

Original Research Article

Formulation and development of colon-targeted mucopenetrating metronidazole nanoparticles

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Abstract

Purpose: To formulation and develop colon-targeted mucopenetrating metronidazole nanoparticles.

Methods: Metronidazole-loaded chitosan nanoparticles with a pH-sensitive polymer, hydroxyl propyl methyl cellulose phthalate (HPMCP), were prepared by ionic gelation technique and then coated with Eudragit S100 by solvent evaporation method. The nanoparticles were optimized using one variable at a time (OVAT) approach. Further, the nanoparticles were evaluated by scanning electron microscopy (SEM) and zeta sizer, as well as for in-vitro release. Muco-adhesion was evaluated by modified bioadhesion detachment force measurement balance and muco-penetration of fluorescein isothiocyanate (FITC) labeled optimized nanoparticles was determined by microscopic technique

Results: Morphological assessment results revealed smooth, spherical particles with homogeneous distribution and polydispersity index (PDI) of 0.213. The optimized formulation showed particle size of 202 ± 27 nm, zeta potential of 26.9 ± 2.4 mV as well as and entrapment efficiency of 79 ± 5.4 %. There was significant difference in drug release between coated (8.46 ± 2.49 %) and uncoated (28.96 ± 4.04 %) nanoparticles at the 5th h in simulated gastric conditions. Muco-adhesion data revealed that uncoated nanoparticles (14.98×103 dyne/cm²) showed higher muco-adhesion detachment force compared to coated (12.34×103 dyne/cm²) nanoparticles. Muco-penetration results confirm the retention (for up to 12 h) of the developed formulation at the target site for enhanced therapeutic exposure of the entrapped drug.

Conclusion: Eudragit S100 coating of chitosan-HPMCP nanoparticles promotes efficient drug targeting and thus provides a strategy for treating mucosal infections. .

Keywords: Metronidazole, pH-sensitive nanoparticles, Hydroxypropyl methylcellulose phthalate, Ionic gelation, Mucoadhesion, Mucopenetration, Intestinal infection

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INTRODUCTION

Amoebiasis is a chronic disorder of intestinal mucosa with organ vulnerability to colon, caused by one celled parasite *Entamoeba histolytica* (*E. histolytica*). Oral route is most preferred because of easy drug delivery in the treatment of such diseases. Metronidazole is a drug of choice in treating amoebiasis. It gets completely absorbed in approximately 1 h after oral administration.

Thus, it is ineffective in conventional delivery system to cure colon infection [1]. Metronidazole acts by disrupting the helical structure of DNA and thus inhibiting nucleic acid synthesis of protozoa. [2]. Most of the drugs are ineffective in complete eradication of the *E. histolytica*, due to poor penetration of the delivery system across the mucus membrane. As the parasites reside and colonize deep into the colonic mucosa and in the intracellular space between the epithelial

cells [3]. Nanoparticles offer many advantages as a delivery system such as easy permeability, high drug loading, no toxicity, resistance to degradation [4], taken up readily in inflammation area, attach and penetrate better to the mucus due to its relatively small mass [5].

Chitosan offer many advantages as a delivery carrier. It is a cationic polysaccharide, thus readily attaches to the negatively charged surface of mucus membrane [6], low toxicity [7], biodegradable [8], antimicrobial, and anti-cancerous [9]. But it undergoes easy dissolution at low pH in stomach [10]. This can be prevented by ionic gelation of chitosan with HPMCP and further coating with Eudragit S100, which dissolves only when the pH is above 7 [11]. Thus the objective of the study was to formulate Eudragit S100-coated pH-sensitive nanoparticles of metronidazole. The nanoparticles were then evaluated for morphology, particle size, zeta potential, PDI, drug loading, *in-vitro* release, muco-adhesion and muco-penetration.

EXPERIMENTAL

Materials

Metronidazole and HPMCP HP55 grade were gifts from La Pharmaceuticals, Ludhiana, Punjab, India. Chitosan (viscosity grade 200-400 mPas), Eudragit S100 and FITC were purchased from Sigma Aldrich, USA. Trehalose dihydrate and dialysis membrane (MW, 10 - 12 K Da) from Hi Media Labs, Pvt Ltd, Mumbai, India. The other materials used were of analytical grade.

Formulation of CS-HPMCP nanoparticles

The nanoparticles were formulated by polyelectrolyte complexation of positively charged chitosan and negatively charged enteric coating polymer HPMCP using modified ionic gelation method with magnetic stirring at room temperature [12]. In brief, different concentrations of chitosan (0.1 - 0.2 % w/v) was prepared in acetic acid (1 % v/v) at pH 5. HPMCP (0.1 - 0.2 % w/v) solution was prepared in sodium hydroxide (0.1 M). This solution was added slowly to chitosan solution containing metronidazole (0.05 - 0.1 % w/v) under magnetic stirring for 30 min at 100 rpm. The pH of final dispersion was kept at 5.5. The dispersion was then centrifuged for 30 min at a speed of 20,000 rpm (42,000 g) at 4 °C. Supernatant was used to measure free metronidazole. Collected nanoparticles were washed using double-distilled water, freeze at - 20 °C in deep freezer, freeze dried in a lyophilizer (Martin Christ model Alpha 1-2 LD plus) using using D (+) 0.5 % w/v of

trehalose dihydrate as a cryoprotectant at - 55 °C at a pressure of 0.01 mm of Hg.

Coating of nanoparticles

The solvent evaporation method using rotary evaporator (Super Fit, Ambala, India) was applied to coat metronidazole nanoparticles with Eudragit S100 [13]. Acetone solution 12 % w/v was used to prepare coating solution. Nanoparticles (100 mg) were dispersed in 1:10 core: coat coating solution. The procedure was carried until sufficient coating was attained. The coated nanoparticles were then dried and weighed.

Evaluation of nanoparticles

Morphology

SEM (ZEISS EVO Series Scanning Electron microscope Model EVO50, Jeol, Japan) was used to determine morphology of nanoparticles. Sample was mounted on the sample holder and gold coating was done for microscopy.

Particle size PDI and zeta potential

Zeta sizer (Beckman Coulter, Delsa nano C) was used to determine particle size distribution, zeta potential and PDI. Samples were dispersed in double distilled water and were sonicated prior to estimation. All measurements were performed in triplicate (n = 3).

Drug entrapment

Nanoparticles obtained were digested for 20 min in acetic acid (2 %) solution using probe sonicator (Misonix, U.S.A.) and then centrifuged for 5 min at 1000 rpm. Metronidazole concentration was estimated in supernatant using UV Spectrophotometer (UV-1700-Pharmaspec Shimadzu, Japan). Drug entrapment (E) of formulations was calculated using Eq 1.

$$E (\%) = \{(X_t - X_s)/X_t\}100 \dots \dots \dots (1)$$

where X_t = total amount of drug added and X_s = amount of drug in the supernatant.

In-vitro release study

In-vitro release was carried out using dialysis membrane (Hi Media, Mumbai, India) in Franz Diffusion cell which was thermostatically maintained at 37 °C ± 1 °C in simulated gastric and colonic fluid. The donor compartment containing 2 ml suspension (equivalent to 10 mg)

of metronidazole, re-suspended by mixing for 5 sec, in Tween 80 (1 % w/v) solution in simulated gastric/colonic fluid [14]. Samples of 1 ml each was taken from receptor compartment at different time intervals and replace with fresh media in equal volume. Samples were diluted where necessary, filtered and analyzed spectrophotometrically at 277 nm.

FITC-conjugated chitosan polymer synthesis

FITC conjugated chitosan was synthesized using a previously reported method [15]. Chitosan was dissolved in 20 ml of acetic acid (0.1 M). It was kept overnight under stirring. Then under constant stirring methanol (20 ml) and 10 ml of FITC solution (2mg/ml in methanol) was added to chitosan solution. The reaction mixture was kept for 3 h in dark room.

Then sodium hydroxide (0.5 M) was added to the reaction mixture till a pH of 8 - 9 to precipitate FITC labeled chitosan. After that it was centrifuged for 10 min at 20,000 rpm. The precipitate then washed thoroughly with deionised distilled water till washing medium was completely free from FITC fluorescence. The FITC conjugated chitosan was then freeze dried.

Preparation of FITC-labeled CS-HPMCP nanoparticles

FITC labeled chitosan nanoparticles were prepared using HPMCP solution as cross-linking agent by a reported ionic gelation method [12]. Briefly aqueous solution of HPMCP (0.2 % w/v) (pH 5.0) in sodium hydroxide (0.1 M), was added with the help of 1 ml syringe into 0.2 % w/v FITC labeled chitosan solution (pH 5) prepared in 1 % v/v acetic acid. The nanoparticles were collected by centrifugation for 30 min at 25,000 rpm (42,000 g), washed and freeze dried.

Bio-adhesion detachment force study

The bio-adhesive performance of 10 mg Eudragit S100 coated and uncoated nanoparticles was calculated by measuring the force needed to detach the nanoparticles from the pig gastric mucosal tissue using modified [16] bio-adhesion detachment force measurement balance. The bio-adhesion detachment force (BF) in dynes/cm² was calculated using equation (2):

$$BF = (m.g)/A \dots\dots\dots(2)$$

where *m* = weight (in grams) added to the balance to detach the membrane, *g* = acceleration due to gravity (m/s²) and *A* = exposed area of the tissue.

In-vivo mucopenetration study

The animal protocols of the present study was carried out in accordance to the guiding principles of laboratory animal care (NIH Publication No. 85-93, revised 1985) [17] and were approved by Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA) (ref no. IAEC/M17/311/2016) of ISF College of Pharmacy, Moga, Punjab, India. FITC labeled CS-HPMCP (10 mg/2 ml) nanoparticles were administered using oral feeding canula to Wistar rats (n=12). After interval of 5, 8 and 12 h, three animals each time were sacrificed and colon portion from each animal was excised, washed with normal saline and antrum region was fixed () in formalin (10 %), sectioned (10µm) and stained with eosin [18]. Then it was seen under digital microscope (100X) (Motic DMWB series) using Motic Images plus 2.0 software and inverted fluorescent microscope (40X) (Olympus) to analyze the localization, mucoadhesion and mucopenetration of fluorescent nanoparticles.

Accelerated stability studies

A stability study was carried out on the optimized batch (CHP5) to assess the stability of nanoparticles by placing in stability chamber, adjusted at different temperature, i.e., 40 ± 0.5, 50 ± 0.5 and 60 ± 0.5° C, at a relative humidity of 75 ± 5 %, as well as at 25° C and relative humidity of 57.6 ± 0.4 % for a period of 12 weeks. The nanoparticles were thereafter evaluated for physical appearance and drug content.

Statistical analysis

The data are presented as mean ± standard deviation (SD). Where appropriate, differences between groups were evaluated using Student's t-test (two groups) at an alpha level of 0.05 using Prism software 5.04, GraphPad Software Inc, USA.

RESULTS

Preparation and optimization of nanoparticles

The nanoparticles were prepared by ionic gelation technique. Prepared nanoparticles were then coated by solvent evaporation method. The various formulation parameters such as polymer concentration and amount of drug were optimized. The resultant nanoparticles were then characterized for particle size, PDI, zeta potential and entrapment efficiency. The result demonstrated that the concentration of polymer

constituents play a key role in determining the morphology of prepared nanoparticles. The results were presented in Table 1.

On the basis of experimental finding, CHP5 formulation of nanoparticles was selected as optimized formulation on the basis of particle size (202 ± 27 nm), zeta potential (26.9 ± 2 mV), PDI (0.213) and entrapment efficiency (79.8 ± 5.4 %). Further results indicate that drug polymer ratio at 1:1 produced nanoparticles with desired size range and entrapment efficiency. Therefore formulation CHP5 was finely selected and considered for further study. The selected formulation was subjected for coating with Eudragit S100 by solvent evaporation. The coating efficiency was adjudged by *in-vitro* release efficiency (Figure 1)

Morphology

The SEM of freeze dried optimized coated and uncoated nanoparticles at 200x magnification showed smooth, spherical porous particles (Figure not shown). At the concentration of chitosan 0.1 % w/v the particles size was diverging between 202 - 236 nm and at 0.2 % w/v concentration 272 – 344 nm which is shown in the Table 1.

Zeta potential

The zeta potential is mainly affected by chitosan concentration and varied from 26.9 - 49.7 mV when chitosan concentration ranges from 0.1 - 0.2 % w/v. It may be said that availability of protonated amino groups are higher with increasing concentration of chitosan, and for optimized formulation Zeta potential was found to be 26.9 ± 2.4 mV.

Particle size

The minimum size i.e. 202 nm (Table 1) was observed with minimum concentration of chitosan and HPMCP and the maximum size, i.e., 344 nm was observed with maximum

concentration of the polymers. Optimized formulation has particle size of 202.7 ± 27 nm.

Drug entrapment (PDE)

The PDE of the nanoparticles ranged from 28.5 - 86.7 % and was highest at the highest concentrations of chitosan and HPMCP. Drug entrapment showed a linear relationship with polymer concentration.

Drug release

In vitro release performed on coated and uncoated CS-HPMCP nanoparticles to estimate drug release at colonic pH. In the current study, release determined in simulated gastric (0.1 M HCl solution) and colonic fluid (pH 6.8). Metronidazole released from the coated nanoparticles after 2 h in 0.1 M HCl was 5.57 ± 1.34 % whereas in uncoated nanoparticles, it was 16.08 ± 3.51 %.

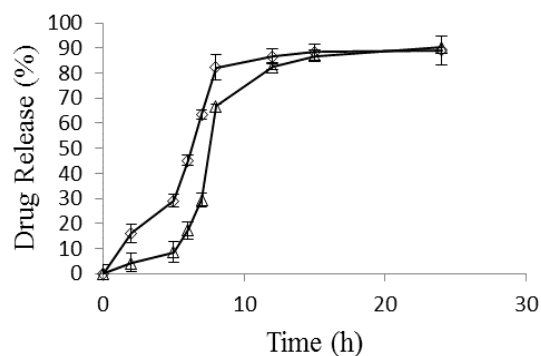


Figure 1: Release profile of optimized Eudragit S100-coated (Δ EU-CS-HPMCP) and uncoated (\diamond CS-HPMCP) nanoparticles

Bioadhesion detachment force

The bioadhesion detachment force studies on optimized nanoparticles ($n=3$) showed detachment force up to 12.34×10^3 dyne / cm^2 for coated and for the uncoated it was up to 14.98×10^3 dyne / cm^2 .

Table 1: Physicochemical properties of nanoparticles

Formulation code	Chitosan (% w/v)	HPMCP (%w/v)	Drug (%w/v)	Particle size (nm)	Zeta potential (mV)	Entrapment efficiency (%)	PDI
CHP1	0.1	0.1	0.05	225±17	32.5±2.3	28.5±4.4	0.219
CHP2	0.2	0.1	0.05	274±23	40.4±4.7	66.5±5.6	0.316
CHP3	0.1	0.2	0.05	236±34	37.6±3.9	34.4±4.2	0.242
CHP4	0.2	0.2	0.05	294±21	47.7±4.2	59.6±4.9	0.268
CHP5	0.1	0.1	0.1	202±27	26.9±2.4	79.8±5.4	0.213
CHP6	0.2	0.1	0.1	272±32	43.6±3.3	75.8±3.3	0.133
CHP7	0.1	0.2	0.1	228±18	49.7±4.3	53.5±3.8	0.322
CHP8	0.2	0.2	0.1	344±37	48.3±3.8	86.7±5.3	0.298

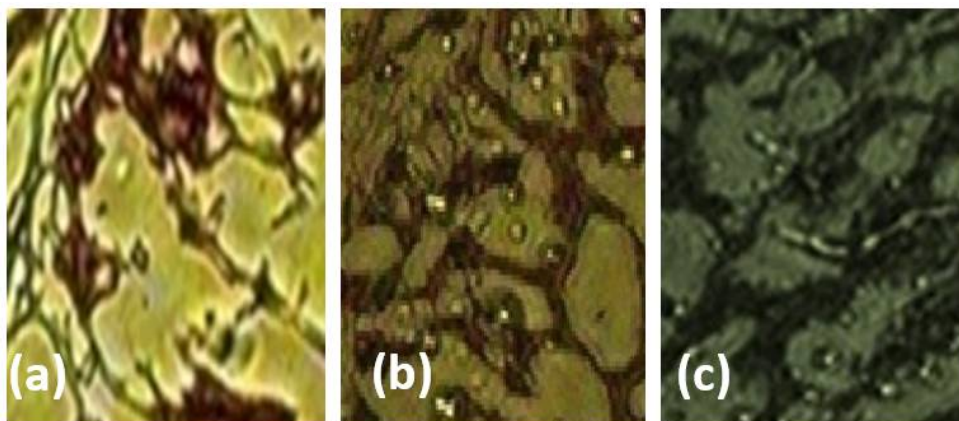


Figure 2: Nanoparticles to the colonic mucosa at 5th, 8th and 12th h, respectively

***In-vivo* mucopenetration**

The *in-vivo* mucopenetration studies of FITC labeled Eudragit S100 coated nanoparticles in Wistar rats revealed localisation and internalisation (Figure 2) of good number of nanoparticles up to 12 h. The histopathology photographs of nanoparticles revealed that in the first five hours the nanoparticles got adhered to the mucosa, and after that the penetration took place [19]. After time period of 8 h and till 12 h, the nanoparticles were seen in the mucus layer and were penetrated deep into mucosa near epithelial cell layer as observed by high fluorescence intensity near the gastric epithelium. The observed muco-penetration is attributed to decline in mucoadhesion because of reduced surface positivity of nanoparticles, which resulted in increased motility in colonic mucosa [20,21].

Stability of nanoparticles

The optimized formulation (CHP5) of nanoparticles was subjected to various temperature and humidity conditions for 12 weeks exhibited no change in colour and appearance. The chemical stability results have shown that the percent drug remaining was found to be 99.35 %, 96.45 %, 92.34 % and 86.57 % at 25 °C, 40 °C, 50 °C and 60 °C respectively. There was statistically insignificant difference in bio-adhesion strength of nanoparticles during 12 weeks at 25 °C and 40 °C. The regression analysis of stability data indicates that the drug degradation follows first order kinetics (Figure 3).

DISCUSSION

Various researchers have prepared chitosan nanoparticles using tripolyphosphate, sodium sulphate, poly- γ -glutamic acid, dextran sulphate,

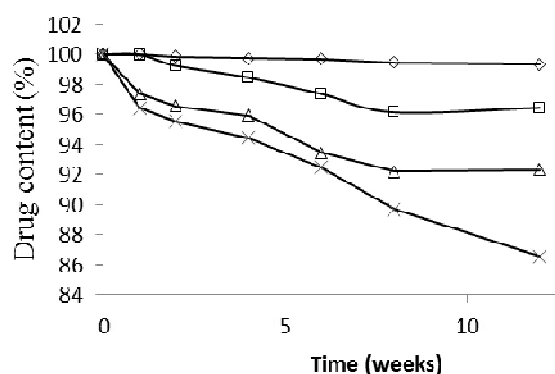


Figure 3: Accelerated Stability Studies of optimized formulation (CHP5) at temperature 25° C (◇), 40° C (□), 50° C (Δ) and 60° C (×)

anionic cyclodextrin [22], Eudragit S100, Eudragit L100 [23] as polyanion. However most of chitosan based nanoparticles have inadequate stability due to its dissociation at low pH. This is because of free amine group protonation of chitosan. This nanoparticles does not reach to small intestine and colon.

Therefore in order to stabilize chitosan nanoparticles HPMCP was used as anionic polymer. Further, the ratio between chitosan and HPMCP is critical to control the particle size and PDI. Additionally, the pH was considered as an important factor since it determines cross-linking degree and also the pH of the delivery site [24].

Zeta potential >25 mV can indicate nanoparticle's stability. In our study zeta potential remained above + 25 mV at the selected pH, thus confirming that the system remains stable and there was no aggregation. In addition to its net positive potential help in initial adhesion of nanoparticles to the surface of gastric mucosa. Amino groups of chitosan interact with mucin glycoproteins, sialic acid and other anionic moieties present on gastric mucosa [25,26]. Coated nanoparticles have comparatively less

mucoadhesive detachment force, which may be due to decrease in surface amino groups [19]. This reduced bioadhesion can facilitate infiltration of nanoparticles to gastric mucosa enhanced penetration and accumulation at the site of infection beneath mucosa.

CONCLUSION

The results of the present study suggest that entrapment of metronidazole in Eudragit S100 chitosan-HPMCP nanoparticles releases maximum content of the drug at colon *in vitro*. Furthermore, due to their very small size, the nanoparticles are capable of infiltrating mucus lining and could thus aid in the eradication of the amoebiasis infection in the gut lining in clinical practice.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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