RESEARCH ARTICLE

The Effect of Colon Targeted Delivery of Celecoxib Loaded Microspheres on Experimental Colitis Induced by Acetic Acid in Rats

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Abstract

Objective: The aim of the present study is to develop colon targeted drug delivery of chitosan microspheres with celecoxib as a model drug. **Materials and Methods:** Mucoadhesive microspheres were prepared using chitosan as a core polymer and coated with Eudragit S-100 by a solvent evaporation technique. The effect of different microsphere formulations was studied. **Results:** *In vitro* release study, core microspheres are found to show burst release in the first 1 h, whereas coated microspheres exhibit a controlled release, and thus suitable for colon target drug delivery. Through an *in vivo* study, we investigated the effect of the coated microspheres in rats exposed to experimental colitis. Colitis was induced in rats by rectal instillation of acetic acid. Rats experienced hemorrhagic diarrheic, body weight loss, rise in myeloperoxidase (MPO), and reduction of reduced glutathione (r-GSH) activity by cyclooxygenase (COX), after 5 days administration of acetic acid. Oral administration of coated microspheres (5 mg/kg) twice-a-day to rats after induction of colitis significantly attenuated the severity of the colonic injury. In addition, there was a significant reduction of MPO and COX levels but arises in the levels of GSH. **Conclusion:** Thus, the study provided the evidence that coated microspheres are beneficial in the treatment of inflammatory bowel disease with minimal side effects.

Key words: Celecoxib, colon drug delivery system, microspheres, nonsteroidal anti-inflammatory, ulcer colitis

INTRODUCTION

The conventional nonsteroidal anti-inflammatory (NSAIDs) drugs such as ibuprofen, diclofenac, indomethacin, and others are reported to inhibit prostanoids generations, and their effects vary in different tissues and organs.[1] Colonic prostanoids generation is increased in both experimental colitis and inflammatory bowel disease (IBD).[2,3] The cyclooxygenase (COX) enzymes catalyze key step in the conversion of arachidonic acid to prostaglandins.[4] Prostaglandins are synthesized by two different forms of COX enzymes: Namely, COX-1 and COX-2.[5] COX-1is a constitutive enzyme which plays a protective role against small intestine and the colon[6,7] mucosal injury through the synthesis of prostaglandins that promote epithelial regeneration. On the other hand, COX-2 expression is dramatically increased in inflammatory states and leads to inflammation pain.[8,9] Based on the above observations, some researchers have attributed the anti-inflammatory action of NAIDS to the inhibition of COX-2, and the harmful effects of NAIDS on the gastrointestinal (GI) mucosa are attributed to the blockage of COX-1 activity.[10,11] This connotation of COX-2

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with inflammatory events led to the development of selective COX-2 inhibitors that are expected to display systematic anti-inflammatory properties without the anticipated GI toxicity.

NSAID drugs are used to inhibited COX activity but were not selective toward COX-1 and COX-2, but celecoxib, a new selective COX-2 inhibitor, is effective to modulate the extent of experimental colitis and, therefore, could also be beneficial in the treatment of IBD.[12] The probable mechanism of action of celecoxib is believed to be due to inhibition of prostaglandin synthesis (through conversion of arachidonic acid), primarily through inhibition of COX-2. It has been shown that at a therapeutic concentration in humans, celecoxib does not inhibit COX-1,[13] which is an important homeostatic protection in the gastric mucosa, kidney. According to recent clinical trials in the patients, celecoxib is associated with a lower extent of the gastric and renal side effects when compared to the other NSAID drugs. This is due to inhibition of the constitutional isomer of COX-1,[14] but long-term use of coxibs increases the risk of serious dose-associated cardiovascular events.

The major challenge in the treatment of ulcerative colitis is the minimization of drug-related side effects by doing site-specific drug delivery to colon. Development of a colon-specific drug delivery system (CDDS), delivering active ingredient in the colon, can lead to direct treatment at the disease site and consequently can decrease the dosing and minimize systemic side effects.[15] These beneficial effects are resulted from increased therapeutic concentration at the target site while restricting systemic absorption. The various primary approaches that are employed to achieve colon-specific delivery include: Prodrugs, pH-sensitive polymers, time-dependent delivery, and colon microflora activated approach.[16-20] Single unit CDDS like coated tablets suffer from the disadvantage of accidental disintegration of the formulation due to manufacturing deficiency or unusual gastric physiology that may lead to increased system bioavailability or loss of local action in the colon.[21] In comparison to unit drug delivery system, colon-specific celecoxib microspheres could minimize the side effects and improve its therapeutic activity. Moreover, the smaller particle size of the microspheres system makes it more uniformly dispersed in the GI tract (GIT), and allows smooth passage through the GIT.[22]

Microparticulate system is one of the promising approaches for controlled drug delivery in specific site action, which can be accomplished using mucoadhesive polymer. Mucoadhesive polymers like chitosan offer extended duration times at the site of drug absorption due to increased contact with absorbing mucous.[23] The factors which are expected to play an important role in determining release profile of the drug incorporated to the coated microspheres include the pH at which Eudragit S-100 coating is soluble, the swelling and degradation behavior of core polymer, drug solubility and diffusion through the core polymer, the interaction between core and coat polymer,[24] if any, and finally the ratio of main and coat polymer employed for the preparation of microspheres.

Taking into account the aforesaid information, celecoxib loaded chitosan microspheres coated with Eudragit S-100 - a pH sensitive polymer which is soluble above pH 7 - was used to prevent drug release from microspheres in the small intestine until they reach the terminal ileum where chitosan ensure a controlled release of celecoxib, following degradation by the abundant colonic microflora. The prepared microspheres were characterized by X-ray diffraction, differential scanning calorimetry (DSC), scanning electron microscope (SEM) for particle size, entrapment efficacy, and *in vitro* drug release. *In vivo* study was conducted to investigate the effect of celecoxib on the inflammatory response (ulcer colitis) caused by intracolonic administration of acetic acid in waster albino rats, and particularly to examine the effect of celecoxib on colon injury and investigation of biochemical marker of the oxidative stress such as myeloperoxidase (MPO) and GSH. Furthermore, COX activity also investigated. To the best of our knowledge, the use of colon targeted celecoxib microspheres on the severity of colitis induced by acetic acid in rats has not been reported, yet.

MATERIALS AND METHODS

Materials

Celecoxib, chitosan, Eudragit S-100 poly (methacrylic acid-co-methyl methacrylate 1:2) were kindly received as gift sample by Dr. Reddy's Research Foundation, (Hyderabad, India), Central Institute of Fisheries Technology, Cochin, India, and Matrix Laboratories (Hyderabad, India), respectively. Glacial acetic acid and Span-80 (sorbitan monooleate) were purchased from S.D-fine chem. Limited (Mumbai, India). MPO assay and reduced glutathione (r-GSH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents, including petroleum ether, acetone, methanol, light liquid paraffin, and heavy liquid paraffin, were of analytical grade, and - supplied by Merck Specialties Pvt. Ltd. (Mumbai, India).

Methods

Preparation of celecoxib-loaded chitosan microspheres

Chitosan microspheres were prepared using emulsion cross-linking method. Weighed amount of chitosan was dissolved in 1% aqueous solution of glacial acetic acid, following dispersion of celecoxib to get a concentration of 2% (w/w). A volume of 3 mL of the resulting solution was then injected with a syringe (Gauge No. 23) into 20 mL of oil phase containing Span-80 (1%, v/v). The continuous stirring was

done using a mechanical stirrer (Remi Motors Ltd, Mumbai, India) to form a w/o emulsion. The external oil phase was composed of heavy and light liquid paraffin in the ratio of 1:1. After 30 min of stirring, 1.5 mL of acetone-saturated glutaraldehyde (8:1) was added to the resultant emulsion. The prepared emulsion was left for stabilization, followed by cross-linking for a period of 8 h. The obtained microspheres were then centrifuged at 4000 rpm, and the residue was washed with petroleum ether, followed by acetone, and dried in hot air oven at 45°C. For optimizing the in-process variable, parameters were adjusted to prepare different formulations [Table 1].

From formulations of P-1 to P-5 the parameters included rotational speed of 1500 rpm and an emulsifier concentration of 1%; wherein the case of R-1 to R-3 the drug-to-polymer ratio was maintained constant at 1:6 with the emulsifier concentration of 1%. In a similar manner, for the formulations of S-1 to S-3 drug-to-polymer ratio were maintained constant at 1:6, with a rotational speed of 1500 rpm.

Encapsulation of core microspheres

Selected core microspheres were coated with Eudragit S-100 using emulsion-solvent evaporation technique. The core microspheres obtained before were dispersed in 2.5 mL of an ethanolic solution of Eudragit S-100 solution (10%, w/v), followed by emulsification in light liquid paraffin (50 mL) containing 1% v/v span-80. 2 mL of ethanol was added dropwise to form a stable emulsion. The emulsification mixture was maintained at 1000 rpm for 3 h using a mechanical stirrer. Encapsulated microspheres were filtered and rinsed with petroleum ether and dried in hot air oven at 40°C. The in-process variable parameters of the coated samples are tabulated in Table 2.

Characterization of microspheres

The thermal analysis of pure celecoxib and optimized microsphere formulation was performed using DSC equipped with a thermal analysis data system (PerkinElmer, USA). 3–4 mg weighed samples were heated in flat-bottom sealed aluminum pans over a temperature range of 40–300°C at a constant rate of 15°C/min under nitrogen purge (50 mL/min) using an empty aluminum pan which was used as the reference pan. X-ray diffractograms of the selected microspheres were carried out on X'Pert Pro MRD X-ray diffractometer (PANalytical BV, The Netherlands) operating at 40 kV and a current of 30 mA at a scan rate of 2°/min over a 2θ range of 10–80°.

The shape and surface morphology of optimized core and coated microspheres were analyzed by SEM (Hitachi S-34000N, Hitachi High-Tech, Tokyo, Japan). The microspheres were mounted on metal stubs and are coated with a 150Å layer of gold. The particle sizes of the microspheres were measured using a Malvern mastersizer (Malvern Instrument Ltd, Malvern Worcestershire, UK). Approximately 100 microspheres were counted for determining the particle size. The distribution of particle size was measured by suspending the microspheres in water.

Drug loading capacity and encapsulation efficiency

Microspheres (10 mg) were dispersed in 0.1 N Hydrochloric acid and allowed to stand for 24 h. The dispersion was then shaken with methylene chloride for extracting celecoxib. The organic extract was then evaporated to dryness, and the residue was dissolved in methanol. The absorbance of the resulting solution was measured at 250 nm using double beam ultraviolet (UV)–visible spectrophotometer (Shimadzu-3600, Japan) for determining the amount of celecoxib present in the microspheres formulation. The percent yield (PY) loading capacity (LC) and entrapment efficiency (EE) were then calculated by the following formula.

$$\text{Percent yield } (\text{PY}) = \frac{\text{Practical mass of microspheres}}{\text{Theoretical mass}} \text{ x } 100$$

$$\text{Loading capacity } (\text{LC}) = \frac{\text{Mass of celecoxib in microspheres}}{\text{Mass of microspheres}} \text{ x } 100$$

$$\text{Entrapment Efficiency } (\text{EE}) = \frac{\text{Mass of celecoxib in microspheres}}{\text{Initial mass of celecoxib}} \text{ x } 100$$

In vitro release of celecoxib-loaded chitosan microspheres

Drug release from the microspheres was determined in simulated GI fluids. Accurately weighed amount of microspheres, equivalent to 30 mg of celecoxib, were added to 450 mL of dissolution medium and the drug release from microspheres was processed using USP rotating paddle dissolution apparatus (Lab India, Disso-8000, India). The mixture was stirred magnetically at 100 rpm and 37°C. The simulation of GI fluid pH variations was accomplished by modifying the pH of the dissolution medium at various time intervals. The initial pH of the medium was maintained at 1.2 for 2 h with 0.1N HCl. Then, the pH of medium was adjusted to 4.5 by adding 1.7 g of KH_2PO_4 and 2.225 g of $Na_2HPO_4.2H_2O$ and sufficient quantity of 1.0 M NaOH. The release rate analysis was followed for every 2 h. After the duration of 4 h, the pH of the dissolution medium was adjusted to 7.4 with 1.0 M NaOH and maintained for 12 h. Measured 2 mL samples were withdrawn at appropriate time intervals and were replaced with fresh dissolution medium. Finally, the samples were subjected to UV-Vis analysis, as described previously. All these release tests were performed in triplicate. The effects of drug-to-polymer ratio on *in vitro* drug release of core and coated microspheres were also evaluated.

Table 1: Processing variable parameters of the prepared chitosan microspheres (rpm, rotations per minute; % (w/v), percentage weight per volume)

Parameters	Process variables	Formulation code
Drug-polymer ratio (constant: 1500 rpm: 1%)	1:2	P1
	1:4	P2
	1:6	P3
	1:8	P4
Rotational speed constant: 1:6; 1%)	1000 rpm	R1
	1500 rpm	R2
	2500 rpm	R3
Emulsifier concentration (constant: 1:6 1500 rpm)	0.5%	S1
	1.0%	S2
	1.5%	S3

Table 2: Eudragit S-100 coated chitosan microspheres with core coat ratio

Core : coat ratio	Formulation code
1:4	CMC1
1:6	CMC2
1:8	CMC3

In vivo studies on microspheres

Experimental animals

Male Wistar rats of 250 ± 25 g were obtained from the animal house facility at National Institute of Nutrition, Hyderabad, India. The rats were maintained under standard environmental conditions with 12 h: 12 h light and dark cycle, at temperature 25 ± 3°C and the relative humidity 45–65 %. The rats provided with standard pellet diet and water *ad libitum*. All the animals were allowed to acclimatize to the laboratory conditions for 7 days prior commencement of the experiment. The animal experiments were performed during the light portion between 8 a.m. and 12.00 p.m. to avoid circadian influence. The experiments were carried out according to the prescribed guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA Reg. No: 1412/A/11/CPCSEA (IAEC No: I/IAEC/LCP/033/2014/WR-24), Government of India, India. The animals were randomly divided into four groups, each group consisting of six animals. These include normal control group, acetic acid control group, vehicle-treated group in which 2% gum acacia was given as a carrier and the drug-treated groups received celecoxib in a dose of 5 mg/kg body wt. The drugs were dispersed in 2 % gum acacia and introduced orally in a volume of 0.5 mL/100 g body wt. The treatment was given twice daily, 24 h after the induction of colitis, and continued for 5 consecutive days.

Induction of experimental colitis in rats

Rats were fasted for 24 h with access to water *ad libitum* after each rat was sedated by giving an intraperitoneal injection of phenobarbitone (35 mg/kg. body wt.). A flexible polyethylene tube with an outside diameter of 2 mm was inserted 8 cm into the colon through the rectum. The polyethylene tube was lubricated with glycerin before entering into rectum. Ulcerative colitis was induced by injecting 2 mL of glacial acetic acid (3% v/v) in 0.9% saline into the colon using polyethylene tube.[25] Injected rats were maintained in a head down position for 2 min to prevent solution leakage. After 24 h of colitis induction, one group of rats was sacrificed under ether anesthesia. Colon was flushed gently with saline and used for macroscopic scoring and biochemical estimation to confirm the induction of colitis.

Evaluation of the disease

Assessment of disease activity index

Disease activity index was quantified with a clinical score through the body weight loss, stools consistency and bleeding of the colon (measured by guaiac reaction, hemoccult) as per Cooper10 procedure as demonstrated.[26] Body weight loss is assessed at 5-point scale as no significant body weight loss; then it was assumed and counted as 0 points and body weight loss of 1–5% as 1 point, 5–10 as 2 points, 10–20% as 3 points, and 20% as 4 points. For stool consistency, the stool was collected in a non-absorbent paper and observed; 0 points were given for regular pellets, 2 points for pasty and semi-formed stools that did not stick to the anus, and 4 points given for liquid stools that did stick to the anus. Bleeding was scored 0 points for no blood in hemoccult, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were added and divided by 3, forming a total clinical core that ranged from 0.0 (healthy) to 4.0 (maximal activity of colitis).

Assessment of colon damage by macroscopic scoring

The severity of colitis was assessed by an independent experienced observer who was blind to the experiment. At postmortem laparotomy, colon (6 cm), extending proximally for 2 cm above the anal orifice, was removed. The tissue was split longitudinally and pinned out onto a card. The macroscopic appearance of the colonic mucosa was scored according to independent observation. An arbitrary scale ranging from 0 to 4 was used as follows: 0=no macroscopic change; 1=mucosal erythema only; 2=mild mucosal edema; slightly bleeding or small erosion; 3=moderate edema; bleeding ulcers or erosions; and 4= severe ulceration/erosion, edema, and tissue necrosis.[27]

Biochemical studies

Colonic tissue samples were homogenized in cold 1 mL of 10 mmol/L Tri-HCl buffer of pH 7.1, using homogenization (Remi Motors Ltd., Mumbai, India). The homogenate sample was used for the measurement of MPO, GSH, and COX activity.

Assessment of colonic MPO activity

MPO activity, an index of polymorphonuclear leukocyte accumulation of several tissues, was determined as described previously.[28] The colon sample weighed (0.5 g) and homogenized in a 10 volume solution of 50 mM of potassium phosphate buffer of pH 7.4, in an ice bath using homogenizer (50 mg tissue/mL). The pellet (containing 95% of the total tissue MPO activity) was re-suspended in an equal volume of potassium phosphate buffer (pH 6.0) and the solution was centrifuged at 20,000 × g for 30 min at 4°C. An aliquot supernatant liquid was used for MPO assay using tetramethylbenzidine and 0.3 H_2O_2 mM as substrate. One unit of MPO activity was expressed as that of converting 1 μ mol of water in 1 min at 22°C. The absorbance change was measured at 655 nm using UV–Visible spectrophotometer (Shimadzu-3600, Japan).

Determination of colonic GSH contents

Colonic tissue GSH concentration was determined using the method previously described,[29] which is based on the formation of relatively stable yellow product when sulphydryl group react with 5, 5-dithio-bis-2-nitrobenzoic acid with GSH present in the tissue. The absorbance was measured at 412 nm with a UV–Vis spectrophotometer (Shimadzu-3600, Japan). The amount of GSH present in the sample was calculated using standard solution of GSH containing 1 mg of GSH/mL of 3% metaphosphoric acid. The increase in the extinction at 412 is proportional to the amount of GSH present.

Assessment of COX activity

COX activity in colon is estimated using a previously described procedure with slightly modifications.[30] The colon sample was homogenized at 4°C in buffer solution containing the following protease inhibitors in a ratio of 5:1 (v/w) (HEPES 20 mM, pH 7.2 + saccarosio 320 mM, DDT 1 mM, STY 10 μg/ml, aprotinin 2 μg/mL, and leupeptin 10 μg/mL). The protein concentration in the homogenates was measured by Bradford assay[31] with bovine serum albumin, used as a standard. Homogenates were incubated at 37°C for 30 min in the presence of excess of arachidonic acid (30 μM). The samples were boiled and centrifuged at 12,000 × g per min. The concentration of 6-keto-PEG 1α present in the supernatant was measured by radioimmunoassay.

RESULTS AND DISCUSSIONS

Celecoxib loaded chitosan microspheres was prepared by emulsion cross-linked method was brownish-yellow colored free-flowing particles. The development of formulation was subjected to various parameters, i.e., polymer concentration, emulsifier concentration, rotational speed, and Eudragit S-100 concentration, to formulate different batches of celecoxib loaded microspheres. The effects of various variables on these characteristics are described as follows:

Particle size

The effects of formulation variables (concentration of polymer, emulsifier, and string speed) on particles size are presented in Table 3. Particle size of the microspheres increases from 8.42 to 10.32 μm with an increase in the concentration of chitosan from 0.5 to 1.5%. With an increase in the concentration of span-80 from 0.5 to 1.5%, the particle size of the microspheres decreased from 11.28 to 8.26 μm. The string speed has also affected particle size. An increase in string speed from 1000 to 2500 led to a decrease in the particle size from 13.63 to 6.52 μm. The increasing core polymer and Eudragit S-100 ratio from 1:4 to 1:8 resulted in increased particle size of the microspheres from 120 to 158 μm.

As the concentration of core polymer increases, the prepared microsphere's particle size increases because the concentration of core polymer increases the viscosity of the core polymer solution. This leads to the larger droplets of the internal phase in the emulsification step for the preparation of microspheres. As the concentration of span-80 increases, the prepared microsphere particle size decreases ascribed to the decrease in the interfacial tension between the aqueous droplets and organic suspension medium. The particle size of microspheres decreases with an increase in the string speed, due to the fact that higher speed affords the required energy and the core polymer solution get dispersed as fine droplets in

the external oily phase. Therefore, the prepared microspheres particles are small in size and have narrow size distribution.

The effect of PY, LC, and EE

The amount of PY, LC, and EE of all the formulations of microspheres are shown in Table 3. As the polymer concentration increased, the percentage of PY increased from 79% to 93%. The percentage of EE increased from 78.51 ± 0.89 to 89.20 ± 0.96, as the drug-to-polymer ratio increased from 1:2 to 1:8. As the concentration of span-80 is increased from 0.5 to 1.5, there was a decrease in the EE from 89.68 ± 0.86 to 86.62 ± 0.72. As the stirring speed increased from 1500 to 2500 rpm, the EE decreased from 90.03 to 87.51%, respectively.

The EE, PY, and LC depend on the concentration of core polymer, span-80, and string speed. EE increases with increasing concentration of core polymer in concordance with previously published reports.[32] The EE decreases with increase in the concentration of span-80 because celecoxib, being a lipophilic drug, is soluble in the external oil phase of the w/o emulsion at a higher concentration of emulsifier (span-80). As the string speed increases, the EE decreases due to very fast diffusion of celecoxib from microspheres, resulting in the loss of celecoxib with a consequent lowering in EE.

X-ray diffraction studies

X-ray diffractograms [supplementary files Figure 1] of celecoxib clearly indicate the presence of a crystalline material having multiple peaks while both polymers, chitosan and Eudragit S-100, were found to be amorphous. While the diffractogram of core microspheres of celecoxib demonstrated the presence of crystalline drug embedded in the amorphous polymer, the diffractogram of coated microspheres showed an amorphous material devoid of any crystallinity. This could be attributed to dilution effect by the amorphous polymers.

Differential scanning calorimetry

DSC thermograms of the celecoxib, core and coating microspheres are represented in supplementary files Figure 2. Pure celecoxib exhibited a melting endotherm at 162°C. Similarly, thermograms for celecoxib without and with coated microspheres demonstrated a depressed, relatively broad endotherm at 168.06 and 178.45°C, respectively, which could be due to the dilution effect of the amorphous polymers.

Surface morphology and particle size distribution

SEM images of core and coated microspheres are represented in Figure 1. They were mostly spherical and had rough surface with an average particle size of 9–14 µm. The rough surface indicates that the surface is associated with drug crystals. In contrast, SEM of the core microspheres of celecoxib in chitosan was mostly spherical and had smooth surface, with average particle size ranging from 1.87 to 3.08 µm.

In vitro release

Core microspheres

In vitro drug release from core, chitosan microspheres were studied in the presence of simulated GI fluids using USP dissolution apparatus. The studies were conducted in 450 mL of the dissolution medium, at stirring rate of 100 rpm and at

Table 3: Average particle size, percentage yield, percent drug content and entrapment efficiency of uncoated and Eudragit S-100 coated chitosan microspheres

Formulation code	Average particle size (µm)	Percentage yield (%)	Loading capacity (%)	Encapsulation efficiency (%)
P1	8.42	78.98 ± 0.93	24.73 ± 0.21	78.51 ± 0.89
P2	8.56	84.85 ± 0.24	15.06 ± 0.09	81.02 ± 0.96
P3	9.62	88.52 ± 0.69	10.76 ± 0.06	83.86 ± 0.56
P4	10.32	95.79 ± 0.96	8.69 ± 0.930	89.20 ± 0.96
R1	13.63	89.0.3 ± 0.42	8.70 ± 0.040	90.03 ± 0.42
R2	9.64	91.56 ± 0.86	8.70 ± 0.060	89.13 ± 0.59
R3	6.52	92.72 ± 0.42	8.51 ± 0.03	87.51 ± 0.46
S1	11.26	90.61 ± 0.32	8.45 ± 0.05	89.68 ± 0.86
S2	9.64	93.49 ± 0.58	8.68 ± 0.08	88.23 ± 0.90
S3	8.28	91.66 ± 0.83	8.50 ± 0.04	86.62 ± 0.72
MC1	120	92.83 ± 0.72	7.8 ± 0.01	92.65 ± 0.22
MC2	140	92. 97 ± 0.25	8.11 ± 0.03	94.87 ± 0.32
MC3	158	93.0.7 ± 0.45	8.01 ± 0.04	95.03 ± 0.14

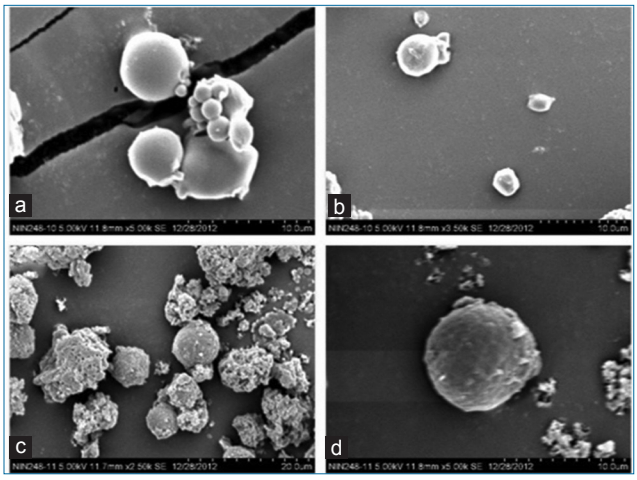

Figure 1: Scanning electron micrographs of celecoxib loaded microspheres (a and b) uncoated and (c and d) coated with Eudragit S-100

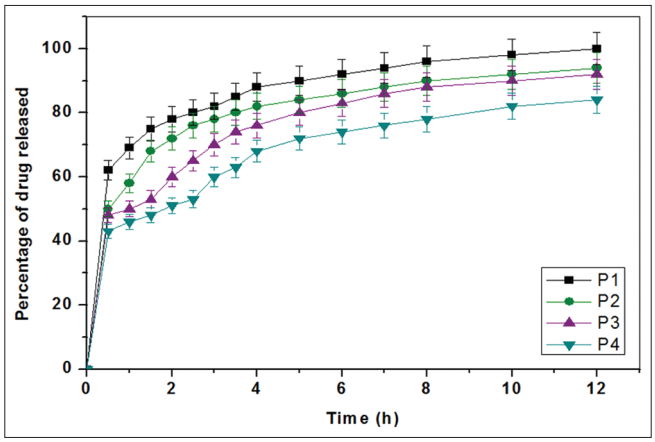

Figure 2: *In vitro* release profiles, showing the effect of drug to polymer ratio on celecoxib release from Chitosan microspheres

37°C. The *in vitro* drug release from microspheres, containing varying ratio of core polymer and drug is presented in Figure 2. Statistical analysis of dissolution profile of drug at the end of 12 h revealed that the P-1 formulation released 100% drug, whereas P-2, P-3, and P-4 formulations released 93.97, 91, and 84%, respectively.

In case of microspheres containing comparatively less proportion of polymer, i.e., P-1 and P-2, there may be a relatively more surface associated drug, indicating faster release. However, for microspheres containing more quantity of core polymer, i.e., P-4, the rate and extent of drug release is quite less as compared to all the other formulations of microspheres, due to the formation of more viscous solution during the drug release which prevents dissolution of the drug and its subsequent release. This result is in accordance with previous reports of colon specific chitosan microspheres.[33]

The drug release from core microspheres showed a burst release in the initial hour as it is clearly evident in Figure 2. About 68–78% of celecoxib was released from core microspheres within 4 h drug, containing drug-to-polymer ratio 1:2–1:8, suggesting the fast release of drug from core microsphere which indicates the celecoxib loaded microspheres released the drug before they reached the colon.[34] These releasing profiles are not suitable for systems targeting to the colon. The release of maximum amount of celecoxib from microsphere is due to the solubility of chitosan in the lower pH (Acidic pH). The release of maximum amount of celecoxib in the upper part of GI tract requires manipulation in the microspheres coated with Eudragit S-100, which is a pH-sensitive polymer having a threshold pH of 7.0

Coated microspheres

The *in vitro* release profiles of the coated chitosan microspheres, in release medium of pH 2, indicate that no drug was released in the first 2 h. When the release studies were conducted in phosphate buffer and pH was gradually increased up to 4.5 %, the drug release started after 4 h, with core to coating ratio ranging from 1:4 to 1:8, and was found to be 13.7 ± 0.1 and 20.4 ± 0.2, respectively [Figure 3]. Drug release decreased with increase in Eudragit s-100 coating ratio because of which its dissolution took longer time. Despite the increase in thickness of coating in microspheres, the release pattern was unaffected due to the increased thickness of celecoxib microsphere. These results are in accordance with earlier reports.[20] The coated microspheres offered delayed and hindered release of celecoxib until it reached the colon. *In vitro,* celecoxib release studies of coated microspheres after 12 h (the pH of the medium beyond 7) released 87.94 ± 5 and 100 ± 62 of celecoxib in case of microspheres of 1:4 to 1:8 coating to core ratios, respectively.

The above obtained *in vitro* drug release studies indicate that coating microspheres successfully protect the drug from degradation in stomach and small intestine, and release maximum drug in the colon region. Celecoxib release rate increased after 4 h since by that time microspheres were exposed to pH above 7.0 which is above the pH at which Eudragit copolymer is soluble. It is expected because Eudragit S-100 is an enteric copolymer made with methacrylic acid-methacrylate, and soluble at pH ≥ 7. After Eudragit was dissolved, then the drug release from microspheres occurred due to swelling of the chitosan, resulting in the formation of a gel. This is followed by drug dissolution and further diffusion through the gel. Our data show higher accumulation of celecoxib at the target site (colon), probably leading to therapeutic enhancement coated microsphere. This would prevent the systemic absorption of celecoxib, thus reducing the cardiovascular side effects of celecoxib.[35]

In vivo evaluation of core microspheres

In the present *in vivo* study, the effect of the celecoxib solution and the celecoxib loaded microspheres was evaluated after

the acetic acid-induced colitis. The acetic acid was used to induce colitis because the acetic acid produces symptoms similar to the human ulcerative colitis. The ulcer formed here shown similarity in respect to many parameters such as histology, eicosanoid production, and excessive oxygen-derived free radicals release by inflamed mucosa.[27]

Figure 4 represent the comparison of macroscopic characteristics of colon after 5 days of colitis induction and 5 days after treatment with the microspheres in comparison to normal colon. Treatment for 5 days with the microspheres significantly reduced the severity of the hemorrhage, and gross lesion score 1.16 was observed. Finally, the colonic damage score (4.1) was assessed and estimated [Figure 5]. In vehicle-treated rats, acetic acid-induced severe colitis was associated with a significant ($P < 0.001$) loss in body weight, and they suffered from general weakness and less activity as shown in Figure 6. Treatment of acetic acid-administered rats, with coated microspheres, significantly prevented the loss of body weight; no illness signs and stool consistency and no bleeding [Tables 4 and 5] appeared in the rats.

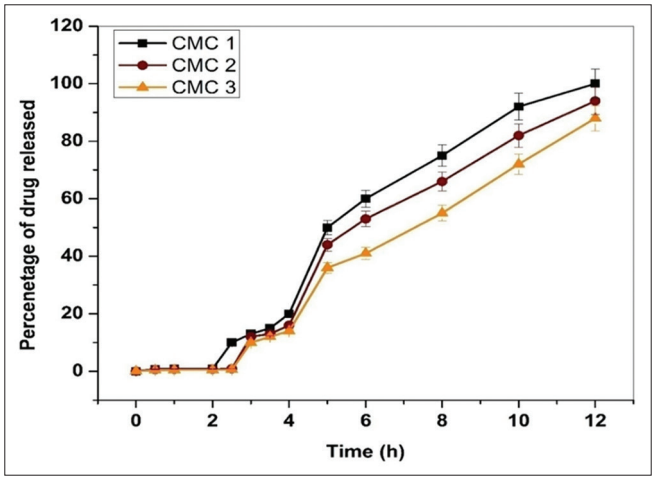

Figure 3: Percentage celecoxib cumulative release from Eudragit coated chitosan microspheres in simulated GI fluids.

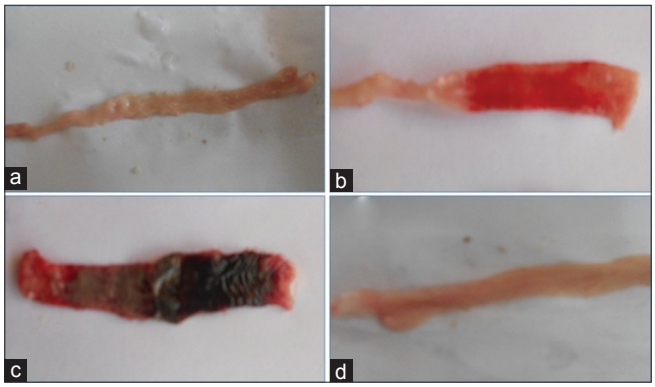

Figure 4: Macroscopic appearance of (a) normal colon, (b) acetic acid treated colon after 24 h and (c) acetic acid treated colon after 5 days (d) acetic acid treated colon after oral administration of microspheres once daily for 5 days. The dose of microspheres was equivalent to 20 mg/kg *n* = 6

In the present investigation, macroscopic study revealed that treatment with the coated microsphere significantly reduced the damage to the colon, induced by acetic acid. The result of present investigation clearly demonstrated that coated microspheres exert a significant attenuation of the extent and severity of the histological signs of cell damage. These results are in agreement with trinitrobenzene sulfonic acid (TNBS)-induced rat colitis.[36] Furthermore, Cuzzocrea group[12] reported that potential protective effect of celecoxib reduces the degree of colitis caused by dinitrobenzene sulfonic acid. Macroscopic examination is considered as one of the sensitive and reliable indicators of the exactness and level of ulcerative colitis.[37] Hence, significant attenuation of the macroscopic changes indicates potential of the coated microsphere in the treatment of ulcerative colitis.

Results were expressed as mean ± S.E.M (N = 6). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test. ***P< 0.001 as compared to the

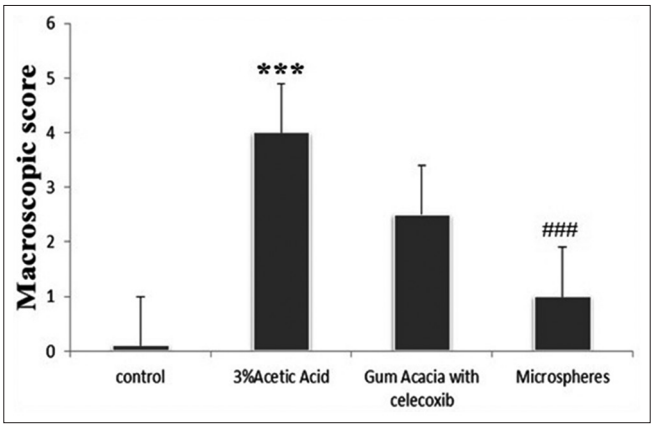

Figure 5: Macroscopic scoring of rat colon biopsy of normal colon, acetic acid-treated colon and acetic acid-treated colon after oral administration of microspheres once daily for 5 days. The dose of microspheres was equivalent to 20 mg/kg *n*=6.

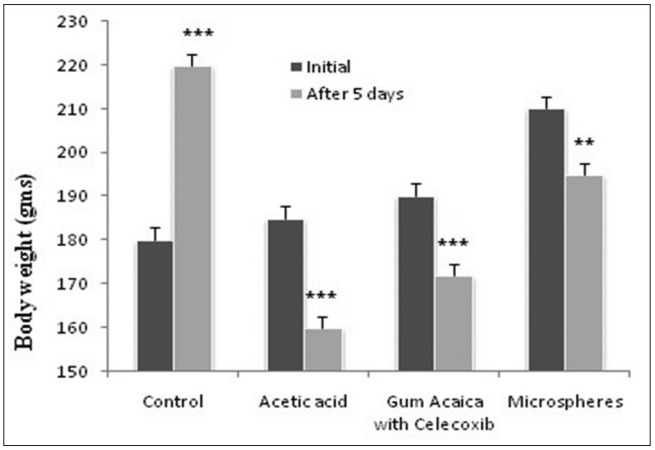

Figure 6: The effect of acetic acid-induced colitis on body weight loss of rats before and after the oral administration of microspheres once daily for 5 days. The dose of microspheres was equivalent to 20 mg/kg. *n* = 6

Table 4: Scoring based on stool consistency

Groups	Rats no	Scoring based on stool consistency					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control	6	0.0	0.0	0.0	0.0	0.0	0.0
3% Acetic Acid	6	2.16 ± 1.16***	3.5 ± 0.22***	3.83 ± 0.16***	4 ± 0.0***	4 ± 0.0***	4 ± 0.0***
2% Gum acacia with celecoxib	6	2 ± 0.25	3.33 ± 0.21	3.16 ± 1.16	3.16 ± 0.16	3 ± 0.25	2.83 ± 0.16
Microspheres	6	1.16 ± 0.16##	2 ± 0.25###	1 ± 0.0###	1 ± 0.0###	1 ± 0.0###	1 ± 0.0###

Results were expressed as mean±S.E.M (n=6). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test. ***P<0.001 as compared to the normal control group, ###P<0.05 as compared to the acetic acid group

Table 5: Scoring based on stool blood

Groups	Rats no	Scoring based on stool blood					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control	6	0.0	0.0	0.0	0.0	0.0	0.0
3% acetic acid	6	1.16 ± 0.166***	2 ± 0.25***	2 ± 0.25***	3 ± 0.25***	4 ± 0.0***	4 ± 0.0***
2% Gum acacia with celecoxib	6	1.5 ± 0.22	1.83 ± 0.16	2 ± 0.25	2.16 ± 0.16	2 ± 0.21	2 ± 0.21
Microspheres	6	1.16 ± 0.16	1.5 ± 0.22	1 ± 0.0###	1 ± 0.0###	1 ± 0.0###	1 ± 0.0###

Results were expressed as mean±S.E.M (n=6). Data were analyzed by one way analysis of variance (ANOVA) followed by Tukey test. ***P<0.001 as compared to the normal control group, ###P<0.05 as compared to the acetic acid group. SEM: Standard error of the mean

normal control group and ###P<0.05 as compared to the acetic acid group.

Results were expressed as mean ± S.E.M (n = 6). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test. ***P < 0.001 as compared to the normal control group and ###P < 0.05 as compared to the acetic acid group.

Effect of coated microspheres on MPO and GSH in the colon

The colitis caused by acetic acid showed a significant (P < 0.05) increase in MPO and a significant decrease in GSH concentration. The levels of MPO activity in the tissues displayed a statistically significant difference among the four tested groups (P < 0.01) as shown in Table 6. By performing pairwise comparisons among these four groups, we can infer that the mean value of acetic acid control group significantly increased (P < 0.001) as compared to saline control group. On the other hand, after treatment with the coated microsphere, a significant decrease in MPO activity was observed as compared to acetic acid control group (P < 0.001). Table 6 summarizes that the GSH concentration in the colon tissues was significantly decreased after induction of colitis as compared to normal control group (P < 0.001). GSH is marker of oxidative stress, and it is decreased when there is an increase in reactive oxygen species (ROS). After treatment with coated chitosan microspheres, there was a substantial increase in the GSH concentration as compared to acetic acid control group (P < 0.001).

There is clear indication that improved formations of ROS play an important role in IBD.[38] Increasing ROS cause cellular oxidative stress by cross-linkage proteins, lipids, and nucleic acid, which then cellular dysfunction, damage and finally death. This further leads to the formation of superoxide ion (O_2^-) by activated neutrophils through NADPH oxidase. Superoxide ion (O_2^-) causes reduction of molecular oxygen to the peroxide ion radical through the enzyme MPO.

Quantitatively, the principal free radical in tissues is superoxide anion (O_2^-), which is converted to H_2O_2 by superoxide dismutase.[39] The enzyme MPO cause reduction of superoxide anion (O_2^-) to molecular oxygen. In this study, we found an increase in MPO activity in the colon was decreased by treatment with coated microspheres. The result obtained, in the present study, from coated microspheres treated acetic acid-induced colitis are in a good correlation with the previous result,[40] which showed that COX-2 inhibitor such as coated microspheres decrease cellular penetration and inflammation of the colon.

It has been largely accepted that both GSH and MPO activity are significant modulators of oxidative stress, its play an important role for tissue damage and IBD. ROS is known to be major cause of IBD. The tri-peptide GSH (L-γ-glutamyl-L-cysteinylglycine, GSH) is the most important intracellular thiol-based antioxidant. As ROS is scavenged by GSH, the deficiency of the GSH is leads to excessive ROS production. This was evaluated using the acetic acid model to test the antioxidant potential of 5-aminosalicylic acid.[41] Decrease in GSH reflects the consumption of tissue thiols. GSH is non-protein sulfahydral compound which decrease is reported in

Table 6: Effect of celecoxib loaded coated chitosan microspheres on MPO levels in the colonic tissue of rats, r-GSH content in the colonic tissue of rats and COX activity

Treatment	Rats no	Dose (mg/kg.bd wt)	MPO levels (units/mg tissue)	r-GSH content (nmol/g tissue)	6-Keto-PGFF1α (pg/mg)
Normal control	6	_	0.48 ± 0.02	1209.12 ± 6.15	214 ± 2.04
3% Acetic acid	6	_	1.09 ± 0.06***	860.63 ± 12.23***	420 ± 5.23***
2% Gum acacia	6	_	1.07 ± 0.04	860.32 ± 4.32	421 ± 3.32
Microspheres	6	5	0.54 ± 0.02###	1123.74 ± 2.53###	214 ± 1.53###

Results were expressed as mean±S.E.M (n=6). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test. ***P<0.001 as compared to the normal control group, ###P<0.05 as compared to the acetic acid group. MPO: Myeloperoxidase, COX: Cyclooxygenase, r-GSH: Reduced glutathione

the accumulation of free radical. Grisham and group have been reported that[42] a reduction in GSH levels following the use of TNBS as a model of experimental colitis. They proposed that the level of GSH is capable of preventing damage in tissues when the antioxidant was neutralized by the liberated ROS. Sulfahydral compounds are among the most important antioxidants. They have a role in the maintenance of cellular proteins and lipid in their functional states. As these are endogenous biochemical of human body, their consumption leads to the toxic effect of oxidative damage resulting in increased membrane and cell damage. Treatment of rats, in this study, was made with coated microspheres by increasing the GSH levels in colonic mucosa. Therefore, it may be suggested that the increased levels of GSH, which we had observed, may be explained to the free radical scavenging capacity of celecoxib from coated microspheres.

Effect of coated microspheres on COX activity

Endogenous prostaglandin E_2 is produced by mononuclear cells in the lamina propria and independent on COX-2 expression. It modulates the intestinal immune response, including differentiation of T cells and the production and release of proinflammatory cytokines. The COX activity in acetic acid-induced colitis was assessed by measuring the increase in the formation of 6-keto-prostogladin F1α in the colon from acetic acid-treated rats. The amount of 6-keto-prostogladin F1α found in the colon from acetic acid treated rats was 420 ± 18 pg/mg/tissue. The amounts of 6-keto-prostogladin F1α in colon were significantly decreased in the colon for the acetic acid-treated rats, which were pretreated with celecoxib loaded chitosan coated microspheres are shown in Table 6.

Determination of colon 6-keto-prostogladin F1α activity, as we now performed, represents COX activity. Therefore, the significant decrease in activity of colon 6-keto-prostogladin F1α by coated microsphere treatment confirms the important role of COX-2 in colon inflammation. This observation is different from the claim that COX-1 makes an important contribution to inflammatory responses.[43]

Our results showed elevation of prostanoid generation in the colonic mucosa of rats with ulcerative colitis and Crohn's diseases. Prostaglandins that contribute to the inflammatory process are derived exclusively from COX-2. Thus, COX-2 inhibitors would exhibit the same anti-inflammatory and analgesic effect. The admiration of the COX-2 inhibitor (celecoxib coated microspheres) produces a significant decrease in tissue prostaglandin production. Therefore, treatment with selective COX-2 inhibitors may reduce the inflammation in experimental colitis by reducing the levels of prostaglandin.

CONCLUSION

From the results of the present study, it can be concluded that celecoxib microspheres, prepared using chitosan as core polymer and Eudragit S-100 as a coating, could be used for a colon targeting drug. The prepared coated microspheres could increase the residence of celecoxib in the colon and avoid celecoxib release in the upper portion of the gut. *In vivo* results demonstrate that celecoxib is a selective COX-2 inhibitor and protective in experimental colitis. These results revealed that treatment with coated microspheres reduces oxidative stress associated with experimental colitis. Therefore, this will provide strong evidence for the celecoxib loaded coated microspheres for colon target delivery, as a new method for the treatment of ulcerative colitis.

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SUPPLEMENTARY

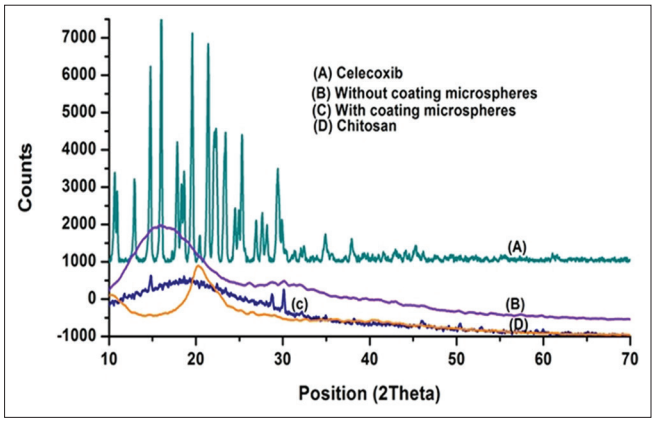

Figure 1: X-ray diffractograms of celecoxib, chitosan, with and without EudragitS-100 coated microspheres.

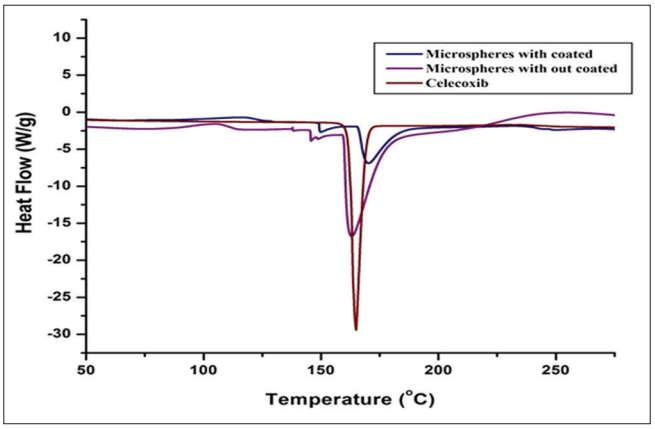

Figure 2: DSC thermogram of celecoxib, with and without Eudragit S-100 coated microspheres