



Development of Tinidazole Loaded Polymeric Nanoparticles Formulation and its Characterization

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Authors' contributions

This work was carried out in collaboration between both authors. Author KTD designed work, collected literature, performed the work and wrote first draft of the manuscript. Author BSV guided to perform the work, analyzed the results searches. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i1831318

Editor(s):

(1) Dr. Mohamed Fathy, Assiut University, Egypt.

Reviewers:

(1) Reem About Assi, Universiti Sains Malaysia, Malaysia.

(2) Suguna Perumal, Kyungpook National University, South Korea.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/66955>

Original Research Article

Received 15 January 2021

Accepted 20 March 2021

Published 30 March 2021

ABSTRACT

Introduction: The development of safe drug delivery systems for a therapeutic agent with less side effects and more bioavailability to the targeted site is very vital in drugs formulation. Tinidazole (TZ) is a drug used to treat giardiasis, amebiasis for colon infections and other infections also such as trichomoniasis, bacterial vaginosis. But the oral bioavailability for the current using drugs low. So, the current study was aimed to develop colon targeted drug delivery system for Tinidazole (TZ) with polymeric nanoparticles (NPs).

Methodology: The nanoparticles formulations of TZ were prepared with modified ionic gelation method using chitosan and hydroxypropyl methylcellulose phthalate (HPMCP) are in different combinations by magnetic stirring method followed by temperature modulated solidification. The solvent evaporation method applied to coat TZ nanoparticles with Eudragit S100. The prepared TZ nanoparticle were studied to evaluate physicochemical properties, *In-vitro* drug release, mucopenetration and *In-vivo* mucoadhesive studies were carried out.

Results: The results of study indicate, 1:1 ratio of chitosan and HPMCP formulation of

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nanoparticles provides better spatial interaction between them and TZ with spherical porous and the particles size was diverging between 202 - 236 nm. *In vitro* release of TZ followed Higuchi and first order equations better than zero order equation. The drug release results of nanoparticles formulations of TZ indicate that the NPs have potential as a drug delivery system compare to uncoated TZ and coated nanoparticles have comparatively less mucoadhesive detachment force.

Conclusion: In conclusion, the study was an evidence to use nanoparticles in colon targeted drug delivery systems for better bioavailability of drugs at targeted site and the biodistribution properties of drugs using nanoparticle will be depend on their composition, particle size and their adhesive abilities.

Keywords: Tinidazole; nanoparticles; drug delivery system; formulation; bioavailability.

1. INTRODUCTION

The emerging of new diseases in recent times around the world are demanding to develop new drug therapeutics to control [1,2]. The usage of current drugs also giving doubt about their efficacy and are becoming resistance towards different diseases, failing in treat them and are may cause for the emerging of new resistant organisms [3,4]. The available drugs around the world have different route of administration i.e., parentals and enteral for treatment and their efficacy dependent on their quantity (Bioavailability) to reach the targeted site after administration [5-7]. The bioavailability of drugs depends on drugs' physiochemical and biological properties and the different ingredients used in their formulations for safe drug delivery system to targeted sites [5,8,9]. So, the development of safe drug delivery systems for a therapeutic agent with less side effects and more bioavailability to the targeted site is very vital in their formulation [10,11]. In order to overcome this situation innovative drug delivery systems are developing through colloidal drug carriers such as nanoparticles, microspheres, liposomes, polymer micelles [12]. These molecules work as carriers for new therapeutic drugs in formulations in the achievement of more bioavailability at targeted sites for their intended use. With development of this nanotechnology, development of targeted drug delivery system obtained more desirability in growth of new formulations to current drugs and new drugs [13,14].

The colon infections are one of the major diseases in the current world people [15]. There were different colon infections; colitis can be caused by viruses, bacteria, and parasites, Inflammatory bowel disease, Ischemic colitis, Allergic reactions and Drug-induced colitis [16-18]. These infections are directly or indirectly causing the mortality [15]. Different medications were available to treat the colon infection

depends on infections' symptoms [19-21]. Tinidazole (TZ) (1*H*-imidazole, 1-[2-(ethylsulfonyl) ethyl]-2-Methyl-5-nitro-1-[2-Ethylsulfonyl)Ethyl]-2- methyl-5-nitroimidazole), is one of such drugs used to treat giardiasis, amebiasis for colon infections and other infections also such as trichomoniasis, bacterial vaginosis [22,23]. Current marketed preparations of tinidazole are conventional immediate release tablets and thus do not provide localized effect in colon. Thus, there is a need to develop colon specific oral drug delivery systems for Tinidazole with minimum drug release before it reaches the colon and immediate release as soon as it reaches the colon.

2. MATERIALS AND METHODS

2.1 Chemical and Drugs

Tinidazole was obtained as free sample from M/s Albert David Ltd., Ghaziabad, India. Chitosan was procured from Yarrow Chem Products, Mumbai, India. Hydroxypropyl Methylcellulose (HPMCP) was obtained from Amrutha Organics, Hyderabad, India. The other chemicals and solvents used in current research are analytical grade.

2.2 Drug polymer Compatibility Studies using Fourier Transform Infrared Spectroscopy (FTIR)

FTIR absorption spectra of pure drug, all the polymers used, and the combination of drug and polymers were taken to confirm the identity of the drug and to detect the interaction of the drug with the excipients [24].

2.3 Thermal Properties by Differential Scanning Calorimetry (DSC)

Thermal properties of pure drug, optimized formulations and stability studies subjected

samples of optimized formulations were evaluated by Differential scanning calorimetry (DSC) using a Diamond DSC (Mettler Star SW 8.10). The analysis was performed from 500 C to 2000 C temperature range at a rate of 5 0 C min⁻¹ under nitrogen flow of 25 ml min⁻¹ [25,26].

2.4 Preparation of Nanoparticles (NPs)

The ionic gelation method with magnetic stirring at room temp was used in preparation of NPs formulation with TZ (0.05-0.1% w/v) was carried to formulate with different combinations of chitosan (0.1-0.2% w/v) in acetic acid (1%v/v pH5.0), which is one of best polysaccharide in the current world with antibiotic activity, hemostatic agent and improves the drug delivery and HPMCP (0.1-0.2%w/v) in 0.1M sodium hydroxide solution (Table 1) which is an cellulose derivative protect the drug from degradation by gastric acids. The completed dispersion was centrifuged at 20000 rpm for 30minutes at 4°C. The supernatant was collected for estimation of TZ and the pellet (bottom material) was washed with double distilled water lyophilized using trehalose dihydrate as a cryoprotectant at -55° C at a pressure of 0.01 mm of Hg. Finally optimized that 1:1 ration of chitosan and HPMCP is most suitable for nanoparticles formulation.

2.5 Nanoparticles Coating

Acetone solution 12% w/v was used to prepare coating solution to coat TZ nanoparticles (dispersed in 1:10) with Eudragit S100 using rota evaporator.

2.6 Nanoparticles Dispersions' Lyophilization (Freeze Drying)

The NPs dispersion was lyophilized to get dry powder using lyophilizer (-49°C and vacuum maintained at 0.120 mBar) and during lyophilization cryoprotectant anhydrous trehalose equivalent to the amount of chitosan was added

to the samples. The samples were stored in desiccator for further study.

2.7 Evaluation of Nanoparticles

The developed NPs formulation was evaluated by different parameters.

2.7.1 Morphology of NPs

Transmission electron microscopy (Hitachi HD-2300) in the phase contrast mode to visualize the internal matrix and shape of individual nanoparticles.

2.7.2 Thermal analysis of NPs

A differential scanning calorimeter (PerkinElmer Diamond DSC, CT, USA) equipped with an intercooler 1P was used to analyze the thermal behavior of NP samples and physical state of the drug in NPs [27]. The thermograms were recorded over a temperature range of 10-350°C at a heating rate of 10°C/min under nitrogen purge gas maintained at a flow rate of 20 ml/min. The thermograms were analysed using Pyris Manager (v 1.3) software.

2.7.3 Droplet size determination of NPs

The droplet size of the resultant nanosuspension was measured in a dynamic light scattering instrument (Nicom 380 ZLS, Particle Sizing Systems, CA) by placing the sample in the path of a Helium Neon laser of wavelength 658 nm at a scattering angle of 90° and a temperature of 23°C.

2.7.4 Zeta potential determination of NPs

Dynamic Light Scattering (DLS) Instrument was used to measure Zeta Potential in the electrophoretic light scattering mode (ELS) by scattering angle at -15.06 and a temperature of 23°C.

Table 1. Formulae of TZ polymeric nanoparticles

Formulation code	Chitosan (%w/v)	HPMCP (%w/v)	TZ (%w/v)
TZ1	0.1	0.1	0.05
TZ2	0.2	0.1	0.05
TZ3	0.1	0.2	0.05
TZ4	0.2	0.2	0.05
TZ5	0.1	0.1	0.1
TZ6	0.2	0.1	0.1
TZ7	0.1	0.2	0.1
TZ8	0.2	0.2	0.1

2.7.5 Drug encapsulation efficiency

Drug encapsulation efficiency studies of NPs using UV-Visible spectrophotometer [27]. It was calculated by subtracting the amount of TZ in supernatant of formulated NPs with TZ after centrifuge in a 100 kDa Amicon Ultra-15 Centrifugal Filter Unit at 7830 RPM for 30 min at 15°C against suitably diluted filtrate of blank NP dispersion at absorbance 265nm from amount originally added to formulation.

2.7.6 *In vitro* drug release studies from NPs

In vitro release profiles of TZ from the lyophilized NPs were obtained by a dissolution test in phosphate buffer solution. A regenerated cellulose membrane (44mm) was loaded with lyophilized TZ NPs and placed in 100ml of phosphate buffer maintained at 37°C with 100rpm. The 4mL of free medium was collected and replaced with fresh phosphate buffer. The collected solution was quantified for TZ against samples prepared as above with free NPs as blank by UV spectrophotometry at 242nm and cumulative release of TZ was calculated based on a pre-generated calibration curve. The mechanism of drug release from the NP's was measured by treating the drug dissolution data with the following different release kinetic models, Zero order release (Cumulative percent drug released Vs time) equation, Higuchi's (Cumulative percent drug released Vs square root of time) equation and Korsmeyer and Peppas (Log cumulative percent drug released versus log time) equation [28].

2.7.7 *In vivo* mucopenetration study

The study was carried out using freshly isolated pig intestinal ileum and is maintained at ice-cold oxygenated phosphate buffer saline (PBS). The mucus sample equilibrated for 20minutes at 37°C in a vibrator to get homogenous dispersion mucus. The mucus was placed in donor chamber of Franz-diffusion cell having dialysis membrane between receptor and donor chambers for support the mucus. Then 2 ml of TZ loaded NPs were added on the surface of mucus and receptor chamber was filled with PBS. Later, at different time intervals 1ml of sample was withdrawn and replaced with equal volume of PBS, conc. Of TZ was determined spectrophotometrically at 277 nm.

2.7.8 *In-vivo* mucopenetration studies

In-vivo mucopenetration study was performed using Fluorescein isothiocyanate (FITC) labeled

chitosan nanoparticles suspension (10mg/2ml) which was administered using oral feeding canula to Wistar rats. The 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 at interval of 5hr, 8hr, 12hr and 24hr colon portion [29-31].

2.7.9 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., $40 \pm 0.5^\circ$ C, at a relative humidity (RH) of $75 \pm 5\%$, as well as at $25 \pm 2^\circ$ C and RH of $60 \pm 0.5\%$ for a period of 12 weeks by evaluated for physical appearance and drug content.

3. RESULTS AND DISCUSSION

3.1 Drug Polymer Compatibility Studies by Fourier Transform Infrared Spectroscopy (FTIR)

The IR spectrum of pure Tinidazole is characterized by principal absorption peaks at 3405.20 cm^{-1} and 3332.50 cm^{-1} (O-H & N-H, stretching vibration broad peaks merged together), 2925.67 cm^{-1} (C-H, stretching), 2925.56 cm^{-1} (C-H, stretching), 1597.13 cm^{-1} (N-H bending vibrations) and 1256.01 cm^{-1} (OH bending and C-O stretching vibrations). Some additional peaks were observed in IR spectrum of physical mixture of TZ and chitosan, which could be due to the presence of the chitosan. Peaks at 2918.68 cm^{-1} (C-H stretching, aliphatic), 1735.75 cm^{-1} (in plane O-H bending) and 1178.05 cm^{-1} (C-O stretching) characterize chitosan. Major frequencies of functional groups of pure drugs remain intact in spectrum of physical mixture containing chitosan. Hence, there is no interaction between drug and lipid used in the study (Figs. 1 and 2).

3.2 Thermal Properties by Differential Scanning Calorimetry (DSC)

The melting range of TZ was observed between $157 - 161^\circ\text{C}$ with capillary method and was comparable with standard range of $158 - 162^\circ\text{C}$ and with DSC it was found to be 160.55°C , the observed values comply with the standard value 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 (Fig. 3).

3.3 Preparation of 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 TZ (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.

pH range of chitosan between pH 2 - 7 and HPMCP between pH 2-7 has been selected and it was found that 5.5 pH was most suitable for nanoparticles formulation between chitosan and HPMCP. Particle size was increased when preparation pH increased further. The effect of mixing ratio between chitosan and HPMCP during formulation of nanoparticles, the different

volumes of HPMCP was added to the chitosan which was kept constant. The minimum particle size was observed with chitosan: HPMCP mixing volume ratio of 1:1 (Table 2).

3.4 Size Determination of NPs

The formulated polymeric nanoparticles are spherical in shape and nanometre size range. The recovery of NPs was found to be 89%. The particle size is a critical parameter for evaluation during and after formulation of NPs. The particle size data of NPs dispersions prepared by modified ionic gelation process by using chitosan as polymer with HPMCP was given in Table 2. A few particles with higher particle size were sometime observed when ionic gelation process is not performed with uniform stirring process. At the concentration of chitosan 0.1% w/v the particles size was diverging between 202-236nm and at 0.2% w/v concentration 272-344 nm (Figs. 4 and 5).

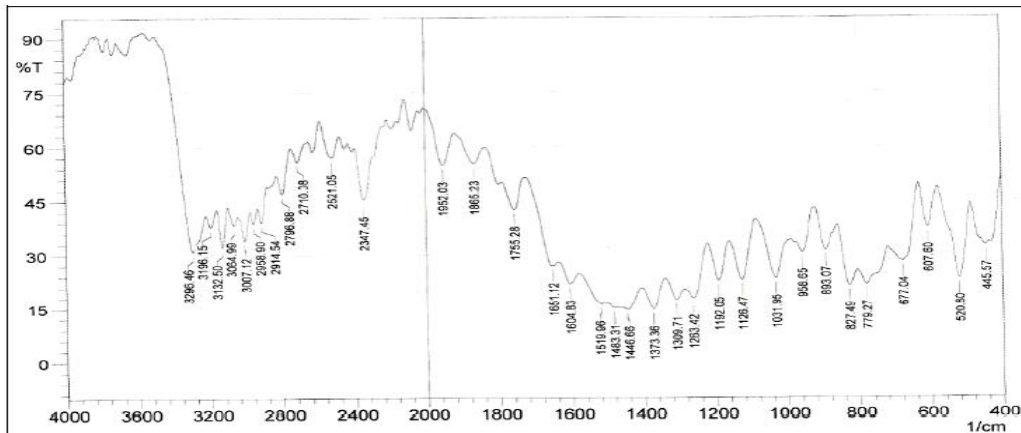


Fig. 1. FT-IR spectrum of tinidazole

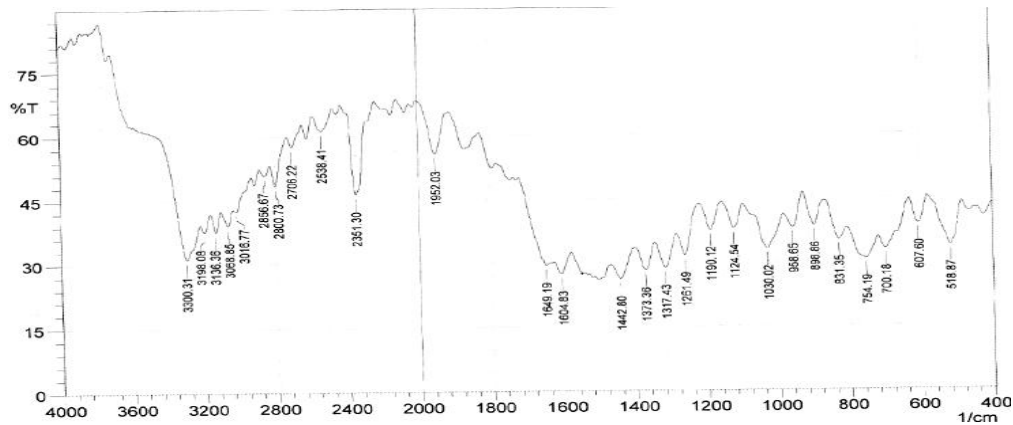


Fig. 2. FT-IR spectrum of coated TZ NPs

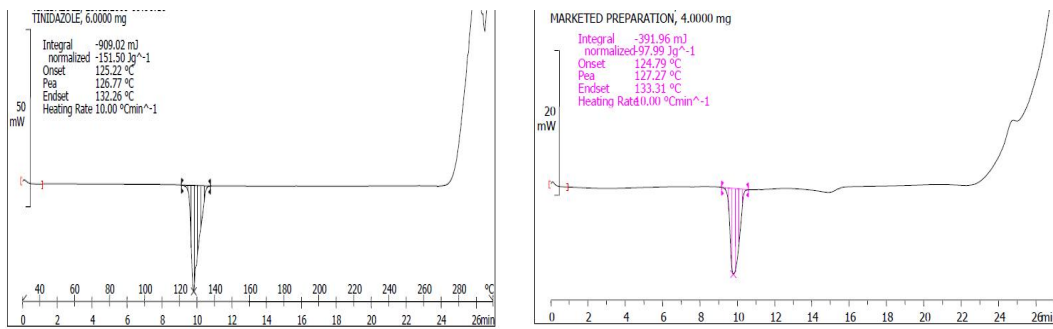


Fig. 3. Thermogram of A) Tinidazole B) Tinidazole formulation

Table 2. Tinidazole polymeric nanoparticles particle size

Formulation code	Chitosan (%w/v)	HPMCP (%w/v)	TNZ (%w/v)	Particle size (nm)
TZ1	0.1	0.1	0.05	225±12
TZ2	0.2	0.1	0.05	273±20
TZ3	0.1	0.2	0.05	239±13
TZ4	0.2	0.2	0.05	298±17
TZ5	0.1	0.1	0.1	200±10
TZ6	0.2	0.1	0.1	272±18
TZ7	0.1	0.2	0.1	221±13
TZ8	0.2	0.2	0.1	345±18

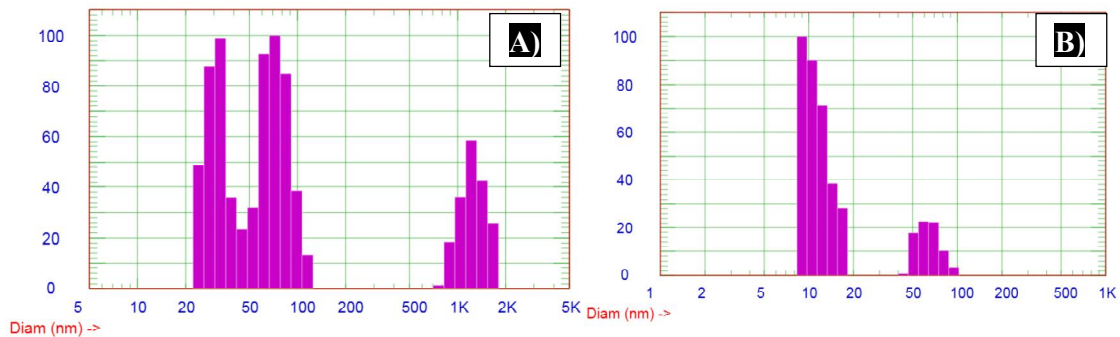


Fig. 4. Particle size distribution of A) uncoated Tinidazole nanoparticles; B) Coated Tinidazole nanoparticles

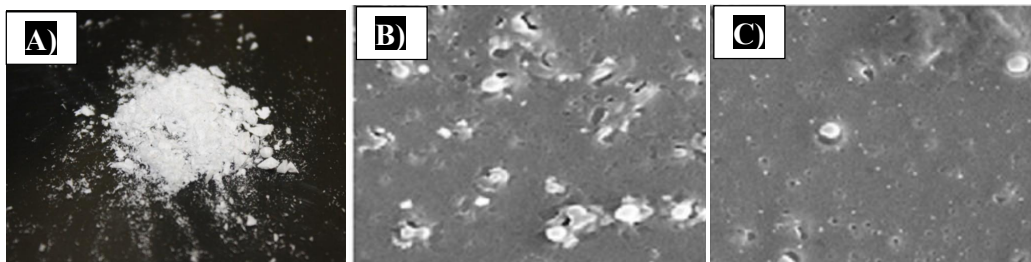


Fig. 5. Scanning electron microscopy images of A) lyophilized Nanoparticles B) Tinidazole nanoparticles without eudragit S 100 coating C) Tinidazole nanoparticles with eudragit S 100 coating

3.5 Zeta Potential Determination of NPs

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 (Fig. 6). It may be said that availability of protonated amino groups is higher with increasing concentration of chitosan, and for optimized formulation Zeta potential was found to be 26.9 ± 2.4 mV and its value >25 mV can be indicating nanoparticles stability. In current study zeta potential remained above + .25 mV at the selected pH, thus confirming that the system remains stable and there was no aggregation (Table 3).

3.6 Drug Encapsulation Efficiency Studies

An indirect method was used to determine the encapsulation efficiency of NPs prepared via low temperature solidification process. The NP dispersion was centrifuged in a 100 kDa Amicon Ultra-15 Centrifugal Filter Unit and the free drug present in the filtrate was analyzed by UV-Visible spectrophotometer (Table 3). The percent drug entrapment of the nanoparticles ranged from 28.5- 86.7% and was highest at the highest concentration of chitosan and HPMCP. Drug entrapment showed a linear relationship with polymer concentration.

3.7 In vitro Drug Release Studies from NPs

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), TZ released from the coated

nanoparticles after 2 h in 0.1M HCl was $5.57 \pm 1.34\%$ whereas in uncoated nanoparticles, it was $16.08 \pm 5.51\%$ (Table 4).

In vitro drug release from redispersed lyophilized NPs was performed in phosphate buffer pH 7.4 by using a dialysis bag. The samples were analysed at 242nm. The TZ release from the NP's was slow and spread over more than 70 h. The slow release indicated that the drug was in the core surrounded by drug free lipid shell. Data of the *in vitro* release was fit into different equations and kinetic models to explain the release kinetics of TZ from NP's. The kinetic models used were zero order, first order, Higuchi, Hixon Crowell and Korsmeyer-Peppas models (Table 5, Figs. 7 A and B).

The bioadhesion 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 15.98×10^3 dynes/cm². Coated nanoparticles have comparatively less mucoadhesive detachment force, which may be due to decrease in surface amino groups. This reduced mucoadhesion can facilitate in infiltration of nanoparticles to gastric mucosa enhanced penetration and accumulation at the site of infection beneath mucosa.

3.8 In-vivo Mucopenetration Studies

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

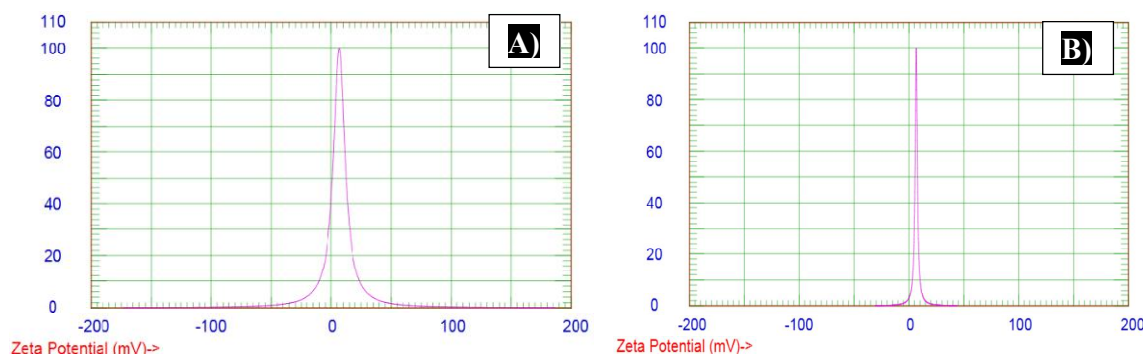


Fig. 6. Zetapotential profile of A) uncoated Tinidazole nanoparticles; B) coated Tinidazole nanoparticles

Table 3. Formulae of tinidazole polymeric nanoparticles with zeta potential, entrapment efficiency

Formulation code	Zeta potential (mV)	Entrapment efficiency (%w/v)	PDI
TZ1	32.5±1.32	27.6±5.3	0.221
TZ2	39.87±2.78	65.7±5.2	0.342
TZ3	35.68±1.23	35.6±5.8	0.235
TZ4	48.36±5.52	58.4±5.9	0.298
TZ5	25.46±2.32	81.2±5.56	0.214
TZ6	45.23±1.47	75.5±5.2	0.145
TZ7	50.23±2.31	52.4±5.7	0.356
TZ8	49.56±2.51	87.6±5.3	0.314

Table 4. In vitro drug release data of the tinidazole from tinidazole nanoparticles

Time (h)	Cumulative% drug released							
	TZ1	TZ2	TZ3	TZ4	TZ5	TZ6	TZ7	TZ8
0	0	0	0	0	0	0	0	0
1	11.20	12.53	15.15	8.23	9.22	16.13	5.27	6.54
2	23.07	31.35	37.89	26.03	19.12	38.81	16.14	15.32
3	40.89	46.3	52.78	33.98	29.03	54.76	26.05	30.01
4	50.86	56.37	63.76	43.93	38.97	71.67	34.01	44.76
5	63.8	73.41	76.73	55.87	48.93	77.73	51.86	66.41
6	72.83	77.66	79.87	68.83	63.85	80.88	69.78	76.45
7	80.89	81.94	83.99	77.87	74.85	84.02	78.78	82.63
8	83.04	84.28	86.15	81.0	78.96	86.17	80.93	84.95
9	85.2	86.62	88.32	83.16	81.11	87.36	82.69	85.31
10	87.36	89.96	90.48	85.32	83.26	88.44	84.25	86.33
11	88.54	91.34	91.67	86	85.42	88.73	85.43	88.02
12	89.72	92.73	92.86	86.68	86.6	89.02	86.69	88.79
13	90.9	92.15	93.55	87.63	87.77	89.68	87.78	89.57

Table 5. Kinetic release data of tinidazole nanoparticles

Formulation Code	Zero order	1 st order	Matrix	Hix. Crow	Peppas
TNZ1	0.883	0.984	0.965	0.974	0.966
TNZ2	0.835	0.985	0.975	0.965	0.954
TNZ3	0.768	0.986	0.963	0.943	0.946
TNZ4	0.912	0.984	0.968	0.976	0.964
TNZ5	0.949	0.986	0.956	0.984	0.986
TNZ6	0.674	0.923	0.945	0.864	0.924
TNZ7	0.936	0.975	0.934	0.965	0.975
TNZ8	0.894	0.967	0.936	0.956	0.953

3.9 Accelerated Stability Studies

The optimized formulation (TZ5) 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 $99.35 \pm 1.12\%$ and $86.45 \pm 1.23\%$, 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 (Table 5). This could be explained by taking surface charge into consideration irrespective of the particle size. There may be less electrostatic interaction between mucin fibre with pegylated nanoparticles which accelerates the process of penetration into the outer mucus and then into the inner mucus, close proximity to the epithelial cells [29]. Also, particles were not cleared with mucus and remained at the site even after the mucus clearance. Pegylated nanoparticles rapidly transported to the mucus as these particles could be able to move in low viscosity channels and do not interact

significantly with mucus [32]. Additionally, pegylation technique also improves the stability of nanoparticles in mucus and it is very important as particles must diffuse through a thick mucus layer in order to treat underlying infection. There were earlier some reports on the development of colon targeted drug delivery systems and development of tinidazole oral drug delivery system. Mukund and Chaudhari were developed colon-targeted delivery system, which uses lactulose and Tinidazole shown protections against pH 1.2 and pH 6.8 upto six-hour study with best results at pH5.0 [33]. Krishnaiah and his team reported *e in vivo* availability of guar gum-based colon-targeted tablets of tinidazole in comparison with immediate release tablets of tinidazole in human volunteers and found *e*

immediate release tablets of tinidazole produced a peak plasma concentration (C_{max} of 3239 ± 428 ng/ml) at 1.04 ± 0.32 hr (T_{max}), whereas colon-targeted tablets produced peak plasma concentration (C_{max} of 2158 ± 78 ng/ml) at 14.9 ± 1.6 hr. The delayed T_{max} , decreased C_{max} , and K_a , and unaltered bioavailability and elimination half-life of tinidazole from guar gum-based colon-targeted tinidazole tablets [34]. By comparing with other earlier reports and current study was first in development of tinidazole loaded NPs with chitosan and results confirm the novel concept of mucopenetration by decreasing or shielding the cationic charge on polymers like chitosan by modifying the surface chemistry of such polymers in order to improve the motility of nanoparticles in mucus membrane.

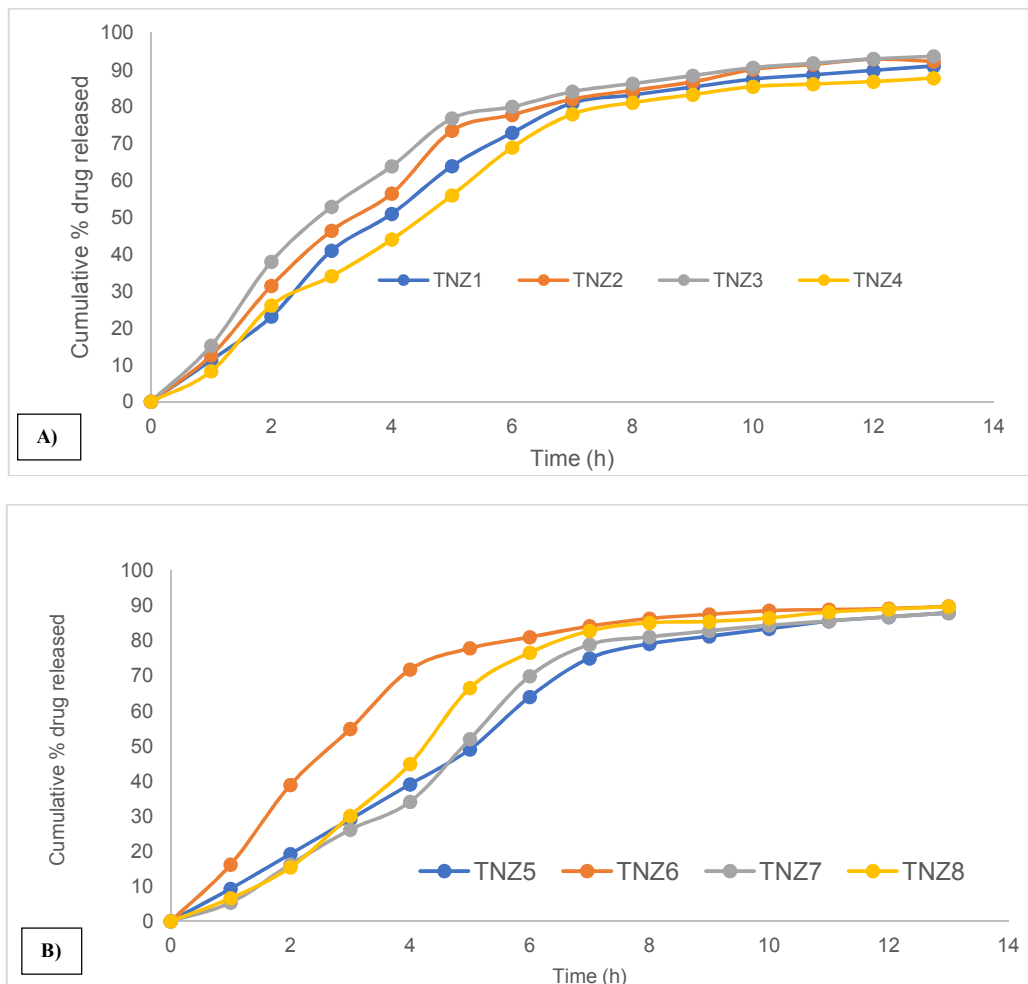


Fig. 7 A and B: *In vitro* drug release profile of Tinidazole nanoparticles

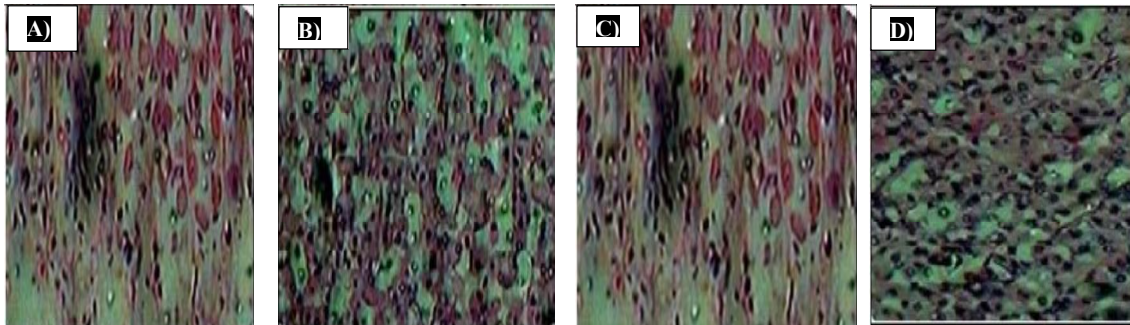


Fig. 8. Histopathological study of FITC labelled chitosan nanoparticles
A) 5th B) 8th C) 12th D) 24th hours

4. CONCLUSION

The current study was aimed to develop Tinidazole NPs using magnetic stirring method followed by temperature modulated solidification and successfully developed TZ loaded nanoparticle with NPs of low nm size using lipid chitosan and nontoxic surfactants like eutdragit S100. The developed TZ loaded NPs were evaluated by different physiochemical parameters and were produced efficient results and also *In vitro* release of TZ followed Higuchi and first order equations better than zero order equation. The current study results for TZ NPs indicate that the NPs 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.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGMENTS

The authors are thankful to authorities of MAM college of Pharmacy for providing the necessary facilities

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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