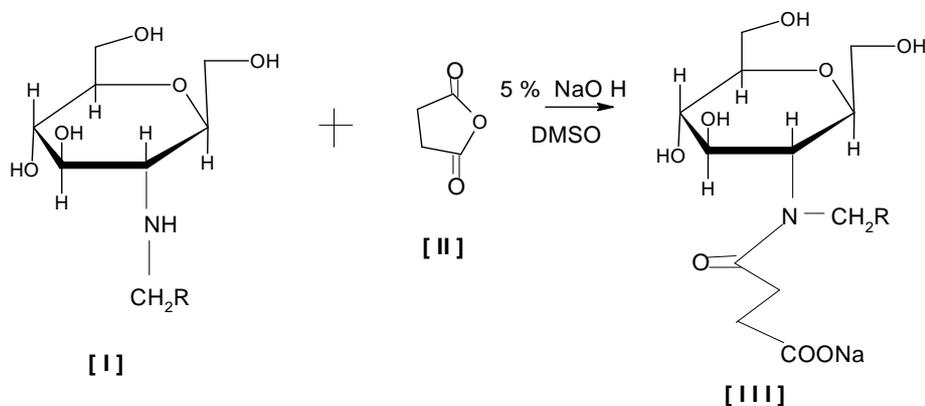


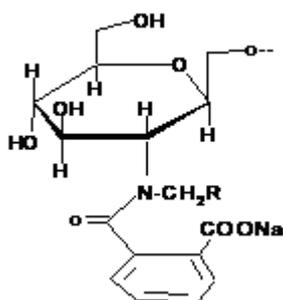
Figure 2 Syntheses of N-alkyl chitosan phthalate derivatives

2.2. Methods

Synthesis of N-alkyl Chitosan derivatives

Chitosan solution 1% w/v was prepared by dissolving 2.0 g of chitosan into 1.0 % v/v aqueous acetic acid (Figure 1.a). The aldehyde solution was added to the chitosan solution at room temperature with stirring for 30 min and adjusted the solution pH 4.5. To this solution, 10 % v/v aqueous solution of sodium borohydride (NaBH_4) was added, and then the solution was stirred for 1 hr. Precipitants of the N-alkyl chitosan derivatives were obtained and pH was adjusted at 10. The precipitants were washed with distilled water. Unreacted aldehyde and the inorganic products were soxhlet-extracted with ethanol and diethyl ether for 2-7 days, respectively. The resulting N-alkyl chitosan derivatives were filtered out and dried for 48 hr under vacuum at 40°C .

Table 1. Chitosan succinate derivatives and their formulation batches



Formulation	code	Succinate derivative	R
F ₁		N-methyl chitosen succinate	H
F ₂		N-ethyl chitosen succinate	CH ₃ -
F ₃		N-butyl chitosen succinate	CH ₃ CH ₂ CH ₂ -
F ₄		N-diethyl chitosen succinate	(CH ₃) ₂ N-
F ₅		N-diethyl chitosen succinate	(C ₂ H ₅) ₂ N-

Synthesis of N-alkyl chitosan succinate and N-alkyl chitosan phthalate derivatives

1.0 g of N-alkyl chitosan (see Figure 1 & Figure 2) was dissolved in dimethyl sulfoxide (20 ml). 1.0 g of succinic anhydride (compound II) or phthalic anhydride (compound IV) was added in the diluted solution and stirred at 60°C for 30 min (Figure 2). The pH of the mixture was adjusted at 5.0 with controlled addition of 5 % w/v aqueous sodium hydroxide solution resulted to get the precipitate of the polymer derivative (Figure 2 & Figure 3). The precipitate was collected by filtrations and dispersed in 50 ml of water and adjusted the dispersion pH 10-12 with 5 % w/v aqueous sodium hydroxide to give a pale yellow solution. The solution was dialyzed using dialysis membrane at room temperature for 2-3 days and lyophilized. The lyophilized products (III) and (V) were recovered (Figure 2 & Figure 3).

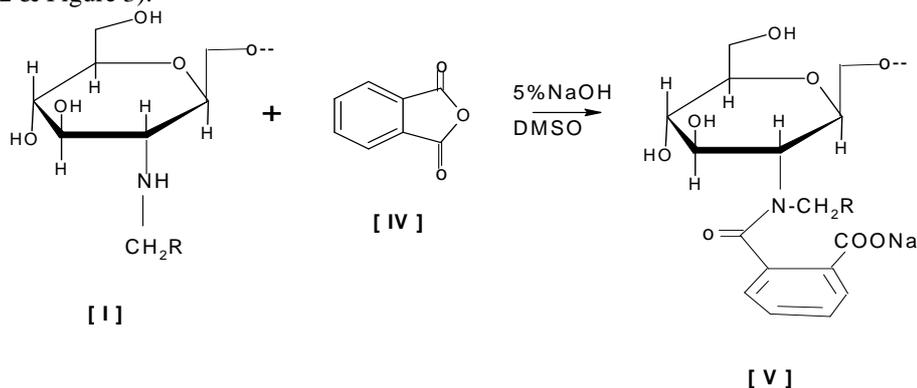


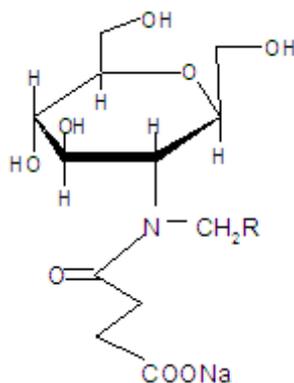
Figure 3. Synthesis of N-alkyl chitosan succinate and N-alkyl chitosan phthalate derivatives

FTIR spectral analysis

The derivatization of chitosan was analyzed using FTIR spectroscopy method (shimadzu Japan). The chitosan derivative was used powder form. The pellets for FTIR were prepared with KBr, press (spectra lab Mumbai) using mixture of the drug and KBr in 1:10 ratios. The spectra were recorded over the wave number range of 4000 to 500 cm^{-1} .

Preparation of microparticle of metronedazol

Microparticles containing metronedazol was prepared by emulsion solvent evaporation method. The drug (1.0 g) was dispersed in 10 % w/v solution of the derivative polymer in solvent mixture of acetone: ethanol (8:2). The resulting dispersion was extruded through a syringe (no. 20) in 100 ml of liquid paraffin in a 250 ml beaker under stirring at 1000 rpm using a propeller stirrer (Remi, Mumbai, India). After 15 min, glutaraldehyde (25 % v/v aqueous solution) was added and stirring was continued for 4 hr to evaporate the solvent at room temperature and to form microparticles of metronedazol. Microparticle thus, obtained were filtered and washed several times with cyclohexane to remove traces of oil (Table 1 & Table 2). They were finally washed with water to remove excess of glutaraldehyde. The microparticle were then dried at room temperature (25 °C and 60 % RH) for 24 hr.

Table 2. Chitosen phthalate derivatives and their formulation batches

Formulation	code	Phthalate derivative	R
F ₆		N-methyl chitosen succinate	H
F ₇		N-ethyl chitosen succinate	CH ₃ -
F ₈		N-butyl chitosen succinate	CH ₃ CH ₂ CH ₂ -
F ₉		N-diethyl chitosen succinate	(CH ₃) ₂ N-
F ₁₀		N-diethyl chitosen succinate	(C ₂ H ₅) ₂ N-

Determination of particle size

The particle size of the microspheres was determined by using optical microscopy method. Approximately 100 microparticles were counted for particle size using a calibrated optical microscope (Labomed CX RIII, Ambala, India).

Surface Morphology

The surface morphology of microparticles was examined by means of a scanning electron microscope (Hitachi, Tokyo, Japan). The microparticles were fixed previously on a brass stub using double-sided adhesive tape and then were made electrically conductive by coating, in a vacuum, with a thin layer of platinum (approximately 3 to 5 nm) for 100 seconds at 30 W.

Determination of drug loading and encapsulation efficiency

Accurately weighed (100 mg) grounded powder of beads was soaked in 100 ml phosphate buffer (pH7.5) and allowed to disintegrating completely for 4 h. The resulting solution dispersion was sonicated using probe sonicator (UP 400 s, Dr. Hielscher GmbH, Germany) for 30 minutes and then filtered through 0.45 µm filter. The polymeric debris was washed twice with fresh phosphate buffer to extract any adhered drug and the drug content was determined by spectrophotometrically at 252 nm against constructed calibration curve. The encapsulation efficiency (EE) was calculated according to the relationship:

$$\% EE = \frac{\text{Calculated drug content}}{\text{Theoretical drug content}} \times 100$$

Percent mucoadhesion

Albino rats (450-500 g, male) were fasted overnight and dissected immediately after being sacrificed. The protocol of the study was approved by the animal ethical committee of the department. The stomachs of the rats were removed, cut into pieces 2 cm long and 1 cm wide and rinsed with 2 ml of physiological saline. One hundred milligram microspheres of each were scattered uniformly on the surface of the stomach mucosa. Then, the mucosa with the microspheres was placed in a chamber maintained at 93% relative humidity and room temperature. After 30 min, the tissues were taken out and fixed on a polyethylene support at an angle of 45°. The stomach was rinsed with

buffer (SGF or Pthalate buffer pH 3.4) solution (pH 1.2) for 5 min at a rate of 22 ml/min. The microspheres remaining at the surface of gastric mucosa were then counted, and the percentage of the remaining microspheres was calculated. The experiment was performed in triplicate. The following formula was used for the determination of % binding:

$$\% \text{ Binding} = \frac{\text{initial wt of microspheres} - \text{wt of unbound microspheres}}{\text{initial wt of microspheres}} \times 100$$

***In Vitro* drug release**

The *in vitro* release of metronedazol from the prepared microparticles was determined using a US Pharmacopoeia dissolution apparatus. Microparticles equivalent to 100 mg metronedazol were taken in cellulose dialysis bag (previously soaked in dissolution medium for 3 h) containing 3 ml of dissolution medium and tied to the paddle. The *in vitro* release studies were conducted at a paddle rotation of 100 rpm in 900 ml of phosphate buffer (pH 7.4 and 37 °C). An aliquot of the release medium (5 ml) was withdrawn through a sampling syringe attached with 0.2 mm filter at predetermined time intervals (0.5, 1, 2, 3, 4, 5, and 6 hours), and an equivalent volume of fresh dissolution medium, which was pre-warmed at 37 °C was replaced. Collected samples were then analyzed for metronedazol content by measuring the absorbance at 276 nm using an ultraviolet spectrophotometer (Shimadzu 1601PC). *In vitro* drug release studies were also conducted separately in simulated gastric fluid (pH 1.2) for 2 hr in an identical manner as described above. *In vitro* release studies were performed in triplicate in an identical manner [10, 11].

Stability of microspheres in simulated gastric fluid

The stability of chitosan microspheres was determined with method of Berthold *et al.*, 1996 [12] with slight modification. The microspheres solution (0.8 % w/v) was prepared in simulated gastric fluid and the suspension was incubated for 60 min. The optical density (OD) of the sample was measured at 500 nm .

RESULTS

The derivatives of succinate and phtalate were synthesis in order to modify chitosen solubility for the colon targeted release of the drug. The synthesis derivatives of chitosen were investigated as a colon targeted drug carrier through the design of micro particle formulation .

Table 3: Evaluations data of microparticles of succinate and phtalate derivative of chitosen

Formulation (code)	Particle size (µm) ^{a,b,n}	% Encapsulation efficiency	% drug content	% transmission
F ₁	110±0.5	83±1.5	42±0.4	15.0
F ₂	100±0.2	95±1.4	52±0.3	8.0
F ₃	112±0.4	76±1.2	48±0.4	20.0
F ₄	114±0.8	89±1.6	39±0.5	23.0
F ₅	120±0.6	83±1.2	40±0.6	25.0
F ₆	105±0.4	75±1.2	57±0.2	33.0
F ₇	95±0.8	89±1.4	62±0.5	10.0
F ₈	101±0.4	80±1.6	64±0.6	21.0
F ₉	111±0.8	74±1.4	50±0.5	17.0
F ₁₀	118±0.2	73±1.3	48±0.7	15.0

a = Mean ± SD

b n = 200

c n = 3

FTIR spectral analysis: The derivatives of chitosen were confirmed by FTIR spectroscopic method. Spectral peaks of the derivatives polymer were inferences for presence of certain group, like N-methyl chitosen (Figure 4 a) band 634.9 cm⁻¹ represent the presence of C≡H bending vibration of alkynes or monosubstituted alkynes. 1411 cm⁻¹ showed O=H bending vibration , band 1075.8 cm⁻¹ was due to C-O-C stretching vibration, Band 1648.3 cm⁻¹ C=O stretching vibration, band 2360.9 cm⁻¹ showed superimposed O-H and NH³⁺ stretch bands and band 346.6 cm⁻¹ N-H stretch of primary amine.

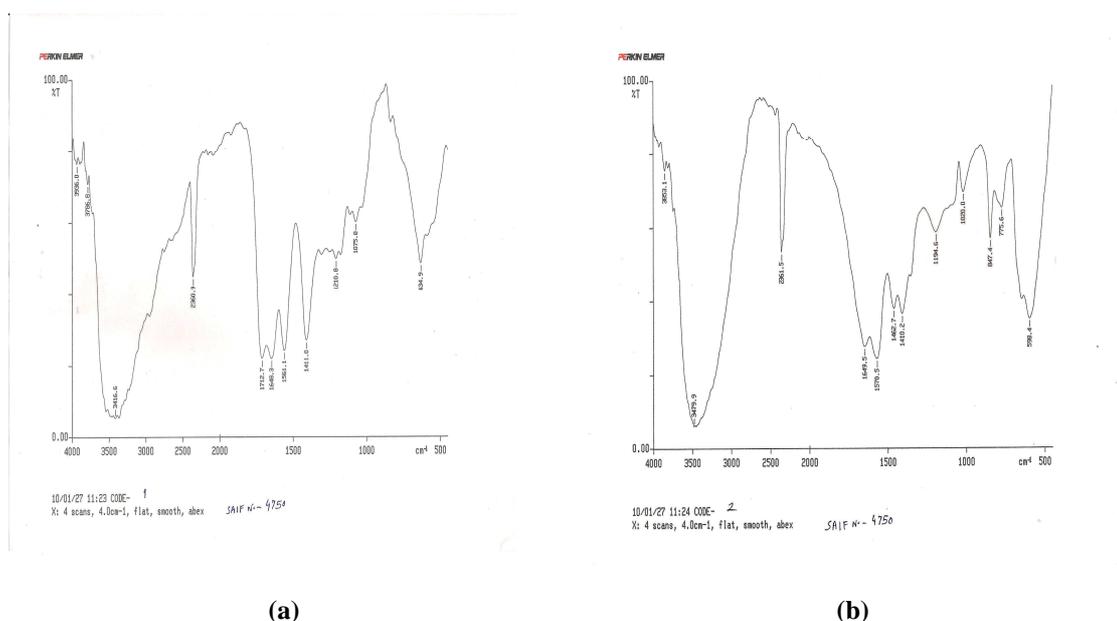


Figure 4. FTIR curve of: (a) N-methyl chitosan and (b) N-methyl chitosan phthalate

N- Methyl chitosen phthalate: Figure 4. b is showed that band 1194.6 cm^{-1} for C-O stretch vibration whereas 1020 cm^{-1} showed for C-N stretch vibration (aliphatic amines), Band 3479.9 cm^{-1} represented N-H stretching , 775.6 cm^{-1} represented for C-H bending vibration, 1570.5 cm^{-1} N = O asymmetric stretch .

N- methyl chitosen phthalate metronidazol microparticle: Band 1019.2 cm^{-1} shows C-C bending vibration and band, 1657.6 cm^{-1} shows C= C stretch vibration band 1237.2 cm^{-1} C-O stretch vibration (Figure 5).

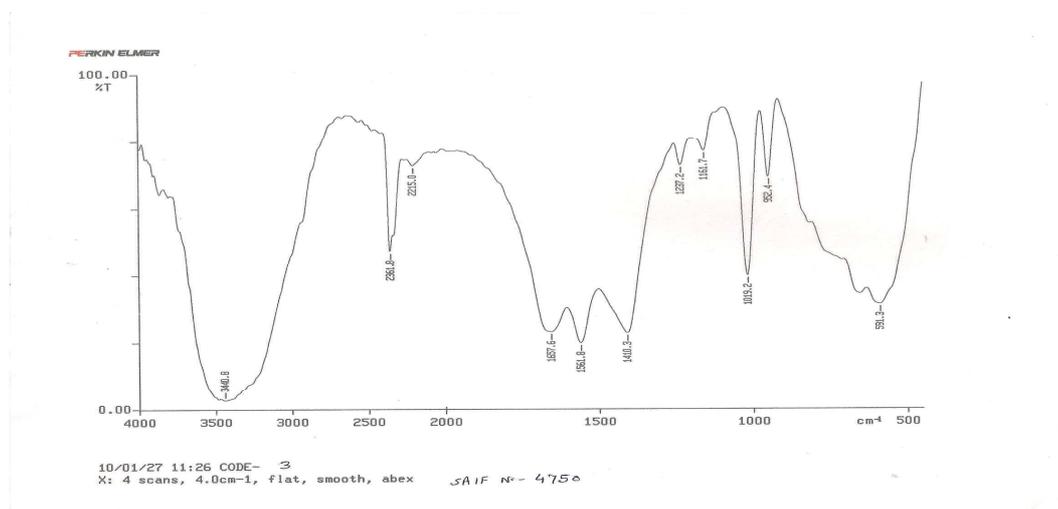


Figure 5. FTIR curve of N-methyl chitosan phthalate metronedazol microparticle

Morphology study: Morphological examination was performed by using a scanning electron microscope (SEM). Scanning electron micrograph revealed that micro particles of batch F₂ were spherical in shape white translucent in appearance (Figure 6).

Particle size: Particle size of formulated microparticle is given in table 3. Particle size of microparticle varied 65 ± 0.2 - $85\pm 0.6\text{ }\mu\text{m}$. The maximum particle size of succinate derivative microparticle was found to be $85\pm 0.6\text{ }\mu\text{m}$ (batch F₄). Particle size of phthalate derivative was in order of $60\pm 0.8\text{ }\mu\text{m}$ (batch F₇) to $83\pm 0.2\text{ }\mu\text{m}$ (batch F₁₀).

Encapsulation efficiency and drug content: Batch F₈ was found comparative highest drug loading ($64\pm 0.6\%$ w/w) whereas batch F₄ found lowest drug loading ($39\pm 0.5\%$ w/w). The encapsulation efficiency of batch F₂ showed was found $95\pm 1.4\%$ while that of batch F₁₀ was found lesser encapsulation efficiency ($73\pm 1.3\%$).

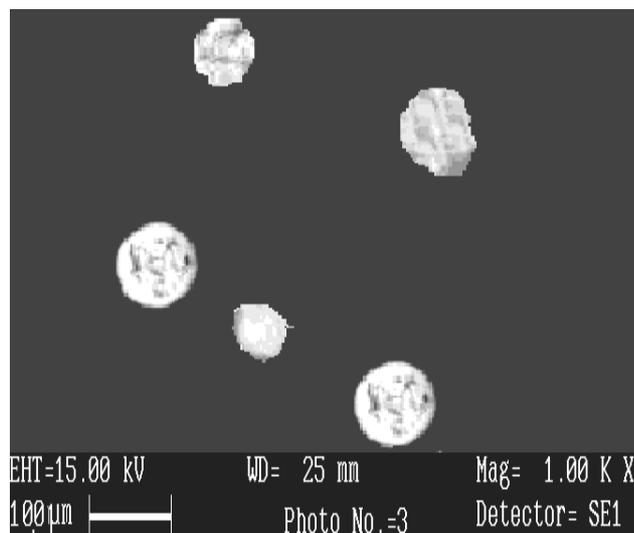


Figure 6 Scanning electron photomicrograph of metronedazol loaded microparticle of batch F₂.

% Mucoadhesion: Mucoadhesion studies were carried out to ensure the adhesion of the formulation to the mucosa for a prolonged period of time (Figure 7). The binding capacity of plain chitosen drug loaded microparticle (batch F) and derivative batch (F₂ and F₇) were evaluated in SGF solution (pH 1.2) and phosphate buffer solution (PBS) of pH 7.5. The mucoadhesion of batch F₂ was found $75\pm 1.2\%$ (PBS) in contrast to SGF ($70\pm 1.4\%$) while that of batch F₇ was found $65\pm 1.2\%$ (PBS) and $58\pm 1.6\%$ (SGF) mucoadesition. Chitosen reference batch F microparticle was found minimum mucoadhesion: $56\pm 1.5\%$ (PBS) and $45\pm 1.2\%$ (SGF).

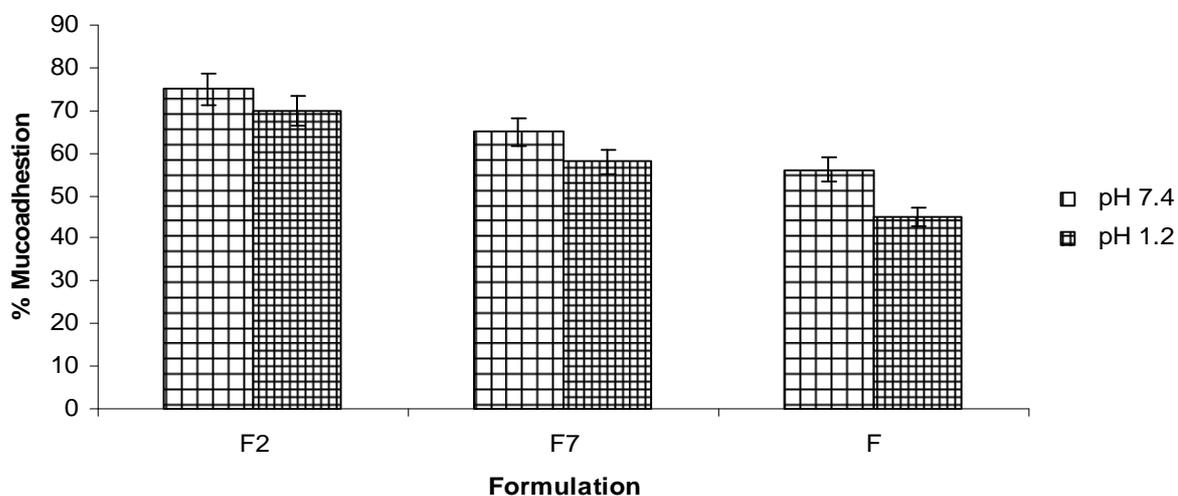


Figure 7 Comparative mucoadhesion study of microspheres batch

In vitro dissolution study: In vitro dissolution study was carried for batch F₂, F₇ and reference batch F of microparticles in SGF (pH 1.2) and PBS (pH 7.4). The drug release in SGF solution was found $26\pm 1.6\%$ (batch F₂), $30\pm 1.2\%$ (batch F₇), $50\pm 1.8\%$ (batch F) upto 2 h of the study (Figure 8). Metronedazol was release in PBS (Figure 9) was $73\pm 1.4\%$ (batch F₂), $62\pm 1.6\%$ (batch F₇), and in reference formulation was $50\pm 1.4\%$ (batch F) up to 2 h of the study.

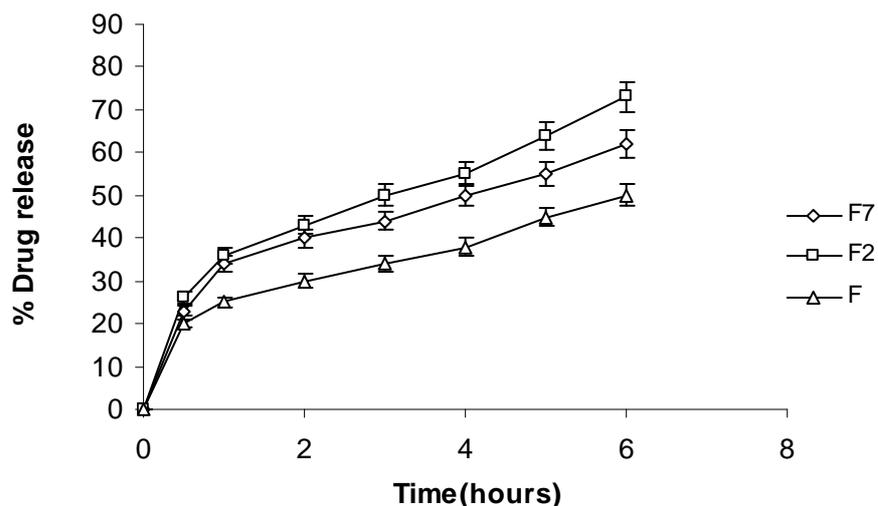


Figure 8 Comparative dissolution in simulated gastric fluid (pH 1.2)

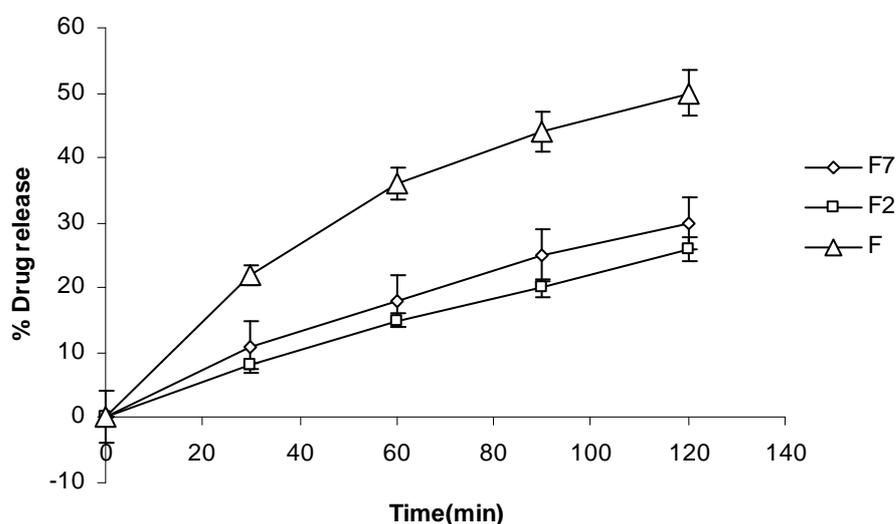


Figure 9 Comparative dissolution in phosphate buffer solution (pH 7.5)

Stability of microspheres in simulated gastric fluid

Chitosan is soluble in acidic pH, therefore, the purpose of carrying out this study was to determine the effect of the synthesis chitosen derivative solubility in the acidic pH, which in turn reflects the stability at acidic pH. The minimum optical density of microspheres was found in batch F₂ (8.0 %) and batch F₇ (10 %) whereas, other batches of the formulation were found to be higher (Table 3).

DISCUSSION

The drug loaded microparticle was prepared by solvent evaporation technique. Presence of carboxylic groups (COO⁻) in synthetic derivative of chitosen microparticle, render the formulation nature as pH stimuli sensitive in order to facilitated the drug release in sustained pattern in to colon of the gastrointestinal tract.

FTIR study: On reaction of phthalate and succinate anhydride with alkyl substituted nucleophiles amino group of chitosen, ring structure of the anhydride was opened and form n-alkyl succinate and phthalate derivative of chitosan. The degree of pthalation and succination was directly proportional to concentration of phthalic or succinic anhydride. The FTIR spectral analysis (Fig. 2 & Fig.3) was revealed for derivatization of chitosan. It is known from the literature that the characteristic bands of chitosan 1655 cm⁻¹, 1560 cm⁻¹, 1380 cm⁻¹ [13].

Surface morphology: SEM microphotograph was found that beads are spherical in shape with smooth surface. The morphology of the microparticle was uniform spherical and F₂ microparticles appeared as white, translucent and rigid.

Particle size: The mean particle size of the microparticles prepared in the present study evaluated and infrence was drawn that particle size of succinate derivative used microparticles was larger in size compare to fumarate chitosen derivative microparticles. This was emphasized that succinate derivate impart more micro viscosity than fumarate chitosen derivative and resulted in bigger microparticles.

Encapsulation efficiency and drug content: The metronedazol has been successfully encapsulated and the microparticles were predominantly round and smooth and hence this method is economical regarding encapsulation efficiency. A microparticle was tested for the drug content and was found to be satisfactory. The result was indicated that there is little wastage of the drug.

% Mucoadhesion: The research using experimental animals adhered to the principal of laboratory animal care. Mucoadesition was maximum in batch F₂ in phosphate buffer solution. Significance difference ($p < 0.05$) in mucoadhesion of SGF fluid and phosphate buffer may be due to more affinity of the polymer toward glycoprotein of gastrointestinal .Mucoadhesion take place between mucosa and polymer which is dependent on polymer structure and charge. It is proposed that polymer chain interpenetrate the mucin leading to the adhesion.

In vitro dissolution study: The rate and extent of the drug release from micro particle batch was significantly different in the studies batchs (batch F₂, F₇ and F) in dissolution medium of SGF and PBS. This could be attributed due to presence of COO⁻ groups in the derivatives. Hence derivative polymer microparticle remains unionized to the drug and polymeric derivative in the gastric fluids and disintegrates or dissolves in the higher pH intestinal environment consequently allows the release of the drug in the small intestine. Increase carboxylic group concentration in the derivative polymer which retards drug release in acid pH and enhance drug release in basic medium. The drug release in acidic pH was very fast and not sustained long duration (Fig.4) whereas in PBS medium ; drug was released with sustained pattern up to 6 hr (Fig. 5) in the order of batch F₂>batchF₇>batch F. This is due to presence of different drug carrier polymer (as N-ethyl chitosan succinate, N-ethyl chitosen phthalate and plain chitosen) in batch F₂, batch F₇, batch F in the respective batch. The polymer increased the density of drug-polymer matrix and also an increase in the diffusional path length that the drug molecules have transverse [14]. Hence N-ethyl chitosen succinate was an optimized derivative of the polymer for the study and promised for loading of other drugs needed for clinical effect in the colon.

Stability of microspheres in simulated gastric fluid

The stability of all the batches of microspheres was determined by measuring the transmission after the microspheres had been exposed to SGF solution. The acid instability of the derivative chitosan microspheres led to the dissolution of the microspheres, and the sample became more transparent. Since the decrease in optical density is directly dependent on the disintegration of the microspheres, transmission is a measure of the concentration of none disintegrates microspheres. A low transmission indicates high stability, and a high transmission implies that the microspheres dissolved in SGF. As shown in Table 1, Batch F₂ was showed minimum optical density whereas other batch showed comparative high value of optical density.

CONCLUSION

The prepared microparticle of metronedazol using N-ethylsuccinate chitosan as a carrier polymer was investigated an effective drug vehicle for pH sensitive delivery of the drug in side colon segment of gastrointestinal tract. Optimized batch of the formulation was encapsulated higher percentage of the drug and sustained drug release from the microparticle over a period of at least 6 hr in the alkali pH of the phosphate buffer solution. *In vivo* mucoadhsive efficiency of the microparticle was satisfactory; microparticles were adhered in mucosa for extended periods. The results clearly indicated that N-ethylsuccinate chitosan based formulation containing metronedazol have a promising potential for delivering of the drug at the colon site and may be very useful for effectively eradication of colon infections like cure of amoebiosis and other colon infections .

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REFERENCES

- [1] Capan Y., Jiang G., Giovagnoli S. *AAPS PharmSciTech.* **2003**, 4: E28.
- [2] Schmidt P.C., Niemann F. *Drug Dev Indust Pharm.* **1992**, 18, 1969-1979.
- [3] Bechard S., Levy L., Clas S. *Int J Pharm.* **1995**, 114, 205-213.
- [4] Felton L.A., Hasse M.M., Shah N.H. *Int J Pharm.* **1995**, 113,17-24.
- [5] Ashford M., Fell J.T., Attwood D., Sharma H., Woodhead P.J. *Int J Pharm.***1993**, 95,193-199.
- [6] Sharma H.L., Sharma K.K. *Principle of Pharmacology*,1st ed. New Delhi: Paras medical publisher; **2007**, pp. 844-847.
- [7] Mura C., Nácher A., Merino V., Merino-Sanjuan M., Carda C., Ruiz A., Manconi M., Loy G., Fadda A.M., Diez-Sales O. *In J Pharm.* **2011**, 1, 145–154.
- [8] Rekha M.R., Sharma C.P. *Tren Biomater Artificial Orga.* **2008**,21,107-115.
- [9] Rasso G., Gavini E., Jonassen H., Zambito Y., Fogli S., Breschi M.C., Giunchedi P. *J. Pharm Scie.* **2009**,98,4852-65.
- [10] Park W.L. *J Pharm Scie Technol.* **1995**,49, 13–19.
- [11] Ha J.H., Kim S.H., Han S.Y., Sung Y.K., Lee Y.M., Kang I.K., Cho C.S. *J Control Rele.***1997**,49, 53–262.
- [12] Berthold A., Cremer K., Kreuter J. *STP Pharm Scie.* **1996**, 6, 358-364.
- [13] Shigmasha Y., Matsura H., Sashiwa H., Samato H. *Int. J. Biolo. Micromolec.* **1996**, 18, 237-242.