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RESEARCH PAPER

Synthesis and Properties of Dextran–Nalidixic Acid Ester as a Colon-Specific Prodrug of Nalidixic Acid

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ABSTRACT

Dextran–nalidixic acid ester (dextran-NA) with a varied degree of substitution (DS) was synthesized as a colon-specific prodrug of nalidixic acid (NA). Solubility in water (mg/ml) of dextran-NA with DS (mg NA/100 mg dextran-NA) of 7, 19, or 32 was 57.57 (equivalent to 4.00 mg NA/ml), 0.53 (equivalent to 0.10 mg NA/ml), or 0.03 (equivalent to 0.01 mg NA/ml), respectively, and that for NA was 0.03 at 25°C. To ensure the chemical stability of dextran-NA at conditions similar to those of the stomach and small intestine, dextran-NA was placed in a solution of pH 1.2 hydrochloric acid buffer or pH 6.8 phosphate buffer and incubated at 37°C; no NA was detected during the 6 h of the incubation period, which indicated that dextran-NA might be chemically stable during the transit through the gastrointestinal tract. Degree of depolymerization (%) by dextranase determined by the 2,4-dinitrosalicylic acid (DNS) method at 37°C for dextran-NA with DS of 7, 19, or 32 was 81, 68, or 8, respectively, in 8 h, and that for dextran was 91. When dextran-NA (equivalent to 50 μg of NA) with a DS of 7 or 17 was incubated with cecal contents (100 mg) of rats at 37°C, the extent of NA released in 24 h was 41% or 32% of the dose, respectively. NA was not liberated from the incubation of dextran-NA with the homogenate of tissue and contents of the small intestine.

KEY WORDS: *Colon-specific delivery; Colon-specific prodrug; Dextran; Dextran–nalidixic acid ester; Nalidixic acid.*

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INTRODUCTION

Numerous publications have appeared during the last decade on the delivery of therapeutic agents specifically to the colon (1–4). One approach for the design of an orally administered colonic delivery system is the development of pharmaceutical preparations such as pH-dependent or time-dependent coating (5). Development of a prodrug is another way to deliver drugs specifically to the colon, with a polymeric or water-soluble substance employed as a colon-specific carrier to prevent absorption of prodrugs in the upper intestine. Polysaccharides, azo polymers, water-soluble amino acids, and mono- or disaccharides are frequently used colon-specific carriers (6–11). Once delivered to the colon, the prodrug is presumed to be activated by the enzymes that originate from the microbes in the large intestine to liberate the parent drugs.

Colon-specific delivery of a therapeutic agent is desirable for the efficient treatment of diseases developed at the colonic site and for those drugs in which absorption through the large intestine is more beneficial (12–15). Basically, the colon is not considered a suitable site of absorption for most drugs considering many hindering factors, such as small surface area, low motility, high viscosity of intestinal contents, low fluidity of the lipid membrane, and the existence of tight junction. Despite these hindering factors, the large intestine could be the preferential site of absorption for therapeutic peptides and proteins if we consider the positive aspects of the large intestine as the site of absorption, such as long transit time, low peptidase activity, and responsiveness to the action of absorption enhancers (16).

Dextran is a polysaccharide of linear α-1,6-glucopyranose chain with α-1,3-glucopyranose branching. It is degraded readily by endodextranase, which is produced by the bacteriods residing only in the colon (17). Release of the drug molecules from the dextran-drug conjugates does not take place in the upper intestine, presumably because the steric hindrance of the polymer matrix prevents the enzymatic action (18). Release of the drug takes place only from the oligomerized dextran-drug conjugate, which is formed by depolymerization of the dextran matrix by the endodextranase in the colon, where the bacterial count is very high (1,18). Stability in the upper intestine and bioactivation in the colon, along with limited inter- or intraspecies variations in the composition and activity of dextranase, make dextran a suitable carrier for the colon-specific delivery of therapeutic agents (1).

Nalidixic acid (NA) is an antimicrobial agent effective for treatment of urinary tract infections. After oral administration, it is well absorbed from the upper intestine. If such an antimicrobial agent is delivered to the colon, it is applicable for the treatment of intestinal infections and has reduced systemic side effects. In the present study, dextran–nalidixic acid ester (dextran-NA) was synthesized and its potential as a colon-specific prodrug of NA was examined. Chemical stability, solubility, effect of degree of substitution (DS) on the depolymerization by dextranase, and release of NA after incubation with the contents of various intestinal segments of rats were investigated.

EXPERIMENTAL

Materials and Instruments

Dextran (molecular weight 82,000), dextranase (*Penicillium* sp.), carbonyldiimidazole (CDI), NA, and 2,4-dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Company (St. Louis, MO) and were used as received. All other chemicals and solvents were reagent grade. Ultraviolet (UV) and infrared (IR) spectra were recorded on a Shimadzu UV-2101 PC spectrophotometer (Shimadzu, Tokyo, Japan) and a Bomem MB 100 Fourier transform infrared spectrophotometer (Bomem, Quebec, Canada), respectively. An Orion 320 pH meter (Orion, Boston, MA) was used for pH measurements. Thin-layer chromatography was carried out on silica gel 60 Kieselguhr F_{254} (Merck, Darmstadt, Germany). An Eyela Mazela-Z tissue homogenizer (Tokyo Rikakikai, Tokyo, Japan) was used for homogenization of the contents and tissues of the gastrointestinal tracts of rats, and a Hanil Supra K-22 centrifuge (Hanil Instrument, Seoul, Korea) was used for centrifugation.

Analysis of Nalidixic Acid

Concentration of NA was determined either by UV spectrophotometry or by high-performance liquid chromatography (HPLC) (19). For UV spectrophotometry, a standard calibration curve was constructed from the results obtained from the solution of NA (5–20 ppm) in 0.1 N NaOH by measuring the absorbance at 335 nm. The HPLC system consisted of models 305 and 306 pumps, a 117 variable UV detector, a model 234 autoinjector, a model 805 manometric module, and a model 811C dynamic mixer from Gilson (Middleton, WI). A symmetry C_{18} column (Waters, 250 × 4.6, 5 μm) with a guard column (20 × 3.9) was used. The mobile phase consisted of acetonitrile and 0.2 M phosphoric acid (1:1, v/v) and filtered through 0.45-μm membrane filter before use. The

mobile phase was eluted at a flow rate of 1.0 ml/min, and the eluate was monitored by measuring the absorbance at 330 nm at a sensitivity of AUFS 0.01. The retention time of NA was 5.6 min. Gilson 712 software was employed for the data analysis. A calibration curve was constructed from the peak area versus the concentration of a standard solution of NA (1–20 ppm) in 0.1 N NaOH.

Preparation of Dextran–Nalidixic Acid Ester

N,N′-Carbonyldiimidazole (1.5 g, 9.2 mmol) was added in small portions to a solution of NA (1.4 g, 6.2 mmol) in 50 ml of anhydrous chloroform and stirred mechanically for 3 h at room temperature. Chloroform was removed by flash evaporation, and the resulting residue was dissolved in 30 ml of dimethylsulfoxide (DMSO). Triethylamine (2 ml) was added to this solution, and 1 g of dextran was dissolved in 20 ml of DMSO and added by drops. After stirring for 24 h at room temperature, the reaction mixture was poured into a solution of ethyl ether (250 ml) and acetone (50 ml). The resulting precipitates were collected by suction filtration, washed with 20 ml of chloroform to remove free NA, washed again with 50 ml of acetone, and dried in a vacuum desiccator. The infrared spectrum showed peaks at 1720 cm^{-1}, 1620 cm^{-1}, and 810 cm^{-1}.

Degree of Substitution

Dextran-NA (10 mg) was hydrolyzed in 10 ml of 1 N NaOH solution at 80°C for 1 h, and the resulting NA was analyzed by UV spectrophotometry as described above. The DS (mg of NA bound per 100 mg of dextran-NA) was determined from the calibration curve.

pH Stability and Solubility of Dextran–Nalidixic Acid Ester

Dextran-NA (1 g) was placed in 50 ml of pH 1.2 hydrochloric acid buffer or pH 6.8 phosphate buffer and incubated for 6 h at 37°C. A 0.1-ml portion of the reaction mixture was removed at an appropriate time interval, and the concentration of NA was determined by UV spectrophotometry. To determine the solubility of dextran-NA, 100 mg of the sample and 1 ml of distilled water were placed in a microtube, which was shaken vigorously on a shaker for 24 h at 25°C. After centrifugation, a 900-μl portion of the supernatant was placed in a microtube, 100 μl of 10 N NaOH were added, and this mixture was hydrolyzed at 80°C for 1 h. The resulting NA was analyzed by UV spectrophotometry as described above, and the concentration of NA was determined from the calibration curve. From the dilution factor and the DS value of the sample, the solubility of dextran-NA was calculated.

Degree of Depolymerization by Dextranase

Degree of depolymerization by dextranase was determined by the DNS method (20). The DNS reagent was prepared by dissolving 5 g of DNS in 100 ml of 2 N NaOH and adding sodium potassium tartrate tetrahydrate solution (150 g in 250 ml of distilled water) and distilled water to adjust the final volume to 500 ml.

A 200-μl portion of maltose solution in 0.1 M, pH 5.4 acetate buffer and 600 μl of DNS reagent was mixed, boiled for 5 min, and cooled for 10 min; the absorbance at 540 nm was measured. A calibration curve was constructed from the results for maltose solutions (0.093 mg/ml to 0.750 mg/ml).

Dextran or dextran-NA (DS 7, 19, or 32) dissolved in 0.1 M pH 5.4 acetate buffer (equivalent to 3.14 mg of dextran/ml) was incubated with dextranase (15 dextranase units [DU]/ml) at 37°C. At an appropriate time interval, it was treated with DNS reagent according to the preceding procedure, and the amount of terminal reducing sugar was determined from the calibration curve.

Release of Nalidixic Acid After Incubation with the Homogenate of Stomach and Small Intestine of Rats

A male Sprague-Dawley rat was anesthetized by ether, and a midline incision was made. Sections of stomach and small intestine were collected separately and homogenized, and the homogenate was diluted to half concentration with pH 4.5 isotonic acetate buffer for the stomach and with pH 6.8 isotonic phosphate buffer for the small intestine. We adopted the pH values for stomach and small intestine as 4.5 and 6.8, respectively, which are the reported pH values normally found in the rat gastrointestinal tract (21,22). A 200-μl portion of this homogenate was placed in a microtube and 800 μl of dextran-NA solution (equivalent to 50 μg of NA) in pH 6.8 isotonic phosphate buffer were added; the mixture was incubated for 6 h at 37°C. At an appropriate time interval, it was centrifuged at 5000 rpm for 3 min. To the 100-μl portion of the supernatant, 900 μl of methanol were added, vortexed for 2 min, and centrifuged for 5 min at 10,000*g*. A 200-μl portion of the supernatant was used for HPLC, and the concentration of NA was obtained from the calibration curve.

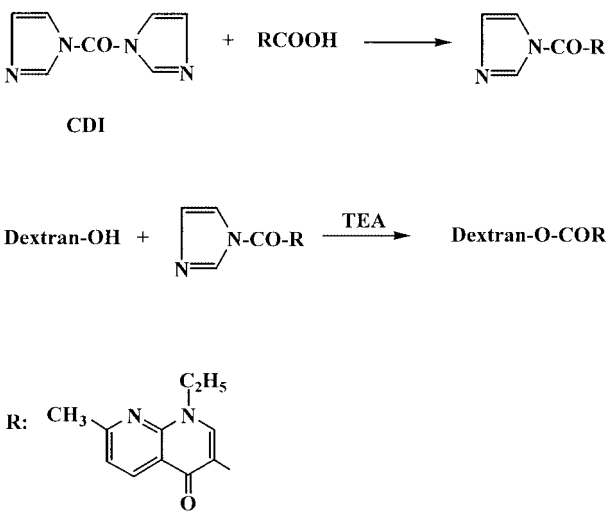
Release of Nalidixic Acid After Incubation with the Cecal Contents of Rats

The cecal segment of the intestine was cut open, and the contents were collected separately in a glove box in which air was previously displaced by nitrogen. The cecal contents (0.1 g) were placed in a microtube, 900 μl of dextran-NA solution in pH 6.8 isotonic phosphate buffer (equivalent to 50 μg of NA) were added, and the solution was mixed and incubated at 37°C under nitrogen atmosphere. At an appropriate time interval, the sample was centrifuged at 5000 rpm for 3 min. To a 100-μl portion of the supernatant, 900 μl of methanol were added; this was vortexed for 2 min and centrifuged for 5 min at 10,000*g*. A 20-μl portion of the supernatant was used for HPLC, and the concentration of NA was obtained from the calibration curve.

RESULTS AND DISCUSSION

Preparation of dextran-NA was achieved in two steps as shown in Scheme 1. NA was reacted with CDI to form imidazolide of NA, which was reacted with the solution of dextran in the presence of triethylamine as a catalyst. The IR spectrum of dextran-NA showed a typical ester carbonyl peak at 1720 cm^{-1} along with peaks that originate from NA and dextran. Dextran-NA with various DS was prepared by changing the ratio of dextran and NA.

To verify the chemical stability of dextran-NA in transit through the stomach and small intestine, dextran-NA with various DS was placed in a buffer solution of pH 1.2 or pH 6.8 at 37°C and incubated for 6 h in consideration of the transit time through the upper intestine and the pH of the stomach or small intestine, respectively. No free NA was detected regardless of the DS value, which suggested that dextran-NA is chemically stable in such conditions. The water solubility of dextran-NA was determined at 25°C; the results are given in Table 1. Solubility (mg/ml) of dextran-NA in water with DS of 7, 19, or 32 was 57.57 (equivalent to 4.0 mg NA/ml), 0.53 (equivalent to 0.10 mg NA/ml), or 0.03 (equivalent to 0.01 mg NA/ml), respectively, and that for NA was 0.03 at 25°C. As expected, the water solubility decreased drastically as the DS increased.



Scheme 1. Preparation of dextran-NA.

It is reported that dextran–carboxylic acid esters are not hydrolyzed by the esterase in the upper intestine due to the steric hindrance of the dextran matrix, and hydrolysis by the esterase takes place once the dextran backbone is degraded into smaller portions (16). Since the dextran backbone degrades in the large intestine by the endodextranase of microbial origin (17), dextran-linked esters might be stable in the upper intestine and release free carboxylic acid after the backbone is degraded into smaller pieces in the large intestine. Since the release of the drug takes place only from the oligomerized dextran-drug conjugate, depolymerization is a prerequisite condition for the dextran-drug conjugate to be a colon-specific prodrug. It is therefore important to investigate the effect of DS on the degree of depolymerization by the dextranase and decide the adequate range of DS for the dextran-linked colon-specific prodrug.

For dextran-NA, the effect of DS on the degree of depolymerization was investigated by the DNS method, for which the amount of isomaltose produced by the endodextranase was determined. The results are shown in Fig. 1. The degree of depolymerization (%) of dextran-

Table 1

Solubility of Dextran–Nalidixic Acid (NA) at 25°C in Water

	Solubility	
DS[a]	Dextran-NA mg/ml	Equivalent of mg NA/ml
7.5	57.57	4.03
19.2	0.53	0.11
32.0	0.03	0.01
Nalidixic acid	—	0.03

[a] Degree of substitution, defined as milligrams NA per 100 mg dextran-NA.

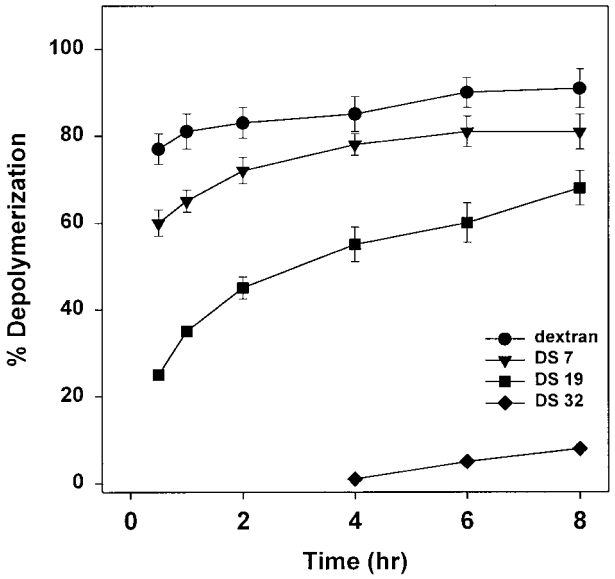


Figure 1. Effect of DS on the depolymerization (%) of dextran-NA by dextranase determined by DNS method at 37°C. Data are mean ± SE (n = 5).

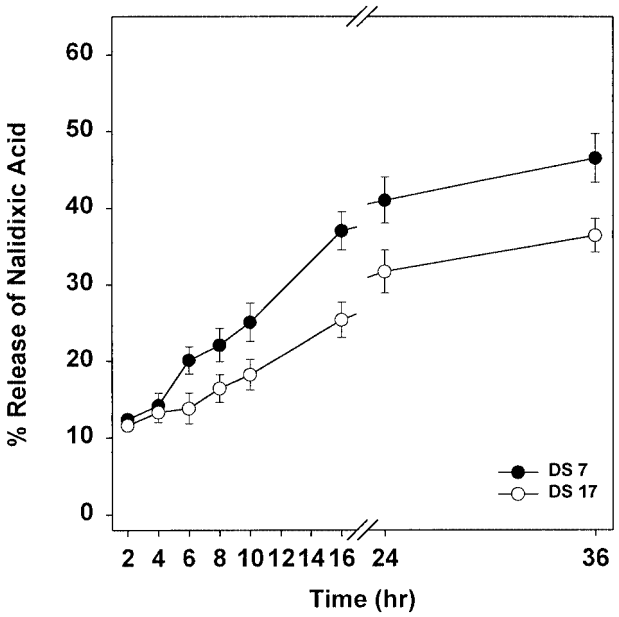


Figure 2. Release of NA during incubation of dextran-NA (equivalent to 50 μg of NA) in 1.0 ml of 10% (w/v) cecal contents (100 mg) in isotonic phosphate buffer (pH 6.8) at 37°C. Data are mean ± SE (n = 5).

NA with DS of 7, 19, or 32 was 81, 68, or 8, respectively, in 8 h and that of dextran was 91. The result indicates that dextran-NA with DS higher than 20 may not be suitable as a colon-specific prodrug of NA.

Dextran-NA with DS of 7 or 17 (equivalent to 50 μg of NA) was incubated with 100 mg of cecal contents in pH 6.8 isotonic phosphate buffer at 37°C; the results are shown in Fig. 2. The degree of NA released for dextran-NA with DS of 7 or 17 was 22% or 16% in 8 h and 47% or 36% in 36 h, respectively. In experiments in which dextran-NA was incubated with homogenates of tissue and contents of the stomach or small intestine, no NA was detected from the incubated sample.

In summary, dextran-NA with varied DS was prepared by changing the ratio of the dextran and NA. Solubility of dextran-NA with DS of 7 and 19 increased greatly in comparison with that of free NA. Dextran-NA was chemically stable at pH 1.2 or pH 6.8 and did not release NA on incubation with the homogenates of tissue and the contents of the stomach and small intestine of rats, but released NA on incubation with the cecal contents of rats. Even though the weight of the drug bound per unit matrix is large for dextran-NA with high DS, it has poor solubility and shows a low degree of depolymerization by the dextranase, which may be the hindering factor for the release of NA by the cecal contents. For dextran-NA, a DS higher than 10 may not be suitable if we consider the solubility, the degree of depolymerization by dextranase, and the release of NA by the cecal contents.

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