TITLE: Colon Targeted Protein Nanoparticles loaded Suppositories : Effective against Intestinal Parasites

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Running Title: Protein Nanoparticles: Fabrication and Evaluation

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ABSTRACT

Purpose: The aim of the present investigation was to formulate protein nanoparticles loaded suppositories for colon targeting of metronidazole (MZ), to achieve sustain release effect.
Methods: Protein nanoparticles were formulated via desolvation technique by utilizing 2³ factorial design which results into eight formulations. The synthesized protein nanoparticles were characterized for different physicochemical and in vitro parameters viz. particle size, surface morphology, entrapment efficiency and zeta potential, drug- excipients compatibility studies.
Results: The formulated protein nanoparticles were found to be spherical in shape and have an average size in the range of 300.7 nm to 504.8 nm. Based on the results obtained, F7 was found to be the optimized formulation that was loaded into the suppository base. Furthermore, suppositories were also characterized for several parameters like content uniformity, weight variation and liquefaction time.
Conclusion: Resultant, suppositories were free from pits, fissures and cracks. The in-vitro release data of metronidazole protein nanoparticles (MZ-PNPs) loaded suppositories were compared with the suppositories loaded with active ingredient only i.e. metronidazole. Screening against Phereetima posthuma was also conducted. The results of in vitro drug release testing proved that protein nanoparticle loaded suppositories is a better approach, compared to pure metronidazole loaded suppositories. Release kinetic study concluded that
the formulation follows higuchi’s equation i.e. having a biphasic release pattern. The efficiency of the formulated dosage form was evaluated using Indian earthworms, *Pheretima posthuma* (*P. posthuma*).

**Keywords:** Protein nanoparticles, Metronidazole, Factorial design, Ethanol desolvation technique, Release Kinetics, Indian earthworms.

**INTRODUCTION**

In present scenario, efforts are being made to achieve more controlled drug distribution throughout the body with lesser side-effects. Drugs which are not able to attain the desired therapeutic level are being incorporated into different carrier systems varying from micro to nano range. Proteins derived nanoparticles are bio-degradable, non-antigenic, metabolizable. Due to the defined primary structure of proteins, they can contribute covalent drug attachment. Recently Albumin, legumin and gelatin are being greatly used in these preparations. Due to its biodegradable and non-toxic nature, it have become the most projecting macro-molecular carrier, these are widely used to prepare nanospheres and nanocapsules. It is a major protein which provides benefits for ease of preparation in desired sizes and presence of reactive groups (thiols, amino or carboxylic groups) ligand binding for covalent linkage. Here albumin acts as a retardant agent i.e. helps to achieve prolonged release. The advantages mentioned above gives authors the basis for using albumin for preparing nanoparticles of Metronidazole (MZ). MZ is a colon targeting drug, effective against *E. histolytica* and *E. giradialamblia*. The pharmacokinetic profile of MZ indicates that the largest amount of active ingredient is absorbed from the upper part of the intestine, furthermore, protein may also get detoriate at gastric pH. Thus, suppositories are chosen as dosage form to prevent drug and Protein nanoparticles (PNPs) from detoriation. In addition, albumin is highly soluble at pH 7.4 which is one of the reasons that rectal route was chosen so that, it is facile to release the drug at the targeted area. Suppositories should have effective mucoadhesive property for their retention of drug on rectal mucosa.

The aim of the present investigation was to fabricate metronidazole loaded protein nanoparticles-based suppositories and to evaluate its efficacy against parasitic infections. These MZ-PNPs suppositories were also compared with MZ loaded suppositories to evaluate its in vitro efficacy and release kinetics pattern of the fabricated formulation.

**MATERIALS AND METHODS**

**Materials**

Metronidazole was procured from CDH Pvt. Ltd., New Delhi, India. Albumin was purchased from Sigma Aldrich Chemicals Pvt Ltd., New Delhi, India. Ethanol, glutaraldehyde, cocoa butter was purchased from CDH Pvt. Ltd., New Delhi, India.

**Methodology**

**Statistical Design**

$^2$^3$^$ full Factorial Design was used for fabrication of protein nanoparticles. This design signifies the two levels (high & low) with 3 independent factors and 2 dependent variables (particle size and entrapment efficiency). In the present work, three independent factors were used i.e. Albumin concentration, Glutaraldehyde concentration and Stirring Speed.
factorials levels were used and coded as -1 and +1. The factorial design and formulation table has been summarized in Table 1 and Table 2.

**Table 1** 2² Factorial Design: Factors and levels for Protein Nanoparticles Formulation

<table>
<thead>
<tr>
<th>S. No</th>
<th>Factors</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1 (Low)</td>
</tr>
<tr>
<td>A</td>
<td>Albumin (mg)</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>Glutaraldehyde (%w/v)</td>
<td>0.11 ml</td>
</tr>
<tr>
<td>C</td>
<td>Stirring Speed (rpm)</td>
<td>6500</td>
</tr>
</tbody>
</table>

**Table 2** Formulation Table for development of metronidazole loaded protein nanoparticles

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Albumin (mg)</th>
<th>Glutaraldehyde (%w/v)</th>
<th>Stirring Speed (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>100</td>
<td>0.11 ml</td>
<td>6500</td>
</tr>
<tr>
<td>F2</td>
<td>100</td>
<td>0.9 ml</td>
<td>7500</td>
</tr>
<tr>
<td>F3</td>
<td>150</td>
<td>0.11 ml</td>
<td>6500</td>
</tr>
<tr>
<td>F4</td>
<td>150</td>
<td>0.9 ml</td>
<td>7500</td>
</tr>
<tr>
<td>F5</td>
<td>150</td>
<td>0.11 ml</td>
<td>7500</td>
</tr>
<tr>
<td>F6</td>
<td>100</td>
<td>0.9 ml</td>
<td>6500</td>
</tr>
<tr>
<td>F7</td>
<td>150</td>
<td>0.9 ml</td>
<td>6500</td>
</tr>
<tr>
<td>F8</td>
<td>100</td>
<td>0.11 ml</td>
<td>7500</td>
</tr>
</tbody>
</table>

**Preparation of metronidazole protein nanoparticles**

Protein nanoparticles were prepared by ethanol desolvation technique using albumin as a polymer/retardant agent. All ingredients were accurately weighed as specified in Table 1. Albumin was dispersed in distilled water to form an aqueous phase while organic part was formed by dissolving metronidazole in ethanol. Aqueous phase was added drop-wise into organic phase. The solution was kept on continuous stirring under magnetic stirring resulting into transparent suspension. Glutaraldehyde was added for initiating cross linking procedure with continuous stirring using magnetic stirrer for 24 h. The process was carried out at room temperature. The resulted solution was sonicated for 5 min and further characterized for different physicochemical and in vitro studies.

**Preparation of metronidazole protein nanoparticles loaded suppositories and metronidazole suppositories**

Metronidazole suppositories were formulated using cocoa butter base. The base was melted using water bath; polysorbate 80 at concentration of 5% w/w was added followed by the addition of metronidazole with gentle stirring to ensure complete mixing. To this melted base, optimized formulation of metronidazole protein nanoparticles (equivalent to 250 mg of
MZ) were added with gentle stirring to ensure uniform mixing. Then this base was poured into the metal molds and allowed to cool at temperature of 4 °C. Suppositories comprising metronidazole (not MZ-PNPs) were also prepared by loading metronidazole into cocoa butter base with uniform mixing. The rest conditions were same as discussed for MZ-PNPs loaded suppositories.

CHARACTERIZATION OF METRONIDAZOLE LOADED PROTEIN NANOPARTICLES

Scanning electron microscopic (SEM)
SEM was used for analyzing particle shape and surface morphology. It was analyzed by scanning electron microscope manufactured by Cart Zeiss EV018. The samples were prepared by lightly sprinkling nanoparticles on double-sided adhesive tape on an aluminum stub. The stubs were then coated with gold to a thickness of 200 to 500 Å under an argon atmosphere using a gold sputter module in a high vacuum evaporator (Quorum SC7620).

Zeta and particle size Analysis
Dynamic light scattering technique was used for Zeta analysis, using a zetamizer (3000HS Malvern Instruments, UK). Samples were prepared to ten times with distilled water to form nanoparticulate suspension. Particle size analysis was performed by Photon correlation spectroscopy.

Drug- excipients Interaction studies
For drug excipients interaction studies, Fourier Transform Infrared Spectroscopy (FTIR) was performed. The sample was mixed with dry potassium bromide powder. Pellets were prepared and kept under pressure to obtain the spectrum. Interaction studies were carried out by taking the individual spectra of pure drug (metronidazole), excipients (albumin) and final prepared protein nanoparticles.

Determination of Drug Entrapment Efficiency
All the preparations were subjected to centrifugation and the supernatants obtained was analyzed for the drug using UV spectrophotometer at 340 nm. Entrapment Efficiency (EE) is calculated by applying the formula,

\[ EE\% = \frac{(Initial\ drug - Drug\ in\ the\ supernatants)}{(Initial\ drug)} \times 100 \]

In vitro drug release studies
These studies were performed in Phosphate buffer saline (PBS) pH 7.4. 2ml of prepared protein nanoparticles were enclosed in dialysis bag 70 (Hi-Media, Mumbai, India) with both ends tied. This bags were kept in the PBS at 37 °C ± 0.5 °C with stirring speed of 50 rpm. At fixed period, 5ml of the samples were taken and replaced by the same volume of freshly prepared PBS (pH 7.4). The samples were withdrawn at regular interval of time with replacement of fresh buffer solution. The samples taken were analyzed by the UV spectrophotometer at 340 nm.

EVALUATION PARAMETERS FOR METRONIDAZOLE PROTEIN NANOPARTICLES LOADED SUPPOSITORIES

Physical analysis
Physical analysis for suppositories was performed; it includes visual examination (color, surface condition and shape), odor and weight.

Weight variation and Mechanical strength determination
The weight variation test was evaluated with reference to the method described in British Pharmacopeia. Average weight 20 suppositories was calculated by weighing them individually. No suppositories should deviate from mean weight by more than 5% except
two, which may deviate by not more than 7.5%. Mechanical strength was measured by using Monsanto hardness tester.

**Content uniformity**

For the evaluation of content uniformity, suppositories were taken and dissolved in 250 ml PBS (pH 7.4). The solution was filtered and content was measured by using UV spectrophotometer at 340 nm.

**Liquefaction time**

The liquefaction time was evaluated by using a test tube having a slender aperture on one side and wider on the other side. This was immersed in water maintained at 37 °C ± 0.5 °C, as the slender end faces toward hot water. Suppository was engrossed from the top of the pipette with a glass rod rested over it. The temperature at which the glass rods just come down and reaches to the end was noted and termed as liquefaction time.

**Disintegration Time**

The disintegration time of suppository was evaluated by USP tablet disintegration (Electro lab, ED 2L) test apparatus. Distilled water was used as a medium maintained at body temperature. Suppository was placed in the metal basket and maintained at 50 rpm. Small quantity of aliquots was withdrawn and replaced with equal quantity of sample and absorbance was measured at 340 nm using UV spectrophotometer.

**In vitro drug release study**

Dissolution method (basket method) was used for determination of in vitro drug release. In vitro test was done for both type of suppositories i.e. MZ suppositories as well as MZ-PNPs suppositories. Suppository was kept in basket and placed into a container having 900 ml of PBS (pH 7.4). The basket was rotated at 50 rpm at a constant temperature i.e. 37 °C ± 0.5 °C. At different time period aliquots were taken and simultaneously restored by equal amount of phosphate buffer. Drug concentration was measured by using UV spectrophotometer at 340 nm.

**Screening against Pheretima posthuma (P. posthuma)**

The optimized MZ- PNPs loaded suppositories were evaluated for their inhibitory activity against intestinal parasites. Indian adult earthworms (P. posthuma) were used for this characterization. The earthworms were collected directly from moist soil and washed with water, so as to clear out dirt and other fecal matter. These removed soil and fecal matter were kept for further use to maintain artificial physiological and anatomical resemblance, thus to create and provide the arena of intestinal roundworm parasite (Ascaris lumbricoids) present in human beings. For screening earthworm were released into Petri plates (containing previously preserved soil and fecal matter obtained while cleaning the earthworms) along with formulated suppositories in solution form. Time taken for paralysis and death of earthworms were observed. Paralysis time was based on earthworm’s behavior with no movements when were shaken vigorously. The time of death was calculated with no movements when dipped in water (50-60 °C temperature), following with faded body color of the earthworms.

**RESULTS AND DISCUSSIONS**

The formulated protein nanoparticles were evaluated for several physicochemical properties viz. particle size, surface morphology, zeta potential, in vitro drug release study. The optimized formulation of protein nanoparticles was loaded to a suppositories base and evaluated for weight variation, disintegration, liquefaction time and in vitro release study. SEM
SEM concluded the particles to be spherical in shape, as shown in Figure 1 and the particle size for all the formulation was found to be below 300 nm. The particles were found to have uniform size distribution and were not conglomerated.

**Determination of Particle Size and Zeta Potential Measurement**

The particle size was in the range of 300.7 nm to 504.8 nm. Out of all the formulation F4 was concluded as the optimized formulation, having the smallest size and optimum PDI of 300.7 nm and 0.190 respectively. The results have been depicted in Table 3. The result obtained from particle size analysis suggested that increment of stirring speed tends to decrease the average particle size. But further increment results into increase in the particle size with the special mention to the formulation with high cross-linking agent. As, it has been suggested that as the stirring speed increases, kinetic energy also increases, resulting in variable surface charges which leads for the formation of agglomerates. Small sized nanoparticles are effective for targeted drug delivery, which allows the drug for efficient uptake by a variety of cell types and drug accumulation at target site. The PDI value was found to be 0.190 implying a narrow and favorable particle size distribution (PDI< 0.5) [29]. Zeta potential of the optimized formulation was found to be -29.2 mv. Particles having zeta potential in the range of +40 mv to -40 mv are considered to be stable. The result signifies the stability of protein nanoparticles with respect to surface charge [22].

**Table 3 Results showing particle size, Poly dispersity index (PDI) and Entrapment Efficiency Percentage**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Batch</th>
<th>Particle Size (nm)</th>
<th>PDI</th>
<th>Entrapment Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>F1</td>
<td>465.2</td>
<td>2.62</td>
<td>58.76±0.30</td>
</tr>
<tr>
<td>2.</td>
<td>F2</td>
<td>437.0</td>
<td>2.10</td>
<td>61.43±1.30</td>
</tr>
<tr>
<td>3.</td>
<td>F3</td>
<td>504.8</td>
<td>1.83</td>
<td>66.23±0.61</td>
</tr>
<tr>
<td>4.</td>
<td>F4</td>
<td>300.7</td>
<td>0.190</td>
<td>85.73±0.56</td>
</tr>
<tr>
<td>5.</td>
<td>F5</td>
<td>348.9</td>
<td>0.43</td>
<td>70.76±0.61</td>
</tr>
<tr>
<td>6.</td>
<td>F6</td>
<td>328.5</td>
<td>0.31</td>
<td>72.83±0.20</td>
</tr>
<tr>
<td>7.</td>
<td>F7</td>
<td>365.6</td>
<td>0.93</td>
<td>93.10±0.30</td>
</tr>
<tr>
<td>8.</td>
<td>F8</td>
<td>450.9</td>
<td>1.97</td>
<td>59.90±0.20</td>
</tr>
</tbody>
</table>

**Drug Excipients Interaction studies**

No interaction was detected during FTIR studies between metronidazole and polymers used. IR spectra of pure drug i.e. metronidazole, albumin and the mixture of these compounds has been illustrated in the Figure 2.

**Drug Entrapment Efficiency**

EE% of all the formulations have been described in Table 3. On the basis of results obtained, it can be concluded that F7 was having the highest entrapment efficiency i.e. 93.10±0.30%. It was observed that percent entrapment efficiency varies with change in stirring speed and concentration of cross-linking agent. From the results it can be concluded that increase in the stirring speed leads to decrease the particle size, thus reducing the percentage drug entrapment efficiency but gets increased on increasing the cross-linking agent concentration [23-25].

**In Vitro Drug Release Study of Metronidazole Protein Nanoparticles**

In vitro drug release for all the formulation have been depicted in Figure 3. From the graph it was observed that F4 and F7 attains the highest drug release for the time period of 18h. Based
on the outcomes, it can be concluded that due to small size of particles, sustain release effect was observed during *in vitro* release study.

**CHARACTERIZATION OF METRONIDAZOLE PROTEIN NANOPARTICLES LOADED SUPPOSITORIES**

The formulated MZ-PNPs suppositories were free from any cracks and fissures. Suppositories were evaluated on the basis of visual examination; the results have been summarized in Table 4. The suppositories were found to have Ogive shaped with smooth texture; having yellowish white in color. The weight variations for the formulated suppositories were found to be in acceptable limit as provided in British pharmacopoeia, furthermore suppositories showed optimal mechanical strength which is a necessary part for handling and transportation purpose. The results are compiled in the Table 4.

*Table 4* Compiled results of characteristics for suppositories parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical Shape</td>
<td>Ogive shaped</td>
</tr>
<tr>
<td>Surface condition</td>
<td>Smooth</td>
</tr>
<tr>
<td>Color</td>
<td>Yellowish white</td>
</tr>
<tr>
<td>Odor</td>
<td>Odorless</td>
</tr>
<tr>
<td>Mechanical strength (Kg/cm³)</td>
<td>1.8±0.97</td>
</tr>
<tr>
<td>Liquefaction time (min)</td>
<td>27±0.12</td>
</tr>
<tr>
<td>Disintegration Time (min)</td>
<td>6.98±0.71</td>
</tr>
<tr>
<td>(37.5±0.5°C)</td>
<td></td>
</tr>
</tbody>
</table>

Readings were taken in triplicate n=3 (mean ± S.D)

**Weight variation**
The weight variation study for all the suppositories were found to be less than 5% and according to British Pharmacopoeia this obtained percentage is acceptable, indicating that mold calibration was ideal. The results have been shown in Table 4.

**Content uniformity**
The content uniformity was within the permissible range (97% to 105%) indicating uniformity of drug dispersion in suppositories. The results have been shown in Table 4.

**Mechanical strength**
The formulated suppositories were found to acquire acceptable mechanical strength. The results have been compiled in Table 4.

**Liquefaction time**
The liquefaction time of the prepared suppositories was found to attain the time period of 27 min. The results have been depicted in Table 4 and are discussed in the discussion part.

**Disintegration time**
According to British Pharmacopoeia the disintegration time for suppositories should be less than 60 min and the results show the same. The average disintegration time was found to be 6.98 min depicted in Table 4.
In vitro release of metronidazole protein nanoparticles - suppositories and metronidazole loaded suppositories

The in vitro release of MZ suppositories showed that about 50% of the drug releases within 25 minutes of drug administration whereas, it took 4 hours for MZ-PNPs loaded suppositories to release its 50% of the drug. For MZ-PNPs loaded suppositories 90% of the drug releases for 12 hours defining a sustained release pattern. It was also observed that MZ-PNPs loaded suppositories gets immediately melts with body temperature and a burst release of drug release followed by sustain release was seen due to presence of protein nanoparticles. The comparative release graph of MZ loaded suppositories and MZ-PNPs loaded suppositories have been depicted in Figure 4.

For release kinetics, the model or equation that best fits the release data was evaluated by correlation coefficient (r) and the value of n (for Korsmeyer Peppas’s Equation). It was found that the in vitro release from formulated MZ-PNPs loaded suppositories was best explained by higuchi’s equation with the highest linearity (R²=0.920), followed by zero order (R²=0.885) and first order (R²=0.643), showed in Figure 5. The value of n=1.139 (greater than 0.45) obtained from korsmeyer peppas’s equation shows that the model follows anomalous (non- Fickian) diffusion process. The release kinetics helps the authors to indicate the higuchi’s equation, as the best suited model, demonstrating a biphasic release pattern having an initial burst release followed by a slow sustained release pattern.

Screening against Pheretima posthuma (P. posthuma)

Screening of MZ-PNPs loaded suppositories against intestinal parasites was conducted on Indian adult earthworm (P. posthuma) because of its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings. The result obtained showed that the formulated dosage form is able to act against these earthworms. The analyzation of paralysis time was based on earthworms’ behavior with no movements while shaken vigorously. Formulated suppositories in the solution form take 6.50±0.05 minutes for death of earthworms, while 3.47± 0.05 minutes were taken for paralysis of earthworms. The results obtained showed its effectivity against Pheretima posthuma.

CONCLUSION

The ability MZ-PNPs loaded suppositories was demonstrated by the results obtained from various evaluation parameters viz. particle size, poly dispersity index, entrapment efficiency and in vitro release profile. The in vitro release study confirmed the prolongation of the drug release time. It can be concluded that the effective and safe dosage form was prepared with reduced side effects for inhibiting parasitic infections. The prepared MZ-PNPs suppositories provide an effective targeted delivery of drug with the release for 12 h. Release kinetics results showed the best fitted model (higuchi’s equation) signifying the phenomenon of sustain release effect of fabricated formulation.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.
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REFERENCES


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