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# *In vitro* Evaluation of Dextran-5-aminosalicylic Acid Conjugate as a Polymeric Colon-specific Prodrug of 5-aminosalicylic Acid

**Yunjin Jung, Hyunchu Jeon, Deakyu Choi and Young Mi Kim*†**

*Laboratory of Biomedicinal/*Medicinal Chemistry, College of Pharmacy, Pusan National University, Pusan 609-735, Korea*

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**ABSTRACT** – Dextran-5-aminosalicylic acid conjugate (dextran-5-ASA) was in vitro-evaluated as a polymeric colon-specific prodrug of 5-aminosalicylic acid (5-ASA). Chemical stability of dextran-5-ASA in the pH 1.2 or 6.8 buffer solutions was investigated at 37 for 6 hrs. The dextran backbone was not degraded and no 5-ASA release was detected. Moreover, dextran-5-ASA neither liberated 5-ASA in the homogenates of the small intestine of rats nor was transported across Caco-2 cell monolayers, suggesting no significant loss of dextran-5-ASA during transit through the upper intestine. Furthermore, incubation of dextran-5-ASA in 10% cecal contents of rats released about 37% and 55% of 5-ASA bound to dextran in 8 hr and 24 hr, respectively. While that with either esterase or dextranase failed to liberate 5-ASA from the polymeric prodrug, incubation of dextran-5-ASA with both esterases and dextranse released 5-ASA up to about 24% of 5-ASA bound to dextran. These results suggest that, after oral administration of dextran-5-ASA, the polymeric prodrug is delivered specifically to and releases 5-ASA in the large intestine, and reveal that the 5-ASA release by cleavage of the ester bond requires precedent depolymerization of the dextran backbone.

**Key words** – Dextran-5-aminosalicylic acid conjugate, Colon-specific prodrug, 5-Aminosalicylic acid, Dextran, Polymeric prodrug, Inflammatory bowel disease

For the efficient treatment of diseases which develop locally at the colonic site to avoid systemic absorption and reduce side effects, delivery of orally administered drugs specifically to the colon is desirable. Development of a prodrug is a way to deliver drugs specifically to the colon. In prodrug approaches, polymeric or water-soluble carrier is employed to prevent absorption of prodrugs in the upper intestine. After delivered to the colon, the prodrug is presummed to be activated by the enzymes originated from the microbes which are especially abundant in that portion of the alimentary canal.[1] Numerous publications have appeared during the last decades.[2-5]

Dextran is a nonstarch polysaccaride of linear α-1,6-glucopyranose chain with α-1,3-glucopyranose branching. It is nonimmunogenic and biocompatible and has been studied widely as a parenteral polymeric drug carrier.[6] It is degraded readily by dextranase which produced by the bacteriods residing only in colon. Release of the drug molecule from dextran-drug conjugate does not take place in the upper intestine, presumably because the steric hindrance of the polymer matrix prevents the enzymic action there. Release of the drug is reported to take place only from the oligomerized dextran-drug conjugate, which is formed by depolymerization of dextran matrix by endodextranase in the colon where the bacterial count is very high.[6,7] Inter- or intra-species variations in the composition and activity of dextranase are very limited. These are very desirable properties for dextran to be adopted as a colon-specific carrier.[2]

Currently no curative therapeutic agents are available and consequently the therapeutic goal in active IBD is to bring rapidly the patient into a hopefully long lasting remission and to prevent any relapses.[8,9] For induction and maintenance of remission, various drugs can be used including aminosalicylates, glucocorticosteroids and immunomodulators. Among the therapeutic agents, 5-aminosalicylic acid (5-ASA) is the mainstay for inducing and maintaining remission in mild to moderate active UC and CD.[8,10] However, 5-ASA itself is not adequate to be used for treatment of IBD because it is absorbed rapidly and extensively through the upper intestine before it reaches to the colon.[8] In addition, systemically absorbed 5-ASA is reported to induce nephrotic syndrome.[11] For this reason, 5-ASA is formulated as sustained-release preparations or prodrugs for colon-specific delivery.[12,13]

In this study, we suggest that, while chemically and enzymatically stable in the upper intestine, dextran-5-ASA conjugate is depolymerized and releases 5-ASA from the depolymerized dextran-5-ASA conjugate in the large intestine.

†본 논문에 관한 문의는 이 저자에게로
Tel : 051)510-2807, E-mail : ymikim@pusan.ac.kr

## Materials and Methods

### Materials and Instruments

Dextran, dextranase (Penicillium sp.), esterase, 5-aminosalicylic acid (5-ASA), 2,4-dinitrosalicylic acid (DNS) and anthrone were obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals and solvents were reagent grade. UV was taken on Shimadzu UV- 2101PC spectrophotometer (Shimadzu, Tokyo, Japan). An Orion 320 pH meter (Allometric, Inc., TX, USA) was used for the pH measurements. Gel fillteration chromatography was carried out using Sephadex G-75 (Pharmacia, Uppsala, Sweden). A Eyela Mazela-Z tissue homogenizer (Eyela, Tokyo, Japan) was used for homogenation of tissue and contents of intestinal tracts of rats and a Hanil Supra K-22 centrifuge (Hanil, Seoul, Korea) was used for centrifugation. Dextran-5-ASA was synthesized in our laboratory. In this experiment, dextran-5-ASA (DS 15, MW 40600) was used. Degree of substitution means amount (mg) of 5-ASA bound to 100 mg of dextran-5-ASA.

### Methods

### Buffer solutions

Buffer solution A, B, or C was prepared as describerd in USP XXIII.

Buffer A: Hydrochloric acid buffer (pH 1.2), Buffer B: 0.2 M citrate buffer (pH 3).

Buffer C: 0.1 M acetate buffer (pH 5.4), Buffer D: Isotonic phosphate buffer (0.1 M sod. phosphate dibasic and 0.15 M sod. phosphate monobasic were mixed to give pH 6.8), Buffer E: Phosphate buffer (5.0 mM sod. phosphate dibasic and phosphoric acid were mixed to give pH 6).

### HPLC analysis

Concentration of 5-ASA was determined by HPLC (Chungi *et al.*, 1989). 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. The mobile phase consisted of 10% methanol in buffer E and 0.5 mM tetrabutylammonium chloride and filtered through 0.45 um membrane filter before use. A mBondapack $C_{18}$ ($300 \times 3.9$) column was eluted with the mobile phase at a flow rate of 1 ml/min and at a pressure of about 2000 psi. The column eluent was monitored at 254 nm with a sensitivity of AUFS 0.01 and a Gilson 712 software was employed for data analysis.

### DNS method

DNS reagent solution was prepared by dissolving dinitrosalicylic acid (5 g) in 2 N NaOH (100 mL) and distilled water (250 mL). To this solution, sodium potassium tartrate tetrahydrate (150 g) was dissolved and the volume was adjusted to 500 mL with distilled water (Bronsted *et al.*, 1995). Maltose solution in buffer C (0.093 mg/mL-0.75 mg/mL), 200 μL was mixed with 600 μL of DNS reagent solution, boiled for 5 min, cooled for 10 min and measured the absorbance at 540 nm by UV spectrophotometer. A calibration curve was made from the results. A portion of the incubated sample was treated with DNS reagent solution according to the same procedure, and the amount of terminal reducing sugar was deduced from the calibration curve.

### Cell Culture and transport experiment

Caco-2 cells between passages 75 and 90 were cultured routinely in DMEM and were supplemented with a 1% non-essential amino acid solution, 10% FBS, benzylpenicillin G and streptomycin sulfate at 37°C under an atmosphere of 95% air and 5% $CO_2$. Cells were harvested by treatment with trypsin/EDTA at 37°C before reaching confluence, were resuspended in DMEM, and were finally seeded at a density of about $1 \times 10^4$ cells/cm$^2$ on a TransWell insert filter (Costar, Cambridge, MA). The culture medium was placed into the apical (0.5 mL) and basolateral (1 mL) chambers. Cells were allowed to reach confluence and to differentiate for 3 weeks before use in the experiment. The TEER of the filter-grown monolayers reached a value of at least 600 W cm$^2$ before use in the experiment. Dextran-5-ASA was dissolved in transport media (HBSS; 9.8 g/L, HEPES; 2.38 g/L, Sodium bicarbonate; 0.35 g/L, Glucose; 1.95 g/L) to prepare a test solution (125 μg equivalent of 5-ASA/mL). The basolateral chamber was bathed in 1 mL of the transport media, and 0.5 mL of the test solution was added to the apical chamber. Samples were collected from both chambers every 20 min up to for 1 h. After collecting samples, the basolateral chamber was then replaced with the fresh transport media. Dextran-5-ASA conjugate in each sample was analyzed by measuring the absorbance at 307 nm.

### Size exclusion chromatography of dextran-5-ASA after incubation with dextranase

Dextran-5-ASA in buffer C (4 mg/mL) and dextranase (10 DU/mL) were placed in a microtube and incubated with shaking at 37°C. At appropriate time interval, it was placed in boiling water to inactivate the enzyme, centrifuged and evaporated. The residue, thus obtained, was dissolved in 100-200 μL of 0.2 M citric acid, loaded on a column packed with Sephadex G-75 (bed volume 30 mL), and eluted with buffer B at a rate of

0.2 mL/min. Each fraction was diluted with buffer C, and amount of dextran was determined by adding 600 uL of anthrone reagent (35 mg of anthrone reagent in 100 mL of concentric sulfuric acid) to 300 uL of eluent of each fraction, standing for 30 min at 0, and measuring the absorbance at 630 nm.

**Release of 5-ASA from dextran-5-ASA conjugate upon incubation with esterase and/or dextranase**

Dextran-5-ASA in buffer C (4 mg/mL) were placed in a microtube and incubated with dextranase (10 DU/mL) at 37°C. Three hours later, pH of the reaction solution was adjusted to 8 using 0.01 M NaOH followed by addition of esterase. Three hours later, it was placed in boiling water to inactivate the enzyme, centrifuged. Free 5-ASA in the supernatants was analyzed by a HPLC. In separate experiments, dextran-5-ASA was incubated with either dextranases and esterase for 6 hr and free 5-ASA was analyzed as aforementioned.

**Release of 5-ASA from dextran-5-ASA in the contents/ homogenates of various segments of gastrointestinal tract of rat**

To examine 5-ASA release from dextran-5-ASA in the homogenates of the small intestine, a half dilution of the homogenates of the small intestine of rats in buffer D, 0.2 mL, was placed in a microtube and added 0.8 mL of dextran-5-ASA solution in buffer D (120 μg equivalent of 5-ASA/ 0.8 mL), and the mixture was incubated for 6 hrs at 37°C. At appropriate time interval, it was centrifuged at 5,000 rpm for 3 min. To the 0.1 mL of the supernatant, 0.9 mL of methanol was added, vortexed for 2 min, centrifuged for 5 min at 10,000 × g. The amount of 5-ASA in 20 μL of the supernatant was analyzed by HPLC. To examine 5-ASA release from dextran-5-ASA in the contents of the large intestine, the cecal contents, 0.1 g was placed in a microtube which was previously displaced by nitrogen. To each microtube, 0.9 mL of dextran-5-ASA solution in buffer D (120 μg equivalent of 5-ASA/0.9 mL) was added and incubated at 37°C. At appropriate time interval, the sample was centrifuged at 5,000 rpm for 3 min. Concentration of 5-ASA in the supernatant was analyzed as aforementioned.

## Results

**Dextran-5-ASA conjugate neither is permeable to Caco-2 cell monolayer nor releases 5-ASA in the homogenates of the small intestine**

For efficient delivery of orally administered dextran-5-ASA conjugate to the large intestine, the polymeric conjugate should not be absorbed and release 5-ASA during the transit through the upper intestine. First, we examined whether dextran-5-ASA conjugate was chemically and enzymatically stable in the upper intestine. The polymeric conjugate was 6 hr-incubated in the pH 1.2 or pH 6.8 buffer solution, representing pH of the stomach and small intestine, and the release of 5-ASA and depolymerization of the polymer conjugate were monitored. Neither release of 5-ASA or depolymerization of the polymeric conjugate was not observed in HPLC analysis and DNS method, respectively. To test enzymatic stability in the upper intestine, the same experiment was done in the homogenates of tissue and contents of the small intestine. No 5-ASA release was observed. We next examined whether the systemic absorption of the polymeric conjugate could be limited in the upper intestine. To do this, the transport of dextran-5-ASA conjugate across Caco-2 cell monolayers was tested. The transport of the dextran conjugate across Caco-2 monolayers was monitored by measuring the absorbance at 307 nm in both apical and basolateral chambers. No change in the levels of the dextran conjugate was detected in both chambers. These results suggest that the dextran conjugate passes the upper intestine without significant loss.

**Dextran-5-ASA conjugate releases 5-ASA in the cecal contents of rats**

To examine whether dextran-5-ASA would liberate the active ingredient, 5-ASA, after delivery to the large intestine, the conjugate was incubated with the cecal contents of rats and the release of 5-ASA was monitored. As shown in Figure 1, the amount of 5-ASA released in the 10% cecal contents was 44 μg (37%) and 66 μg (55%) in 8 hrs and 24 hrs, respectively.

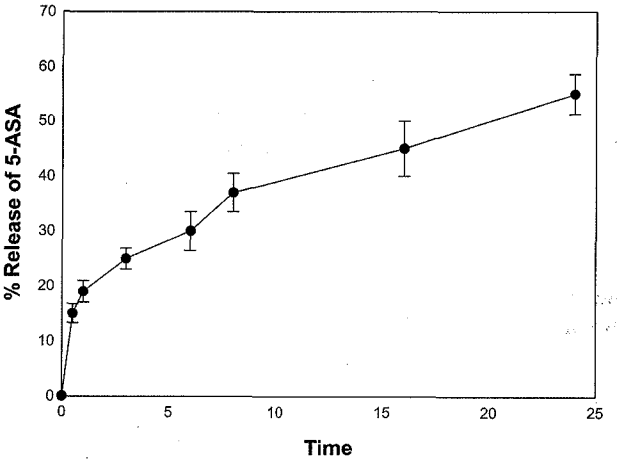


**Figure 1**—Release of 5-ASA during incubation of dextran-5-ASA (equiv. to 120 μg of 5-ASA) in the ten-fold diluted cecal contents. Data are mean ± S.E. (n = 5).

To test whether the 5-ASA release was dependent on microbial enzymes in the large intestine, the same experiment was done with autoclaved cecal contents, which is appropriate to test microbially-triggered activation of a colon-specific prodrug.[14] In contrast to the above result, 5-ASA was not detected up to for 8 hr upon incubation of dextran-5-ASA with the autoclaved cecal contents.

**Depolymerization of dextran-5-ASA conjugate is prerequisite for enzymatic release of 5-ASA from the dextran conjugate**

Our data demonstrate that although esterases are abundant throughout the digestive tract, the ester bond between 5-ASA and dextran is cleaved (deesterified) to liberate 5-ASA only in the large intestine where microbial dextranases exist. This suggests that the ester bond may not be hydrolyzed by esterases in the upper intestine due to the steric hindrance conferred by the dextran backbone, and hydrolysis by esterases takes place only after the dexran backbone is degraded to smaller sizes by the endodextranases of microbial origin in the large intestine. To test this hypothesis, we first examined whether dextranase was able to depolymerize the modified dextran, dextran-5-ASA. The depolymerization was accessed chemically and chromatographically by the DNS method and size exclusion chromatography, respectively, following incubation of the dextran conjugate with dextranase. Table I showed that the depolymerization occurred and appeared to be complete in 2.5 hr. In parallel with the result, the maxima of the distribution curve moved to the fraction of lower molecular weight as the depolymerization proceeded (Figure 2). To examine whether the depolymerization preceded the enzymatic deesterification for release of 5-ASA from the dextran conjugate, the dextran conjugate was incubated with esterase and/or dextranase and the release of 5-ASA was monitored. Consistent with our hypothesis, while incubation with either esterase or dextranase failed to deesterify dextran-5-ASA resulting in no release of 5-ASA, 5-ASA was released up to about 24% of 5-ASA bound to dextran upon incubation of the dextran conjugate with both esterases and dextranase.

**Table I**–*Depolymerization (%) of Dextran-5-ASA by Dextranase*[a]

| Time (hr) | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
|---|---|---|---|---|---|---|
| Depolymerization (%)[b] | 80 | 93 | 96 | 97 | 100 | 100 |

[a]: The amount of sample containing 2.52 mg equivalent of dextran was incubated with dextranase (15 DU/mL) at 37°C.
[b]; The degree of depolymerization was determined by DNS method at the indicated time interval.

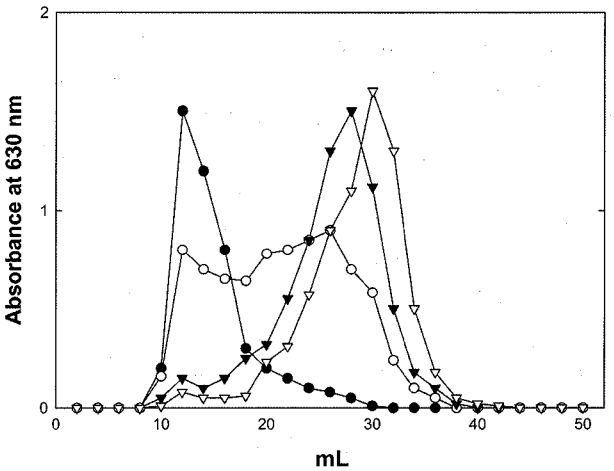


**Figure 2**–Size exclusion chromatography of dextran-5-ASA after incubation with dextranase (10 DU/mL) at 37°C. 0 (●), 0.5 (○), 30 (▼), 180 (▽) min.

## Discussion

Our data demonstrate that dextran-5-ASA conjugate is chemically and enzymatically stable in the upper intestine and shows very low permeability across Caco-2 cell monolayers. In contrast, the conjugate is depolymerized and releases 5-ASA in cecal contents, which is dependent on microbial enzymes.

We suggest that dextran-5-ASA is delivered to the large intestine without significant loss in the upper intestine. This argument is supported by the data showing that 1) dextran-5-ASA conjugate was stable in not only pH 1.2 and 6.8 buffer but also the homogenates of the small intestine, 2) the transport of dextran-5-ASA across CaCo-2 cell monolayers was not detectable, which is consistent with the fact that dextran with M.W 40000 is not permeable to Caco-2 cell monolayers.[2]

Our data demonstrating that 5-ASA was liberated from dextran-5-ASA conjugate upon incubation in the cecal contents strongly suggest that the conjugate delivered to the large intestine should release the active ingredient 5-ASA. The release of 5-ASA was dependent on microbial enzymes as the 5-ASA release did not occur in the autoclaved cecal contents. The 5-ASA release (deesterification) seems to require precedent depolymerization of the dextran backbone. This is illustrated by showing that while that with either esterase or dextranse failed to afford free 5-ASA, incubation of dextran-5-ASA with both dextranase and esterase liberated free 5-ASA. The dextranase-mediated depolymerization of dextran-5-ASA was visualized by size exclusion chromatography along with DNS method.[15] The dextranase-mediated depolymerization is likely to reduce the steric hindrance probably resulting in exposure of

the ester bond, which renders it susceptible to esterase. The prerequisite of depolymerization also explains why dextran-5-ASA conjugate is stable (no release of 5-ASA) in the homogenates of the small intestine where there exist abundant esterases but no dextranase. Since it was reported that the rate of the depolymerization decreases as DS and hydrophobicity of a drug coupled to dextran increases,[16-17] utilization of dextran as a colon-specific carrier seems to be suitable for hydrophilic drugs or for drugs whose effective doses are not very high if they are hydrophobic.

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