



THERAPEUTIC POTENTIAL OF BIODEGRADABLE MESELAMINE MICROSPHERES IN THE TREATMENT OF ULCERATIVE COLITIS

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ABSTRACT

The use of microparticles (MPs) for targeted oral drug delivery to the inflamed gut tissue in inflammatory bowel disease was examined. Such a strategy of local drug delivery would be a distinct improvement compared with existing colon delivery devices for the ulcerative colitis. An experimental colitis was induced by acetic acid to Wistar rats. Meselamine, a front line drug in the treatment of ulcerative colitis, was incorporated within poly (lactic-co-glycolic acid) i.e. PLGA microspheres, which were administered once a day orally and rectally for five consecutive days. A clinical activity score, colon body weight ratio, myeloperoxidase activity and ulcer index were determined to assess the inflammation. All microparticulate formulations proved to be as efficient as compared to the drug in suspension in mitigating the experimental colitis. The clinical activity score, colon body weight ratio, myeloperoxidase activity and ulcer index decreased significantly ($p < 0.05$), after the oral administration of meselamine microspheres suspension. The free drug, meselamine, suspension does not found effective in terms of clinical activity, myeloperoxidase activity and ulcer index, as is evident from the non significant decrease in clinical parameters. The meselamine microspheres further proved their potential in reducing the inflammation proved by the histopathology of the resected colon from every group of animals. This new delivery system enabled the drug to accumulate in the inflamed tissue with higher efficiency than when given as suspension of drug. The microspheres deposition in the inflamed tissue should be given particular consideration in the design of new carrier systems for the treatment of inflammatory bowel disease.

Key words: Microspheres, Ulcerative colitis, PLGA, Meselamine, Clinical activity.

INTRODUCTION

The ordinary treatment of inflammatory bowel disease requires the frequent intake of anti-inflammatory drugs at high doses, which causes the absorption of those drugs from the small intestine, leading to significant adverse events. Therefore, several strategies have been followed such as the development of prodrugs that deliver drugs specifically in the large bowel after cleaving the active part from the hydrophilic carrier by specific bacterial enzymes in the colon¹ and the development of solid dosage forms that release the drug in the colon in dependence of the physiological environment². The administration of drugs by rectal route is also currently used. However, it is not effective when the inflamed tissues are located in the upper parts of the colon.

Although prodrugs lead to reduced adverse effects, a more comfortable dosage frequency cannot be achieved. Sustained drug release devices, e.g., pellets, capsules, or tablets, delivering the drug specifically in the colon for a longer time period have been developed. However, their efficiencies seem to be decreased in many cases due to the diarrhea, a symptom of inflammatory bowel disease that enhances the elimination and reduces the possible drug release time³. Thus, a carrier system that delivers the drug specifically and exclusively to the inflamed regions after oral administration for a prolonged period would be desirable. Such a system could reduce side effects significantly in the case of conventional chemical anti-inflammatory compounds.

Because microspheres can be designed to control drug release after oral administration, the development of such microspheres seems to be promising primarily to reduce the dosage frequency. In the case of colitis, a strong cellular immune response is known from the inflamed regions, i.e., in general, an increased presence of neutrophils, natural killer cells, mast cells, and regulatory T cells, which have an important role in the pathophysiology of inflammatory bowel disease⁴.

Moreover, it has been reported that microspheres and nanoparticles can be efficiently taken up by macrophages⁵. Thus, it may be expected that particle uptake into those immune-related cells or the disruption of the intestinal barrier function could allow the accumulation of the particulate carrier system in the desired area⁶. A subsequent increase of residence time that would be postulated for

microparticles and nanoparticles compared with existing drug delivery systems allow a dose reduction. Indeed, it has been demonstrated that microparticles containing dexamethasone showed previously promising results in colitis-induced mice⁷. In addition, the successful oral administration of drugs with strong adverse effects pertaining to the high doses such as meselamine and rolipram may be a new medical approach.

The aim of this project is to test in vivo targeting potential of microspheres to the inflamed tissue. The suitability of microspheres for targeted drug delivery to the inflamed gut tissue in inflammatory bowel disease i.e. ulcerative colitis will be examined. This is an attempt to design and fabricate microspheres of salicylates like 5-amino salicylic acid encapsulated in biodegradable polymers such as PLGA, which may shown slow release and are targeted to inflamed colon in ulcerative colitis is examined in experimental animal models. To compare the efficiency of the microparticles treatment, different groups of rats received suspension of both, free anti-inflammatory drug and microparticles. The mitigating effect of all formulations was determined by a clinical score system, the colon/body weight ratio, myeloperoxidase activity and ulcer index.

MATERIALS AND METHOD

The biodegradable polymer Poly (DL- lactide-co-glycolide) i.e. PLGA 50:50 (Molecular weight 10,000) was purchased from Boehringer Ingelheim, Germany. PVA (Polyvinyl alcohol) and Meselamine (5-aminosalicylic acid) was purchased from Hi-media, Mumbai. All other chemicals and reagents are of analytical grade purchased from Sigma Chemicals, Mumbai and Merck Chemicals, Mumbai. All animal experimental protocols are approved by *Institutional Ethical Committee* clearance bearing the number 817/04/ac/CPCSEA.

EXPERIMENTAL

Preparation of Biodegradable Microspheres

The preparation of microspheres was based on emulsification solvent evaporation method. Fix amounts of poly (lactic-co-glycolic acid) (125 mg) and meselamine (50 mg) were dissolved in the solvent blend consisting of 5ml methylene chloride, 5ml DMSO, 10 ml acetone. This solution was slowly poured into 50 ml of 2% w/v polyvinyl alcohol and an oil/water-emulsion was formed by

homogenizer for 5 min. After evaporation of the solvent, microspheres were recovered by centrifugation at 55,000g for 30 min and washed with distilled water. The washing step was repeated once before microspheres were resuspended in distilled water and lyophilized overnight.

Microspheres were analyzed for their size distribution and their surface potential using Photon Correlation Spectroscopy (PCS) (Malvern S4700 PCS System, Malvern Instruments, Ltd, Malvern, UK) and a Zetasizer II (Malvern Instruments, Worcestershire, UK), respectively. Meselamine entrapment within microspheres as well as the in-vitro drug release kinetics was determined by UV/VIS Spectroscopy (Schimadzu UV/VIS Spectrophotometer, Japan).

Evaluation of Therapeutic Potential of Microspheres

Therapeutic potential of meselamine microspheres in the treatment of ulcerative colitis was determined by using experimentally induced ulcerative colitis model in rats. All animal experiments were carried out in accordance with the *Recommendations in the Guide for Care and Use of Laboratory Animals*. The study was conducted at Sudhakar Naik Institute of Pharmacy, Pusad, Dist. – Yavatmal (M.S.) after obtaining *Institutional Ethical Committee* clearance bearing the number 817/04/ac/CPCSEA.

Method of Inducing Experimental Colitis

The acetic acid induced colitis model was selected as well recognized experimental model that allows the induction of colitis at an exact location. A model of diffuse colitis in rats induced by intraluminal colonic instillation or serosal application of dilute acetic acid was described by MacPherson and Pfeiffer⁸; Ritzpatrick et al⁹ tested the anti-inflammatory effects of various drugs on acetic acid induced colitis in the rat.

Wistar rats of either sex aged 12-15 weeks and weighing about 230-250 gm were used for the study. All animals were housed in an air conditioned room at 22±3°C, 55±5% humidity, 12 hours light/dark cycle and allowed to free access to water and laboratory chow for the duration of study. To induce the model of inflammation in rat colon, the animals were arbitrarily separated into treatment group (n=6/group), fasted 24 hrs with free access to water and then lightly narcotized with ether. A soft polyethylene canula fitted with adapter of 8cm length was used.

The open end of tube was sealed and running length was perforated at dist of 0.5cm at 90° apart from each other. This specially designed polyethylene catheter was then inserted rectally into colon such that the tip of catheter was 8cm proximal to anus. The 1ml of 10% acetic acid was then installed into the lumen by syringe fitted to adaptor. After 10 sec, 3 washings with normal saline each of 0.5 ml were given for 10 sec. A control group received 0.5ml of 0.9% (w/v) saline administered as above procedure. The rats were housed without treatment for 48 hrs to develop full inflammatory bowel diseases i.e. colitis model.

During treatment period i.e. from 3rd day onwards since acetic acid installation, all animals received 1ml (orally/rectally) 0.5mg/kg of body weight microparticles suspension once daily for five days. The oral administration was performed by gavage, where as rectal administration was done by 8cm intrarectal catheterization providing microspheres directly to the inflammation site. The control group received only saline (Colitis control), drug free microparticles (MP control), standard drug suspension and one group received drug loaded microspheres suspension as mentioned above. During the period of treatment all animals are allowed for the free access of lab chow and water. All animals are under observation for the determination of clinical activity score.

All animals were sacrificed 24 hrs after last drug and microspheres administration and colons were resected and weighed. The therapeutic potential of microparticles was evaluated with respect to following parameters –

- i) Clinical activity score
- ii) Colon body weight ratios

- iii) Myeloperoxidase activity
- iv) Macroscopic assessment (Ulcer Index)
- v) Histopathological assessment.

Clinical Activity Score

Colonic activity was quantified with clinical score accessing weight loss, stool consistency and rectal bleeding as described by Hartmann, et al¹⁰ and Lamprecht, et al¹¹. No weight loss was counted as 0 points, 1-5% as a 1 point, 5-10% as 2 points, 10-20% as 3 points and >20% as 4 points. For stool consistency 0 points was given for well formed pellets, 2 points for pasty and semiformed stools that does not stick to anus and 4 points were given for liquid stools that sticks to anus. Bleeding was scored as 0 points for no blood, 2 points for positive findings and 4 points for gross bleeding. The mean of these scores was forming the clinical score ranging from 0 (healthy) to 4 (maximum activity).

Determination of Colon/Body Weight Ratio

The rats were sacrificed and abdomen was opened and the distal colon was rapidly excised and opened longitudinally along the mesenteric ridge. The fecal contents were removed and the colon was washed with 0.9% (w/v) saline and placed with mucosal surface upward over glass plate or slide chilled with ice. The ratio of 8cm segment distal colon weight was calculated as index of colonic tissue edema as described by Yue, et al¹²

Myeloperoxidase Activity (MPO Activity)

The measurement of Myeloperoxidase activity is the first quantitative assessment to quantify the severity of colitis. It is reliable index of inflammation caused by infiltration of activated neutrophils to the inflamed tissue. Activity was analyzed by using std. method given by Krawisz, et al.¹³. Briefly, distal colon specimen was minced in 1ml of HTAB buffer (0.5% in 50mM phosphate buffer) on ice and homogenized. The homogenate was sonicated for 10 sec. (Microson™ XL 2007, Ultrasonic Cell Disruptor, Microsonix Inc., USA), freeze-thawed three times and centrifuged at 12,000 rpm for 3 min (Eppendorf AG 22331, Germany). Myeloperoxidase activity in the supernatant was measured spectrophotometrically. Supernatant (0.1 ml) was added to 0.167 mg/ml of *o*-dianisidine hydrochloride and 0.0005% hydrogen peroxide, and the change in absorbance at 460 nm was measured. One unit of myeloperoxidase activity was defined as the amount that degraded 1µmol of peroxidase per minute at 25 °C. The results were expressed as U/mg tissue as given by Jagtap, et al¹⁴.

Assessment of Macroscopic Ulceration (Ulcer Index)

Gross mucosal damage was scored on 0-3 grade scale by single observer blind to treatment as described by Rodrigue, et al,¹⁵. Damage was scored as follows: Score 0 represents no damage, score 1 localized hyperemia with slight or minimal ulceration, and score 2, linear ulcers and one or two regions with ulcer of 1-2 cm and score 3 for severe ulceration (regions with ulcers > 2cms). The macroscopic ulceration can also be determined by calculating the ulcer index of every group. The ulcer index was determined by opening the stomach on greater curvature and the scores were given 0 to 3 depending upon the severity of ulcers (normal colored colon = 0, red coloration = 0.5, ulcer spots <3 = 1, ≥3 but ≤5 = 2 and ulcer spots > 5 = 3). Ulcer index (U_i) was then calculated from the above scorings as follows:

$$U_i = U_n + U_s + U_p \times 10^{-1}$$

Where, U_n is the average of number of ulcers per animal,

U_s is the mean severity of ulcer score and

U_p is the percentage of animals with ulcer incidence.

Histological Evaluation

After scoring, two representative tissue samples were excised from each colon and maintained in 10% (v/v) formaldehyde for the microscopic studies. When visible ulceration or inflammation was present, at least one of the samples from affected region was taken.

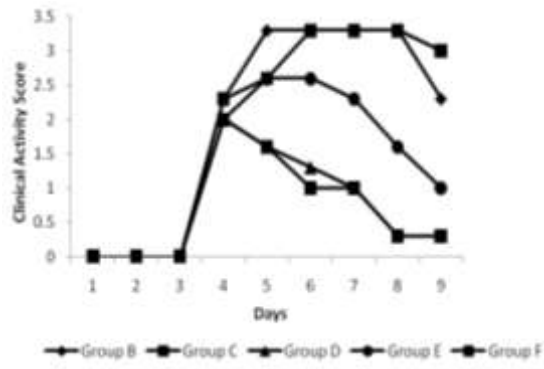


Figure -1: Clinical activity score during treatment. (n= 6) [MP control (Group C) being similar to the colitis control (Group B) Group D and Group F shows significant, $P < 0.01^{**}$, decrease in clinical activity compared with colitis control rats given saline] Error bars are not shown for clarity reasons.

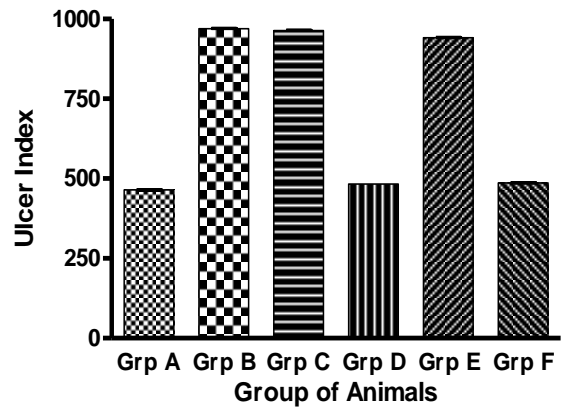


Figure -4: Ulcer Index after treatment [Each bar is an average value \pm S.D. of six animals of each group. MP treated group (Group D) orally and rectally (Group F) shows significant lowering, $p < 0.01^{**}$, ulcer index than the average value of the control group]

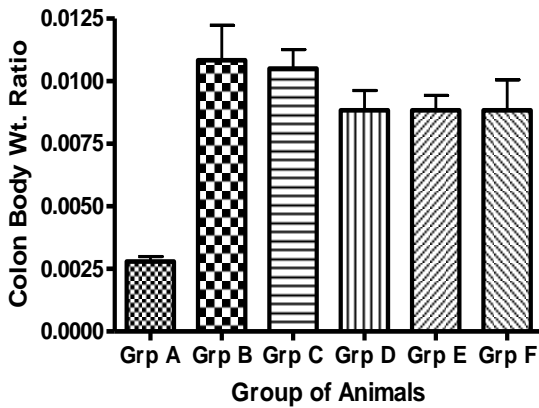


Figure -2: Colon body weight ratio after treatment [Each bar is an average value \pm S.D. of six animals of each group. MP orally treated group (Group D) shows significant lowering, $p < 0.05^*$, of colon/body weight ratio than the average value of the control group (Group B)]

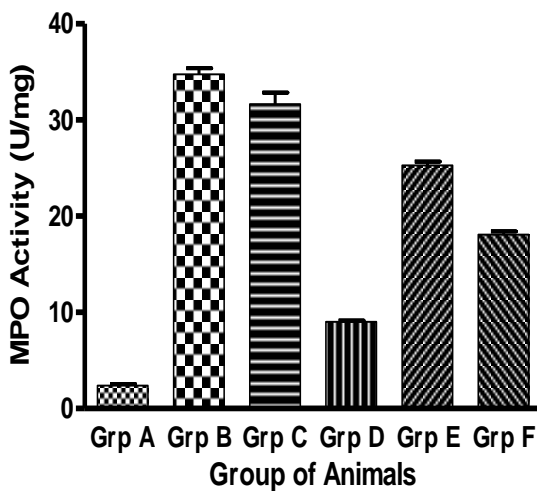


Figure -3: Myeloperoxidase activity after treatment [Each bar is an average value \pm S.D. of six animals of each group. MP orally treated group (Group D) and rectally treated (Group F) shows significant lowering, $p < 0.01^{**}$, of MPO activity than the average value of the control group]



Figure -5: Histopathological slide of normal control group (Group A)

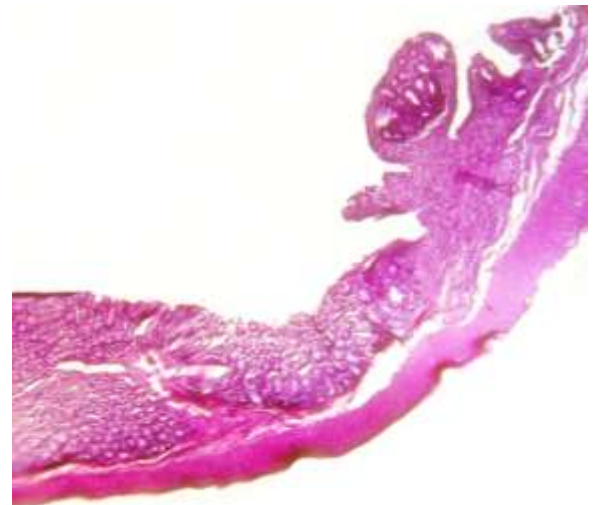


Figure -6: Histopathological slide of colitis control group (Group B)



Figure -7: Histopathology slide of group receiving blank microspheres (Group C)



Figure -8: Histopathology slide of group receiving meselamine microspheres orally (Group D)



Figure -9: Histopathology slide of group receiving free meselamine suspension (Group E)

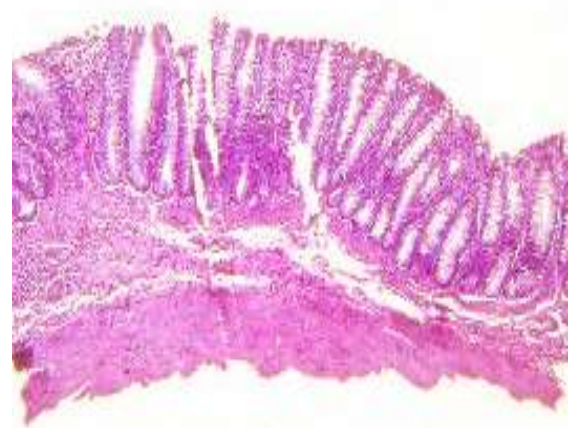


Figure -10: Histopathology slide of group receiving meselamine microspheres rectally (Group F)

The samples were processed routinely and embedded in paraffin blocks. Sections of 5 μ m were stained with heamatoxylin and eosin. Microscopic assessment was done and slides were interpreted.

Statistical Analysis

The mean value \pm standard deviation (S.D.) was calculated for each parameter. Results were statistically analyzed by descriptive statistical methods (ANOVA and Dunnet's Multiple Comparison Test). $P < 0.01^{**}$ and $P < 0.005^{*}$ indicates statistical significance. MS Windows based *Graph Pad Prism Software* is used for statistical analysis.

RESULT AND DISCUSSION

The microspheres prepared with poly [DL-lactide-co-glycolide], a biocompatible and biodegradable polymer were characterized in terms of particle size, drug content, surface potential, encapsulation efficiency, and drug release (Table 1). Microspheres prepared by the emulsification solvent evaporation method had a spherical shape, submicrometer size, and were relatively monodispersed.

Discrete spherical microspheres having a mean diameter of about 1.15 μ m to 1.5 μ m with low standard deviations (monodispersity) were produced. The small polydispersity index suggested that the size distribution of the products is fairly monomodal. The above-mentioned monodispersed size distribution and excellent redispersibility of microspheres indicate that the surface of PLGA microspheres is stabilized by some reasons to prevent aggregation. The most likely reason to explain the findings might be the adsorption of PVA to PLGA microparticles.

To evaluate the therapeutic value of meselamine-containing microspheres, the effect of the carrier system was studied on preexisting colitis. All animals received an intrarectal application of acetic acid except the healthy control group. Before this time point, animals showed no clinical problems.

After inducing the experimental colitis the clinical score increased rapidly and consistently for the next 3 days for all groups (Fig. 1). The inflamed tissue showed an extremely increased mucus production in the area of distal colon compared with the histology of healthy gut sections from the control group. Significant damages of the intestinal tissue, e.g., ulceration, have been observed. Starting from day 4, rats received orally either meselamine suspension or meselamine microspheres suspension daily for five consecutive days, only the colitis control group received saline instead. The clinical activity score was used to evaluate the severity of the colonic inflammation and the colitis control group proved to be an excellent model of inflammation as evidenced by the highly increased clinical activity.

All drug-receiving groups showed a decrease of inflammation severity after a lag time of 24 to 48 h. The difference between drug-treated groups and colitis controls became significant ($P < 0.05^*$) on day 8. During the whole meselamine treatment period the clinical activity was lowered by free drug and by the two drug carrier formulations as well. After the 5 days without drug treatment, the free drug group does not show the significant reduction in the clinical activity, whereas for the microsphere groups continuously reduced clinical activity scores was observed.

On day 9 (24 h after the last drug administration), the first series of animals was sacrificed and colon/body weight ratio and myeloperoxidase activity were determined to quantify the inflammation. The drug-treated groups showed a distinct decrease in the colon/body weight ratio compared with the colitis control group (Fig.2). The difference between free drug and the microparticles formulations was significant. Furthermore, the myeloperoxidase activity in samples obtained from the inflamed colonic tissue was examined (Fig. 3).

Here also, an enormous difference between meselamine microspheres-treated group, MPO activity = 9.00 U/mg ($P < 0.05^*$) and colitis control groups (MPO activity = 34.75 U/mg) was found. As observed previously, significant differences for the mitigating effect of all meselamine-receiving groups were observed. Whereas, the colitis control group, showed a continuously strong colonic inflammation reflected by a high clinical activity score.

The macroscopic changes includes marked thickening of the intestinal wall associated with hemorrhages, epithelial exfoliation and ulcer that exceed 2 cm were seen. A narrowing of the lumen of the colon adjacent to the inflamed sites with a proximal dilation of the bowel was also seen in the acetic acid-treated rats, but generally the colon was not perforated. As evident by the ulcer index (Fig. 4) which was calculated on the basis of scores correspond to the severity of ulcer, colitis control group having ulcer index, $U_i=970.00$ which is quite high as compared to normal group having ulcer index value, $U_i= 464.40$. Microsphere treated group both orally and rectally having ulcer index values as $U_i=482.90$ and $U_i=486.50$, respectively which are significant ($P < 0.05^*$) as compared to the group receiving drug suspension having ulcer index value $U_i=941.70$ which is just short of colitis control group.

Histopathological changes of the colon in case of inflammatory response included appearance of local hyperemia, and mild to severe inflammatory cellular infiltrate including polymorphonuclear leucocytes, eosinophils, lymphocytes and granulocytes can be observed. The inflammation was spread over the mucosa, submucosa, muscle layer and included serosa. Normal control group A, representative slide (Figure 5), shows normal colonic mucosa with regularly formed colonic folds covered by intact mucosa. Only occasional leukocytes are present in the lamina propria of the mucosa.

Histological examination of colon sections from control rats showed no signs of inflammation. The histopathological features of group B, colitis control group (Figure 6), indicates invigilation of mononuclear cells, glands appears exhausted along with focal lymphocytic aggregation. Five days after induction of colitis the group C (Figure 7) administered with blank microparticles showed evidence of mucosal congestion, and hemorrhagic ulceration in mucosa and submucosa. In other word, it has the same histological features like the acetic acid treated group.

Treatment of rats with the meselamine microspheres suspension receiving orally, Group D, significantly attenuated the extent and severity of the histological signs of cell damage (Figure 8). It has normal appearance and shows no signs of ulceration or bleeding.

Histopathological studies confirmed the anti-inflammatory potential exerted by the microspheres on inflamed colonic tissues, as evident from the representative slide. Free drug meselamine suspension treated group E (Figure 9), even after the daily dosing, there is still some signs of inflammatory response such as degree of edema, but no cross-mucosal damage.

Also note the difference in colon cross-sectional area, which is partially reversed by meselamine. Histological characteristics of rectally treated drug loaded microspheres suspension group F (Figure 10) shows the inflammation of submucosa along with hyper active elongated glands. Because of accumulation of lymphocytes, histiocytes, and fewer neutrophils there is marked crypt destruction. The inflammatory process is limited to the mucosa and submucosa, severe colonic inflammation, characterized by a dense inflammatory cell infiltrate in all colonic wall structures and widespread mucosal sloughing. These inflammatory signs might be due to the frequent insertion of polyethylene catheter for the dosing that may cause the physical damage to the ulcers there by enhancing the inflammation process.

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Table - 1: Optimization Parameters of Microspheres

Sample Number	Method of Preparation	Particle size (μm)	Polydispersity Index (PI)	Zeta Potential (mV)	Drug Content (%w/w)	%Drug Entrapment
Sample A	SESD	1157.1 \pm 1.9	0.166	-58.4 \pm 1.4	1.7 (1.8; 1.9)	24.2 (23.6; 24.9)
Sample B	MSESD	1135.0 \pm 1.3	0.059	-49.2 \pm 0.7	1.5 (1.6; 1.4)	62.0 (63.0; 61.0)
Sample C	PPT	1558 \pm 2.7	0.356	-55.1 \pm 0.9	1.4 (1.4; 1.4)	14.5 (14.2; 14.8)

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