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Research Article

Macromolecular Prodrugs. XVI. Colon-Targeted Delivery—Comparison of the Rate of Release of Naproxen from Dextran Ester Prodrugs in Homogenates of Various Segments of the Pig Gastrointestinal (GI) Tract

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We have determined initial rates of naproxen formation from dextran-naproxen ester prodrugs incubated in homogenates of various segments of the pig GI tract. Drug liberation proceeded 15–17 times faster in cecum and colon homogenates than in aqueous pH 7.4 buffer or homogenates of the small intestine. The degree of conjugate substitution did not affect the liberation rates, whereas enhanced drug activation was observed with decreasing molecular size of the carrier dextran. During incubation in colon homogenates the average molecular weight of the dextran prodrugs decreased. The mechanism of drug activation from the prodrugs may therefore involve an initial depolymerization step of the dextran chains by dextranases secreted from bacteria in the pig colon. The generated small fragments then serve as substrates for esterases and other hydrolases.

KEY WORDS: dextran ester prodrugs; naproxen regeneration; activation in pig gastrointestinal (GI) tract homogenates; dextranases.

INTRODUCTION

In an associated paper (1) we have reported on the bioavailability of naproxen from orally administered dextran-naproxen ester prodrugs ranging in molecular weight of the carrier dextran from 10,000 to 500,000. Naproxen regeneration in the pig GI tract below the ileum may involve one or more enzyme systems. The results suggested that the first step of the drug activation involved depolymerization of the dextran chain probably mediated by dextranases. The present study focuses on the mechanism of drug liberation from the dextran prodrugs in the pig GI tract.

EXPERIMENTAL

Materials

The dextran fractions T-150 and T-250 was obtained from Sigma (USA) and Pharma Cosmos, Viby (Denmark). The other dextran compounds were purchased from Pharmacia, Sweden. The dextran-naproxen ester prodrugs were synthesized and characterized as previously described (1).

Analytical Procedures

Quantitation of parent naproxen released from the various dextran conjugates in the hydrolysis studies was performed by using a reversed-phase HPLC procedure. A column, 125 × 4.6 mm, packed with Spherisorb ODS-1 (5-μm particles) was eluted with a mobile phase consisting of methanol–0.02 *M* phosphate buffer, pH 2.5 (65:35, v/v). The flow rate was maintained at 1.0 ml min^{-1} and the column effluent was monitored at 271 nm. The HPLC apparatus was composed of a Hitachi L-6200 Intelligent pump, an Hitachi L-4000 variable-wavelength detector, a Reodyne Model 7125 injection valve, an Hitachi 655A-40 auto sampler, and an Hitachi D2000 chromato-integrator. Quantitation of the drug compound was done from peak area measurements in relation to those of external standards chromatographed under the same conditions.

Quantitation of intact dextran naproxen ester conjugates was done employing a high-performance size exclusion chromatography procedure [HP(SEC)] (2). The chromatographic system consisted of an Hitachi Model 655A-11 solvent delivery pump, equipped with a variable-wavelength Hitachi F1000 fluorescence detector, a Rheodyne Model 7125 injection valve with a 20-μl loop, and an Hitachi Model D2000 chromato-integrator. The column, 250 × 8 mm, was packed with spherically shaped Nucleosil Diol 7-OH particles (7 μm) (Mackerey-Nagel, F.R.G.). During chromatography, the column was protected by a small precolumn packed with nucleosil Diol and by a silica saturation column positioned between the pump and the injection valve. The

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latter column was packed with LiChroprep SI 60, 15–25 μm (Merck, F.R.G.). The mobile phase was a mixture of acetonitrile–0.05 *M* phosphoric acid (30:70, v/v). The flow rate was set at 1 ml min^{-1} and the column effluent was monitored at excitation and emission wavelengths of 330 and 360 nm, respectively. In addition to the detection of free and conjugated naproxen, the HP(SEC) method was used to estimate the average molecular weights of the dextran conjugates as a function of time in the GI tract homogenates.

Preparation of GI Tract Homogenates

A male albino rabbit and a female pig (Danish landrace/Yorkshire) weighing approximately 3 and 45 kg, respectively, were used. The animals were given standard diets before they were killed. About 30 min was required to excise the various parts of the gastrointestinal tract from each animal. The GI tract segments with their content were cut into small pieces, weighed, pooled in glass vials, and stored at −20°C. After thawing the tissue homogenates were prepared by suspending each GI tract segment in twice the volume of cold 0.9% sodium chloride. The mixture was homogenized and centrifuged at 5000*g* in a refrigerated (4°C) centrifuge. The resulting supernatant was frozen immediately in 2-ml portions. Due to the length of the pig colon (close to 2 m), homogenates of the proximal (colon I) and the distal (colon II) part were prepared separately. Colon samples without content were obtained after carefully rinsing the tissue with 0.9% sodium chloride.

Kinetic Measurements

The reaction solutions were kept at 37 ± 0.2°C in a constant-temperature water bath. The initial velocities of naproxen formation were in most cases monitored after adding 1000 μl of a 0.2 *M* phosphate buffer, pH 7.40, containing 40 ± 4 mg of the individual dextran conjugate to 1000 μl of a homogenate solution preheated to the temperature of study. Aliquots (200 μl) were withdrawn and deproteinized with 600 μl of methanol. The mixtures were vortexed and centrifuged at 10,000*g* for 4 min. The methanolic supernatants were analyzed for liberated drug by using the HPLC apparatus equipped with an auto sampler. During the sampling period, ranging from 6 to 60 min, six to eight samples were taken at suitable intervals. Naproxen was stable in the precipitation media for more than 24 hr as evidenced by HPLC analysis.

For the HP(SEC) experiments a stock solution of a dextran T-70 naproxen ester prodrug in 0.2 *M* phosphate buffer, pH 7.40, was prepared (3.3 mg ml^{-1}). The reactions were initiated by adding 1000 μl of the stock solution to 2000 μl of the pure colon I homogenate or to equal volumes of the homogenate containing 50 mg of glucose and 5, 10, or 50 mg of parent dextran T-70. At appropriate intervals 200-μl samples were taken and added to 400 μl of a 20% (w/v) trichloroacetic acid solution. After vortexing the mixtures were centrifuged at 10,000*g* for 4 min. Due to the limited stability of the dextran ester conjugates in the precipitation solution (2) HP(SEC) analysis was carried out immediately after the sample preparation.

RESULTS

To compare the initial rates of naproxen formation from various dextran prodrugs, GI tract homogenate samples derived from the same bulk homogenate preparation were employed. The initial rate studies were limited to an overall degradation of the dextran ester prodrugs of less than 2%. The drug per se was not metabolized significantly after incubation for 24 hr in the GI tract homogenates as evidenced by HPLC analysis. Relatively high substrate concentrations of the various dextran derivatives (about 20 mg ml^{-1}) were used to ensure proper HPLC quantitation of liberated naproxen. In the assessment of the relative stability of the individual conjugates in the different segments of the GI tract, the concentration of the prodrugs was kept constant, at 20 ± 2 mg ml^{-1} (Fig. 1). The apparent linear relationship between the initial velocity, v_i, and the amount of a dextran T-70 prodrug with DS 5.6 (range, 13–36 μg ml^{-1}) in the pig colon I homogenate with its content can be described by the following equation:

$$v_i(\mu g\ ml^{-1}\ hr^{-1}) = 2.09\ c\ (\mu g\ ml^{-1}) + 28.7 \quad (1)$$

The v_i values determined for dextran T-70 prodrugs (derived from the same parent dextran fraction) with DS (degree of substitution) of 3.7 and 9.9, respectively, differ only slightly from the value found for the conjugate with a DS of 5.6. Thus it appears for DS below 10 that the degree of substitution of the dextran derivatives has only minor effects on drug activation in the pig colon.

The initial velocities of naproxen liberation were determined after incubation of a dextran T-70-naproxen ester prodrug (DS 8.3) in homogenates prepared from various segments of the GI tract of pigs with their content. The initial velocities have been calculated for a substrate concentration of 20 mg ml^{-1}. In a few cases the v_i values were corrected by using Eq. (1). As seen from Table I naproxen is liberated much faster in the cecum and colon homogenates than in the stomach and small intestine reaction mixtures. For compar-

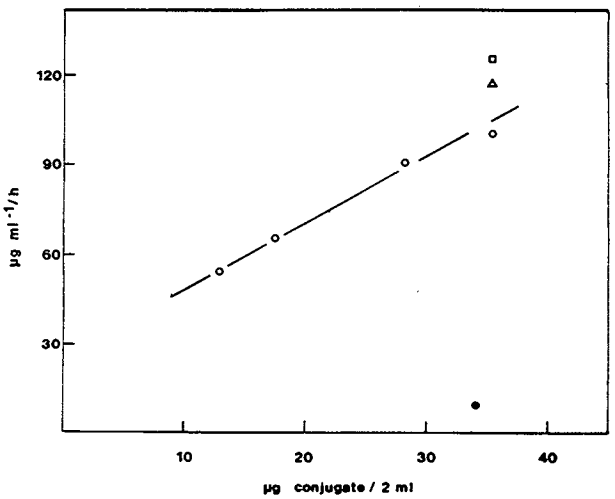


Fig. 1. Initial velocities of naproxen formation from a dextran T-70-naproxen prodrug (DS 5.6) as a function of the substrate concentration in a pig colon homogenate at 37°C, with the content (○) and without the content (●). Identical experiments carried out for dextran T-70 conjugates with DS 3.73 (△) and DS 9.90 (□).

ison the initial rate of drug regeneration was also determined in aqueous phosphate buffer pH 7.40. The calculated v_i value of 6.6 μg ml^{-1} hr^{-1} is similar to those obtained in the duodenum, jejunum, and ileum segments, reflecting that drug activation from the conjugates in the small intestine proceeds exclusively by pH dependent hydrolysis. In the stomach homogenate (pH 7.40) naproxen is released almost twice as fast as in aqueous buffer pH 7.40. The 12- to 17-fold v_i increase in the cecum and the colon compared to the aqueous buffer suggests an enzyme-mediated release of naproxen. The data from identical experiments carried out in rabbit homogenates are further included in Table I, showing similar pattern of drug release over the various GI segments, although drug activation in rabbit colon homogenate proceeds more slowly. The latter feature might account for the difference in bioavailability of naproxen from the conjugates in the rabbit [60%(3)] and in the pig [almost 100% (1)]. The rate of drug liberation in pig cecum and colon homogenates (with their content) decreased progressively as a function of increasing molecular weight of the dextran carrier molecule (Table II). Direct evidence for the occurrence of fragmentation of the dextran backbone is provided by HP(SEC) analysis of the dextran prodrugs after incubation in the colon homogenates. In Fig. 2 a molecular weight calibration curve for the dextran prodrugs is depicted. The retention times refer to high-performance size exclusion chromatography of the conjugates on Nucleosil Diol. Thirty percent (v/v) acetonitrile was incorporated into the aqueous mobile phase in order to eliminate adsorption phenomena. The observed linear relationship in the molecular weight range 10,000–70,000 can be described by the equation

$$\log M_w = -0.32\, r_t + 6.85 \qquad (r = 0.995,\ n = 4) \quad (2)$$

where r_t refers to the retention time of the individual conjugate.

In one experiment a dextran T-70 prodrug was incubated in a colon homogenate medium and sampled at various times. Using Eq. (2) it was calculated that after 6.8 hr the average molecular weight was changed from approximately 72,000 to 45,000, whereas the M_w of the main fraction after 24 hr was about 26,000. Further, the reduction of the peak height of the conjugate was accompanied by an increase in the peak width. Based on peak area measurements, the degradation kinetics approximated a zero-order reaction. The results indicate a breakdown of the dextran chains (most likely effected by dextranases) while naproxen is still covalently attached to the carrier, since only the drug is responsible for the fluorescence detector response. In colon homogenates containing various amounts of a parent dextran sample the decrease in molecular weight of a dextran T-70 prodrug, as expressed by the increase in retention time, was followed as a function of time by using the HP(SEC) procedure (Fig. 3). In the reaction mixture containing a 50-fold excess of parent dextran, little change of r_t was observed for more than 6 hr, and very little naproxen was liberated during

Table 1. Initial Velocities of Naproxen Formation (v_i) After Incubation of a Dextran T-70-Naproxen Ester Prodrug with DS 8.3 in Homogenates Prepared from Various Segments of the GI Tract of Rabbits and Pigs with Their Content (37°C)

Homogenate/ buffer[a]	Rabbit, v_i (μg ml^{-1}/hr)	Pig		
		v_i (μg ml^{-1}/hr)	pH (start)	pH (end)
Stomach	—	11.2	7.3	7.5
Duodenum	7.1	7.4	7.4	7.4
Jejunum	6.0	7.1	7.4	7.4
Ileum	6.3	8.1	7.3	7.5
Cecum	65.1	86.5	7.4	7.5
Colon I	32.6	107.0	7.4	7.4
Colon II	—	111.2	7.4	7.4
0.1 *M* phosphate, pH 7.40	6.6	6.6	—	—

[a] The reaction solutions: 33% homogenate–0.2 *M* phosphate buffer, pH 7.4 (1:1, v/v).

Table II. Initial Velocities of Naproxen Formation (v_i) After Incubation of Dextran-Naproxen Ester Prodrugs, Varying in Molecular Size, in Pig Homogenates of Cecum and Colon with Their Content (37°C)

Naproxen conjugate[a]	Pig cecum homogenate,[b] v_i (μg ml^{-1}/hr)	Pig colon I homogenate,[b] v_i (μg ml^{-1}/hr)
Dex T-1 (DS 2.2)[c]	272.4	248.1
Dex T-10 (DS 6.5)	210.2	224.0
Dex T-20 (DS 6.4)	138.0	—
Dex T-40 (DS 5.6)	108.1	—
Dex T-70 (DS 5.6)	101.2	100.6
Dex T-250 (DS 6.9)	94.3	78.9
Dex T-500 (DS 8.0)	84.8	65.1

[a] The concentration of the conjugates was 20 ± 2 mg ml^{-1}.
[b] The reaction solutions: 33.3% homogenate–0.2 *M* phosphate buffer, pH 7.4 (1:1, v/v).
[c] DS, degree of substitution.

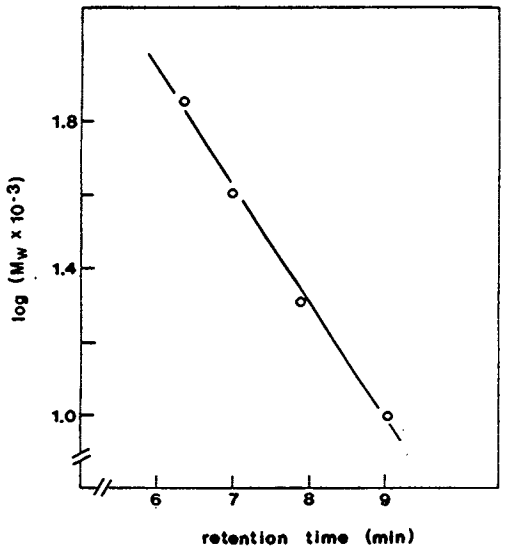


Fig. 2. Molecular weight calibration curve for dextran-naproxen ester prodrugs (DS 5.6–6.4). The retention times refer to high-performance size exclusion chromatography [HP(SEC)] of the conjugates on Nucleosil Diol.

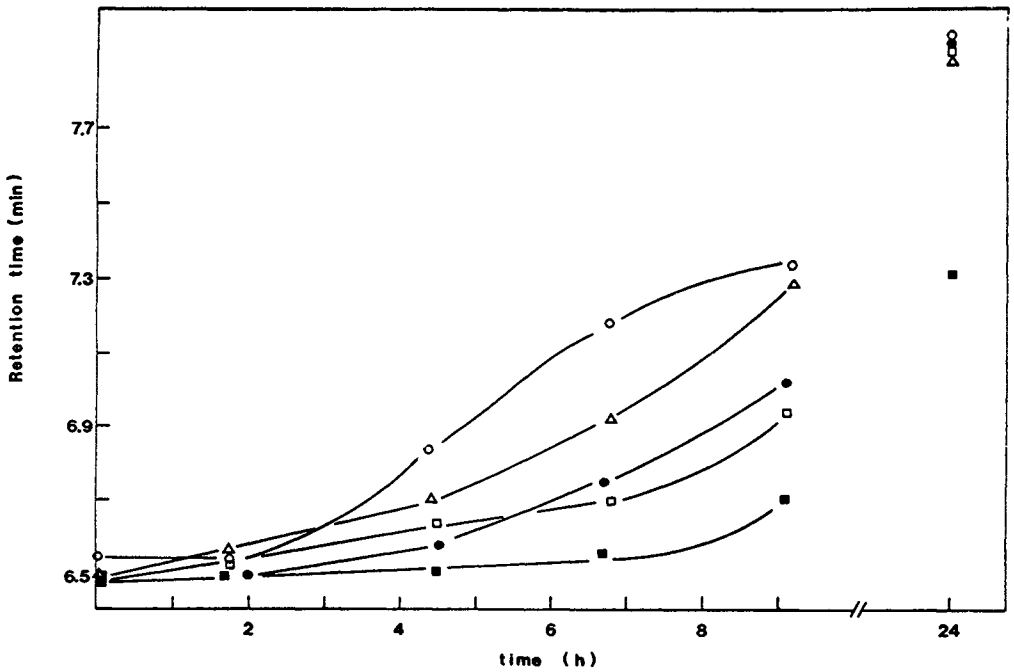


Fig. 3. Retention times from HP(SEC) analysis of a dextran T-70 conjugate (DS 5.6) after incubation in pig colon I homogenates (1 mg ml^{-1}) at 37°C. (○) Colon I homogenate–0.2 *M* phosphate buffer, pH 7.4 (2:1); (△) do but contains 5 mg parent dextran T-70; (□) do but contains 10 mg parent dextran T-70; (■) do but contains 50 mg parent dextran T-70; (●) do but contains 50 mg glucose.

this period. Coadministration of glucose also slowed down the rate of depolymerization of the dextran prodrug.

To identify the source of dextranase activity in the pig colon, identical degradation experiments were performed using colon homogenates with and without the content, respectively. In the latter medium the initial rate of naproxen formation was small and almost equal to the rate of drug activation in aqueous phosphate buffer, pH 7.4 (Fig. 1). In addition, no change in molecular size of the prodrug, using the HP(SEC) procedure, was observed. Thus, fermentation of the dextran prodrugs takes place in the colon content, presumably brought about by dextranases secreted from the microbial flora.

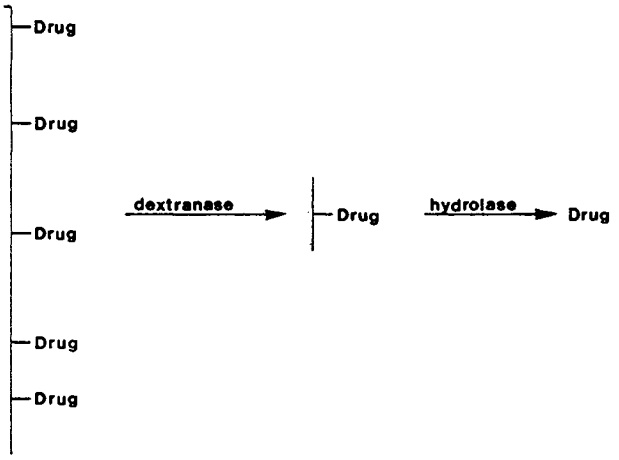
DISCUSSION

The data presented in Table I indicate that naproxen is released from the prodrugs predominantly in the pig cecum and colon. Previous *in vivo* studies showed that (i) the naproxen absorption profiles after oral administration of the dextran prodrugs exhibited a characteristic lag time of drug appearance in the systemic circulation of about 2–3 hr (1) and (ii) analysis of the distribution of dextran prodrugs along the GI tract at various times after the conjugate administration revealed that after 4 hr free naproxen as well as prodrug was found only in the cecum and the colon (4). In the bioavailability studies (1) the prodrugs were given mixed with food and the pigs were fed during each blood sampling. Whereas the presence of food may influence the pH of the various segments of the GI tract to varying degrees, all the GI tract homogenates were adjusted to pH 7.4 by the addition of a phosphate buffer. Concerning the degradation experiments performed in the stomach homogenate this procedure might at least partly be justified because the pH of the anterior portion of the pig stomach is approximately 4.3 (5), and it increases in the presence of food. Pig small intestine pH values of 6.0 (proximal part) and 7.5 (distal part) have been reported (5), whereas the pH of the colon content was close to seven.

Kinetic models for the description of enzymatic hydrolysis of polysaccharides have been developed (6). These somewhat theoretical considerations appear not easily applicable to the complex drug liberation enzyme systems operating in the pig colon. However, some characteristics of the activation might be deduced from the performed experiments. Apparently the fragmentation of the prodrug dextran chains constitutes the rate-limiting step in the naproxen absorption process (1). The HP(SEC) results indicate that the disappearance of the conjugate in the colon homogenates approximates a zero-order reaction. Because of the observed positive slope of the plot of initial velocity versus substrate concentration (Fig. 1), strict saturation kinetics are, however, not achieved.

Bacterial dextranases are classified as belonging to either the exo or the endo type, dependent on their ability to release glucose and combinations of glucose di- and trimers or to cleave dextrans into polymeric fragments of progressively decreasing size, respectively (7). Although competitive inhibition of enzyme activity exerted by glucose (Fig. 3) has been observed in a dextran-exo-dextranase system (8), the changes of prodrug molecular weight in the colon homogenate resemble an endo-dextranase-mediated reaction. Exo-dextranases alone had very little effect on the molecular weight distribution of a dextran T-250 sample over a period

of about 10 hr (9). On the other hand, in the colonic environment dextran depolymerization might very well be afforded by a combination of the two types of enzymes. Further, the addition of an α-glucosidase to a dextranase system resulted in a synergistically enhanced rate of degradation of a T-2,000 dextran derivative (10). This observation might indicate that the dextran splitting enzyme system is even more complex, since the colon is rich in bacterial glucosidases (11). As to the mechanism of drug activation from the conjugate (Scheme I) we have speculated that the dextran



Scheme I

chains are initially cleaved randomly by dextranases. The progressive increase in fragmentation results in a diminished efficiency of steric protection of the dextran drug bond, and consequently sufficiently small fragments will become substrates for esterases and other hydrolases residing in the colon.

A prerequisite for the dextran prodrug approach to provide colon site-specific delivery of various drug compounds is that the dextranase system is relatively insensitive to chemical modification of the dextran carrier. Results of several *in vitro* experiments indicate that dextranase catabolism of a variety of dextran derivatives tolerates a moderate degree of drug substitution. In general, dextranase efficiency is diminished with increasing ligand load (12). Endodextranase action is abolished for dextran ethyl carbonate ester derivatives with a degree of substitution (DS = mol ligand/mol anhydroglucose) above 0.8 (13). Positively charged dextran derivatives (14) and negatively charged compounds (12,15) are substrates for dextranases, although the depolymerization reactions proceed more slowly compared to native dextran. Even degradation of cross-linked dextran particles effected by α-1,6-glucosidases has been reported (16).

It remains to be established whether dextrans are able to deliver other carboxylic acid drugs and therapeutic agents containing other types of functional groups selectively to the colon. However, the dextran prodrug approach is promising because (i) many different enzymes are secreted from gastrointestinal microorganisms (17), (ii) Bacteroides species (some of which secrete dextranases) account for approximately 20% of the human fecal flora (18), and (iii) Bacteroides species are present in the fecal flora of patients suffering from ulcerative colitis and Crohn's disease (19).

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