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Synthesis, *in-vitro* characterization and pharmacological evaluation of colon specific conjugates of naproxen and polysaccharides

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Abstract

The aim of the study was to prepare site specific drug delivery of naproxen using polysaccharides by the formation of glycosidic linkage which are hydrolysed by the microflora present in colon. This approach prevents drug release in the upper gastrointestinal environment. Due to the minimal degradation of conjugates in upper gut, the *in vitro* drug release in SGF, SIF and SCF was found up to 3.82±0.03%, 12.35±0.06% and 92.23±3.57% respectively.

Keywords: Colon specific drug delivery, conjugates, naproxen

1. Introduction

Various drug delivery approaches have been explored for successful delivery of drugs to the target site. However, the oral route of administration is considered to be the most convenient and preferred route for a sustained as well as controlled drug delivery system^[1]. Various drug delivery strategies have been employed to trigger the release of drugs to the large intestine; however, they do not reach the site of action in appropriate concentrations. Thus, to ensure an effective and safe therapy for the large bowel diseases, the colon specific drug delivery system is considered to be the preferable approach^[2]. Targeting of drugs to the colon offers several potential therapeutic advantages, like increasing the systemic absorption of poorly absorbed drugs and effective treatment of the colonic diseases such as amoebiasis, ulcerative colitis, Crohn's disease, colorectal cancer, *etc.* This delivery system can be also used in certain conditions where drugs should be delivered after a lag time, like in chronopharmacotherapy of diseases showing Circadian rhythms in their pathophysiology^[3]. Several available approaches for targeting the drug selectively to the colon include pH sensitive polymers, time dependent dosage forms, use of carriers degraded by enzymes produced by colonic bacteria, prodrug based approaches, bioadhesive and osmotically controlled drug delivery systems^[4]. Colon specific drug delivery (CDDS) are advantageous owing to the fact that, in comparison with conventional dosage forms, CDDS provide a more consistent and reproducible transit through the gastrointestinal tract (GIT). Moreover, they also provide more uniform drug dispersion in the GI tract, which results in more homogeneous drug absorption. This helps in predicting gastric emptying, increases colon residence time, decreases local irritation^[5]. Naproxen [(S)-2-(6-methoxynaphthalen-2-yl)propanoic acid], is a non selective COX inhibitor widely used as an analgesic in the treatment of rheumatoid arthritis and colitis. It can be used in FAP and in colon cancers due to its inhibitory action on COX-2 enzymes^[7, 8]. Moreover, because of the same mode of action, it shows synergistic action with that of anticancer drugs. The polysaccharides as carrier of NSAIDs to target colon is much better approach by formation of glycosidic conjugates. It is biocompatible, biodegradable, non-toxic and shows mucoadhesive properties as well. Due to its mucoadhesion nature, its residence time in the colon can be increased, which subsequently results in maximum bioavailability^[9]. Taking the above information into account, a study was designed for the preparation and characterization of naproxen conjugates for the colon drug delivery system^[10].

2. Methods and Materials

2.1 Materials

Naproxen was procured as gift samples from Microlab Pvt. Ltd., Bangalore India. Polysaccharides like dextran was purchased from Himdia, Mumbai, India; chitosan was obtained as gift sample from Central Institute of Fisheries Technology, Cochin, India.

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Pectin and Inulin was purchased from Loba Chemical Pvt. Ltd, Mumbai. All other chemical and reagent were used of analytical grade.

2.2 Methods

2.2.1 Preparation of Drug Conjugates¹¹⁻¹³

2.2.1.1 Chemical modification of naproxen

(a) Reduction of Naproxen

The weighed naproxen (0.03 mole) and LiAlH₄ (0.03 mole) in diethyl ether were refluxed in a water bath for 2 hr. Then it was cooled, filtered and washed with 10 mL diethyl ether for three times and finally washed with water and dried. Yield 72%; M.P. 148-151 °C.

(b) Chlorination of 2-(6-methoxynaphthalen-2-yl) propanol

The compound 2-(6-methoxynaphthalen-2-yl) propanol (0.015 mole) and thionyl chloride (1.6 mL, 0.015 mole) were stirred at 0 °C for 1 hr and then refluxed for 2 hr cooled and poured on to ice cold water. The compound was washed with distilled water several times to remove any unreacted thionyl chloride and recrystallized using purified with ethanol-water and dried. Yield 64%; M.P. 158-159 °C.

2.2.1.2 Conjugation of modified naproxen with polysaccharides

(a) Synthesis of Dextran – Naproxen Conjugate (DNp)

To the solution of dextran in 25 mL of DMF in a round bottom flask, 2-(6-methoxynaphthalen-2-yl) 1-chloropropane (0.008 mole), mercuric bromide (0.008 mole) and yellow mercuric oxide (0.004 mole) were added, stirred and refluxed for 2 hr. The obtained suspension was filtered and the filtrate was dried in a rotatory vacuum evaporator at 80 °C; 100 rpm; 400 mm Hg. The product was recrystallized using methanol-distilled water (1:2), filtered using Whatmann filter paper, washed with distilled water and then dried. Yield 70%; M.P. 238-240 °C.

(b) Synthesis of Pectin – Naproxen Conjugate¹⁻³ (PNp)

To a solution of pectin in 25 mL DMF in a round bottom flask 2-(6-methoxynaphthalen-2-yl) 1- chloropropane (0.008 mole) was added slowly with stirring. Then mercuric bromide (0.008 mole) and yellow mercuric oxide (0.004 mole) were added, stirred and refluxed for 2 hr. The obtained suspension was filtered, dried in a rotatory vacuum evaporator at 75 °C; 100 rpm; 300 mm Hg, recrystallized using methanol-distilled water. The product was filtered using Whatmann filter paper and dried. Yield 51%; M.P. 237-239 °C.

(c) Synthesis of Inulin – Naproxen Conjugate¹⁻³ (INp)

Inulin was dissolved in minimum quantity of water and to it 2-(6-methoxynaphthalen-2-yl) 1- chloropropane (0.008 mole) in 25 mL DMF was added. Then mercuric bromide (0.008 moles) and yellow mercuric oxide (0.004 mole) were added, stirred and refluxed for 2 hr. The obtained suspension was filtered, dried in a rotatory vacuum evaporator at 90 °C; 100 rpm; 400 mm Hg and recrystallized using methanol- distilled water. Yield 56%; M.P. 241-243 °C.

(d) Synthesis of Chitosan-Naproxen Conjugate¹⁻³ (CNp)

Chitosan dissolved in minimum quantity of 2% glacial acetic acid, 25 mL DMF containing (0.008 mole) 2-(6-methoxynaphthalen-2-yl) 1- chloropropane was added under stirring. Then mercuric bromide (0.008 mole) and yellow

mercuric oxide (0.004 mole) were also added to the solution, stirred and refluxed for 2 hr and then finally stirred at room temperature for 12 hr. The obtained suspension was filtered, dried in a rotatory vacuum evaporator at 90 °C; 100 rpm; 400 mm Hg and recrystallized using methanol-distilled water. The product was filtered using Whatmann filter paper and dried. Yield 52%; M.P. 249-251 °C.

3. Characterization

3.1 Thin Layer Chromatography (TLC): the purity of the compound was checked by TLC using silica gel G, suitable solvent systems and detecting agent; iodine vapors

3.2 FT-IR and ¹HNMR : The IR spectrum of the synthesized conjugates was recorded on jasco V -530 FTIR in potassium bromide (anhydrous, IR grade).The ¹HNMR spectrum of conjugates was recorded in DMF, using a ¹HNMR Varian Mercury 300 Hz, with super conducting magnet.

3.3 Swelling Study of Conjugates^[14]

Accurately weighed amount of each of the conjugates (W₀) was dipped in the swelling medium SGF pH 1.2, SIF-1 pH 4.5, SIF-2 pH 6.8 and SCF pH 7.0. for 2 hr. At predetermined time intervals, the gel was removed from the swelling medium, blotted with filter paper to remove excess water from the gel surface, and the swollen hydrogels (W₁) was weighed. The swelling ratio (SR) is calculated according to the following equation. The results are given in table 1.

$$SR = \frac{W_1 - W_0}{W_0}$$

Where W₁- the weight of the swollen gel
W₀- the weight of the conjugate

3.4 In-Vitro Drug Release Studies

In-vitro drug release studies were carried out according to Sounders and Ellenbogen (1985)^[11] extraction techniques using USP dissolution test apparatus (apparatus 2). The dissolution studies were carried out in different dissolution medium (900 mL) including simulated colonic fluid (4.0%w/v), which was stirred at 100 rpm at 37±0.2 °C^[15]. Samples were withdrawn periodically and compensated with an equal volume of fresh dissolution media. The drug content in the withdrawn samples was estimated spectrophotometrically at λ_{max} 271 nm for conjugates of Naproxen. The results were given in fig1.

4. In-Vivo Animal Study^[16]

4.1 Ulcerogenic Activity

The ulcerogenic activity was determined by the Rainsford's cold stress method, which is used to determine ulcerogenic potency of a drug at a ten times higher dose. Albino rats were distributed in healthy control, standard group and test groups. Doses of each of the synthesized compounds were first calculated on equimolar basis of pure drug and then were converted into ten times higher doses. Formulation of synthesized compounds and standard drug were administered orally. After oral administration of 5 mL of the aqueous drug suspensions (at 10 times the normal dose), the animals were stressed by exposure to -15° for 1 h. The animals were placed in separate cages, to ensure equal cold exposure. After 2 hrs

of drug administration, the rats were sacrificed using isoflurane anaesthesia, the stomach and duodenum were dissected out of the body along with the first 5 cm of the intestine, then rinsed with saline and the contents of the stomach were emptied. The stomach and the intestine were then excised open along the greater curvature and gently wiped clean with a swab dipped in saline. The mucosal damage was examined grossly using a magnifier. A score for the ulcer was studied similar to pyloric ligation induced ulcer model [17]. The results were given in table 3.

Scoring of ulcer will be made as follows :

Normal stomach/intestine.....	(0.0)
Red coloration.....	(0.5)
Spot ulcer.....	(1.0)
Hemorrhagic streak.....	(1.5)
Ulcers.....	(2.0)
Perforation.....	(3.0)

Mean ulcer score for each animal will be expressed as ulcer index (UI):

$$UI = (UN + US + UP) \times 10^{-1}$$

UN = Average of number of ulcer per animal

US = Average of severity score

UP = Percentage of animal with ulcer

4.2 Colonic edema study

A section of inflamed colon of healthy control, colitis control, standard control and test control group on twelfth day in TNBS induced animal model after treatment with naproxen and its conjugates, was weighed then dried in an oven (80 °C) for 24 hr and then reweighed to determine the wet-to-dry weight ratio, an indicator of colon edema [17]. The results were given in table 2.

5. Result and Discussion

5.1 Results of Thin layer Chromatography

Reduced compound of naproxen, the purity of the compound was checked by TLC using silica gel G, solvent system; n-hexane: ethyl acetate (8:2 v/v), detecting agent; iodine vapors. Only one spot was obtained, R_f - 0.64.

After chlorination of 2-(6-methoxynaphthalen-2-yl) propanol, the purity of the compound was checked by TLC using silica gel G, solvent system; n-hexane: ethyl acetate (8:2 v/v), detecting agent, iodine vapors. Only one spot was obtained, R_f - 0.66.

The purity of Dextran – Naproxen Conjugate was checked by TLC using silica gel G, solvent system; chloroform: methanol: formic acid (5:1:0.5), detecting agent; iodine vapors. Only one spot was obtained, R_f - 0.67.

The purity of Pectin – Naproxen Conjugate was checked by TLC using silica gel G, solvent system; chloroform: methanol: formic acid (5:1:0.5), detecting agent, iodine vapors. Only one spot was obtained, R_f - 0.71.

The purity of Inulin – Naproxen Conjugate was checked by TLC using silica gel G, solvent system; chloroform: methanol: formic acid (5:1:0.5), detecting agent, iodine vapors. Only one spot was obtained, R_f - 0.64.

The purity of Chitosan-Naproxen Conjugate was checked by TLC using silica gel G, solvent system; chloroform: methanol: formic acid (5:1:0.5), detecting agent; iodine vapors. Only one spot was obtained, R_f - 0.69.

5.2 Structural elucidation

Reduced compound of naproxen IR (KBr) spectrum: 3422.62

(OH str), 3038.27(CH str aromatic), 2939.73(CH str, aliphatic), 2874.33 (CH str, aliphatic), (1604.5, 1570, 1460, 1410.81, C=C str in benzene) 1223.15(C-O-C str) cm^{-1} . The disappearance of peak at 1727.27 cm^{-1} , and appearance of peak at 3422.62 cm^{-1} (m; OH_{str}), confirms that the carboxyl group has been reduced to methyl hydroxyl group. ¹H NMR; (ppm): (ppm): 1.53 (CH₃, 3H, d), 1.9 (CH, 1H, q), 3.7 (OCH₃, 3H, s) 7.1 - 7.3 (benzene, m), 4.5 (OH, 1H, s).

After chlorination of 2-(6-methoxynaphthalen-2-yl) propanol, IR (KBr) spectrum: 3038.27(CH str aromatic), 2939.73(CH str, aliphatic), 2874.33(CH str, aliphatic), (1604.5, 1570, 1460, 1410.81 C=C str in benzene), 1223.15 (C-O-C str), 701.71(Cl str) (cm^{-1}).

IR: 701.71 cm^{-1} (Cl_{str}), confirms the formation of chloro derivative of Naproxen. ¹H-NMR (ppm): 1.53 (CH₃, 3H, d), 1.9 (CH, 1H, q), 3.7 (OCH₃, 3H, s) 7.1 - 7.3 (benzene, m) 3.2(CH₂, 2H, d).

IR spectrum of Dextran – Naproxen Conjugate was shown some characteristic peaks at 3462.73(m; OH_{str}), 3038.27(CH str aromatic), 2939.73(CH str, aliphatic), 2874.33(CH str, aliphatic), (1604.5, 1570, 1460, 1410.81 C=C str in benzene), 1179.15(glycosidic linkage str), 866.49 cm^{-1} (cm^{-1}) IR: The disappearance of Cl str peak at 701.71 cm^{-1} and appearance of peak at 1179.15 cm^{-1} (m; characteristic peaks in glycoside); ¹H NMR: δ 3.36 ppm (d) indicate formation of glycosidic bond between drug and polysaccharide.

IR spectrum of Pectin – Naproxen Conjugate was shown some characteristic peaks at 3449.91(m; OH_{str}), 3038.27(CH str aromatic), 2939.73(CH str, aliphatic), 2874.33(CH str, aliphatic), 1749.52(C=O str), (1604.5, 1570, 1460, 1410.81 C=C str in benzene), 1223.63(C-O str), 1170.15(glycosidic linkage str), (cm^{-1}) IR: The disappearance of Cl str peak at 701.71 cm^{-1} and appearance of peak at 1170.15 cm^{-1} (m; characteristic peaks in glycoside); ¹H NMR: δ 3.54 ppm (d) indicate formation of glycosidic bond between drug and polysaccharide.

IR (KBr) spectrum of Inulin – Naproxen Conjugate was shown some characteristic peaks at: 3508.41(m; OH_{str}), 3038.27(CH str aromatic), 2938.73(CH str, aliphatic), 2874.33(CH str, aliphatic), (1604.5, 1570, 1460, 1410.81 C=C str in benzene), 1223.63(C-O str), 1178.15(glycosidic linkage str) (cm^{-1}) The disappearance of Cl str peak at 702.63 cm^{-1} and appearance of peak at 1178.15 cm^{-1} (m; characteristic peaks in glycoside); ¹H NMR: δ 3.52 ppm (d) indicate formation of glycosidic bond between drug and polysaccharide.

IR spectrum of Chitosan-Naproxen Conjugate was shown some characteristic peaks at 3538.31(m; OH_{str}), (3390.31, 3308.67 NH str), 3038.27(CH str aromatic), 2939.73(CH str, aliphatic), 2874.33(CH str, aliphatic), (1604.5, 1570, 1460, 1410.81 C=C str in benzene), 1223.63(C-O str), 1169.25(glycosidic linkage str), (cm^{-1}) IR: The disappearance of Cl str peak at 701.71 cm^{-1} and appearance of peak at 1169.25 cm^{-1} (m; characteristic peaks in glycoside); ¹H NMR: 3.63 ppm (d) indicate formation of glycosidic bond between drug and polysaccharide.

5.3 Swelling Index

Drug release from swellable conjugates depends on the degree of gelation, hydration, chain relaxation and erosion of the polymer. Swelling studies were performed in simulated fluids to evaluate drug release kinetics of DNp, PNp, INp and CNp and 6.01±0.19, 4.67±0.03, 3.70±0.12 and 2.65±0.09 swelling ratio were found respectively. Conjugates synthesized with dextran showed higher swelling than the conjugates with

chitosan in SGF.

DNp, PNp, INp and CNp showed 7.48 ± 0.33 , 7.20 ± 0.35 , 6.99 ± 0.18 and 4.08 ± 0.12 swelling ratio respectively in SIF II. Drug conjugates with pectin and dextran showed higher swelling than conjugates with chitosan in SIF II.

DNp, PNp, INp and CNp showed 6.89 ± 0.31 , 6.01 ± 0.33 , 4.98 ± 0.25 and 3.47 ± 0.12 swelling ratio respectively in SIF I. Swelling studies of drug conjugates with pectin and dextran

showed higher swelling than conjugates with chitosan in SIF I.

Swelling studies of the drug conjugate were also performed in SCF the swelling ratio 29.36 ± 1.39 , 24.98 ± 1.25 , 26.32 ± 1.23 and 34.81 ± 1.22 were found in case DNp, PNp, INp and CNp respectively. Drug conjugates with chitosan showed maximum swellability while conjugates with pectin showed minimum swellability in SCF.

Table 1: Swelling Ratio of the Synthesized Conjugates in Simulated Fluids

Conjugates	Simulated gastric fluid	Simulated intestinal fluid I	Simulated intestinal fluid II	Simulated colonic fluid
DNp	6.01 ± 0.19	6.89 ± 0.31	7.48 ± 0.33	29.36 ± 1.39
PNp	4.67 ± 0.03	6.01 ± 0.33	7.20 ± 0.35	24.98 ± 1.25
INp	3.70 ± 0.12	4.98 ± 0.25	6.99 ± 0.18	26.32 ± 1.23
CNp	2.65 ± 0.09	3.47 ± 0.12	4.08 ± 0.12	34.81 ± 1.22

Values represent Mean \pm SD, n=6

5.4 In-Vitro Drug Release

In-vitro drug release studies showed drug release upto $3.82 \pm 0.03\%$ in SGF, $12.35 \pm 0.06\%$ in SIF and $92.23 \pm 3.57\%$ from conjugates in SCF which confirm the stability of drug conjugates in SGF and would have more potential for colon specific delivery. The drug release studies in various dissolution media were observed. The initial drug release in SGF might be due to the fact that a small number of charges present in SGF might have allowed a faster drug release as a result of higher solvent penetration into the polymeric network. This would have resulted into a faster rate

of polymer hydration in acidic pH of SGF. Once the outer layer is hydrated/gelled, it acts as a barrier for the drug release and the drug then slowly diffuses out independent of pH. The drug release in SIF I and SIF II could be attributed to the fact that there might be an ion exchange phenomenon between these fluids, but release of drug from conjugates in colon is due to presence of microfloral enzymes which degrade polysaccharide as well as hydrolysis of glycosidic linkage in conjugates. The results of this study revealed that the conjugates would be suitable for colonic delivery system by the formation of conjugates of naproxen with polysaccharides.

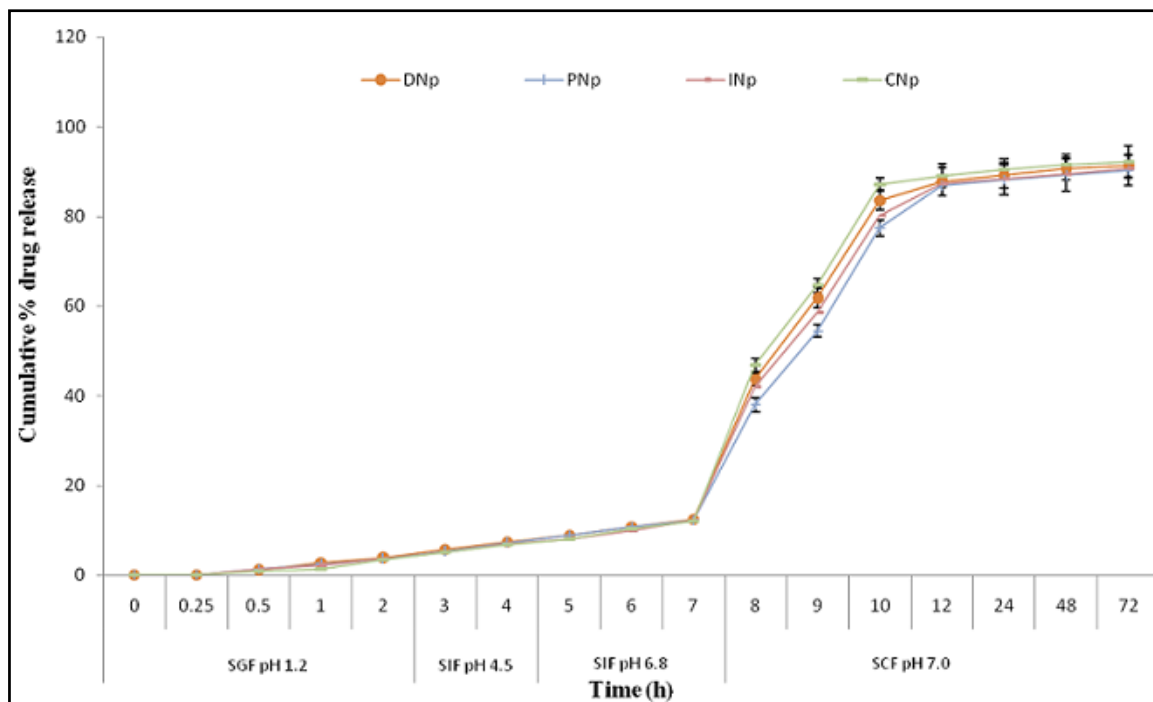


Fig 1: Cumulative % drug release from conjugates Colonic edema study

A section of affected colon was weighed, dried in an oven (80 °C) for 24 hr and then reweighed to determine the wet-to-dry weight ratio, an indicator of colon edema. this pharmacological evaluation shows that the wet-to –dry weight ratio of animals of test groups was very close to healthy group. This study confirms better anti-inflammatory activity of conjugates as compared to parent drug. The Wet/dry weight ratio of Healthy, Colitis control, Naproxen, DNp, PNp, INp and CNp was 4.2 ± 0.22 , 6.4 ± 0.23 , 6.2 ± 0.24 , 4.50 ± 0.19 , 5.12 ± 0.22 , 4.62 ± 0.23 and 4.30 ± 0.19 respectively.

Table 2: Wet/Dry Weight Ratio of the Albino Rats Colon

S. No.	Groups	Wet/dry weight ratio
1	Healthy	4.2 ± 0.22
2	Colitis control	6.4 ± 0.23
3	Naproxen	6.2 ± 0.24
4	DNp	4.50 ± 0.19
5	PNp	5.12 ± 0.22
6	INp	4.62 ± 0.23
7	CNp	4.30 ± 0.19

Values represent mean \pm SD (n=6)

5.5 *In-vivo* ulcerogenic activity

The ulcerogenic activity was determined by the Rainsford's cold stress method, which is used to determine ulcerogenic potency of a drug at a ten times higher dose. Albino rats were distributed in healthy control, standard group and test groups. Doses of each of the synthesized compounds were first calculated on equimolar basis of naproxen (20 mg /kg) and then were converted into ten times higher doses. The conjugates of drug shown very less ulcer index as compared to pure drug which were Healthy(2.12±0.57), Naproxen(35.32±0.54), DNp(4.82±0.22), PNp (5.71±0.19), INp(8.45±0.16) and CNp (3.62±0.24).

Table 3: Ulcerogenic activity of drug/formulations

S. No.	Group	Ulcer Index
1	Healthy	2.12±0.57
2	Naproxen	35.32±0.54
3	DNp	4.82±0.22
4	PNp	5.71±0.19
5	INp	8.45±0.16
6	CNp	3.62±0.24

6. Conclusion

Polysaccharide conjugates of naproxen have the potential to deliver naproxen in colon with maximal concentration, providing the protection against upper gut environment. The naproxen conjugates established its efficacy an anti-inflammatory prodrug moiety in experimentally induced colitis that had insignificant ulcerogenic potential. Conclusively an appropriate dosage form of conjugate can be considered as therapeutically efficacious system for treatment of colitis, with reduced gastric intolerance.

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