



Formulation and Characterization of Metronidazole Loaded Polymeric Nanoparticles

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Abstract

This research describes the preparation of metronidazole (MNZ) polymeric nanoparticles by using chitosan intended for colon-specific delivery. The polymeric nanoparticles were prepared by the ionic gelation method. The nanoparticles were evaluated for their in vitro drug release properties. Scanning electron microscopy was used for morphology observation. The nanoparticles exhibited mucoadhesive properties, which diminished with increasing drug content. The nanoparticles with a particle size range between 200 and 300 nm exhibited excellent mucoadhesive properties. The results show that the formulated nanoparticles have succeeded in controlling the release of (MNZ) over a 12-hr period. In conclusion, the release of MZ was found to be dependent upon the composition of the nanoparticles, the ratio of the components and possible particle size, as well as bioadhesive ability.

Keywords

Metronidazole, chitosan, nanoparticles, bioadhesion, colon-specific.

INTRODUCTION:

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In most cases (conventional dosage forms), only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biochemical properties¹. Therefore, developing a drug delivery system that optimizes the pharmaceutical action of a drug while

reducing its toxic side effects *in-vivo* is a challenging task. One of the approaches is the use of colloidal drug carriers that can provide site-specific or targeted drug delivery combined with optimal drug release profiles. Among these carriers' liposomes and nanoparticles have been the most extensively investigated. Considerable research is being directed towards developing polymeric nanoparticles for drug delivery.

Polymeric nanoparticles which possess a better reproducibility and stability profiles. Nanoparticles have attracted a lot of attention of the pharmaceutical scientist in the drug delivery system due to versatility in targeting tissues, accessing deep molecular targets and controlling drug release². Polymeric materials such as chitosan (CS) and poly-D,L-lactide-co-glycolide (PLGA) are used for synthesis of biodegradative nanoparticles. CS is a natural polysaccharide derived by partial deacetylation of chitin. Properties such as biodegradability, non-toxicity and good biocompatibility make it suitable for use in biomedical and Pharmaceutical formulations.

Metronidazole [2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol] is an amoebicide, antiprotozoal and antibiotic effective against anaerobic bacteria and certain parasites. It is one of the most preferred drugs of choice for intestinal amoebiasis, giardiasis, trichomoniasis, bacterial vaginosis, surgical infections and duodenal ulcer associated with *Helicobacter pylori* infections, etc. The drug is to be delivered to the colon for their effective action against *E. histolytica* wherein the trophozoites reside in the lumen of the caecum and large intestine and adhere to the colonic mucus and epithelial layers³. However, the pharmacokinetic profile of metronidazole indicates that the drug is completely and promptly absorbed after oral administration reaching a concentration in plasma of about 10 mg/mL approximately 1 h after a single 500 mg dose. The administration of this drug in conventional tablet dosage form provides minimal amount of metronidazole for local action in the colon, still resulting in the relief of amoebiasis however, associated with unwanted systemic effects. Therefore, the objective of current investigation was to develop colon targeted metronidazole loaded nanoparticles⁶. This is because colon targeted drug

delivery system of metronidazole is associated with the following added advantages.

- 1.98-100% bioavailability with 7-8 h half-life and hepatic metabolism of metronidazole
- 2.High physiochemical stability of metronidazole
- 3.Feasible analytical methodology (UV-spectrophotometer) for *in-vitro* studies of the developed delivery system²¹

MATERIALS:

Pure Metronidazole was obtained by M/s Albert David Ltd. Ghaziabad, ex gratis. Chitosan was purchased from Yarrow Chem Products, Mumbai. HPMCP was purchased from Hyderabad, India. All other materials and reagents used in this study were of analytical grade of purity.

Preparation of NP'S:

Preparation of nanoparticles by modified ionic gelation process

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 temp. In brief, different conc. 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 MNZ (0.05 - 0.1 % w/v) under magnetic stirring for 30 min at 100 rpm while maintaining 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 MNZ. 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 D (+) 0.5 % w/v of trehalose dihydrate as a cryoprotectant at -55°C at a pressure of 0.01 mm of Hg⁴. The formulae were given in Table 1.

Table 1: Formulae of MNZ polymeric nanoparticles

Formulation code	Chitosan (% w/v)	HPMCP (% w/v)	MNZ (% w/v)
MNZ1	0.1	0.1	0.05
MNZ2	0.2	0.1	0.05
MNZ3	0.1	0.2	0.05
MNZ4	0.2	0.2	0.05
MNZ5	0.1	0.1	0.1
MNZ6	0.2	0.1	0.1
MNZ7	0.1	0.2	0.1
MNZ8	0.2	0.2	0.1

Table 2: The value of n characterizes the release mechanism of drug by Korsmeyer Peppas kinetic model.

S. No.	Diffusion exponent (n)	Drug Release Mechanisms
1	$0.45 \leq n$	Fickian diffusion
2	$0.45 < n < 0.89$	non-Fickian transport
3	$n = 0.89$	Case-II transport
4	$n > 0.89$	Super case-II transport

Table 3: Formulae of MNZ polymeric nanoparticles

Formulation code	Particle size (nm)	Zeta potential (mV)	Entrapment efficiency (%w/v)	PDI
MNZ1	225±12	32.5±1.32	27.6±4.3	0.221
MNZ2	273±20	39.87±2.78	65.7±4.2	0.342
MNZ3	239±13	35.68±1.23	35.6±4.8	0.235
MNZ4	298±17	48.36±4.52	58.4±3.9	0.298
MNZ5	200±10	25.46±2.32	81.2±4.56	0.214
MNZ6	272±18	44.23±1.47	74.5±3.2	0.145
MNZ7	221±13	50.23±2.31	52.4±3.7	0.356
MNZ8	345±18	49.56±2.51	87.6±4.3	0.314

Table 4: Kinetic release data of MNZ loaded NPs

Order type	Correlation coefficient
Zero order	0.960893
First order	0.98366
Higuchi	0.996752
Korsemeyer-peppas	0.992623
Hixson crowell	0.977311

Coating of Nanoparticles

The solvent evaporation method using rotary evaporator was applied to coat MNZ nanoparticles with Eudragit S100. 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 10 % of coating was attained. The coated nanoparticles were then dried and weighed⁵.

Evaluation parameters for nanoparticles:

Lyophilization of nanoparticle dispersion:

The nanoparticle dispersions were lyophilized (freeze dried) to obtain a dry powder. A particular NP dispersion was placed in a 100 kDa Amicon Ultra-15 centrifugal filter unit and centrifuged at 7830 RPM for 30 min at 15°C temperature in a centrifuge 5430R (Eppendorf AG, Hamburg, Germany) using rotor F-35-6-30 to isolate the NPs. The NPs obtained on top of the filter were re-dispersed in appropriate amount of deionized water and the cryoprotectant anhydrous trehalose equivalent to the amount of chitosan was added. The mixture was then placed in a fast freeze flask (Labconco, MO, USA) and then frozen quickly at -75°C. The frozen samples were lyophilized for 72 hours in the lyophilizer (Free Zone 2.5 liter benchtop freeze dry system, Labconco, MO, USA). The temperature was kept at about -49°C and vacuum maintained at 0.120 mbar. The lyophilized

samples were collected and stored in a desiccator for further characterization⁶.

Zeta potential determination of NPs⁹

Samples were prepared for zeta potential analysis by diluting 1 ml of the NP dispersion with 10 ml deionized water. DLS instrument was used to measure Zeta Potential but in the electrophoretic light scattering mode (ELS). The NP dispersion and re-dispersed lyophilized NP samples were placed in a standard glass cuvette for measurement. The scattering angle was set at -14.06 and a temperature of 23°C.

Morphology of NPs¹⁰

Transmission electron microscopy (TEM) was used in the morphological analysis of metronidazole loaded CS nanoparticles. The nanoparticles suspensions were spread on a glass plate and dried at room temperature. The dried nanoparticles were then coated with gold metal under vacuum and then examined.

Drug encapsulation efficiency studies of NPs¹¹

A 12 ml sample of freshly prepared drug loaded NP dispersion was centrifuged in a 100 kDa Amicon Ultra-15 Centrifugal Filter Unit at 7830 RPM for 30 min at 15°C temperature using centrifuge 5430R. The amount of the unincorporated drug was measured by suitably diluting the filtrate and measuring its absorbance at 265 nm using single beam UV

spectrophotometer against suitably diluted filtrate of blank NP dispersion. Encapsulation efficiency was calculated by subtracting the amount of drug in the filtrate from the amount of drug originally added to the formulation.

In vitro drug release studies from NPs^{21,22}

In vitro release profiles of MNZ from the lyophilized NPs were obtained by a dissolution test in phosphate buffer solution (USP phosphate buffer, release medium, 0.2 M, pH 7.4). Regenerated cellulose membrane (dialysis membrane with molecular weight cut off of 12-14 kDa, Fisher brand® regenerated cellulose dialysis tubing, 44 mm diameter) was used. Lyophilized MNZ loaded NPs equivalent to 2 mg of MNZ and 10 ml phosphate buffer (pH 7.4) was placed into a dialysis bag that immersed into 100 ml phosphate buffer solution and the system was maintained at 37°C under mild agitation of 100 rpm in a reciprocal shaking bath. At predetermined time intervals, aliquots of the release medium (4 ml) were withdrawn and assayed for drug release and replaced by 4 ml of fresh buffer. An assembly similar to the one described above was also prepared for the blank lyophilized NP (to be used as blank for UV spectrophotometry). MNZ in the release medium was quantified by UV spectrophotometry at 242 nm against the blank and cumulative release of MNZ was calculated based on a pre-generated the calibration curve¹².

The results of kinetic treatment applied to dissolution profile of best formulation were given in **Table 4**. *In vitro* drug release, Higuchi and Peppas's data for all formulations were shown in graphs **Figure 8-12**.

In-vitro mucopenetration studies^{19,20}

For mucus sample, pig intestinal ileum (freshly isolated) was taken from local slaughter house. It was kept in ice-cold oxygenated phosphate buffered saline (PBS) prior to sample processing. The colon part was taken and rinsed thoroughly with PBS. The mucus was then harvested through gentle scraping and was divided into 500 mg aliquots and preserved at -20° C before starting the experimentation¹³.

The mucus (400 mg) was equilibrated at constant temperature of 37° C for 20 min in a vibrator to form homogenous dispersion of mucus. Then it was placed on the donor chamber of Franz-diffusion cell with dialysis membrane located between donor and receptor compartment to support mucus. Then 2 ml of MNZ loaded chitosan nanoparticles were added on the surface of mucus. The receptor chamber was filled with PBS. After fixed interval of time 1ml of sample was withdrawn and replaced with equal

volume of PBS, conc. of MNZ was determined spectrophotometrically at 277 nm.

In-vivo mucopenetration studies^{14,15,16}

In-vivo mucopenetration study was performed using FITC labeled chitosan nanoparticles suspension (10 mg / 2 ml) which was administered using oral feeding canula to Wistar rats (n=12). After interval of 5 , 8, 12and 24 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 (10 µm) and stained with eosin. 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 and mucoadhesion of fluorescent nanoparticles.

Accelerated stability studies^{17,24,25}

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° C, at a relative humidity (RH) of 75 ± 5 %, as well as at 25 ± 2° C and RH of 60 ± 0.5 % for a period of 12 weeks (Badawi *et al.*, 2011). The nanoparticles were thereafter evaluated for physical appearance and drug content.

RESULTS AND DISCUSSION

The melting range of MNZ was observed between 157 -161° C with capillary method and was comparable with standard range of 158-162° C and with DSC it was found to be 160.55° C (**Figure 1**), the observed values complies with the standard values given in the official monograph (BP), which confirmed the identity and purity of the drug. The observed λ_{max} was 277 nm compared to the standard λ_{max} of 278 (**Figure 2**). The FTIR Spectroscopy was performed on MNZ and shown in **Figure 3**. The interpretation was done using standard wave number ranges with observed wavenumber along with assignment of specific chemical group are listed in **Table 5**, which confirmed the presence of MNZ.

The polymeric nanoparticles of MNZ with chitosan were prepared as per the modified ionic gelation method. The solvent evaporation method was used to coat Eudragit S100 on the surface of MNZ nanoparticles.

Various factors such as polymer weight ratios, polymer mixing ratios, pH of polymers, effect of sonication time during mixing influences the ionic interaction. Chitosan (0.1-0.2 %), HPMCP (0.1-0.2 %) and MNZ (0.05-0.1 %) concentration selected for further studies and it was optimized that 1:1 ration

of chitosan and HPMCP is most suitable for nanoparticles formulation.

The formulated polymeric nanoparticles are spherical in shape and nanometre size range. The recovery of NP was found to be 89%. The lyophilized powder of nanoparticles was given in **Figure 4**. The particle size is a critical parameter for evaluation during and after formulation of NPs. The particle size data of NP dispersions prepared by modified ionic gelation process by using chitosan as polymer with HPMCP was given in **Table 5**. A few particles with higher particle size were sometime observed when ionic gelation process is not performed with uniform stirring process.

The TEM of freeze dried optimized coated nanoparticles at 200x magnification showed smooth, spherical porous particles were depicted in **Figure 14-15**. 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 3**. The minimum size i.e. 202 nm 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 particle sizes the shown in **Figure 6**. The drug entrapment follows the linear relationship with respect to chitosan concentration as the polymer concentration increases, the entrapment efficiency of the nanoparticles increases.

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

The chemical reaction between FITC and chitosan was confirmed by IR spectrum. FITC labelled chitosan was used in preparation of FITC labelled CS-HPMCP nanoparticles and the optimized formulation showed the particle size of 272 ± 20.8 nm.

The bio adhesion detachment force studies on optimized nanoparticles (n=3) showed detachment force up to 12.34×10^3 dynes / cm² for coated and for the uncoated it was up to 14.98×10^3 dynes / cm². Coated nanoparticles have comparatively less mucoadhesive detachment force, which may be due to decrease in surface amino groups. This reduced muco-adhesion can facilitate in infiltration of nanoparticles to gastric mucosa enhanced

penetration and accumulation at the site of infection beneath mucosa.

Stability of nanoparticles

The optimized formulation (CHP5) of nanoparticles was subjected to two different temperatures 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 ± 1.12 % and 86.45 ± 1.23 %, at 25 °C and 40° C respectively. There was statistically insignificant difference in bio-adhesion strength of nanoparticles for 12 weeks. The regression analysis of stability data indicates that the drug degradation follows first order kinetics.

Data of the *in vitro* release was fit into different equations and kinetic models to explain the release kinetics of MNZ from NP's. The kinetic models used were zero order, first order, Higuchi, Hixson Crowell and korsmeyer-peppas models. The drug release data of the selected NP formulation was given in **Table 4** and shown in **Figure 8 to 12**.

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FITC labelled Pegylated chitosan nanoparticles showed internalization of the particles more at 5 and 8 h. This may be due to reduction of surface charge which accelerates the adhesion and penetration of the nanoparticles in to the mucus. Study conducted at 12 h and 24 h showed good number of fluorescent particles in the inner mucus layer.

The optimized formulation (MNZ5) of nanoparticles was subjected to two different 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 ± 1.12 % and 86.45 ± 1.23 %, at 25° C and 40° C respectively. There was statistically insignificant difference in bio-adhesion strength of nanoparticles during 12 weeks. The regression analysis of stability data indicates that the drug degradation follows first order kinetics (**Figure 9**).

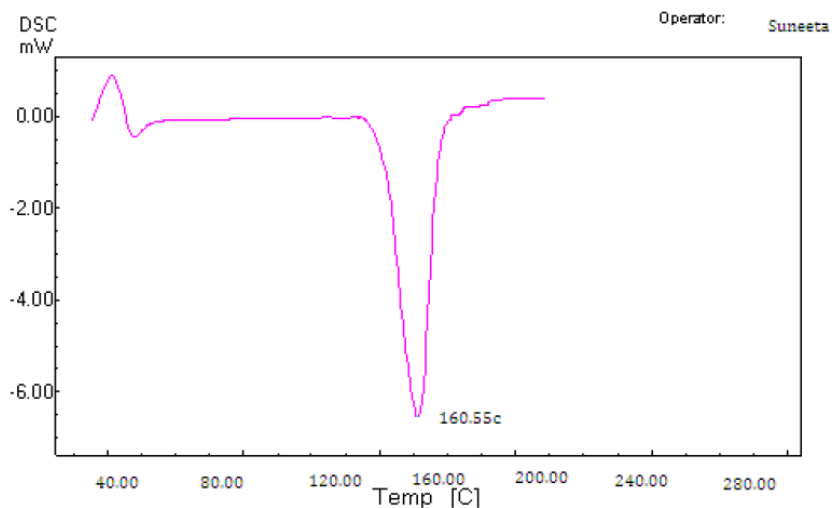


Figure 1: DSC thermogram of MNZ

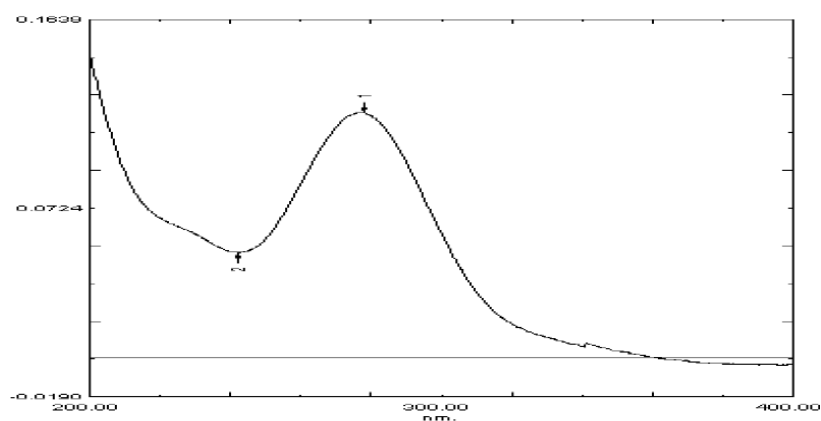


Figure 2: Scan spectrum of MNZ

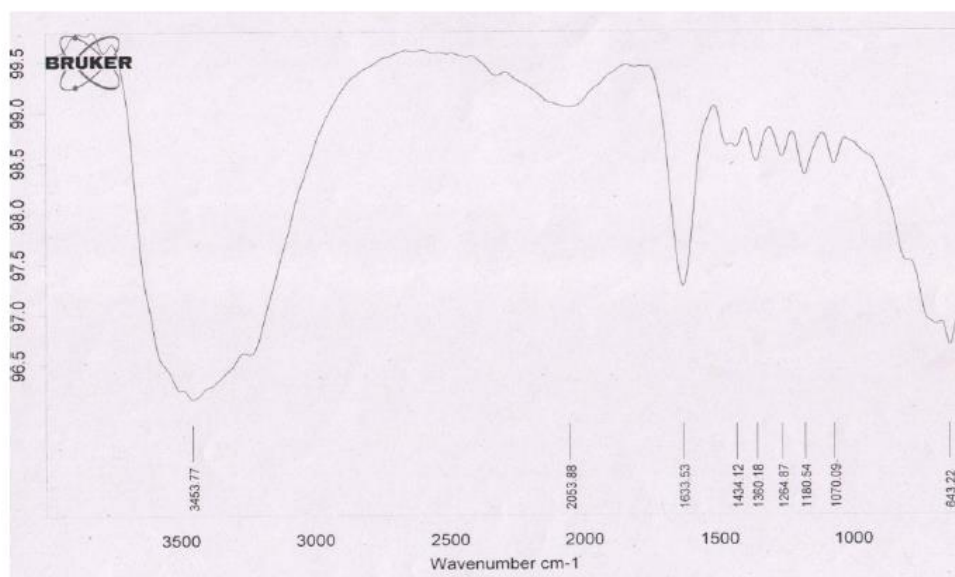


Figure 3: FTIR spectrum of MNZ

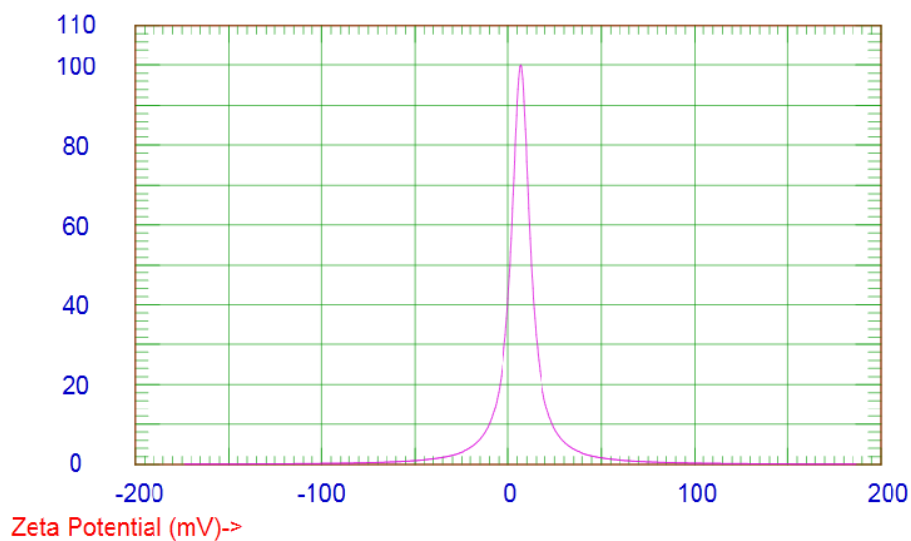


Figure 4: Zeta potential profile of coated MNZ NPs

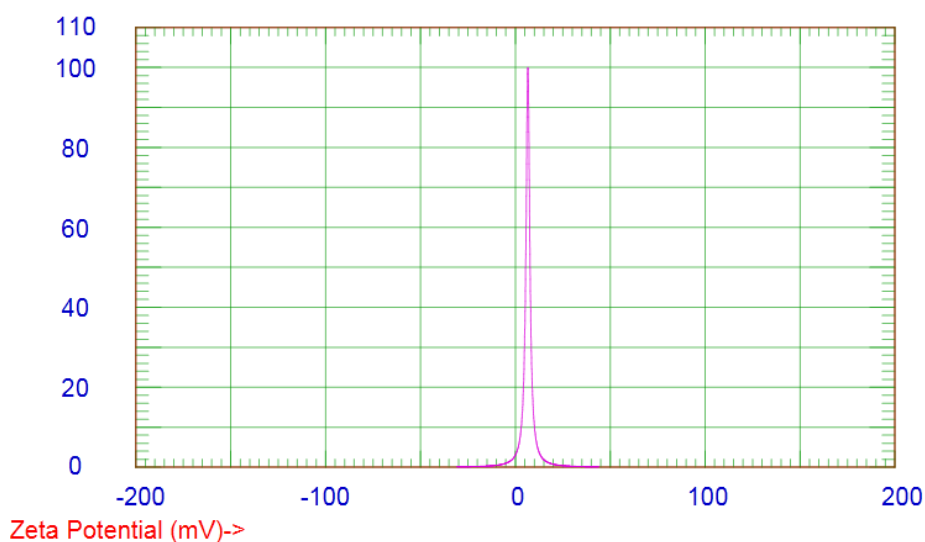


Figure 5: Zeta potential profile of coated MNZ NPs

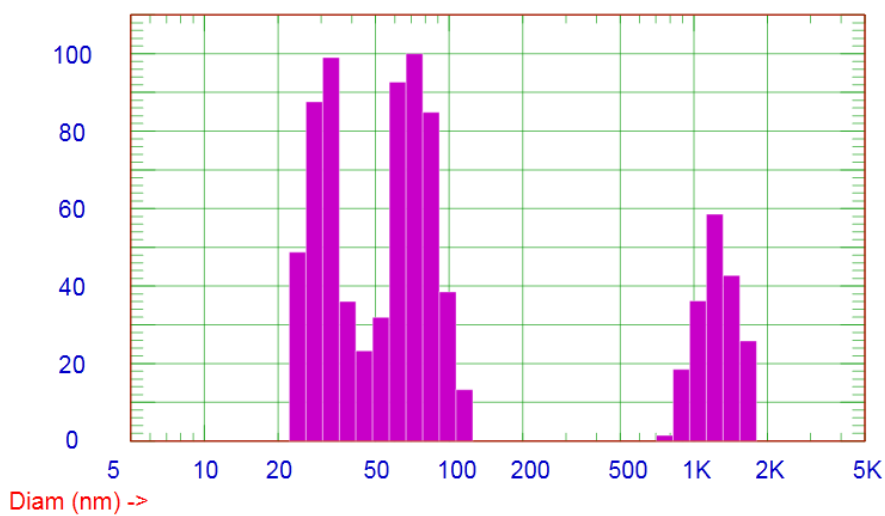


Figure 6: Particle size distribution of uncoated MNZ NPs

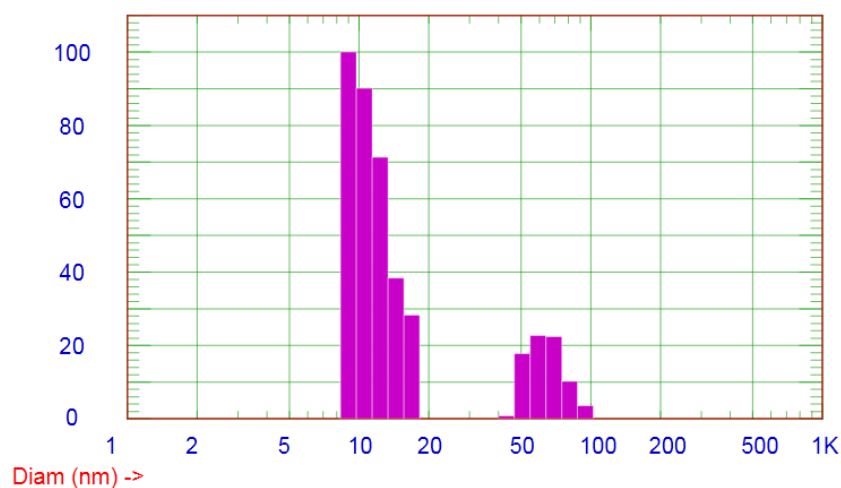


Figure 7: Particle size distribution of coated MNZ NPs

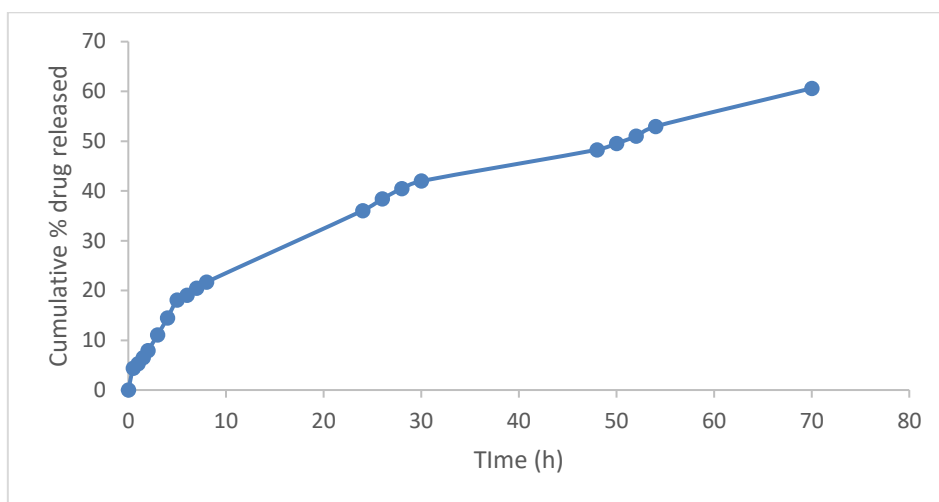


Figure 8: Zero order profile of MNZ loaded redispersed lyophilized NPs prepared via temperature modulated solidification process

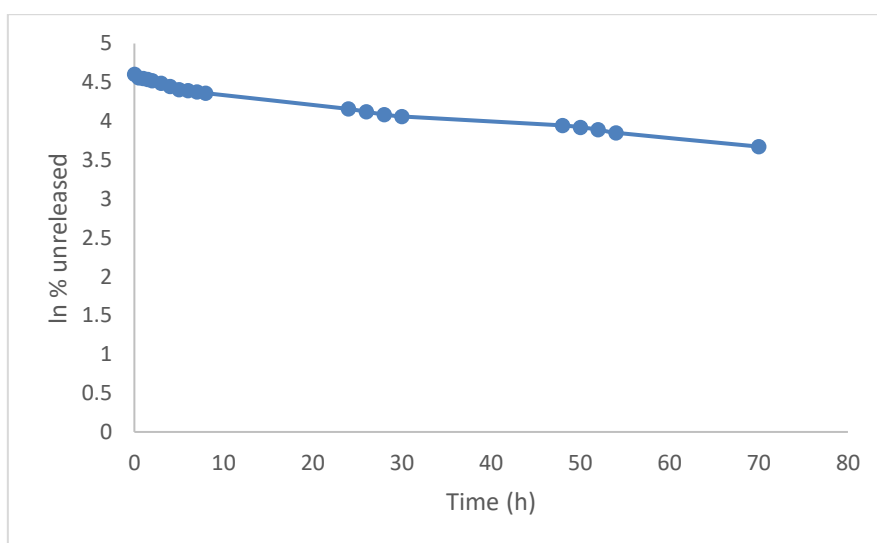


Figure 9: First order profile of MNZ loaded redispersed lyophilized NPs prepared via temperature modulated solidification process

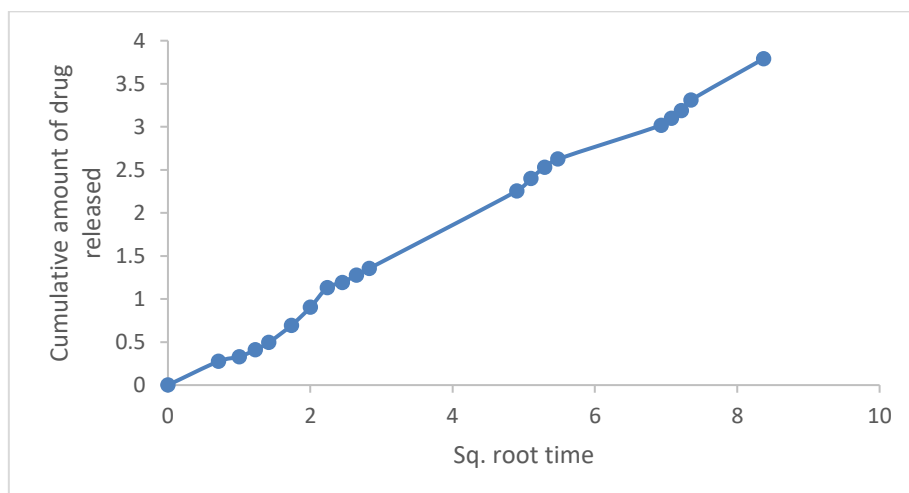


Figure 10: Higuchi order profile of MNZ loaded redispersed lyophilized NPs prepared via temperature modulated solidification process

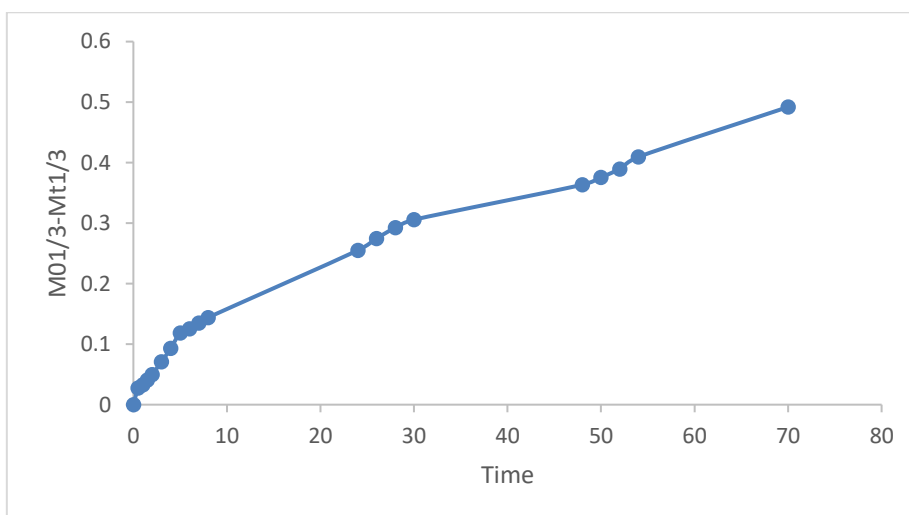


Figure 11: Hixson crowell profile of MNZ loaded redispersed lyophilized NPs prepared via temperature modulated solidification process

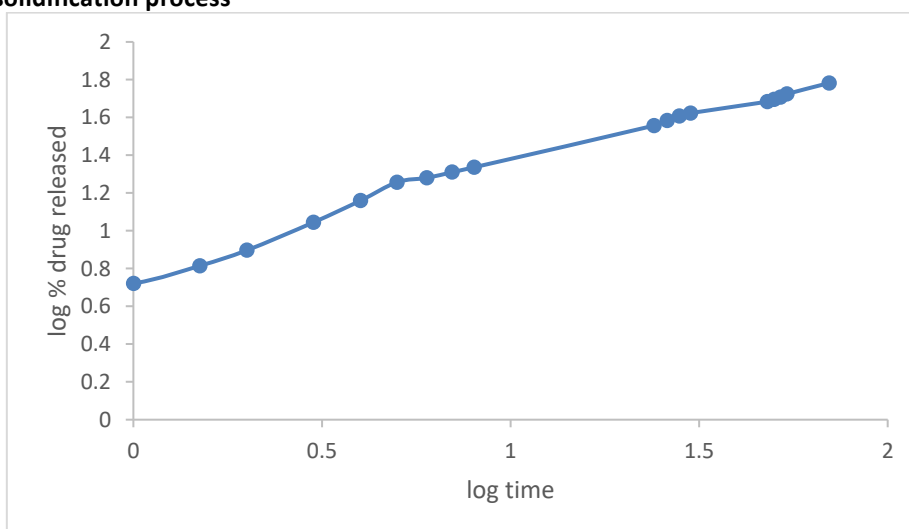


Figure 12: Korsmeyer-peppas profile of MNZ loaded redispersed lyophilized NPs prepared via temperature modulated solidification process



Figure 13: Photograph of lyophilized NPs

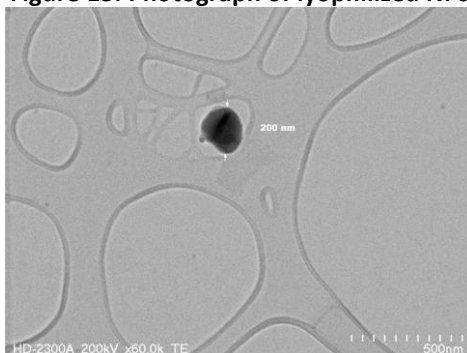


Figure 14: TEM image of MNZNPs without eudragit S 100 coating

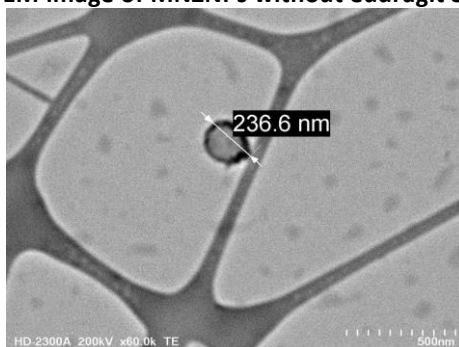
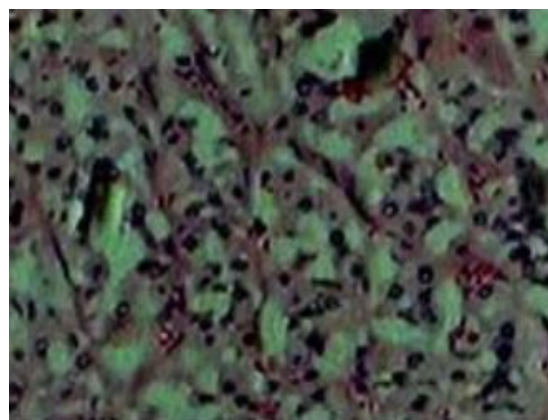
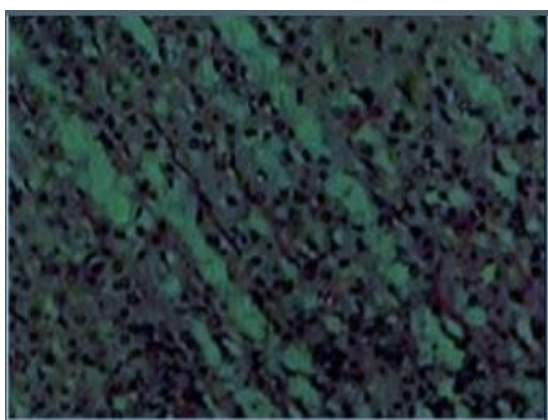


Figure 15: TEM image of MNZNPs with eudragit S100 coating



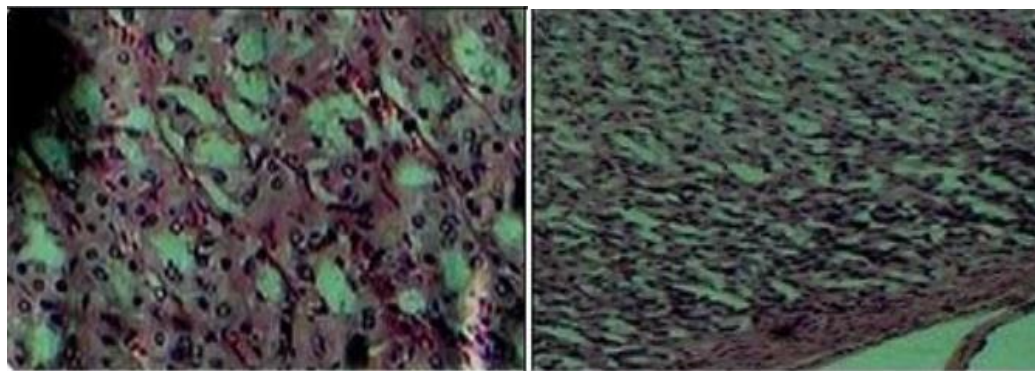


Figure 16: Histopathological study of FITC labelled chitosan nanoparticles a) 5h b) 8th c) 12th d) 24th hour

CONCLUSION:

A successful attempt was made to develop MNZNP. There are numerous preparation methods available for producing NP. In the present study MNZ loaded NP were prepared by magnetic stirring method followed by temperature modulated solidification. This method is simple, easy and is suitable to produce NP of 50-200 nm size ranges. Lipophilic drugs like MNZ can be successfully loaded in lipid chitosan by using nontoxic surfactants like tween 80. *In vitro* release of MNZ followed Higuchi and first order equations better than zero order equation. The results for MNZ indicate that the NP have potential as a drug delivery system. Furthermore, they may have utility for site specific drug delivery since the small size of the particle and its biodistribution properties may allow their delivery to target sites. Sustained release of nanoparticles might extend the circulation time of drug will suitable for reducing the initial hypotensive peak and prolong the antihypertensive effect.

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