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Gastrointestinal distribution and absorption behavior of Eudragit-coated chitosan–prednisolone conjugate microspheres in rats with TNBS-induced colitis

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Abstract

Conjugate of chitosan and succinyl-prednisolone, termed Ch–SP, was synthesized, and Ch–SP microspheres (Ch–SP-MS) and Eudragit L100-coated Ch–SP-MS (Ch–SP-MS/EuL) were prepared using Ch–SP. Ch–SP-MS and Ch–SP-MS/EuL had a mean size of 1.5 and 26.6 μm, respectively, and a drug content of 4.6 and 3% (w/w), respectively. Prednisolone (PD) was released very slow in JP 14 first fluid (pH 1.2), and gradually in JP 14 second fluid (pH 6.8). The addition of cecal or colonic content did not accelerate the release. Rats with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis were used in animal studies. Gastrointestinal distribution and plasma concentration were investigated by oral administration of PD alone and Ch–SP-MS/EuL. For PD alone, PD was distributed at the stomach and small intestine, and disappeared from the gastrointestinal tracts within 8 h. When administering Ch–SP-MS/EuL, the drug was distributed mainly in the lower intestine between 3 and 24 h. Plasma concentration was much lower in Ch–SP-MS/EuL than in PD alone, suggesting lower toxic side effects of Ch–SP-MS/EuL. Thus, Ch–SP-MS/EuL delivered PD specifically near the diseased site and PD was released gradually, with much less plasma concentration of PD. Ch–SP-MS/EuL are suggested as a useful delivery system to the site of inflammatory bowel disease.
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Keywords: Eudragit L100-coated chitosan–prednisolone conjugate microspheres; TNBS-induced colitis; Gastrointestinal distribution; Plasma concentration; Drug release

1. Introduction

Inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn's disease is becoming a significant problem as a severe, chronic and refractory disease with the increase in the westernized lifestyle (Fiocchi, 2002). IBD is thought to be an inappropriate immune response, and appears to be caused by various factors including the individual genetic background and environments affecting enteric flora and the intestinal immune system. 5-Aminosalicilic acid (5-ASA) and steroids are still the most common drugs for the treatment of IBD (Brzezinski et al., 1995; Ardizzone and Bianchi, 1998; Gionchetti et al., 2003; Kesisoglou and Zimmermann, 2005). For 5-ASA, salazosulfapyridine is a prodrug of 5-ASA, and releases 5-ASA in the colon based on reductive action by colonic bacteria (Klotz, 1985). Pentasa acts as a delayed release system of 5-ASA, and specifically releases the drug around the colonic region (Larouche et al., 1995). These are used clinically in mild or moderate disease conditions, but steroids and immunosuppressive drugs are required for the treatment of more severe IBD (Gionchetti et al., 2003). However, the treatment with steroids or immunosuppressive agents is often accompanied by toxic side effects, which are mainly based on systemic absorption, that is, non-specific biodistribution. Therefore, the specific delivery of drugs to diseased sites is important, and new drugs or novel dosage forms have been developed (Campieri et al., 1997; Rutgeerts, 1998; Friend, 2005).

Recently, for the delivery of 5-ASA, non-steroidal and steroidal anti-inflammatory drugs to intestinal diseased sites, much attention has been paid to micro- or nano-particulate dosage forms, because they can be delivered efficiently and retained long in the lower intestine (Jani et al., 1989; Watts et al., 1992; Desai et al., 1996; Sakuma et al., 1999; Shimoda et al., 2001). Micro- and nano-particles with a diameter of less than

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10 μm, not subject to elimination by diarrhea, were retained well at the site of colitis with a thicker mucous layer (Lamprecht et al., 2000, 2001a). Small particles can penetrate the mucus layer more deeply. Furthermore, microparticles of several hundred nanometers to several micrometers are subject to uptake by leukocytes such as macrophages (Tabata and Ikada, 1988; Tabata et al., 1996; Van Der Lubben et al., 2001), which appear in large numbers at IBD sites. In these microparticulate dosage forms, release control was also important. The control of drug release involves diffusion through polymer matrices and/or degradation of polymers by enzymes of bacteria and leukocytes. Biodegradable micro- or nano-particles showing a gradual drug release of anti-inflammatory drugs were reported to exhibit good efficacy in IBD animal models (Nakase et al., 2000; Lamprecht et al., 2001b). Chitosan capsules were reported to be useful to improve the efficacy of 5-ASA; chitosan capsules containing 5-ASA efficiently delivered the drug to the large intestine and showed effective release around the diseased sites due to degradation of the capsules (Tozaki et al., 1999, 2002). Furthermore, enteric-coated tablets or pellets (Khan et al., 2000; Sinha and Kumria, 2003; Akhgari et al., 2005), and various prodrugs (McLeod et al., 1993, 1994; Yano et al., 2002; Wiwattanapatapee et al., 2003) have been developed for colon-specific delivery.

We developed chitosan–prednisolone conjugate microspheres (Ch–SP-MS) to deliver the drug to the diseased sites and achieve gradual drug release (Onishi et al., 2005). This is based on the concepts of localization at the diseased sites by microparticles and controlled release by ester hydrolysis. Ch–SP-MS, reported previously, showed excellent particle size and satisfactory drug content as a specific delivery system for the treatment of IBD, and Eudragit coating of Ch–SP-MS was useful to protect the morphological characteristics of the original Ch–SP-MS at gastric pH and to achieve good regeneration of Ch–SP-MS under intestinal pH conditions (Oosegi et al., in press). Drug release from Eudragit-coated Ch–SP-MS was suppressed at gastric pH and in the initial step at pH 6.8, and subsequently promoted at pH 6.8. These release patterns were suitable for the specific delivery of prednisolone (PD) at IBD sites (lower intestine). The particle characteristics and in vitro release profiles were similar when either Eudragit L100 or Eudragit S100 was used. In this study, in order to clarify in vivo behavior of Eudragit L100-coated Ch–SP-MS (Ch–SP-MS/EuL), their drug release in fluid containing intestinal content, and gastrointestinal transit and systemic absorption after oral administration to rats with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (Morris et al., 1989) were examined.

2. Materials and methods

2.1. Materials

Prednisolone (PD), prednisolone 21-hemisuccinate (SP) sodium salt (SP-Na) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Chitosan (Ch) (viscosity grade = 1000 (5 g/l, 20 °C), deacetylation degree = 80% (mol/mol)) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Eudragit L100 was obtained from Rohm GmbH Chemische Fabrik (Darmstadt, Germany). Sorbitan sesquioleate (SO-15) was purchased from Nikko Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Animals

Six-week-old male Wistar rats (200 g) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan), and bred on the breeding diet MF (Oriental Yeast, Tokyo, Japan) with water ad libitum at 23 ± 1 °C and relative humidity of 60 ± 5%. They were used for the experiments soon after purchase. The experimental protocol was approved by the committee on Animal Research of Hoshi University, Japan. The animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, Japan. The rats were fasted for 48 h before the induction of ulcerative colitis. TNBS (20 mg) dissolved in 0.25 ml of 50% (v/v) ethanol was instilled into the colon 7 cm from the anus through a catheter. Three days after TNBS treatment, the rats, which weighed 80–100% of that immediately before TNBS instillation, were selected as an ulcerative colitis animal model.

2.3. Preparation of conjugate microspheres and their Eudragit coating

The conjugate of Ch and SP, named Ch–SP, was prepared by the carbodiimide coupling of the amino groups of Ch and the carboxyl group of SP as reported previously (Oosegi et al., in press). Briefly, 5 ml of aqueous solution containing SP–Na (40 mg) was added to 85 ml of aqueous solution of Ch (120 mg) with the pH adjusted to pH 5.5 with 1 M HCl aqueous solution and 1 M NaOH aqueous solution, and 5 ml of aqueous solution containing EDCI (200 mg) was added. The resultant mixture was stirred at 900 rpm under ice cooling for the first 5 h and at room temperature for 19 h. Then, 5 ml of water containing 200 mg EDCI was added, and the resultant mixture was stirred at 900 rpm at room temperature for 24 h. The final mixture was poured into a four-fold volume of acetone. The precipitate was separated by centrifugation at 3000 rpm for 5 min, and washed using a mixture of acetone and water (4:1, v/v). The final precipitate was suspended in 60 ml of water, and lyophilized to obtain Ch–SP powder.

Ch–SP microspheres (Ch–SP-MS) were prepared by emulsification and subsequent evaporation. Namely, Ch–SP (50 mg) were dissolved in a 1% (v/v) acetic acid aqueous solution, and added to 150 ml of liquid paraffin containing 1, 2 or 3% (w/v) SO-15, which was stirred at 1200 rpm at 70 °C. Stirring was continued at 70 °C for 20 min, and at 80 °C for another 10 min. The resultant emulsion was sonicated at 80 °C under 28 kHz (100 W) for 10 min using an ultrasonicator VS-100III Sunpar (Iuchiseieido, Japan), stirred at 400 rpm at 100 °C for 1 h, and then cooled to the room temperature. After the addition of the same volume of *n*-hexane, the mixture was centrifuged at 3000 rpm

for 5 min to separate the precipitate, which was washed with *n*-hexane and dried in a desiccator to obtain Ch–SP-MS.

Eudragit-coated Ch–SP-MS (Ch–SP-MS/EuL) were prepared as follows. After Ch–SP-MS (130 mg) were suspended in 1 ml of Eudragit L100 (130 mg) solution in methanol, the suspension was added to 50 ml of liquid paraffin containing 2% (w/v) SO-15, and stirred at 600 rpm at 40 °C. The mixture was stirred at 40 °C under reduced pressure until the methanol was evaporated completely, and then cooled to room temperature. After the addition of the same volume of *n*-hexane, the resultant mixture was centrifuged at 3000 rpm for 5 min to obtain the precipitate, which was washed with *n*-hexane, and the final precipitate was dried in a desiccator to yield Ch–SP-MS/EuL.

2.4. Measurement of particle shape and size and drug content

Powder samples of Ch–SP-MS and Ch–SP-MS/EuL were thinly coated with platinum using a JEOL JFC-1600 Auto Fine Coater (JEOL, Japan) and observed using a JEOL JSM-5600LV scanning electron microscope (JEOL, Japan), and micrographs were taken. The particle size and size distribution were examined by measuring the Green diameters of 100 microparticles chosen at random from the micrographs.

The drug contents of Ch–SP-MS and Ch–SP-MS/EuL were analyzed by referring to the previous study (Onishi et al., 2005). Briefly, the sample (2 mg) was put into a 0.1 M NaOH aqueous solution (10 ml), incubated for 10 min at 45 °C and centrifuged at 3000 rpm for 5 min. The content of PD was determined by the measurement of UV absorption of the supernatant at 246 nm using a Beckman DU 640 spectrophotometer (Beckman, Japan).

2.5. Morphological analysis of microparticles in different pH media

Ch–SP-MS/EuL were placed in 5 ml of JP 14 first fluid (pH 1.2) or JP 14 second fluid (pH 6.8) at a concentration of 50 μg PD equiv./ml, and incubated by horizontal shaking at 100 rpm at 37 °C. At 0.25, 1.5 and 4 h, an aliquot sample was withdrawn. After the sample was centrifuged at 3000 rpm for 5 min, the precipitate was washed gently with water, and dried in a desiccator. The resultant solid was observed by scanning electron microscopy (SEM) to examine its size and shape.

2.6. In vitro drug release studies

In vitro release experiments were carried out using JP 14 first fluid, JP 14 second fluid, and a suspension of cecal or colonic content in JP 14 second fluid. The suspension of cecal or colonic content was prepared as follows. Three days after TNBS was instilled in rats in the same manner as shown above, they were sacrificed by the inhalation of an excessive amount of ethyl ether. Cecal or colonic content was collected, weighed, and diluted at the concentration of 20% (w/v) using JP 14 second fluid. Then, the content suspension (20%, w/v) was homogenized using a glass homogenizer with a Teflon pestle. After the homogenized suspension was filtered with gauze, the supernatant was used as the incubation medium of cecal content suspension or colonic content suspension. Ch–SP-MS and Ch–SP-MS/EuL were placed into 5 ml of each medium at a concentration of 50 μg PD equiv./ml. Each mixture was incubated by horizontal shaking at 100 rpm at 37 °C. At appropriate time points, the mixture was centrifuged at 3000 rpm for 5 min, and an aliquot sample (100 μl) was withdrawn from the upper layer and stored in freezer at −20 °C until analysis. After each sampling, the incubation mixture was gently stirred, and incubation was continued. The incubation time was defined as the time that the mixture was immersed in a water bath at 37 °C. Each sample was diluted three-fold by addition of the HPLC mobile phase, and assayed for PD by HPLC.

2.7. High performance liquid chromatography (HPLC)

The amount of PD in the sample solution was determined by HPLC. The HPLC system consisted of an LC-10AS pump, SPD-10A spectrophotometric detector, C-R7 chromatopac, SCL-10A system controller, SIL-10A autosampler and CTO-10A column oven (Shimadzu Corp., Japan). The detector was set at 246 nm, and the column oven was set at 30 °C. A Spelcosil LC-18-DB column (4.6 mm in inner diameter × 150 mm in length, particle size 3 μm; SUPELCO, USA) was used as an analytical column. A 22% (w/v) 2-propanol aqueous solution containing 0.1% (w/v) trifluoroacetic acid was used as the mobile phase, and the flow rate was 1.0 ml/min. The absolute calibration curve method was applied to the quantification.

2.8. Investigation of gastrointestinal distribution

Three days after TNBS was instilled in rats in the same manner as shown above, the rats were fasted for 24 h. Then, PD alone or Ch–SP-MS/EuL were orally administered at a dose of 5 mg PD equiv./kg as a suspension in saline (1.5 ml per rat). At 3, 8 and 24 h after administration, rats were sacrificed by the inhalation of an excessive amount of ethyl ether, and the stomach, upper half of the small intestine (upper small intestine or proximal small intestine (SIP)), lower half of the small intestine (lower small intestine or distal small intestine (SID)), cecum, upper one-third of the colon (proximal colon (COP)) and lower two-thirds of colon (distal colon (COD)) were excised. The content in each tissue was collected by washing with phosphate-buffered saline, pH 7.4 (PBS), homogenized using a glass homogenizer with a Teflon pestle, and diluted with PBS into 4 ml for the upper small intestine, lower small intestine and proximal colon, and into 8 ml for the cecum and distal colon. To 100 μl of the content suspension, 100 μl of saturated NaCl aqueous solution, 100 μl of 5% (w/v) phosphoric acid and 4 ml of the mixture of *t*-tributyl methyl ether and pentane (2:3, v/v) were added, and the resultant mixture was shaken vigorously. Then, 3 ml of the organic phase was dried under nitrogen gas at room temperature. After the resultant residue was dissolved in the HPLC mobile phase, the sample was analyzed for PD by HPLC.

As for Ch–SP-MS/EuL, the amount of total (free plus incorporated) PD in the content suspension was further determined as follows. To 100 μl of the content suspension, 11 μl of 0.1 N

NaOH aqueous solution was added, and kept at 45 °C for 10 min to hydrolyze the ester. Then, extraction of PD and preparation of the sample for the HPLC assay were performed as stated above. The obtained sample solution was analyzed by HPLC to determine the total amount of PD in the content suspension.

The recovery ratio from the content of each tissue described above was carried out as follows. Each tissue was excised as stated above except that no drug was administered, and the content was collected in the same way as the tested sample. To 100 μl of the obtained content suspension, PD was added so that the PD concentration was 0.1, 1 and 10 μg/ml, and the mixture was treated in the same manner as the tested sample. The final sample was assayed for PD by HPLC. The recovery ratio was calculated as the ratio of the observed amount to the calculated amount, and used for data correction.

2.9. Gastrointestinal absorption studies

Three days after TNBS was instilled in rats in the same manner as shown above, the rats were fasted for 24 h. Then, PD alone or Ch–SP-MS/EuL were orally administered at a dose of 5 mg PD equiv./kg as a suspension in saline (1.5 ml per rat). Immediately before and 0.5, 1, 2, 4, 7, 12 and 24 h after administration, blood samples (0.3 ml) were withdrawn via the jugular vein under light anesthesia by the intraperitoneal injection of pentobarbital. Plasma was obtained after centrifugation of the blood at 3000 rpm for 10 min, and 100 μl of plasma was treated and assayed for PD in the same manner as the suspension of the tissue content. The recovery ratio was also determined in the same way as the suspension of the tissue content except that plasma of normal rats was used. The data correction was performed with the recovery ratio. Maximal plasma concentration (C_{max}), the time to reach C_{max} (T_{max}), area under the plasma concentration–time curve (*AUC*), mean residence time (*MRT*) and variance of residence time (*VRT*) were calculated for the period of 0 to infinite by the trapezoidal rule using the program MULTI (Yamaoka et al., 1981).

2.10. Statistical analysis

Comparison was performed using the unpaired *t*-test, and significant difference was set as $P < 0.05$.

3. Results

3.1. Particle size and drug content

Particle morphology was compared among Ch–SP-MS prepared using liquid paraffin with 1, 2 and 3% (w/v) SO-15. All Ch–SP-MS showed an almost spherical shape. Their mean particle size was 1.5, 1.9 and 1.9 μm, respectively, with the size distribution of 0.7–2.9, 1.0–3.5, 0.7–4.3 μm, respectively ($n = 100$ for each group). As Ch–SP-MS prepared with liquid paraffin with 1% (w/v) showed the smallest size, they were used in the present study. The morphologies of Ch–SP-MS and Ch–SP-MS/EuL and their size distribution are shown in Fig. 1. Ch–SP-MS/EuL had a mean diameter of 26.6 μm, and their size distribution ranged mostly from 7 to 75 μm. Table 1 summarizes the mean particle size and drug content of Ch–SP-MS and Ch–SP-MS/EuL used in the following experiments. Ch–SP had

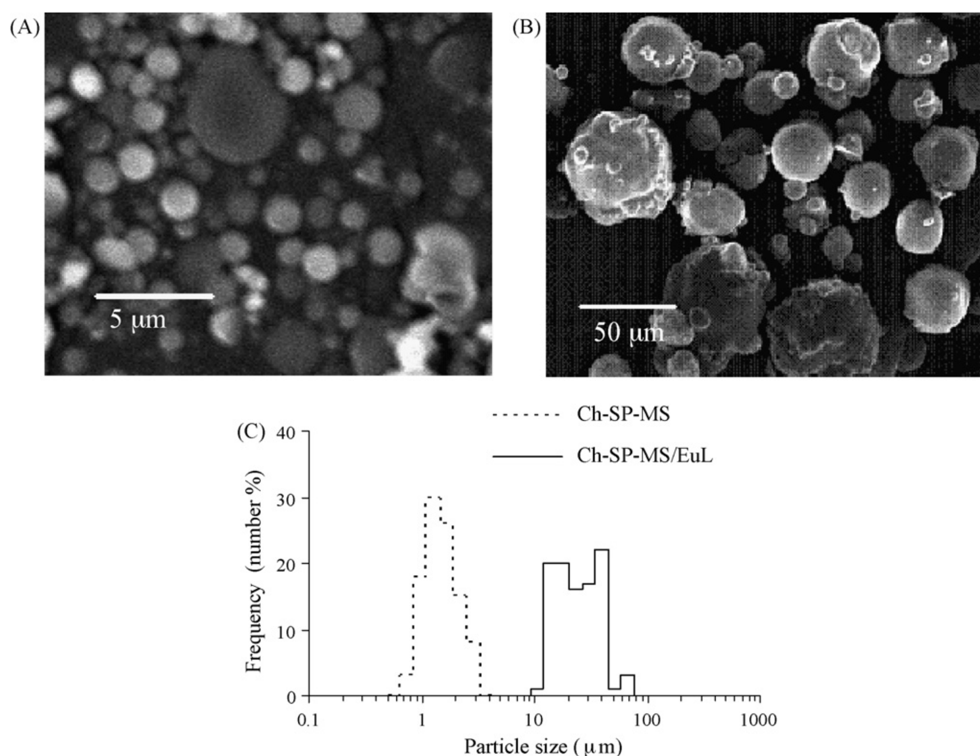

Fig. 1. SEM micrographs of Ch–SP-MS (A) and Ch–SP-MS/EuL (B), and particle size distribution (C). Scale is shown with a white bar in each micrograph. Size distribution was obtained using 100 particles chosen at random.

Table 1
Particle characteristics of Ch–SP-MS and Ch–SP-MS/EuL

Microparticulate formulation	Particle size (μm)[a]	Particle size range (μm)	Drug content (%, w/w)[b]
Ch–SP-MS	1.5 ± 0.5	0.7–2.9	4.60 ± 0.74
Ch–SP-MS/EuL	26.6 ± 10.7	7–75	3.22 ± 0.68

[a] The results are expressed as the mean ± S.D. ($n = 100$).
[b] The results are expressed as the mean ± S.D. ($n = 3$).

a mean PD content of 7.73% (w/w). Ch–SP-MS and Ch–SP-MS/EuL displayed the mean drug contents of 4.60 and 3.22% (w/w), respectively.

3.2. Morphological features in different pH media

Ch–SP-MS/EuL were investigated for changes in particle shape and size in JP 14 first fluid and JP 14 second fluid at 37 °C. At appropriate time points, the microparticles were separated by centrifugation, washed briefly with water and observed by SEM (Fig. 2). In JP 14 first fluid (pH 1.2), the original shape and size were maintained fairly well, though the surface condition changed to some extent. On the other hand, the particle size decreased extensively in JP 14 second fluid (pH 6.8) even at 1.5 h, and the particles were irregularly shaped or slightly larger than Ch–SP-MS. Moreover, most of the microparticles in JP 14 second fluid at 4 h displayed an almost spherical shape, and their particle size was close to the original Ch–SP-MS. The particle size of Ch–SP-MS/EuL was maintained at gastric pH, but changed mostly to several μm within 1.5 h at intestinal pH.

3.3. In vitro release

The drug release profiles from Ch–SP-MS and Ch–SP-MS/EuL were investigated in JP 14 first fluid (pH 1.2), JP 14 second fluid (pH 6.8), and cecal or colonic content suspension in JP 14 second fluid (Fig. 3). Both kinds of microparticles showed very slow drug release in JP 14 first fluid; in particular, the release was suppressed very much in Ch–SP-MS/EuL. The drug release was caused gradually in JP 14 second fluid, in which the release was somewhat faster in Ch–SP-MS than in Ch–SP-MS/EuL. For both kinds of microparticles, the release rate tended to fall to some extent by the addition of cecal or colonic suspension. Namely, for Ch–SP-MS, the mean cumulative release at 6 h was 20, 10 and 11% in JP 14 second fluid, cecal content suspension and colonic content suspension, respectively. As for Ch–SP-MS/EuL, the mean cumulative release at 6 h was 15, 14 and 12% in JP 14 second fluid, cecal content suspension and colonic content suspension, respectively.

3.4. Gastrointestinal distribution

Gastrointestinal distribution–time profiles of PD were investigated after oral administration of PD alone and Ch–SP-MS/EuL to rats with TNBS-induced colitis. The recovery ratios were 82, 85, 86, 85, 84 and 85% for the contents of the stomach, upper small intestine, lower small intestine, cecum, proximal colon and distal colon, respectively. The gastrointestinal transit profiles after oral administration are described in Fig. 4. For PD alone, the drug was detected in the stomach, upper small intestine and lower small intestine at 3 h, but not at 8 or 24 h; that is,

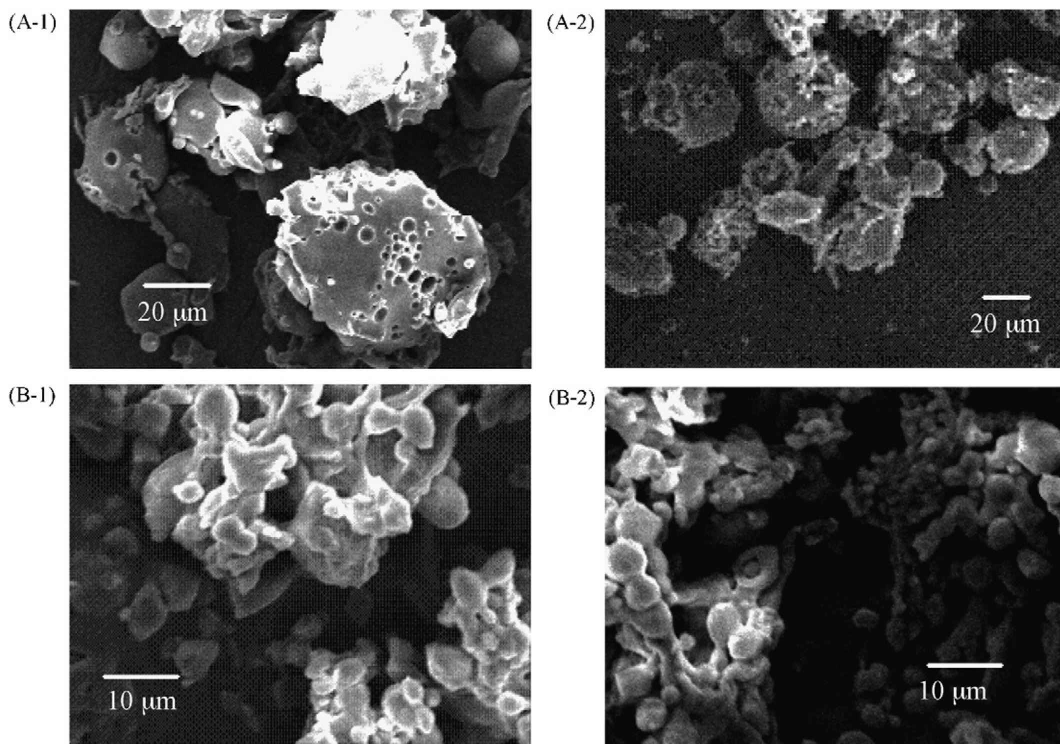

Fig. 2. SEM micrographs and change in particle size of Ch–SP-MS/EuL during incubation in different pH media at 37 °C. (A-1) JP 14 first fluid (pH 1.2), 1.5 h; (A-2) JP 14 first fluid (pH 1.2) 4 h; (B-1) JP 14 second fluid (pH 6.8), 1.5 h; (B-2) JP 14 second fluid (pH 6.8), 4 h.

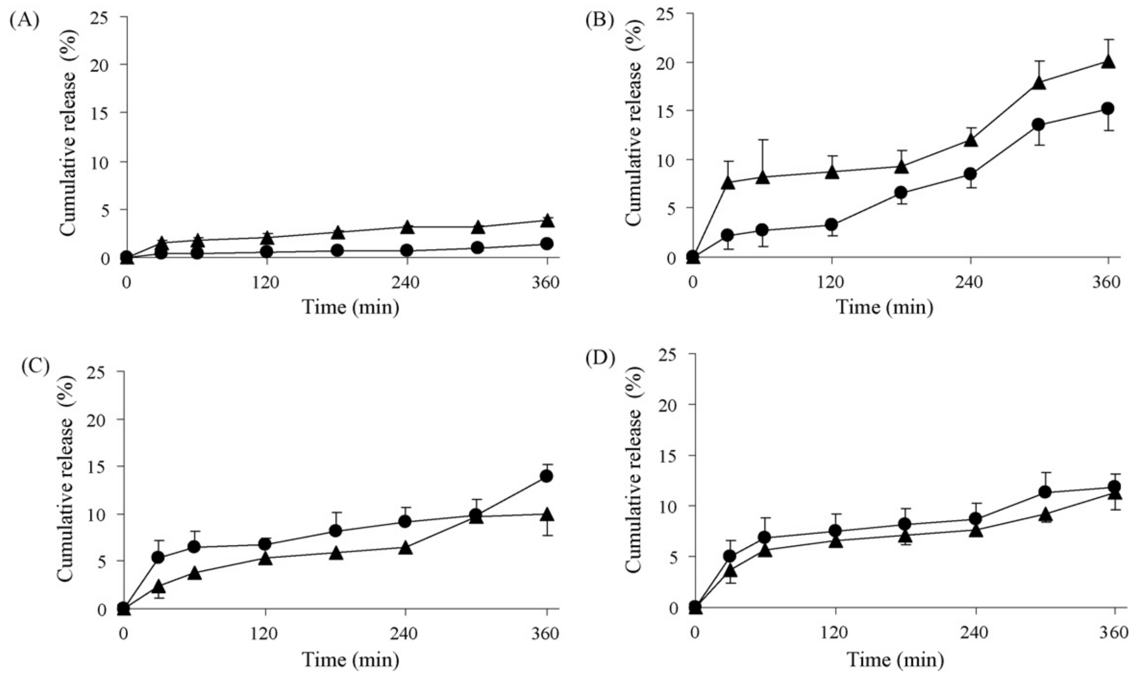

Fig. 3. In vitro release from Ch–SP-MS (▲) and Ch–SP-MS/EuL (●) during incubation in different media at 37 °C. (A) JP 14 first fluid (pH 1.2); (B) JP 14 second fluid (pH 6.8); (C) cecal content suspension at pH 6.8; (D) colonic content suspension at pH 6.8. The results are expressed as the mean ± S.D. ($n = 3$).

PD was eliminated fairly fast from the gastrointestinal tract. On the other hand, for Ch–SP-MS/EuL, free PD was observed in the cecum and colon at 3 h, and in the colon at 8 and 24 h; however, the amount of detected PD was several μg in each tissue at 3 h, and 1.1 μg or less in the colon at 8 and 24 h. The combined PD was observed mainly in the lower small intestine at 3 h, when the amount of total (free and incorporated) PD was 263 μg there. At 8 h, the combined PD were detected mainly in the lower small intestine and cecum, where the amounts of total PD were 40 and 141 μg, respectively. The amount of total PD distributed in the observed tissues decreased very much at 24 h. Only several μg of total PD were detected mainly in the lower small intestine to proximal colon at 24 h. Ch–SP-MS/EuL supplied free PD in the colon for a long time, at least 3–24 h after administration.

3.5. Plasma concentration and pharmacokinetics

After oral administration of PD alone and Ch–SP-MS/EuL to rats with TNBS-induced colitis at 5 mg PD equiv./kg, the plasma concentration–time profiles were obtained as shown in

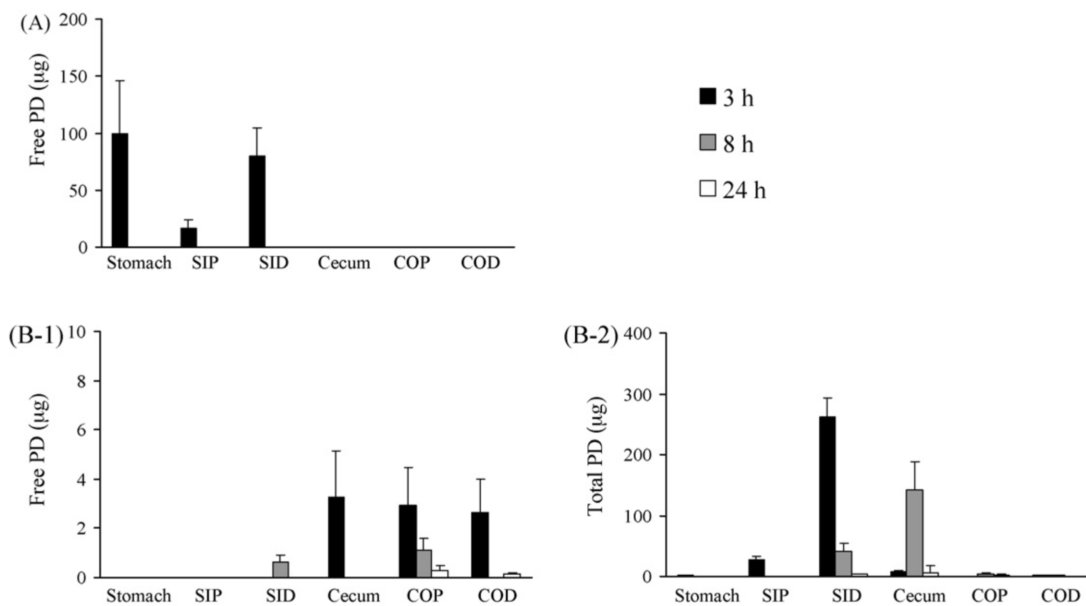

Fig. 4. Gastrointestinal transit after oral administration of PD alone and Ch–SP-MS/EuL to rats with TNBS-induced colitis. (A) Free PD for PD alone; (B-1) free PD for Ch–SP-MS/EuL; (B-2) total PD for Ch–SP-MS/EuL. SIP: proximal small intestine (upper half of small intestine); SID: distal small intestine (lower half of small intestine); COP: proximal colon; COD: distal colon. The results are expressed as the mean ± S.E. ($n = 3$).

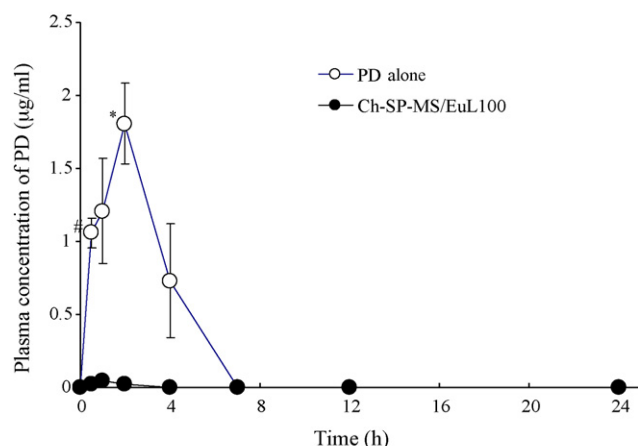

Fig. 5. Plasma concentration–time profiles of PD after oral administration of PD alone and Ch–SP-MS/EuL to rats with TNBS-induced colitis. The results are expressed as the mean ± S.E. ($n = 3$). $^{*}P < 0.05$ vs. Ch–SP-MS/EuL and $^{\#}P < 0.001$ vs. Ch–SP-MS/EuL.

Fig. 5. PD alone exhibited a rapid increase in the plasma level, which reached a maximal plasma concentration (1.81 μg/ml) at 2 h. The plasma concentration declined quickly, and was not observed at 7 h or later. Ch–SP-MS/EuL showed very low plasma levels during observation, and no drug was detected in the plasma at 7 h or later. The pharmacokinetic parameters are described in Table 2. The C_{max} and *AUC* were significantly 35 and 73 times less in Ch–SP-MS/EuL than in PD alone ($P < 0.01$ and 0.05 for C_{max} and *AUC*, respectively). Moreover, T_{max} and *MRT* in Ch–SP-MS/EuL were 1.33 and 1.43 h, respectively, which were somewhat less than in PD alone. *VRT* was approximately 5 times less in Ch–SP-MS/EuL than in PD alone.

4. Discussion

In the present study, Eudragit L100-coated chitosan–succinyl prednisolone conjugate microspheres (Ch–SP-MS/EuL) were examined in vitro and in vivo to elucidate their gastrointestinal behavior. In order to improve the efficacy and toxicity of anti-inflammatory drugs including PD, it is essential to deliver the drugs specifically to the diseased site and to reduce gastrointestinal absorption as far as possible (Tozaki et al., 2002; Yano et al., 2002). Various dosage forms including tablets, capsules, pellets, prodrugs and microparticles have been developed (McLeod et al., 1993, 1994; Tozaki et al., 1999; Khan et al., 2000; Nakase et al., 2000; Lamprecht et al., 2001b; Tozaki et al., 2002; Yano et al., 2002; Sinha and Kumria, 2003; Wiwattanapatapee et al., 2003; Akhgari et al., 2005), and some are used clinically (Klotz, 1985; Larouche et al., 1995). In particular, as steroidal anti-inflammatory drugs such as PD can often exhibit severe toxicity as well as high efficacy, their specific delivery to the diseased site is important to improve efficacy and toxicity. We have developed conjugate microspheres, Ch–SP-MS, by combining the concept of well-controlled release by macromolecular prodrugs and that of good localization and retention by microparticulate formulations in gastrointestinal mucosa (Onishi et al., 2005). As reported previously, Ch–SP (conjugate or macromolecular prodrug) and its microspheres Ch–SP-MS can release PD gradually at intestinal pH, while the release was suppressed very much in gastric pH (Oosegi et al., in press). In addition, Eudragit coating was found useful to protect the size and shape of Ch–SP-MS from stomach pH (Oosegi et al., in press).

Ch–SP, Ch–SP-MS and Eudragit L100-coated Ch–SP-MS (Ch–SP-MS/EuL) showed the mean drug contents of approximately 7.73, 4.60 and 3.22% (w/w), respectively (Table 1). The drug content study was based on quick swelling of the particles and stable UV absorption of PD in alkaline solution (Onishi et al., 2005), in which chitosan and its microspheres did not affect the UV absorption due to their insoluble property in this condition. The difference in drug content between Ch–SP and Ch–SP-MS was considered to be due to the hydrolysis of ester in the preparation process of Ch–SP-MS under high temperature. In Ch–SP-MS/EuL, the drug content was also reduced because of the addition of Eudragit L 100. However, these drug contents were considered suitable for practical use, because the effective dose of PD was not so high for the treatment of ulcerative colitis. The particle shape was almost spherical in both Ch–SP-MS and Ch–SP-MS/EuL. All Ch–SP-MS/EuL were larger than Ch–SP-MS (Fig. 1), indicating that all Ch–SP-MS were coated with Eudragit L100. Namely, most Ch–SP-MS/EuL incorporated a plurality of Ch–SP-MS per particle as shown previously (Oosegi et al., in press). As Ch–SP-MS had a size of 0.7–2.9 μm, they were considered to be suitable for retention in and deep penetration into the intestinal mucosa (Jani et al., 1989; Watts et al., 1992; Desai et al., 1996; Sakuma et al., 1999; Shimoda et al., 2001; Lamprecht et al., 2000, 2001a), and adequate for the uptake by leukocytes such as macrophages (Tabata and Ikada, 1988; Tabata et al., 1996; Van Der Lubben et al., 2001). The morphological behavior of Ch–SP-MS/EuL in the gastric or intestinal conditions was examined in vitro. Their original shape and size were almost maintained in JP 14 first fluid (pH 1.2), though the surface condition was slightly changed (Fig. 2). In JP 14 second fluid (pH 6.8), the Eudragit layer was dissolved fairly quickly (Fig. 2). Even at 15 min, the mean size was 7.1 μm, and less than 4 μm at 1.5 h or later. Therefore, the morphology of Ch–SP-MS/EuL were protected in the stomach, and regenerated Ch–SP-MS readily in the intestine, which was suitable for the efficient delivery of Ch–SP-MS to the intestine.

Table 2
Pharmacokinetic parameters after oral administration of PD alone and Ch–SP-MS/EuL100

Formulation	C_{max} (μg/ml)	T_{max} (h)	*AUC* (μg h/ml)	*MRT* (h)	*VRT* (h^2)
PD alone	1.81 ± 0.23	2.00 ± 0.00	5.97 ± 1.32	2.20 ± 0.30	1.16 ± 0.40
Ch–SP-MS/EuL100	0.05 ± 0.04**	1.33 ± 0.33	0.08 ± 0.07*	1.43 ± 0.32	0.21 ± 0.18

The results are expressed as the mean ± S.E. ($n = 3$). $^{*}P < 0.05$ vs. PD alone. $^{**}P < 0.01$ vs. PD alone.

The release of PD from Ch–SP-MS was almost controlled by the hydrolysis rate of the carboxy ester (Oosegi et al., in press). As reported previously, the release of PD was suppressed very much at the gastric pH (pH 1.2), but caused gradually at intestinal pH (pH 6.8). However, under in vivo conditions, gastrointestinal contents were considered to influence the release rate and pattern. In particular, cecal and colonic contents were considered to be important because they are around the diseased site. Their effects on the drug release were investigated using the content suspension in JP 14 second fluid. As the results, the intestinal contents hardly influenced the release rate and pattern; or rather, their addition tended to lower the release to some extent (Fig. 3). Probably, the carboxy ester was protected from esterase by steric hindrance by the surrounding bulky Ch molecules. Although Ch might be degraded by the microflora, the hydrolysis of the ester hardly seemed to be influenced, probably because Ch oligomers still remained around the ester. Although the degradation of Ch by microflora might be affected by diets, the intestinal contents obtained in the present conditions did not accelerate the drug release. Instead, the liquid or solid components in the content suspensions seemed to interact with Ch–SP-MS, resulting in preventing the cleavage or release to some extent.

Examination of the gastrointestinal transit profiles is essential to evaluate the ability of delivering a drug to the diseased site. As shown in Fig. 4, Ch–SP-MS/EuL delivered Ch–SP-MS efficiently to the lower small intestine, cecum and colon, which are associated with ulcerative colitis. Furthermore, free PD was detected specifically at the area of lower intestine for a long period. Sugimoto et al. (1998) reported that PVA-gel or Ch/PVA-gel microspheres with similar size (a few μm to a few dozen μm) could move fairly fast to lower intestine; namely, they were distributed mainly in lower small intestine at 3 h, more than 60% reached to large intestine within 4.5 h, and most microparticles reached large intestine at 6 h. Therefore, Ch–SP-MS/EuL or regenerated microparticles might show fairly fast gastrointestinal transit. In this experiment, the gastrointestinal content was taken only by washing the inside of the tissue; therefore, the particles within villi or microvilli might not be recovered completely. In fact, as the recovery of the drug at 3 h was near 40%, microparticles might remain within villi and microvilli to some extent. This point will have to be examined in more detail. However, this recovery near the half of dose, distribution of free PD in the lower intestine and the following plasma concentration profiles (Fig. 5) supported that the obtained gastrointestinal profiles should reflect the main distribution of PD (free and incorporated). Therefore, Ch–SP-MS/EuL showed fairly fast gastrointestinal transit and specific delivery of PD to the diseased site. Such specific delivery is also important to reduce toxic side effects, which are caused mainly by gastrointestinal absorption to systemic circulation (Yano et al., 2002). Ch–SP-MS/EuL exhibited much lower plasma levels than PD alone (Fig. 5). C_{max} and AUC were significantly 35 and 73 times less in Ch–SP-MS/EuL than in PD alone, respectively. As drug absorption is generally caused mainly in the small intestine, the localization of PD to the cecum and colon by Ch–SP-MS/EuL was considered to prevent drug absorption. Further, PD release from Ch–SP-MS/EuL was suppressed in stomach and slow in upper intestine, and the transit rate of the particles was fairly fast (Fig. 4). Therefore, only a very small part of the drug was considered to be released around the absorption area, and then most part of the drug reached large intestine where the drug was hardly absorbed. In addition, PD alone might exhibit some gastrointestinal retention due to being administered in a suspension form. These were considered to be the reason for less T_{max} and MRT in Ch–SP-MS/EuL than PD alone. These in vivo analyses demonstrated that Ch–SP-MS/EuL could transfer fairly fast to the diseased site, achieve high localization and gradual release of the drug around the diseased site and cause much less drug absorption. It is suggested that Ch–SP-MS/EuL should be useful for the treatment of IBD by improving the efficacy of PD and reducing its toxicity.

5. Conclusion

Eudragit L100-coated chitosan–succinyl prednisolone conjugate microspheres (Ch–SP-MS/EuL) were examined in vitro and in vivo in order to elucidate their gastrointestinal behavior. Chitosan–succinyl prednisolone conjugate microspheres (Ch–SP-MS) were regenerated fairly fast at intestinal pH, and the drug release rate was controlled mainly by pH, not by the large intestinal content. Ch–SP-MS/EuL showed efficient localization of prednisolone (PD) in the lower small intestine, cecum and colon, and PD was released gradually there for a long period. The gastrointestinal absorption of PD was much less with Ch–SP-MS/EuL than with PD alone, suggesting the reduction of toxic side effects by Ch–SP-MS/EuL. These demonstrated that Ch–SP-MS/EuL should be a useful drug delivery system for the treatment of inflammatory bowel disease (IBD).

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