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Synthesis and *In Vitro* Properties of Dexamethasone 21-Sulfate Sodium as a Colon-Specific Prodrug of Dexamethasone

In Ho Kim, Hye Sik Kong, Bo Im Choi, Young Soo Kim, Hee Jung Kim, Young Wook Yang, Yun Jin Jung and Young Mi Kim
College of Pharmacy, Research Institute for Drug Development, Pusan National University, Busan, Korea

ABSTRACT We synthesized dexamethasone 21-sulfate sodium (DS) as a colon-specific prodrug of dexamethasone and investigated its properties. Introduction of a sulfate group to dexamethasone lowered the apparent partition coefficient from 52.5 to 0.27 in 1-octanol/pH 6.8 phosphate buffer at 37°C. DS was stable on incubation with buffer solutions of varied pH or with the upper intestinal contents of rats at 37°C for 24 h. On incubation with the cecal contents, DS was hydrolyzed by producing dexamethasone over 80% of the dose at 10 h. When DS was incubated with the cecal contents collected from trinitrobenzenesulfonic acid (TNBS)-induced colitic rats, the degree of prodrug hydrolysis and production of dexamethasone amounted to 70% of healthy rats. In comparison with prednisolone, hydrocortisone, and cortisone, dexamethasone was stable against bioinactivation by the cecal contents, a desirable property for the development of a colon-specific prodrug. These results demonstrated that DS might be delivered specifically to the colon as an intact form to produce dexamethasone in high yield, suggesting DS as a potential colon-specific prodrug of dexamethasone.

KEYWORDS Colon-specific prodrug of glucocorticoid, Dexamethasone, Sulfatase, Dexamethasone 21-sulfate sodium, Inflammatory bowel disease

INTRODUCTION

The principal drugs used in the therapy of inflammatory bowel disease (IBD) are the anti-inflammatory agents such as glucocorticoids and 5-aminosalicylic acid derivatives (Friedman and Blumberg, 2004; Robinson, 1998). Glucocorticoids are well absorbed in the upper intestine, and only a limited fraction of the dose is delivered to the inflammatory site in the distal ileum or colon after oral administration. To achieve efficient therapy and limit side effects caused by systemically absorbed drugs, development of colon-specific prodrugs of glucocorticoids has been the target of many studies (Nolen et al., 1995; McLeod et al., 1994; 1993; Haeberline et al., 1993; Friend & Chang,

Address correspondence to Young Mi Kim, College of Pharmacy, Pusan National University, Busan 609-735, Korea; Fax: 051-513-6754; E-mail: ymikim@pusan.ac.kr

1985). The drug molecule is usually coupled to the nonabsorbable polymer or hydrophilic molecule by the linkage, which is stable in the upper intestine and labile in the colon. In this way, absorption in the upper intestine is limited to allow the delivery of the prodrug to the colon, where the active drug is usually released by enzymes originated from microorganisms (Friend, 1991; Sinha & Kumria 2003; Shamat, 1993; Rubinstein, 1990).

Van Eldere et al. (1994) reported on the isolation and identification of intestinal steroid-desulfating bacteria from rats and humans. Huijghebaert et al. (1984) reported that microbial estrone sulfatase activity in the intestinal contents of rats was high in the cecum but very low in the small intestine. These reports suggested that sulfatase in the gastrointestinal tract is of microbial origin and its activity is high in the cecum but very low in the small intestine (Van Eldere et al., 1994; Huijghebaert et al., 1984). On the basis of these reports, we suggested sulfate ester sodium might serve as a promising colon-specific promoiety for glucocorticoids, which possess hydroxyl groups for promoiety attachment.

Previously, we prepared prednisolone 21-sulfate sodium (PDS) as a colon-specific prodrug and investigated its properties. Prednisolone was produced on incubation of PDS with the rat cecal contents, and the concentration increased in earlier period of incubation, passed through a maximum, and decreased as the incubation period extended. Similar patterns were noticed from other reports on the colon-specific delivery system of prednisolone, and the fraction recovered as prednisolone was generally low and variable depending on the colon-specific delivery system (Friend & Chang, 1984; Yano et al., 2001).

No proper explanation was provided in those studies. We suggested that reductive metabolism by the microbial enzymes in the large intestinal contents should be responsible for these results (Jung et al., 2003), considering that reduction is one of the most common metabolic reactions executed by the microbes in the large intestine. Because the reduction of 4, 5 double bond and 3-keto group in ring A of glucocorticoids results in a loss of anti-inflammatory action (Schimmer & Parker, 2001), the stability against reductive metabolism should be a crucial factor for the selection of a proper glucocorticoid for the design of a potential colon-specific prodrug of a glucocorticoid. Although PDS possessed necessary properties required for a colon-specific prodrug, the level of prednisolone in the large intestine was relatively low after oral administration due probably to the reductive metabolism. In this study, we investigated the metabolic stability of several glucocorticoids on incubation with the rat gastrointestinal tract segment contents.

Dexamethasone was quite stable against reductive metabolism, suggesting it as a promising candidate for the development of a colon-specific prodrug of a glucocorticoid. We synthesized dexamethasone 21-sulfate sodium (DS) and assessed its potential as a colon-specific prodrug of a glucocorticoid.

MATERIALS AND METHODS

Chemicals and Animals

Dexamethasone, prednisolone, and sulfatrioxide triethylamine complex (STT) and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO) and were used as received. Solvents for NMR and HPLC were obtained from Merck Inc. (Damstadt, Germany). All other chemicals were reagent grade, commercially available products. Male Sprague-Dawley rats weighing 200–250 g were purchased from Daehan Biotec Co. Ltd. (Daegu, Korea) and housed in the animal care facility at Pusan National University, Busan, Korea. The animals were fasted overnight (16 h) prior to killing for the experiment. Prednisolone 21-sulfate sodium (PDS) was prepared according to the procedure described previously (Jung et al., 2003).

Instruments

IR spectra were recorded on a Bomem MB 100 FT-IR spectrophotometer (Bomem). ^{1}H-NMR spectra were taken on a Varian AS 500 spectrometer, and the chemical shifts were in ppm downfield from tetramethylsilane. Elemental analysis was carried out by an Elemental Analyzer System (Profile HV-3). Melting points were taken on a Mel Tem II (Laboratory Devices, Holliston, MA) and were uncorrected. A Polytron PT 3100 homogenizer, an Eppendorf Centrifuge 5415C (Hamburg, Germany), and a Taitec microincubator M-36 (Japan) were used. The HPLC system consisted of Model 305, 306 pumps, a 117 variable UV detector, a Model 234 autoinjector, a Model 805 manometric module, and a Model 811C dynamic mixer from Gilson.

Analysis of Drugs in the Rat Gastrointestinal Tract Segment Contents

A male Sprague-Dawley rat was anesthetized by diethyl ether, a midline incision was made, and various segments of gastrointestinal tract were obtained. The contents of the proximal small intestine (PSI), distal small intestine (DSI), cecum, and colon were diluted separately with pH 6.8 isotonic phosphate buffer solution (20 w/v%). To a 500-μL portion of the above suspension were added 20, 40, 100, and 200 μL of the stock solution of each drug (50 μg/mL) and appropriate volume of pH 6.8 isotonic phosphate buffer to make the final volume of 1 mL, vortexed for 2 min, and centrifuged at 5000 rpm for 3 min. The supernatant provided standard solutions of each drug in concentrations of 1, 2, 5, or 10 μg/mL in various biological specimens. To a 0.1-mL portion of the standard or blank solution, 0.9 mL of methanol was added to precipitate protein in the sample, vortexed for 2 min, centrifuged for 5 min at 10,000 rpm, and filtered through a membrane filter (0.45 μm). The supernatant (20 μL) was injected on a μBondpak C_{18} column (300 × 3.9 mm) and eluted with acetonitrile/0.067 M, pH 4.5 phosphate buffer (4/6) at a flow rate of 1.0 mL/min. The eluate was monitored by measuring the absorption at 254 nm at a sensitivity of AUFS 0.01. A calibration curve was constructed from the results.

Preparation of DS

STT (5.58 g, 25.0 mmol) was added in portions with stirring to the solution of dexamethasone (3.92 g, 10.0 mmol) in 50 mL of anhydrous benzene and pyridine (1/1) at 56–60°C. The solvent was removed by flash evaporation under reduced pressure, and dexamethasone 21-sulfate triethylammonium was obtained as oily residue. It was dissolved in a minimum amount of water and suspended into a solution of 10% NaCl with mechanical stirring for 1 h. The resulting precipitate, DS, was collected by suction filtration and recrystallized from absolute ethanol. (Overall yield, 4.2 g: 84%). mp: 182°C (dec.); IR (nujol)ν_{max}(OH): 3450 cm^{-1}, (C=O): 1719, 1662 cm^{-1} (C=C): 1611 cm^{-1}, (S=O): 1259, 1034 cm^{-1}; ^1H NMR (D_2O): δ 1.00 (s, 3H, C-18), 1.55 (s, 3H, C-19), 4.90–5.21 (AB q, 2H, C-21), 6.23 (s, 1H, C-4), 6.43 (d, 1H, C-1), 7.54 (d, 1H, C-2); EA for $C_{22}H_{28}FSO_8Na$: Calculated (C; 53.44, H; 5.67, S; 6.48), Found (C; 53.58, H; 5.20, S; 6.65)].

Chemical Stability

A solution of DS (100 μg/mL) was incubated in 0.1 M, pH 1.2 hydrochloric acid buffer, 0.1 M, pH 4.5 acetate buffer, and pH 6.8 isotonic phosphate buffer at 37°C for 24 h. At a predetermined time interval, a 20-μL portion of the solution was removed, and the concentration of dexamethasone or DS was analyzed by HPLC as described previously.

Solubility

Solubility was determined by placing 50 mg of DS in a 1-mL microtube containing isotonic phosphate buffer (pH 6.8) and shaking it for 24 h at 25°C. After centrifugation, 20-μL portion of the supernatant was analyzed by HPLC as described previously.

Apparent Partition Coefficient

To a solution (10 mL) of dexamethasone or DS (100 μg/mL) in pH 6.8 isotonic phosphate buffer presaturated with 1-octanol, 10 mL of 1-octanol presaturated with pH 6.8 isotonic phosphate buffer was added. The mixture was shaken for 10 h and left standing for 4 h at 37°C. The concentration of dexamethasone or DS in the aqueous phase was analyzed by HPLC as described previously. A calibration curve was constructed from the standard solutions (0.01–10 μg/mL) of dexamethasone or DS in pH 6.8 isotonic phosphate buffer. The apparent partition coefficients were calculated by using the equation $(C_o–C_w)/C_w$, where C_o and C_w represent the initial and equilibrium concentration of the drug in aqueous phase, respectively.

Incubation of Drugs With the Rat Gastrointestinal Tract Segment Contents

Contents of various gastrointestinal tract segments were collected in a glove box, which was previously displaced by nitrogen. For incubation with 20% cecal contents, 0.2-g portion of the contents, 0.5 mL of a drug solution (100 μg equivalence in pH 6.8 isotonic phosphate buffer), and appropriate volume of buffer

were mixed to make the final volume 1 mL and incubated at 37°C in a shaking incubator, which was placed in a glove box under nitrogen. For incubation with 10% and 5%, the relative amounts of gastrointestinal tract segment contents were adjusted accordingly. At appropriate time intervals, the sample was centrifuged at 5000 rpm for 3 min. To a 0.1-mL portion of the supernatant, 0.9 mL of methanol was added to precipitate protein in the sample, vortexed for 2 min and centrifuged for 5 min at 10,000 rpm. Concentration of the drug in a 20-μL portion of the supernatant was determined by HPLC as described previously. We adopted pH 6.8, which was the reported pH values normally found in the rat gastrointestinal tract (McLeod et al. 1993; William-Smith, 1965).

Incubation of Drugs With Gastrointestinal Tract Segment Contents Collected From TNBS-Induced Colitis Rat

Inflammation was induced by the method of Yano et al. (2001) and Morris et al. (1989). Before the induction of colitis, the rats were fasted for 24 h but they had free access to water. The rats were lightly anesthetized with diethyl ether. A rubber catheter (OD, 2 mm) was inserted rectally into the colon so that the tip was 8 cm proximal to the anus, approximately at the splenic flexture. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) dissolved in 50% (v/v) aqueous ethanol was instilled into the colon via the rubber cannula (15 mg/0.3 mL/rat). The instillation procedure required about 5 sec to complete. Then the rats were kept in a supine Trendelenburg position for 3 min. Three days after the administration of TNBS, the rats whose body weight was decreased by 0–20% of the initial weight were used for the experiments. Cecal and colonic contents were collected and used for the experiment according to the procedure described previously.

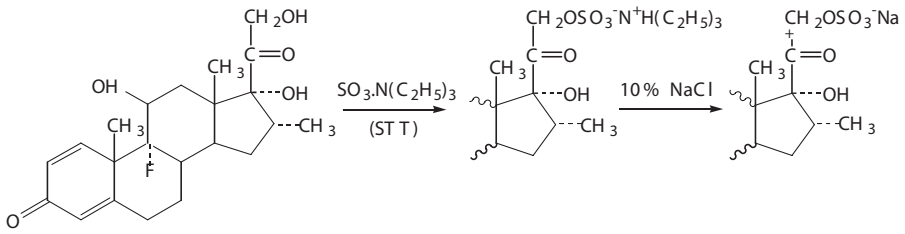
Statistical Analysis

The results are expressed as means ± SE. The nonpaired Student's t-test was used to assess the statistical significance ($P < 0.05$) of results for all measurements.

RESULTS AND DISCUSSION

To be a colon-specific prodrug, accessibility to the colonic site as an intact form and activation in the large intestine is required. We synthesized dexamethasone 21-sulfate sodium (DS) as a colon-specific prodrug of dexamethasone expecting that increased hydrophilicity of the molecule by the introduction of sulfate ester sodium group and limited availability of sulfatase in the upper intestine should allow the delivery of orally administered prodrug as an intact form specifically to the colon, where the active drug is expected to be released by the action of sulfatase of microbial origin (Van Eldere et al., 1994; Huijghebaert et al., 1984). Van Eldere et al. (1994) reported on the isolation and identification of intestinal steroid-desulfating bacteria from rats and humans. These reports might indicate that the sulfate linkage is stable in the small intestine and hydrolyzed by sulfatases originated from microbes in the colon.

Sulfation of dexamethasone proceeded readily by the reaction of dexamethasone and sulfatrioxide triethylammonium complex in anhydrous pyridine to give dexamethasone 21-sulfate triethylammonium, which produced DS in good yield by treating with concentrated sodium chloride solution (Scheme 1). Sulfation of other secondary hydroxy groups was excluded by terminating the reaction before such



SCHEME 1 **Preparation of dexamethasone 21-sulfate sodium.**

products were monitored on TLC. IR spectrum of DS showed two strong absorption peaks from (—S=O) asymmetric and symmetric stretching of sulfonic acid ester at 1259 and 1034 cm^{-1}, respectively. The most characteristic change in ^1H- NMR spectrum of DS was the downfield shift of the two protons on C-21 to 0.5 ppm by the introduction of a sulfate group. Solubility of DS increased greatly (14.4 mg/mL) compared with dexamethasone, which was practically insoluble in pH 6.8 phosphate buffer solution. Apparent partition coefficient of DS and dexamethasone in 1-octanol/pH 6.8 phosphate buffer at 37°C was 0.27 and 52.5, respectively. The low partition coefficient of DS suggested that absorption of DS by way of transcellular passive diffusion might be restricted greatly. When DS was incubated with buffer solutions of pH 1.2 and pH 6.8, which represented pH of the stomach and small intestine, respectively, the concentration of DS remained constant for more than 24 h. When DS was incubated with the contents of stomach and small intestine, DS was not hydrolyzed, showing no change in the concentration of DS for the whole incubation period of 24 h. These results suggested that DS might be nonabsorbable and chemically and enzymatically stable in the upper intestine, and a large fraction of orally administered dose might be delivered to the colon as an intact form.

Considering that 4, 5 double bond and the 3-keto group on ring A of a glucocorticoid are essential for anti-inflammatory action (Schimmer & Parker, 2001), the metabolic stability of a glucocorticoid in the gastrointestinal tract should be a very important factor for the selection of a candidate drug for developing colon-specific prodrugs. We incubated cortisone, hydrocortisone, prednisolone, and dexamethasone with the contents (10%) from various gastrointestinal tract segments of rats and assessed their metabolic stability. As listed in Fig. 1, they were stable on incubation with the upper intestinal contents, with no concentration change for the whole incubation period. With the cecal contents, dexamethasone was quite stable, whereas the concentration of cortisone, hydrocortisone, and prednisolone decreased rapidly as the incubation time extended, exhibiting their metabolic instability. The amount of drug remaining in the medium was 22%, 35%, 53%, and 92% at 3 h and 0%, 8%, 28%, and 85% at 7 h for cortisone, hydrocortisone, prednisolone, and dexamethasone, respectively. Because reduction is one of the common metabolic

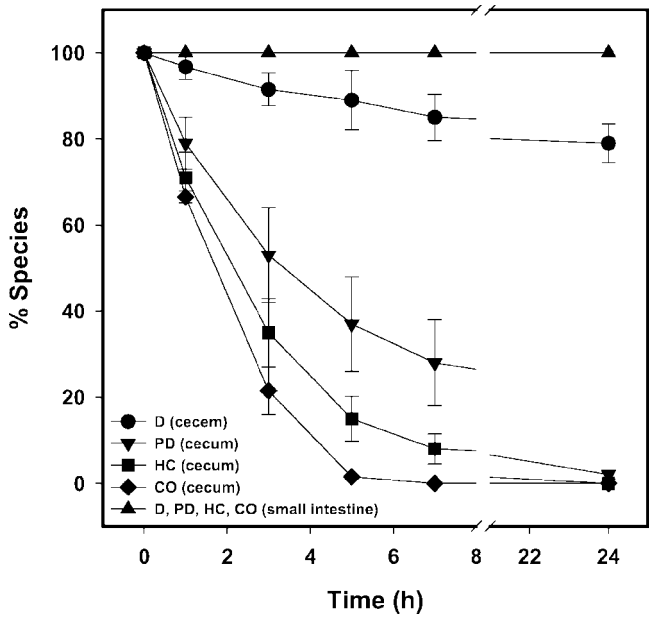


FIGURE 1 **Incubation of cortisone (CO), hydrocortisone (HC), prednisolone (PD), and dexamethasone (D) with the small intestinal and cecal contents of rats (10%) in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

reactions exerted by the microbes in the large intestine (Faigle, 1993), we suggest that diminution in the amount of drugs in the incubation medium might be related to the reduction of the double bond and/or 3-keto group on ring A, which is the only chromophore in the structure of these glucocorticoids detectable by a UV detector of the HPLC system. These results suggested that dexamethasone might be the most promising candidate for the development of a colon-specific prodrug.

Figure 2 shows the results when DS was incubated with the rat gastrointestinal tract segments contents (5%). DS was stable when it was incubated with the contents from proximal small intestine (PSI) and distal small intestine (DSI) and hydrolyzed to release dexamethasone on incubation with the contents of cecum and colon. The fraction released as dexamethasone amounted to 52% and 38% of the dose at 10 h and 62% and 38% at 24 h for cecal and colonic contents, respectively, which indicated that prodrug activation took place most readily in the rat cecum, where the bacterial counts are high as in the human colon.

Figure 3 shows the results when DS was incubated with 5%, 10%, and 20% cecal contents. The fraction released as dexamethasone increased as the concentration of the cecal contents increased. The level of dexamethasone amounted to 52%, 78%, and 84% of the

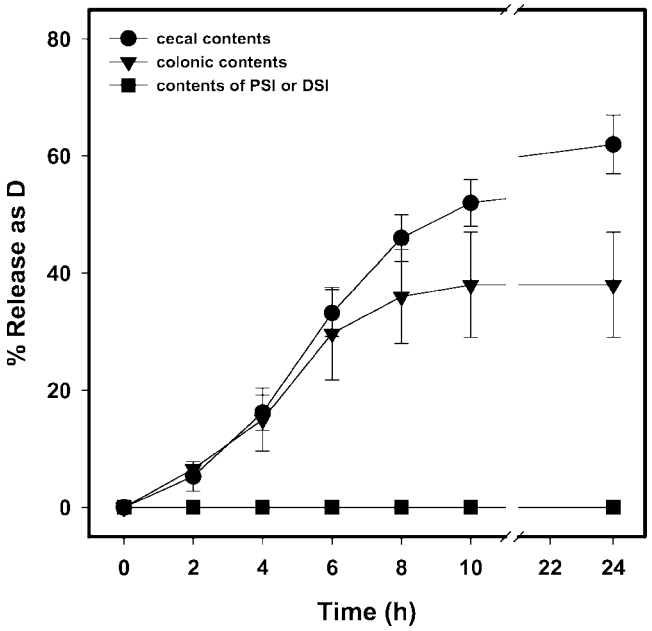


FIGURE 2 **Release profiles of dexamethasone (D) on incubation of dexamethasone 21-sulfate sodium (DS) with the contents (5%) of proximal small intestine (PSI), distal small intestine (DSI), cecum, and colon of rats in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

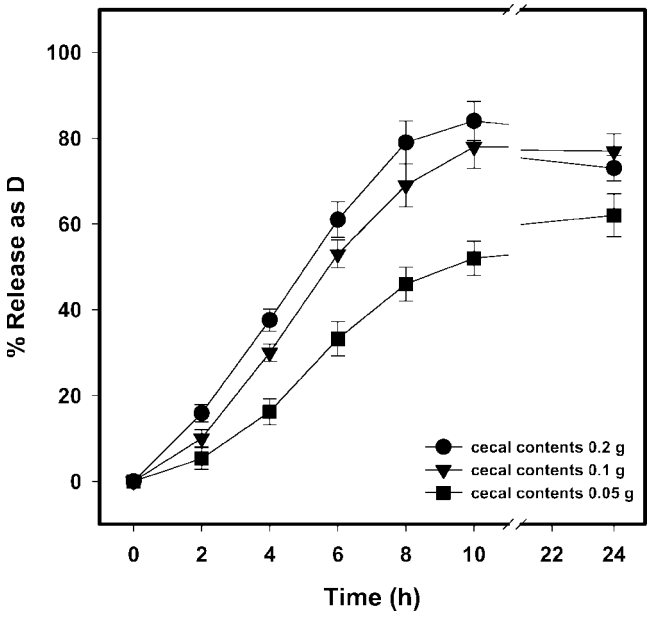


FIGURE 3 **Release (%) of dexamethasone (D) on incubation of dexamethasone 21-sulfate sodium (DS) with varied dilution of the cecal contents of rats in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

dose at 10 h and 62%, 77%, and 73% at 24 h for 5%, 10%, and 20% cecal contents, respectively. As the incubation period extended to 24 h, the level of dexamethasone decreased slightly with 20% cecal contents, which are in accordance with the metabolic stability of dexamethaone (Fig. 1). Effects of cecal contents concentration on hydrolysis of DS and release of dexamethasone were compared with the respective results for prednisolone 21-sulfate sodium (PDS).

As shown in Fig. 4, the rate of DS conversion and the level of dexamethasone in the medium increased as the concentration of the cecal contents increased (left section). In PDS, the level of prednisolone, which passed through a maximum and decreased as the incubation period extended, was no longer in parallel order with the concentration of cecal contents even though the rate of hydrolysis was comparable in both cases (right section).

The difference exhibited by DS and PDS might be related to the relative stability of dexamethasone and prednisolone against reductive metabolism by the cecal contents as shown in Fig. 1. The level of a glucocorticoid in the incubation medium might be related to the relative rates of hydrolysis of the prodrug and reduction of the glucocorticoid, which might be affected by the concentration of the cecal contents. Hydrolysis might dominate in earlier period of incubation to produce and accumulate the glucocorticoid. As the incubation period extends, the amount of prodrug and, consequently, the rate of prodrug hydrolysis decreases. If the glucocorticoid is stable against reductive metabolism, as observed with dexamethasone, the level of glucocorticoid will increase as long as it is produced from hydrolysis of the prodrug. If the glucocorticoid is vulnerable to the reductive metabolism as observed with prednisolone, the level of glucocorticoid will decrease when the amount reduced exceeds the amount produced. These results suggest that hydrolysis precedes the reduction process, which is slower than hydrolysis. If the rate of a glucocorticoid reduction is faster than the rate of hydrolysis of the prodrug, the glucocorticoid may not be detectable in the incubation medium because it will disappear as soon as it is formed. Therefore, the potential of glucocortcoid 21-sulfate sodium as a colon-specific prodrug will be dependent greatly on the relative rate of hydrolysis (bioactivation) and reduction of ring A (bioinactivation) by the microbes in the large intestine.

In this regard, the results suggested that cortisone and hydrocortisone may not be suitable candidates for developing colon-specific prodrugs and DS should be a more promising colon-specific prodrug than PDS.

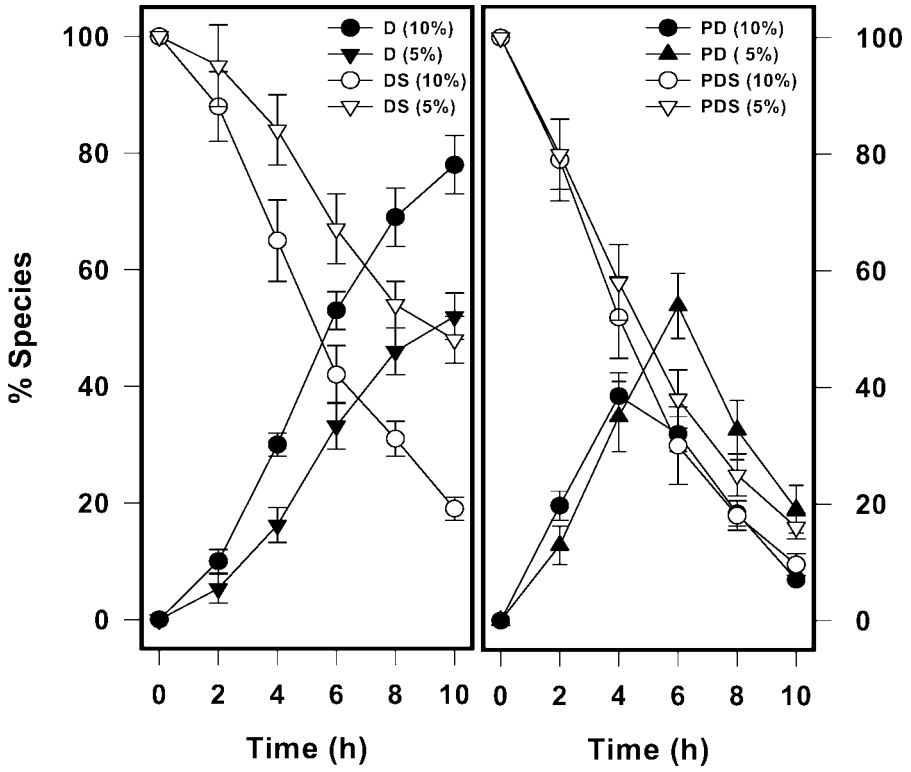


FIGURE 4 **Release of dexamethasone (D) and prednisolone (PD) on incubation of dexamethasone 21-sulfate sodium (DS) and prednisolone 21-sulfate sodium (PDS) with 5% and 10% cecal contents of rats in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

To investigate the effect of bowel inflammation on hydrolysis of the prodrug and production of the glucocorticoid, DS and PDS were incubated with the cecal and colonic contents collected from TNBS-induced colitic rats, and the results were compared with those from healthy rats. The degree of DS hydrolysis and production of dexamethasone for colitic rats amounted approximately to 90% and 70%, respectively, of the healthy rats at 24 h for the cecal contents (Fig. 5) and 60% and 80%, respectively, for the colonic contents (Fig. 6). The degree of PDS hydrolysis and production of prednisolone for colitic rats were comparable with those of the healthy rats for the cecal contents and slightly lower for colonic contents (Figs. 7 and 8). The discrepancy between healthy vs. colitic rats was more pronounced for the colonic contents than the cecal contents, which might be related to the fact that the colon was major inflammatory site. PDS was only slightly affected by the inflammation, and it might be due to the relatively fast PDS hydrolysis in comparison with DS (Fig. 5 vs. Fig. 7 and Fig. 6 vs. Fig. 8).

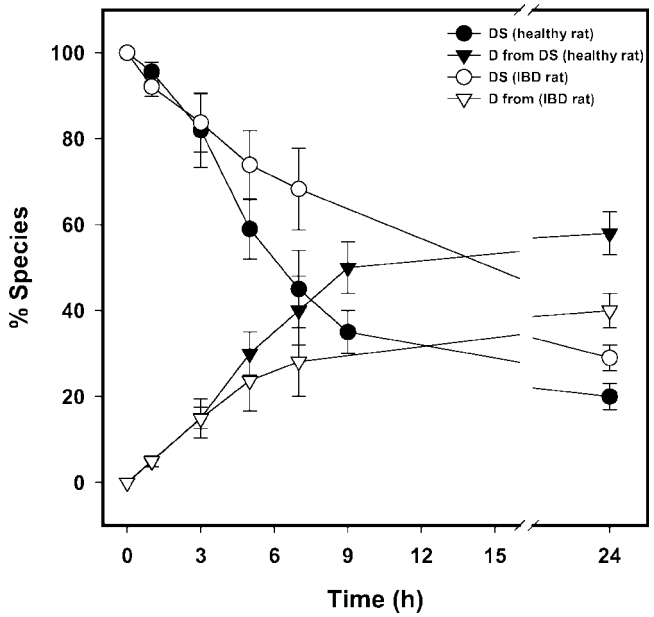


FIGURE 5 **Hydrolysis of dexamethasone 21-sulfate sodium (DS) and release of dexamethasone (D) on incubation with 5% dilution of cecal contents collected from healthy rats or TNBS-induced colitic rats in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

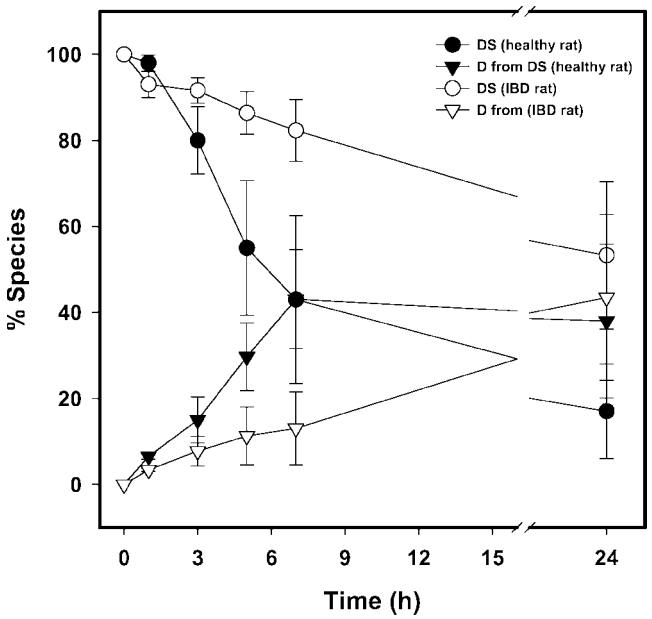


FIGURE 6 **Hydrolysis of dexamethasone 21-sulfate sodium (DS) and release of dexamethasone (D) on incubation with 5% dilution of colonic contents collected from healthy rats or TNBS-induced colitic rats in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

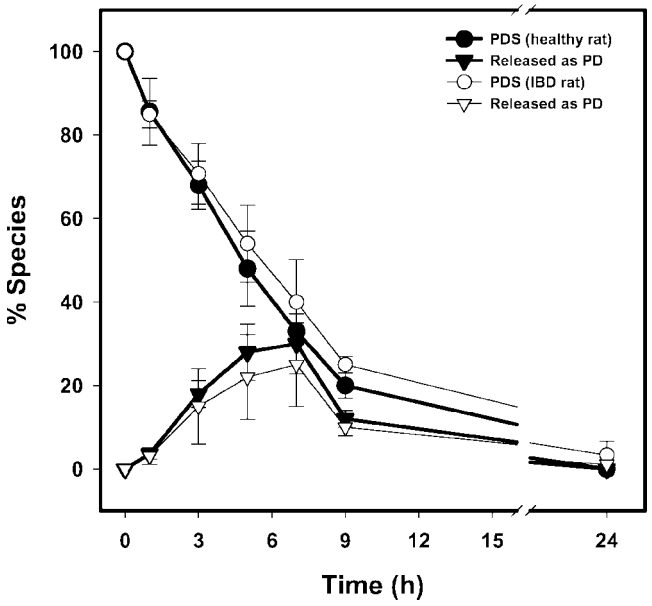


FIGURE 7 **Hydrolysis of prednisolone 21-sulfate sodium (PDS) and release of prednisolone (PD) on incubation with 5% dilution of cecal contents collected from healthy rats or TNBS-induced colitic rats in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

To summarize, dexamethasone was stable against bioinactivation by the cecal contents, a crucial property for the development of a colon-specific prodrug.

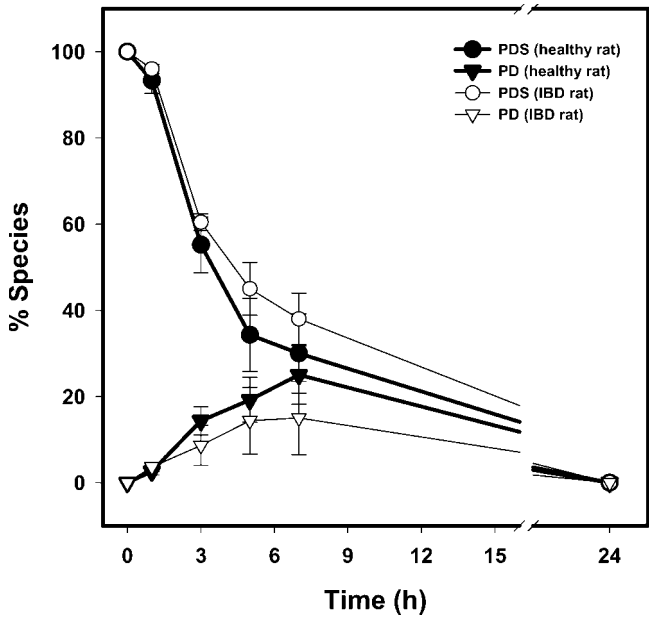


FIGURE 8 **Hydrolysis of prednisolone 21-sulfate sodium (PDS) and release of prednisolone (PD) on incubation with 5% dilution of colonic contents collected from healthy rats or TNBS-induced colitic rats in pH 6.8 phosphate buffer at 37°C. Data are means ± SE (n = 6–10). P < 0.05.**

DS exhibited characteristics required for a colon-specific prodrug such as low partition coefficient, stability under the conditions of upper intestine and production of dexamethasone by the cecal contents. DS might be nonabsorbable and stable in the upper intestine, and a large fraction of orally administered DS might be delivered to the colon as an intact form and liberate dexamethasone. We suggest DS as a promising colon-specific prodrug of dexamethasone. Further studies are in progress.

ACKNOWLEDGMENT

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