IN VIVO EVALUATION OF BUDESONIDE MICROSPHERES FOR COLON SPECIFIC DRUG DELIVERY

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ABSTRACT

Objective: The aim of the present study was to evaluate the in vivo behaviour of budesonide microspheres for colon targeted delivery.

Methods: In vivo therapeutic effect was evaluated using trinitrobenzene sulfonic acid (TNBS) induced colitis in rats. The therapeutic effect was assessed by determining the damage score, clinical activity score, colon/body weight (C/B) ratio and myeloperoxidase (MPO) activity measurement. The data were compared with standard drug 5-aminosalicylic acid (5-ASA). The targeting efficiency of the formulation was assessed by X-ray studies on the rabbit.

Results: The study showed that oral administration of budesonide microspheres exerted an affirmative impact on the colonic ulcer healing by decreasing the area of ulceration, reducing the mass of colon by improving the symptoms of colitis. MPO activity decreased significantly after oral administration of microspheres. Histopathological studies carried out also confirmed the result. The X-ray studies revealed that the formulations were able to target the colon.

Conclusion: The in vivo study confirmed the ability of budesonide microspheres in targeting the colonic region.

Keywords: Budesonide, Colitis, Targeted delivery, Myeloperoxidase, Ulceration, Clinical activity score

INTRODUCTION

Targeted drug delivery into the colon is highly desirable for local treatment of a variety of bowel diseases such as ulcerative colitis, Crohn’s disease, amoebiasis, colon cancer and systemic delivery of proteins and peptide drugs [1, 2]. The pathogenesis likely involves genetic, environmental and immunological factors [3]. Ulcerative colitis is a type of inflammatory bowel disease that affects the lining of the large intestine, colon and rectum. It can be treated more effectively by local delivery of anti-inflammatory drugs such as 5-aminosalicylic acid (5-ASA) and corticosteroids to the colon. Newer corticosteroids with high local activity and low systemic side effects are the drug of choice for the treatment of inflammatory bowel disease [4]. Budesonide is a locally acting corticosteroid and has the highest affinity for glucocorticoid receptor [5]. It is approved as a standard drug for the localized treatment of inflammatory bowel disorder due to negligible oral bioavailability, rapid clearance and no active metabolites [6]. In the present study, the drug budesonide was formulated into microspheres and its targeting efficiency was evaluated in vivo.

MATERIALS AND METHODS

Materials

Budesonide was purchased from Astra Zeneca, UK. Chitosan was received as a gift sample from Central Institute of Fisheries Technology, Kochi. Trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma Chemicals, USA.

5-Aminosalicylic acid and barium sulphate were procured from Apollo Pharmaceuticals, Mumbai and Eudragit-S 100 was obtained from Degussa India Pvt Ltd, Mumbai. All other reagents and chemicals used were of analytical grade.

Methods

Preparation and characterization of microspheres

Chitosan microspheres were prepared using ionotropic gelation process with counter polyanion tripolyphosphate (TPP) [7, 8]. A 2 factor 3 levels full factorial design was used for the formulation of microspheres. The amount of polymer chitosan (2, 4 and 6 %w/v) and cross-linking agent TPP (5, 7.5 and 10%v/v) were selected as independent variables and the amount of drug release, particle size and entrapment efficiency were selected as dependent variables. Chitosan was dissolved by stirring for 60 min in a 2%v/v acetic acid solution to obtain a transparent and homogeneous solution. The drug budesonide (9 mg) was then dispersed in chitosan solution. Microspheres were formed by dropping 10 ml of this bubble free dispersion of chitosan through 22# disposable syringe into gently agitated (magnetic stirrer) 40 ml of TPP solution. The formed microspheres were separated after 2 h by filtration, washed thoroughly with reverse osmosis (RO) water and then air dried.

The prepared microspheres were coated with eudragit S-100 polymer by solvent evaporation method [9]. 50 mg of microspheres were dispersed in 10 ml of coating solution prepared by dissolving 500 mg of eudragit S-100 in ethanol: acetone (2:1). This organic phase was poured in to 70 ml of light liquid paraffin containing 1% w/v span 80. The system was agitated for a period of 3 h with a speed of 1000 rpm. The coated microspheres were filtered after the evaporation of the solvent, washed with n-hexane, and dried. Prepared microspheres were characterized for micrometric properties, entrapment efficiency and in vitro drug release study [10]. The formulation which exhibited good entrapment efficiency and drug release behaviour was selected for in vivo study.

In vivo study

Before commencement of the experimentation on animals, the experimental protocol was subjected to the scrutiny of the Institutional Animal Ethical Committee, and it was approved by the committee. Institutional Animal Ethical Committee, Govt Medical college, Kozhikode has given consent for conducting the animal experiment–No IAEC/08/2013 dtd 12/03/2013.

Induction of experimental colitis

Colitis was induced using trinitrobenzene sulfonic acid (TNBS) [11]. Male abino rats (average weight-200-300 g, 12-15 w, n=6/group) were distributed into six different groups. The animals fasted for 48 h before experimentation and allowed food and water ad libitum.
after the administration of TNBS. To induce inflammation, all groups except healthy control group were treated by the following procedure. After light anaesthesia with ether, the rats were catheterized 8 cm intra-cutaneously, and 500μl of TNBS in ethanol was applied (dose was 150 mg/kg body weight of TNBS in ethanol 50% solution) into the colon via rubber catheter. Animals were then maintained in a vertical position for 30 s, and returned to their cages. For 3 of the rats were housed without treatment, to maintain the development of full inflammatory bowel disease model.

### Treatment groups

Rats were randomized into six different groups, each consisting of six animals. a. healthy control group, b. colitis control group, c. group receiving a suspension of drug (budesonide), d. group receiving a suspension of standard drug (5-ASA), e. group receiving a suspension of formulated microspheres and f. group receiving polymer (chitosan) suspension. The suspension was prepared using normal saline in all cases. The animals received treatment orally once daily for 7 continuous days. Healthy control and colitis control group received only saline. Oral dose for rat was calculated from the dose of human [12]. Animals were sacrificed with carbon dioxide 24 h after the last drug administration. A segment of colon 8 cm long was excised and evaluated.

### Determination of colon/body weight ratio (C/B ratio)

The C/B weight ratio was calculated as an index of colonic tissue oedema [13]. The rats were killed, then the abdomen was opened, and the distal colon was rapidly excised and opened longitudinally along the mesenteric edge. The distal colon specimen was rinsed with isotonic saline. An 8 cm segment showing gross pathological changes was weighed for determining C/B mass ratio.

### Clinical activity score

Colitis activity was quantified with a clinical score assessing weight loss, stool consistency and rectal bleeding [14]. No weight loss was counted as 0 point, 1 to 5% as 1 point, 5 to 10% as 2 points, 10 to 20% as 3 points and >20% as 4 points. For stool consistency, 0 point was given for well-formed pellets, 2 points for pasty and semi-formed stools that did not stick to the anus, and 4 points were given for liquid stools that stick to the anus. Bleeding was scored as 0 point for no blood, 2 points for positive finding, and 4 points for gross bleeding. The mean of these scores forms the clinical score ranging from 0 (healthy) to 4 (the maximal activity of colitis).

### Assessment of macroscopic ulceration (damage score)

Gross mucosal damage [11] was scored on a 0-5 grade scale by two independent observers who were blinded to the treatment. Damage was scored as follows:  

- Score 0 no damage  
- Score 1 localized hyperemia with no ulceration  
- Score 2 linear ulceration with no significant inflammation  
- Score 3 linear ulcers with inflammation at one site  
- Score 4 two or more sites of ulceration and/or inflammation  
- Score 5 two or more sites of ulceration and inflammation or one major sites of inflammation and ulceration >1 cm along the length of colon

### Measurement of myeloperoxidase (MPO) activity

The distal colon specimen (200 mg) was minced in a beaker containing 1 ml of hexadecyl trimethyl ammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0) on ice, transferred to a test tube and homogenized (three times for 30 s each on ice). After homogenization, the homogenizer was rinsed twice with 1 ml of HTAB. The pooled homogenate and washes were sonicated for 10 s, freeze-thawed three times, and centrifuged at 10,000 rpm for 15 min. The supernatant was assayed spectrophotometrically for MPO activity [15]. 0.1 ml of supernatant was combined with 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml O-dianisidine hydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured. One unit of MPO activity is defined as the amount which degrades 1 μmol of the peroxide per min at 25 °C.

### Histopathological study

Tissue samples were excised from each colon and maintained in 10% formaldehyde for histopathological studies [16]. Specimens were fixed in 10% buffered formalin solution embedded in paraffin, stained with haematoxylin and eosin and then subjected to histopathological studies. Microscopic evaluation was performed by a pathologist unaware of the study design.

### Determination of in vivo targeting efficiency

This study was carried out to check the in vivo targeting efficiency of the formulation [17, 18]. Rabbits were selected as an animal model for evaluating the colon-specific delivery. Roentgenography study, a comparatively safer technique was carried out in healthy male albino rabbits. The behaviour of budesonide microspheres in rabbit was observed using a radiographic imaging technique. It involves the use of radio-opaque marker barium sulphate, incorporated in the formulation to determine the position of microspheres. Healthy rabbits fasted overnight and on the next day morning microspheres enclosed in a capsule shell was administered followed by giving 25 ml of water. At different time intervals, X-ray images were taken under the supervision of a radiologist.

### In vivo drug release study

A capsule containing microspheres having 9 mg of drug was given to 24 h fasted rabbit. Blood samples (1 ml) were taken serially from the ear vein of the animals after 0, 2, 6, 8, and 24 h of drug administration. The plasma was separated by centrifugation for 3 min at 13,000 rpm. The protein was extracted by adding 1 ml of dichloromethane to 1 ml plasma. The organic phase was taken in a test tube and evaporated. The residue was dissolved in 2 ml of mobile phase consisting of a water/ethanol mixture (57/43 by volume). The samples were injected into the high-performance liquid chromatography system (HPLC). N-Propyl-p-hydroxy benzoic acid-methanol solution was used as an internal standard. The HPLC system was equipped with a UV detector at 246 nm in a reverse phase column [19]. Presence of drug in the faecal content of rabbit was analysed by UV spectrophotometry [20].

### Statistical analysis

Results were expressed in mean±SD. Data obtained were subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests using Graphpad Instat software.

### RESULTS AND DISCUSSION

Prepared microspheres were characterized for micrometric properties, entrapment efficiency and in vitro drug release study. The formulation exhibited good flow behaviour and had good entrapment efficiency. The microspheres successfully retarded drug release until it entered the colon [10].

### In vivo results

The comparison of the therapeutic effect of budesonide microspheres and its suspension with the standard drug on C/B mass ratio, clinical activity score, body mass change, macroscopic damage score and MPO activity in TNBS induced colitis are shown in tables (1, 2, 3, 4 and 5).

### Colon/Body mass ratio (C/B mass ratio)

#### Table 1: Data of C/B mass ratio in control and treatment groups after 7 d treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>C/B Mass ratio (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control</td>
<td>2.2±0.82</td>
</tr>
<tr>
<td>Colitis control</td>
<td>9.6±0.3***</td>
</tr>
<tr>
<td>Budesonide Suspension</td>
<td>6.2±0.26***</td>
</tr>
<tr>
<td>Standard drug (5-ASA)</td>
<td>6.4±0.18***</td>
</tr>
<tr>
<td>Budesonide microspheres</td>
<td>5.8±0.14***</td>
</tr>
<tr>
<td>Polymer suspension</td>
<td>9.2±0.84***</td>
</tr>
</tbody>
</table>

*** Significant, p<0.001 (with control). $$ significant, p<0.001, (with standard), ANOVA followed by Tukey-Kramer multiple comparison, N=6.
During the treatment period, the signs of colitis started to decrease and the C/B ratio was decreased compared to the colitis control group. The decrease in C/B ratio is due to the anti-inflammatory activity of budesonide. Microspheres show better therapeutic effect than that of the suspension. This may be due to the release of drug from the chitosan polymer in the colonic region. Polymer doesn’t have any effect in controlling colitis. Standard drug treated group reduces the severity of colitis but the effect observed is less than that of microspheres.

The increased activity of microspheres may be due to the localised release of the steroidal drug.

**Clinical activity score and body mass change**

All rats treated with TNBS developed clinical symptoms such as loss of appetite, bloody diarrhoea and significant body mass loss (p<0.001). This could confirm the colitis model.

Gross bleeding was observed in colitis control group, and polymer treated group. Pasty and semi-formed stools were seen in other groups.

### Table 2: Clinical activity score

<table>
<thead>
<tr>
<th>Groups</th>
<th>Stool consistency</th>
<th>Rectal bleeding</th>
<th>Weight loss</th>
<th>Clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colitis control</td>
<td>3.8±0.6</td>
<td>3.6±0.6</td>
<td>3.8±0.2</td>
<td>3.8***</td>
</tr>
<tr>
<td>Budesonide</td>
<td>2.2±1.2</td>
<td>2.4±0.6</td>
<td>3.0±0.8</td>
<td>2.5***</td>
</tr>
<tr>
<td>Microspheres</td>
<td>2.2±0.2</td>
<td>2.0±0.4</td>
<td>3.2±0.6</td>
<td>2.5***$</td>
</tr>
<tr>
<td>Standard drug</td>
<td>2.6±0.5</td>
<td>3.2±0.6</td>
<td>2.6±0.8</td>
<td>2.8***</td>
</tr>
<tr>
<td>Polymer</td>
<td>3.8±0.4</td>
<td>3.6±0.4</td>
<td>3.8±0.2</td>
<td>3.8***</td>
</tr>
</tbody>
</table>

*** Significant, p<0.001 (with control). $ Significant, p<0.001, (with standard), ANOVA followed by Tukey-Kramer multiple comparisons, N=6

### Table 3: Body mass change (%)

<table>
<thead>
<tr>
<th>Groups</th>
<th>% change in body mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>+22.6±0.58</td>
</tr>
<tr>
<td>Colitis control</td>
<td>-25.67±0.63***</td>
</tr>
<tr>
<td>Budesonide</td>
<td>+3.10±0.55***</td>
</tr>
<tr>
<td>Microspheres</td>
<td>+3.67±0.38***</td>
</tr>
<tr>
<td>Standard drug</td>
<td>-4.82±0.43***</td>
</tr>
<tr>
<td>Polymer</td>
<td>-24.77±0.68***</td>
</tr>
</tbody>
</table>

*Body mass change after 7 d treatment, -loss in weight, +increase in weight, *** Significant, p<0.001 (with control), ANOVA followed by Tukey-Kramer multiple comparison, N=6

On day 0, the body mass of rat was 178.0±6.8 g in the healthy control group (n=6) and 180±4.8 g in groups treated with TNBS (n=30), which shows no significant difference between them. The average mass gain in the healthy control group was 22.6±0.58%, but the rates in the colitis group suffered significant body mass loss of -25.67±0.63%. The results in table (3) shows that the polymer group and the colitis control group do not show a significant difference from each other. All other groups differ in the body mass changes from the colitis control group significantly (p<0.001). During the treatment period, the signs of colitis started to decrease in severity for all treated groups except polymer groups. Effects of budesonide microspheres and budesonide suspension are statistically significant with that of standard drug (p<0.001). Colitis can be controlled effectively using budesonide.

**Macroscopic damage score**

The colon in colitis control group was severely damaged, indicated by mucosal hyperaemia, haemorrhage, deep ulcers and necrosis. Daily treatment with budesonide microspheres reduces the damage and shows the significant result (P<0.01) as observed in table (4). Polymer treated group doesn’t have any effect on relieving the symptoms.

### Table 4: Data of macroscopic damage score in control and treatment groups after 7 d treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Macroscopic score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Colitis control</td>
<td>3.0±0.18***</td>
</tr>
<tr>
<td>Budesonide</td>
<td>2.4±0.30***$</td>
</tr>
<tr>
<td>Microspheres</td>
<td>2.5±0.06***$</td>
</tr>
<tr>
<td>Standard drug</td>
<td>2.3±0.30***$</td>
</tr>
<tr>
<td>Polymer</td>
<td>2.8±0.40***</td>
</tr>
</tbody>
</table>

*** Significant, p<0.001, with Healthy control, $ significant, p<0.05, with Colitis control, ANOVA followed by Tukey-Kramer multiple comparison, N=6

**Determination of myeloperoxidase (MPO) enzyme activity**

Myeloperoxidase is an endogenous enzyme in mammalian granulocyte and plays an important role in the initiation and progression of acute and chronic inflammation. The activity of MPO, which is found in neutrophils and in small quantities in monocytes and macrophages can be used for evaluating the degree of inflammation in the intestine. Myeloperoxidase is soluble in hexadecyl trimethyl ammonium bromide and activity is measured with a diansidine-hydrogen peroxide assay.
Table 5: Biochemical analysis of colon tissue homogenates for myeloperoxidase enzyme (MPO) activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>MPO unit/g of wet tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control</td>
<td>0.14±0.36</td>
</tr>
<tr>
<td>Colitis control</td>
<td>0.52±0.42***</td>
</tr>
<tr>
<td>Budesonide Suspension</td>
<td>0.24±0.22***</td>
</tr>
<tr>
<td>Standard drug (5-ASA)</td>
<td>0.22±0.04**</td>
</tr>
<tr>
<td>Budesonide microspheres</td>
<td>0.20±0.16*</td>
</tr>
<tr>
<td>Polymer suspension</td>
<td>0.50±0.26***</td>
</tr>
</tbody>
</table>

*Significant, p<0.05, ** significant, p<0.01, *** significant, p<0.001, with respect to control. ANOVA followed by Tukey-Kramer multiple comparison, N=6.

Histopathological investigation

The histopathological investigation was carried out on the excised colon and the fig. 1, 2, 3, 4 and 5 show microscopic images of the stained specimens. The findings are as follows:

The tissue obtained from healthy control group showed intact colon with normal villi, normal glands and lamina propria fig. (1).

![Fig. 1: Normal colon](image1)

Tissues excised from TNBS administered colitis group showed mucosal ulceration, mild distortion of glands, neutrophilic infiltration and decrease in goblet cells and blunted villi as shown in fig. (2).

![Fig. 2: TNBS induced ulcerative colon](image2)

Tissues excised from microspheres treated group shows less of inflammation, more of goblet cells compared to colitis control group as observed in fig. (3).

![Fig. 3: Site showing healing of ulcer by administration of microspheres](image3)

Blunted villi, distorted glands, increased inflammatory cells in lamina propria are seen in tissues treated with polymer group. Changes are similar to chronic ulcerative colitis as shown in fig. (4).

![Fig. 4: Polymer treated colon](image4)

Fig. (5) Represents standard drug treated group shows slight blunting of villi, no distortion, the normal number of goblet cells and scanty inflammation.

![Fig. 5: Standard drug treated colon](image5)
In vivo targeting efficiency

X-ray studies revealed that the microspheres remained in the stomach for the first 2h as shown in fig. (7). Microspheres get released from the capsule shell by dissolving the gelatin shell and distributed in the small intestine as observed in the fig. (8).

Since the particles are small and the amount of radio-opaque material is also less, the amount of microspheres visible by X-rays is also minimal. Microspheres are visible at 4th h in the small intestine as observed in fig(8), and some are also seen in the X-ray image taken at the 8th h in the large intestinal region [22]. X-ray image at 10th h indicates the presence of microspheres in the colonic region as seen in fig.(10). The observations were confirmed by a veterinary surgeon. It can be concluded that the formulation is able to target colonic region effectively.
In vivo release study

Analysis of blood samples did not show the presence of budesonide in detectable amount while fecal content analysis reveals the presence of the drug. HPLC analysis of budesonide pure drug sample shows two characteristic peaks representing epimer A and epimer B of budesonide in the chromatogram as observed in fig. (11).

Fig. 11: HPLC analysis of pure budesonide sample

Blood sample analysis after 2h of administration of the formulation is shown in the fig. (12). In this characteristic peaks are absent that indicate the absence of budesonide in the blood sample.

Fig. 12: HPLC analysis of blood sample following administration of budesonide microspheres (after 2h)

HPLC analysis of blood samples after 4, 6, 8 and 24 h of administration of the formulation in the rabbit showed the absence of budesonide. Faecal content analysis indicated the presence of the drug.

CONCLUSION

The study showed that oral administration of budesonide microspheres exerted an affirmative impact on the colonic ulcer healing by decreasing the area of ulceration, reducing the mass of colon by improving the symptoms of colitis. MPO activity decreased significantly after oral administration of microspheres. Histopathological studies carried out also confirmed the result. Tissue excised from microspheres treated group shows less inflammation and more goblet cells than colitis control group. In vivo study conducted on rabbits proved that the drug was not present in a detectable amount in the blood sample collected at different time intervals after oral administration of the dosage form while faecal content analysis indicated the presence of the drug. The X-ray

Fig. 9: X-ray image after 8 h

Fig. 10: X-ray image after 10 h
studies revealed that the formulations are able to target the colon. With the permission of human ethical committee, detailed scintigraphic studies are needed to investigate its potential for clinical use.

ACKNOWLEDGEMENT

Thankfully remember Central Institute of Fisheries Technology, Kochi for providing the sample of Chitosan.

CONFLICT OF INTERESTS

Declare none

REFERENCES