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Azo-containing urethane analogues for colonic drug delivery: synthesis, characterization and in-vitro evaluation

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Abstract

A urethane-based analogue containing an azo aromatic linkage in the backbone was synthesized for use in colon-specific delivery systems by reacting toluene-2,6-diisocyanate with a mixture of an aromatic azo diol, (bis-4-hydroxyphenyl)-4,4′-diazobiphenyl, poly(ethylene glycol) (Mn = 4000; number-average molecular weight) and 1,2-propanediol (propylene glycol). The resultant compounds (UR-1 and UR-2) were characterized by IR spectroscopy, ^{1}H NMR spectroscopy, DSC studies, X-ray diffraction studies and molecular weight determination by gel permeation chromatography. The compounds exhibited low molecular weight, lacked film-forming properties and crystallinity in the structure. An in-vitro bacterial degradation test to demonstrate the susceptibility of azo bond to bacterial enzymes was performed using media inoculated with lactobacillus culture. The results indicated degradation of films by azoreductase. In-vitro permeation of 5-aminosalicylic acid was studied in control and lactobacilli-treated films. The permeability of the lactobacilli treated films was significantly increased suggesting the potential of these compounds for application in colonic targeting.

Introduction

Improved drug delivery systems are required for drugs currently in use to treat localized diseases of the colon. Such diseases are most effectively treated by local delivery of anti-inflammatory agents to the large intestine. However, these agents are either delivered inefficiently and unpleasantly by large volume rectal enema or exhibit unacceptable side-effects after oral administration because of absorption in upper regions of the gastrointestinal tract (Friend 1991). Thus, oral colon-specific drug delivery is of increasing importance. A number of different physiological triggers are proposed for targeted release of drugs in the colon. One approach is to utilize the plethora of microorganisms in the lower gastrointestinal tract (Lloyd et al 1994). The ability of microorganisms to selectively metabolize certain carbohydrates or azo groups has been effectively exploited in the development of polymers for colonic drug delivery systems. A universal approach to utilize bacterial degradation of azo bonds to achieve site-specific release has been the basis of the synthesis of polymers suitable for coating (Saffran et al 1986), and use of hydrogels with azo–aromatic cross-links (Bronsted & Kopecek 1992). Earlier studies in the development of azo polymers have shown that when dosage forms coated with polymers reached the colon, the azo groups were reduced to hydrazo groups by the action of azoreductases liberated by intestinal microflora

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(Kimura et al 1992). However, preparation and coating of such cross-linked azo polymers was very difficult because of the poor solubility of commonly used solvents. This suggested a need for the development of soluble, colon-degradable polymers.

This study presents the synthesis of urethane-based analogues containing azo aromatic groups that can be degraded by colonic bacteria. With the aim of synthesizing polymers with film-forming properties and a good solubility profile, azo containing urethane analogues were prepared by reaction of (bis-4-hydroxyphenyl)-4,4′-diazobiphenyl (HPDB), poly(ethylene glycol) (PEG) and toluene 2,4-diisocyanate (TDI). Films cast using these analogues in combination with another film-forming polymer, ethylcellulose (EC) were studied for in-vitro degradation and in-vitro permeation of 5-aminosalicylic acid (ASA).

Materials and Methods

Materials

The aromatic azo diol HPDB was synthesized as indicated in Figure 1. PEG-4000 and 1,2-propanediol (propylene glycol; PG) were procured from S. D. Fine Chemicals Ltd, Mumbai, India. TDI was a gift sample from Expanded Incorporation, Mumbai, India. ASA was a gift sample from Unichem Laboratories Ltd, Mumbai, India. EC (45 cps) was a gift from Crown Chemical Company. All other chemicals and solvents used were of analytical reagent grade. The culture of lactobacilli was obtained by inoculating curd in fluid thioglycolate medium and was maintained anaerobically at 37°C.

IR spectra of the compounds were recorded on a Jasco FTIR 5300 spectrometer using the KBr pellet method. ^{1}H NMR spectra were recorded on a Bruker AMX-500 FT NMR at 298K with tetramethylsilane as an internal standard. The DSC scans were obtained using a differential scanning calorimeter (Shimadzu Thermal Analyser, DT-40) equipped with a monitor and a computerized thermal analyser system 40-1 printer. The instrument was calibrated using indium as a reference standard. Powder X-ray diffraction patterns of the compounds were recorded using a Phillips X-ray diffractometer (PW 1130/00) with a copper target, voltage 45 KV, current 30 mA and a scanning rate of 2° min^{-1}. Ultraviolet spectra were recorded on a Shimadzu UV-160A UV-visible spectrophotometer.

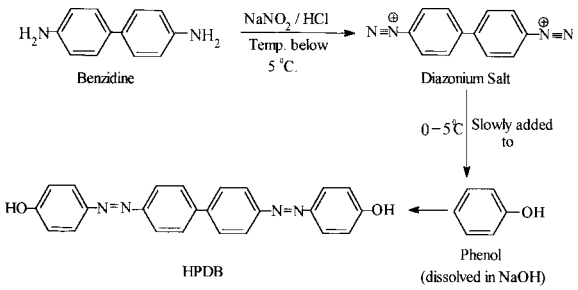

Figure 1 Synthesis of (bis-4-hydroxyphenyl)-4,4′-diazobiphenyl (HPDB).

Preparation of HPDB

HPDB was synthesized as indicated in Figure 1. Benzidine (5 g, 27.13 mmol) was dissolved in a mixture of concentrated hydrochloric acid (14 mL) and water, and the solution was cooled to below 5°C. Chilled solution of sodium nitrite (4.12 g, 59.7 mmol) in water was added slowly to the benzidine solution under continuous stirring and the temperature of the reaction mixture was maintained between 0 and 5°C. The diazonium salt thus formed was maintained in an ice bath at below 5°C. An alkaline solution of phenol (5.11 g, 54.27 mmol) in sodium hydroxide was prepared and cooled to 5°C. Chilled diazonium salt was added dropwise to cold phenol solution under continuous stirring. After complete addition, the reaction mixture was allowed to stabilize in the ice bath for 45 min with occasional stirring. The solution was then filtered under vacuum and the product collected. The product was purified by dissolving it in an aqueous solution of sodium hydroxide (10% w/v) followed by re-precipitation by slow addition of concentrated hydrochloric acid. The precipitated product was recrystallized in a mixture of ethanol and water (90:10), separated by vacuum filtration and finally air-dried.

Preparation of urethane analogue with an azo aromatic linkage

Synthesis of the urethane analogue was carried out as outlined in Figure 2. HPDB (3 g, 27.3 mmol) and PEG (4 g, 1 mmol) were placed in a 100-mL three-necked round-bottomed flask equipped with a mechanical stirrer and a dropping funnel. The flask was evacuated for 12–16 h and then flushed with dry nitrogen gas. PG (5.18 g, 245 mmol) was added to the flask and the contents heated to 120°C in an oil bath with stirring

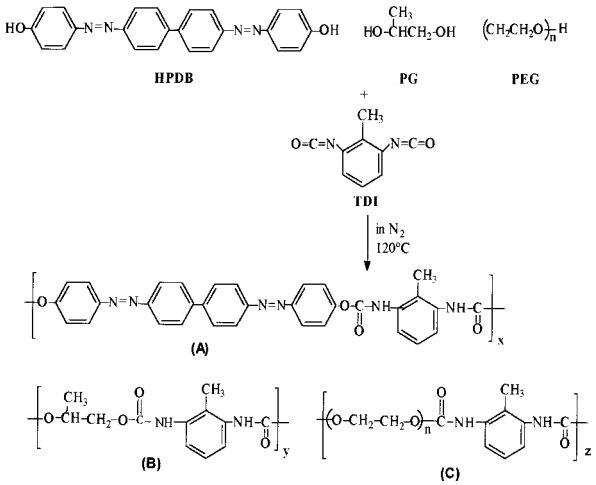

Figure 2 Synthesis of the urethane analogues containing an azo aromatic linkage. HPDB, (bis-4-hydroxyphenyl)-4,4′-diazobiphenyl; PEG, poly(ethylene glycol); PG, propylene glycol; TDI, toluene 2,4-diisocyanate. A, B and C represent portions of the polymer resulting from reaction of TDI with HPDB, PG and PEG respectively.

Table 1 Composition of urethane analogues, UR-1, UR-2 and UR-3.

Analogue	HPDB (mM)	PEG-4000 (mM)	PG (mM)
UR-1	27.3	1.0	245.0
UR-2	27.3	2.0	244.0
UR-3	27.3	4.0	242.0

HPDB, (bis-4-hydroxyphenyl)-4,4′-diazobiphenyl; PEG, poly(ethylene glycol); PG, propylene glycol.

under a nitrogen flow. TDI (13.227 g, 273 mmol) was added to the flask very slowly from a dropping funnel over a period of 1 h. Stirring was continued until the viscosity increased. At the end of the reaction, 10 mL ethanol was added to the reaction mixture to stabilize the terminal isocyanate groups. Completion of the reaction and absence of HPDB and TDI was monitored by thin-layer chromatography (TLC). TDI, HPDB and reaction mixture were spotted on a pre-coated silica plate and the chromatogram was developed using a mixture of chloroform and methanol (9.9:0.1) as mobile phase. The products were purified by dissolving in tetrahydrofuran (THF) and re-precipitation with *n*-hexane. The precipitate was filtered, washed with *n*-hexane and dried at room temperature in air. Urethane analogues with three different compositions were prepared as outlined in Table 1.

Evaluation of film-forming properties and determination of molecular weight

Urethane analogues (UR-1 and UR-2) were dissolved in methanol, water, acetone, dimethylformamide (DMF), THF, methylene chloride and a mixture of methylene chloride and methanol (7:3) to form a 10% w/w solution. These solutions were then poured on different substrates such as glass, teflon and release liner membrane (No. 1022; 3M Company, Germany) and film formation evaluated after the solvent was allowed to evaporate at room temperature. The same procedure was repeated with addition of plasticizers, diethyl phthalate and dibutyl phthalate (10% by weight of compound) to the solution of urethane analogue. After complete evaporation of the solvent, attempts were made to remove films from the substrates.

The molecular weight of compounds was determined by gel permeation chromatography (GPC) on a Waters instrument equipped with an R 401 differential refractometer. A Styragel HR 1, 3 and 4 column (7.8 mm × 300 mm; Waters) was used with THF as eluent. Molecular weights were calibrated with polystyrene standards.

In-vitro bacterial degradation test

Preparation of isolated films

The compound prepared did not show any film-forming properties. Films of the compound were therefore prepared by mixing it with another film-former, EC (45 cps). A 10% w/w of the polymer solution (EC:UR, 4:1) in the mixture of methylene chloride and methanol (7:3) was prepared. The films were cast on a release liner membrane (No. 1022; 3M Company). After complete evaporation of the solvent, the films were removed from the release liner and dried to a constant weight at 45°C.

Bacterial degradation test

Lactobacillus species was selected for degradation studies as these organisms produce and release azoreductase (Van den Mooter et al 1992). The films were incubated for 4–7 days at 37°C anaerobically in the V.P.I. diluent fluid (Van den Mooter et al 1992) medium inoculated with the lactobacillus culture. Films incubated in media without microorganisms under identical conditions were used as controls. After incubation, films were rinsed thoroughly with distilled water, dried and evaluated for in-vitro degradation by visual inspection, microscopy and spectroscopy.

For UV measurements, the untreated films (films without incubation), control films (films incubated in

absence of lactobacilli) and treated films (films incubated in presence of lactobacilli) were dissolved quantitatively in a mixture of methylene chloride and methanol (7:3) and the solution diluted appropriately to give an accurate concentration of approximately 100 μg mL^{-1}. Solutions were scanned on a UV-visible spectrophotometer in the region of 200–800 nm and λ_{max} recorded. Absorptivity (1%, 1 cm) at the respective λ_{max} was calculated for all films. Untreated, treated and control films were also inspected visually and examined under a microscope, (Photomicrograph (Hund Wetzlar), with magnification 10×) for the presence of degradation. Reflecting light was used for observations.

In-vitro permeation studies

To evaluate the effect of azoreductase on the permeability of polymer films, in-vitro permeation of ASA across the films was studied in a Keshary-Chien cell. Films were prepared from a 4% w/w solution of EC and UR-2 (4:1) in a mixture of methylene chloride and methanol (7:3). Films were incubated anaerobically for 2 days at 37°C with lactobacillus culture in fluid thioglycolate medium. Films were then washed with distilled water and mounted in Keshary-Chien cells. The receptor compartment of cells contained phosphate buffer (pH 7.4) and the donor compartment was filled with 2 mL ASA solution (1 mg mL^{-1}) in phosphate buffer (pH 7.4). Receptor fluid was stirred continuously with a magnetic needle and maintained at 37 ± 1°C with a circulating water bath. Samples (1 mL) were periodically withdrawn from the receptor compartment and replaced with the same volume of fresh buffer. Samples were analysed spectrophotometrically at 331 nm after appropriate dilution and the amount of ASA permeated across the film was calculated. The experiments were performed in triplicate and means ± s.d. for each time point were calculated. Similar permeation studies were carried out for control films (films incubated in absence of lactobacillus culture).

Results and Discussion

Preparation of HPDB

HPDB was obtained as a black, shiny, fine powder (mp 285–288°C). Figure 3 shows the IR spectrum of HPDB with characteristic bands at 3408 cm^{-1} (a broad peak), 1468 cm^{-1} and 725 cm^{-1}, that are indicative of the presence of a phenolic hydroxy functionality aromatic azo (-N=N-) group and an aromatic moiety, respectively. Figure 4 gives the DSC scan of HPDB, which shows a peak at 299.7°C. The onset temperature of transition was 259°C, the maximum peak transition temperature was 299.7°C and the recovery temperature was 323.4°C for the peak. The positive value of enthalpy indicates an exothermic change.

Preparation of a urethane analogue with an azo aromatic linkage

Of the three different analogues, UR-3 was obtained as a product with elastic consistency (foam-like structure) which was very difficult to process and therefore not evaluated further. UR-1 and UR-2 were obtained as light brown, fine powders, which were further characterized. The absence of HPDB and TDI in UR-1 and UR-2 was determined by TLC. The TLC plates, when observed under UV light, (254 nm) showed no spots corresponding to R_f values of HPDB and TDI indicating the absence of both these reactants. The solubility of UR-1 and UR-2 was evaluated in different solvents. Both compounds were freely soluble in DMF and THF. UR-2 was freely soluble in methanol, acetone, ether and methylene chloride, whereas UR-1 was only slightly soluble in these solvents. Both compounds were insoluble in water. The solubility profile of UR-2 was better compared with UR-1 probably because of the greater contribution of glycols in the formation of UR-2.

IR spectra of both UR-1 and UR-2 were almost similar and showed peaks at 3308 cm^{-1} (-N-H stretching vibrations), 1716 cm^{-1} (-O- in the urethane linkage), 1597 cm^{-1} (corresponding to -O-C=O stretching in amide group), 1450 cm^{-1} (aromatic azo functionality -N=N-), 1070 cm^{-1} and 1225 cm^{-1} (aryl -O-C linkage and -O-CH_2 linkage), and 768 cm^{-1} (aromatic ring). The IR spectrum of UR-2 is shown in Figure 3. ^{1}H NMR spectra of both polymers in d_6-dimethylsulfoxide revealed a doublet for CH_3 of the PG unit at δ = 1.21–1.23, a singlet for -CH_2O of the PEG unit at δ = 3.51–3.55, a doublet for -CH_2 of the PG unit at δ = 4.07–4.09, a quartet for -CH of the PG unit at δ = 5.01 and multiplets for aromatic rings at δ = 6.95–7.16, 7.51–7.83 and 8.15.

The DSC scans of UR-1 and UR-2 are shown in Figure 4. These thermograms were similar showing characteristic peaks at 277.2°C and 279.1°C in the case of UR-1 and UR-2, respectively. Onset transition temperatures were 222.5°C (UR-1) and 223.1°C (UR-2), maximum peak transition temperatures were 277.2°C (UR-1) and 279.1°C (UR-2), and recovery temperatures were 326.8°C (UR-1) and 328.1°C (UR-2). The negative value of enthalpy for UR-1 and UR-2 indicated an

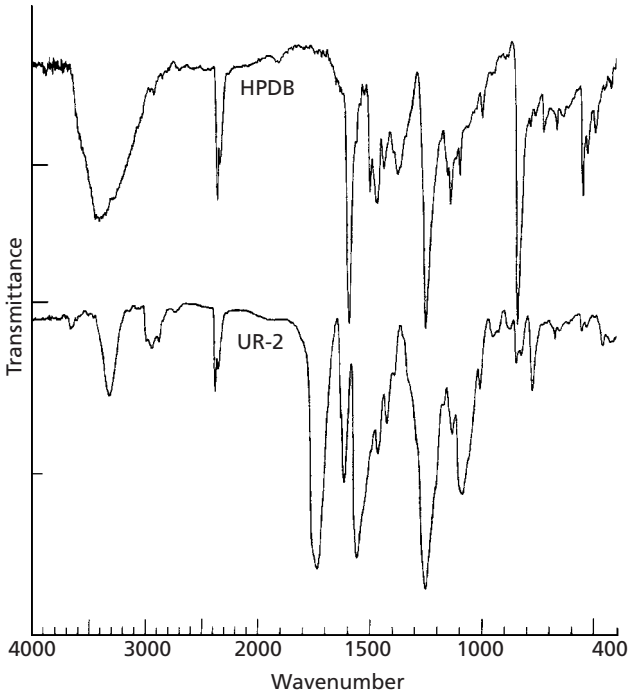

Figure 3 IR spectra of (bis-4-hydroxyphenyl)-4,4′-diazobiphenyl (HPDB) and UR-2.

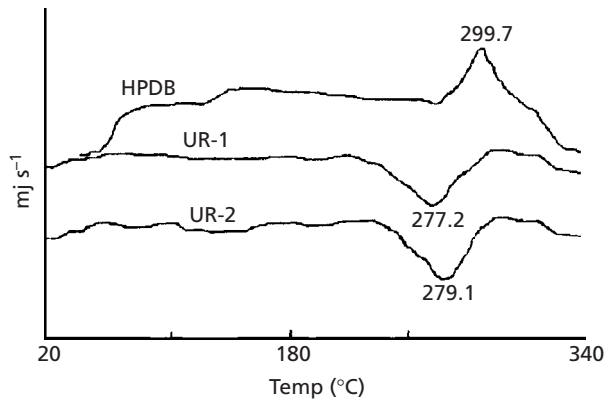

Figure 4 DSC scans of (bis-4-hydroxyphenyl)-4,4′-diazobiphenyl (HPDB) and urethane analogues UR-1 and UR-2.

endothermic change in contrast to HPDB which showed a positive peak with exothermic change. Thus DSC scans of urethane analogues were markedly different from the DSC scan of HPDB indicating its absence in these analogues. The XRD pattern of urethane analogue, UR-2 (Figure 5) showed a diffused spectrum with the absence of any significant peaks. This indicated the

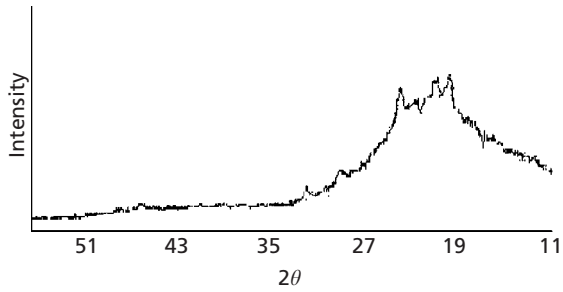

Figure 5 X-ray diffractogram of urethane analogue UR-2.

short range of order and low degree of crystallinity of the analogue.

Evaluation of film-forming properties and determination of molecular weight

No films could be formed by these analogues on any of the substrates used (glass, teflon and release liner) even after the addition of plasticizers. Evaporation of solvents resulted in deposits of weak conglomerates which was suggestive of low molecular weights. This was supported by molecular weight studies. The number- and weight-average molecular weights of UR-1 and UR-2 were determined by GPC. UR-1 was found to have a number-average molecular weight (Mn) of 1663.2 and weight-average molecular weight (MW) of 2085.74. UR-2 had a Mn of 1620.47 and MW 1989.12.

In-vitro bacterial degradation test

Films incubated in a lactobacillus culture showed a colour change from brown to yellow, whereas control films did not show any colour change. Schacht et al (1996) reported a colour change from brown to pink for films composed of polyamides and polyurethanes. The EC:UR (4:1) films were also observed under a microscope after anaerobic incubation for 5 and 7 days. Photomicrographs of films after incubation for 5–7 days indicated loss of integrity, which could be attributed to degradation of the azo linkages of UR-1 or UR-2. As these films were prepared with an impermeable polymer (EC) of a high viscosity grade (45 cps), the films formed were relatively hydrophobic and thick. This could be the reason for the longer time required for the degradation of the azo linkages of the UR-1 and UR-2 components of the films.

UV spectrophotometric studies of untreated, treated and control films were also carried out. All the films showed a peak at λ_{max} 397 nm. The UV scans of EC:UR (4:1) films incubated with or without lactobacilli are

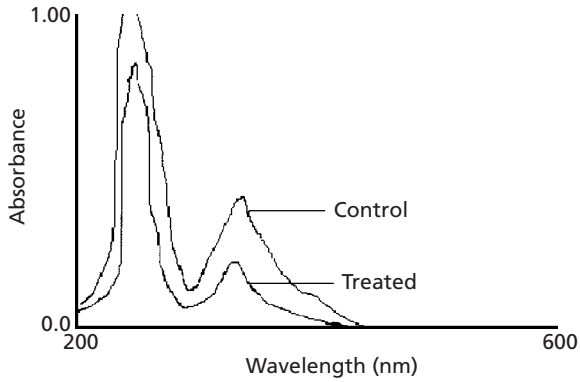

Figure 6 UV scan of EC:UR (4:1) film incubated in presence (treated) and absence (control) of lactobacilli.

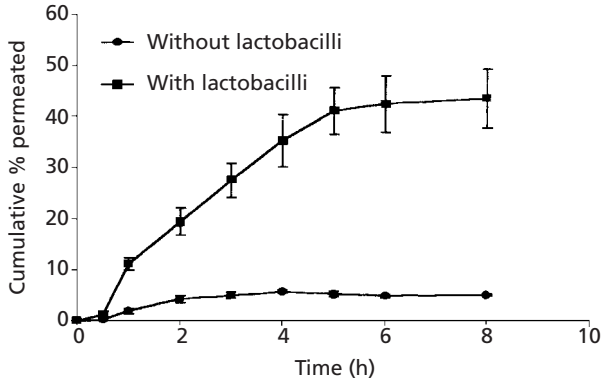

Figure 7 In-vitro permeation of 5-aminosalicylic acid across films of EC:UR-2 (4:1) incubated in fluid thioglycolate medium.

given in Figure 6. Values of absorptivity (1%,1 cm) for untreated films (films without incubation), films incubated in media containing lactobacilli and films incubated in media without lactobacilli were 32.58, 9.16 and 27.46, respectively. The results indicate a small decrease in absorptivity of films incubated in media inoculated without lactobacilli compared with untreated films. Significant reduction in the absorptivity of treated films, to the extent of 66%, was observed compared with control films. The absorptivity of the film is ascribed to the characteristic absorption of the azo aromatic chromophore of the urethane analogues. The reduction of azo linkages by microorganisms would therefore decrease this absorptivity (Kimura et al 1992). Thus, UV spectroscopy results of the in-vitro bacterial degradation tests indicated that azo bonds in the film were reduced by the bacterial enzyme, azoreductase.

In-vitro permeation studies

The permeation of ASA, an anti-inflammatory agent used in the treatment of ulcerative colitis, was performed across the EC:UR-2 (4:1) film in Keshary-Chien cells. Results of the permeation studies are shown in Figure 7. In control films (incubated without lactobacillus culture) only 5.16 ± 0.26% ASA (n = 3) permeated across the films in 8 h, whereas 43.51 ± 5.77% ASA (n = 3) permeated across treated films (incubated in presence of lactobacillus culture). These results suggest that during incubation in presence of lactobacilli, azoreductase activity on the UR-2 component of the films resulted in a significant increase in permeability. These results are in accordance with previous reports which have shown that permeability of caffeine from azo-containing methacrylate polymers was significantly enhanced by bacterial degradation of the azo polymer (Van den Mooter et al 1992). Further, Shantha et al (1995) showed that the release of 5-fluorouracil from azo polymeric hydrogels was faster and was dependent on the concentration of azo polymer in the hydrogel.

In conclusion, the urethane analogues synthesized in the present study, although devoid of film-forming properties on their own, still possessed significant potential for use in colonic drug delivery in combination with other standard film-forming polymers.

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