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Chondroitin sulfate: A potential biodegradable carrier for colon-specific drug delivery

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Summary

Chondroitin sulfate, a soluble mucopolysaccharide utilized as a substrate by the bacteroid inhabitants of the colon, was cross-linked and formulated in a matrix form with indomethacin as a drug marker. Three levels of cross-linkage were used. Cross-linkage was characterized qualitatively by IR spectral analysis and by UV absorbance shifts in hydroalcoholic solutions; it was also characterized quantitatively by comparing the amount of methylene blue remaining after adsorption (at equilibrium) onto the cross-linked product vs that remaining after adsorption onto untreated chondroitin. The indomethacin release kinetics from the various formulations was analyzed in phosphate-buffered saline (PBS) with and without rat caecal content at 37°C under a CO_2 atmosphere. In separate experiments the caecal content was sonicated to cause lysis of the bacterial cell membranes. The drug release profiles indicated a constant rate of biodegradation in the presence of rat caecal content, and diffusion-driven release from the matrix's surface area in PBS control. Prolonged incubation in PBS with rat caecal content increased drug release, and by 28 h the released indomethacin levels were significantly higher than those in the PBS controls. The maximal cumulative percent release values for the PBS controls were: 30.1 ± 10.0, 19.7 ± 15.0, 9.0 ± 4.1 for the three levels of cross-linkage of the chondroitin sulfate carriers, respectively, while those for the caecal content media were 71.0 ± 19.0, 48.7 ± 35.0, and 22.5 ± 7.9, respectively. It was concluded that the indomethacin release from these systems was dependent upon the biodegradation action of the caecal content. The linear correlation between the degree of cross-linkage and the amount of drug released in the presence of caecal content suggests that drug release in the colon can be controlled by adjusting the relative amounts of the variously cross-linked chondroitin sulfate formulations in the matrices.

Introduction

Designing orally administered colonic delivery systems is necessarily complicated because the colon, located at the end of the alimentary canal, is difficult to access. Nevertheless, if drugs could be targeted to the colon, it would be possible to treat colon disorders like inflammatory bowel diseases locally, reducing systemic absorption and avoiding painful treatments (enemas). It would also increase the efficiency of those drugs, like steroids, that can be absorbed in the large intestine.

The content of the large intestine is nearly 30% microorganisms. As a result, drug metabo-

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lism is effected by both endogenous enzymes, located in the intestinal mucosa, and enzymes of the bacterial inhabitants of the human gastrointestinal tract (Scheline, 1968). The realization that the enzymes of microorganisms in the human colon may hydrolyze prodrugs and other molecules (e.g., laxatives) to active therapeutics has led to increased research activity in the area of microbially controlled drug delivery to the colon (Rubinstein, 1990).

A well known demonstration of the use of this concept is the delivery of 5-aminosalicylic acid in the alimentary canal by sulfasalazine or Olsalazine. Most of the dose of these drugs remains intact in the stomach and in the small intestine. In the colon, however, bacterial azo reduction splits the prodrugs and the active moiety, 5-aminosalicylic acid, is released locally (Willoughby et al., 1982; Klotz, 1985). As an alternative approach to the azo-linked prodrugs, glycoside prodrugs can be used. Galactose, glucose, or cellobiose, known to serve as substrates for colonic bacteria, were linked to selected steroid drugs commonly used in the treatment of inflammatory bowel disease (Friend and Chang, 1985; Tozer et al., 1991). In other studies, dextran ester prodrugs of naproxen and ketoprofen were reported to be hydrolyzed in the pig, releasing their drug content primarily in the caecum and colon (Harboe et al., 1989; Larsen et al., 1989; Larsen and Jensen, 1991).

Peptide drugs, like insulin, cannot be directly administered orally, since before they can be absorbed they are digested by brush border peptidases. Recently, it was reported that such peptide drugs can be administered orally if they are first coated with an azopolymer (Saffran et al., 1986, 1988, 1990). This coating, susceptible to cleavage by the colonic bacteria, protects the drug until it passes the stomach and the small intestine, and reaches the large bowel. A sustained pharmacologic response was observed when delivery systems coated with co-polymer containing azo bonds were orally administered to rats, and later to dogs.

Chondroitin sulfate (Fig. 1) is a soluble mucopolysaccharide (Wastenson, 1971; Toledo and Dietrich, 1977), which is utilized as a substrate by

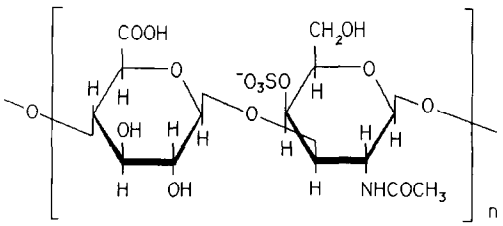

Fig. 1. Chondroitin sulfate.

the bacteroid inhabitants of the large intestine, mainly by *Bacteroides thetaiotaomicron* and *B. ovatus* (Salyers, 1979). Periplasmic enzymes are probably responsible for chondroitin breakdown: apparently, an outer membrane receptor binds chondroitin sulfate and brings it into contact with enzymes like chondroitin-sulfate-lyase (Salyers and O'Brien, 1980).

In the human colon, the natural sources of chondroitin sulfate are sloughed epithelial cells or dietary meat. We suggest, therefore, that chondroitin sulfate could be used as a colonic drug carrier. Such use would depend on its persistence as a solid dosage form in the physiological environment of the stomach and small intestine. Since natural chondroitin sulfate is readily water soluble it might not protect its load successfully. However, cross-linked chondroitin would be less hydrophilic and thus would provide a better shield.

We have previously shown that the release of indomethacin from a cross-linked chondroitin matrix can be enhanced in the presence of rat caecal content as compared to its low release rate in non-caecal buffered medium (Rubinstein et al., 1992a). The objectives of the present study were: (a) to fully characterize chondroitin products with three different degrees of cross-linkage, and (b) to compare the release profiles of indomethacin from matrices prepared from the different cross-linked products, and assess their potential use as colonic drug delivery systems using rat caecal content.

Materials and Methods

All materials and reagents were purchased from Sigma, St. Louis, MO, unless otherwise

mentioned in the text. All solvents were analytical or HPLC grade.

Cross-linking chondroitin sulfate and product characterization

Chondroitin sulfate type A (ChS) was mixed with 1,12-diaminododecane using dicyclohexylcarbodiimide (DCC) as a catalyst (Sheehan, 1955). The molar ratio of DCC to diamino reagent was 1.1:1. The molar ratios of ChS to the diamino reagent were 7:3, 5:5, or 4:6. The purification procedure for the cross-linked products has been described elsewhere (Rubinstein et al., 1992a).

The batch to batch uniformity of the products was ascertained by scanning their absorbance spectra spectrophotometrically (Uvikon 930, Kontron Instruments, Switzerland) in 1% w/v hydroalcoholic solution (1 part water, 2 parts ethanol) (Fig. 2). Qualitative IR analysis was performed after dispersing each product in KBr discs (Fig. 3). The extent of cross-linking was assessed by

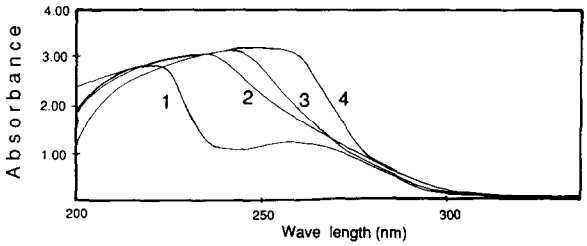

Fig. 2. Typical UV spectrum of chondroitin sulfate and cross-linked chondroitin sulfate products in hydroalcoholic solutions. (1) Chondroitin sulfate, (2) ChS_{70}, (3) ChS_{60}, (4) ChS_{55}.

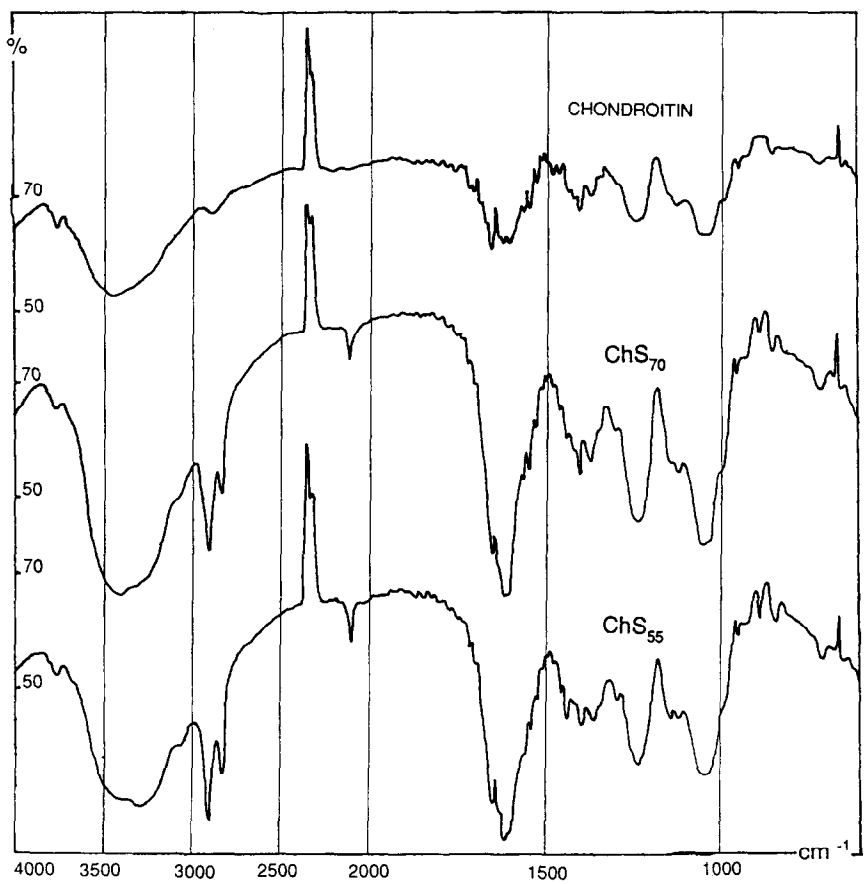

Fig. 3. IR spectra of chondroitin sulfate and cross-linked chondroitin sulfate products (ChS_{70} and ChS_{55} are shown).

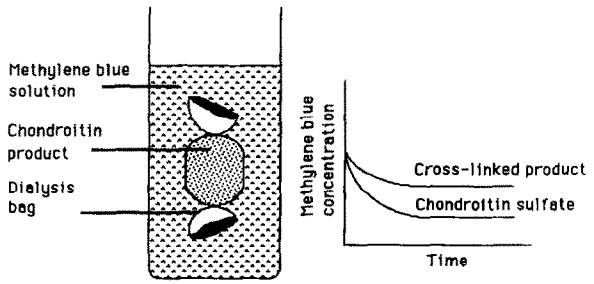

Fig. 4. Schematic presentation of the adsorption system used to determine the RMN values (left). The plot (right) demonstrates the way RMN values of the various products were measured as compared to chondroitin.

measuring the amount of methylene blue which was adsorbed as a result of cation exchange. Samples of 10 mg of natural or variously cross-linked ChS were immersed in a dialysis bag (Spectra/por 6 × 30 mm, mol. wt cut-off 12000–14000 (Spectrum, LA)). The dialysis bags were mounted in glass beakers, each containing 15 ml of 0.1% methylene blue in veronal buffer at 37°C (Fig. 4). The methylene blue, in turn, diffused into the bag and adsorbed onto the dispersed powder. The disappearance of chromophore from the methylene blue solution outside the dialysis bag was monitored spectrophotometrically (Uvikon 930, Kontron Instruments, Switzerland) at 665 nm at predetermined time intervals. Equilibrium was determined when no further reduction in methylene blue concentration was noted (25 h). The differences in the absorbance at the beginning and at equilibrium were monitored. The difference for a system containing cross-linked product was divided by that obtained in the system containing untreated ChS. This value multiplied by 100 was determined as the relative methylene blue adsorption number (RMN), as follows:

$$\mathrm{RMN} = \frac{(A_0 - A_m)}{(A_0 - A_c)} \times 100 \qquad (1)$$

where A_m is the absorbance value of methylene blue solution at equilibrium in the system containing cross-linked chondroitin sulfate, A_c represents the absorbance value of the methylene blue solution containing untreated chondroitin sulfate, and A_0 is the initial absorbance value of the methylene blue solution before any adsorption occurred. The various cross-linked chondroitin products are referred as ChS_{RMN}.

Calculated in this way, the RMN value for the ChS treated with diamino reagent in a ratio of 7:3 was 68.6 ('ChS_{70}'), for that treated in a ratio of 5:5, the RMN was 60.5 (ChS_{60}), and for that treated in a ratio of 4:6, the value of RMN was 54.4 (ChS_{55}).

Matrix preparation

Indomethacin is a water-insoluble drug which we have used as a drug marker. The cross-linked chondroitin dry powder was sieved through 40 mesh sieve, and mixed with indomethacin at a ratio of 9:1 w/w. Matrices, each weighing 200 mg, were then double-pressed, using a Perkin Elmer manual press.

Caecal content medium

Our aim was to use the rat caecum as a specific 'mini-fermenter' model. 24 h prior to the release experiments, five Sabra rats (Lutsky et al., 1984), weighing 200–300 g and maintained on a normal diet, were intubated with teflon tubing. To induce the chondroitin sulfate-lyase enzymes postulated to be in the caecum, we administered 1 ml of 20% w/v ChS aqueous solution directly to the rats' stomachs through the teflon tubing which was afterwards removed. 30 min before the drug release experiments the rats were killed by an overdose of sodium pentobarbital i.p. injection. The caecum was ligated at two ends (2 cm distance), cut loose, and immediately removed from the rat body. The formed caecal bag was then opened, its content weighed, pooled, and suspended in phosphate-buffered saline (PBS, pH 7) to give a final caecal dilution of 1.25% w/v. As the caecum is a naturally anaerobic environment, we maintained anaerobiosis by carrying out this last step under CO_2.

Drug release experiments

Two types of drug release studies were performed: one with sonicated rat caecal content ('sonicate'), and the other with intact caecal content. The first type lasted 5 h, with sampling

times of 0, 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min. In the second type of drug release experiments only whole bacteria were involved as we did not use any 'sonicates.' These studies lasted 28 h, with sampling times of 0, 3, 6, 9, 12, 14, 21, 24 and 28 h. The sonicates were prepared as follows: the caecal content suspensions were sonicated for 3 min using an ultrasonic processor (Heat systems – Ultrasonic, Inc, W-380) in an ice bath before they were diluted to 1.25% w/v with PBS (pH 7). This was done to break the bacterial cell walls so that a drug release experiment could be performed in the presence of higher enzyme levels. This experimental protocol was based on the findings that the location of colonic bacterial chondroitin sulfate-lyase is periplasmic (Salyers and O'Brien, 1980). The release experiments were performed in PBS (pH 7), with added caecal content, or sonicated caecal content. Each experiment was accompanied by a control study which was performed according to the same protocol but included as a dissolution medium PBS (pH 7) only.

Each drug release experiment represented a different batch of the cross-linked chondroitin, and was performed in duplicate in 100 ml sealed glass vials which were shaken at 80 rpm in a 37°C water bath under a CO_2 atmosphere. With the exception of the studies that included rat caecal sonicates, we tested three different batches for each of the three levels of cross-linkage with different pools of caecal content, and for each level we reported the average of the results of the three experiments. An indomethacin assay was performed on 1 ml samples withdrawn in triplicate at predetermined time intervals. Each time, 3 ml of PBS was added back to the system to maintain constant volume and pH.

Indomethacin analysis

1 ml samples were acidified with 200 μl of 0.4 N HCl, and extracted with 1 ml ethyl acetate containing 0.2 mg% of flufenamic acid as an internal standard. The mixture was vortexed and then centrifuged for 3 min at 3400 rpm. Aliquots of 500 μl of the organic phase were evaporated, and the residue was redissolved in a 50:50 mixture of phosphate buffer, pH 7.5 and acetonitrile. 20 μl of the solution were injected into the HPLC system (Hewlett Packard 1050 pumping system, Jasco 875 Intelligent UV/Vis detector, Hewlett Packard 3365 ChemStation Data analyzer and Hewlett Packard analog-digital 35900C Dual Channel Interface Convertor). The wavelength was 280 nm, and the column was 5 μm, 250 × 4.6 mm RP-18 (LiChroCART 250-4, E. Merck, Germany).

Statistical analysis

A one-way paired t-test was performed at each time point to analyze the significance of the differences between the amounts of indomethacin released in rat caecal content vs the controls. It was necessary to use a paired t-test because we were interested in comparing the amounts of drug released for the same batch of carrier matrices at each time point simultaneously for the experimental and control groups. The one-way test was selected because the specific degradation of cross-linked chondroitin sulfate was always expected to result in an increase in the release of the drug in the presence of caecal content. A difference was considered to be statistically significant when the p value was less than 0.05.

Results

The spectral analyses of the various types of modified chondroitin sulfate are presented in Figs 2 and 3, demonstrating that a reaction between the diamino reagent and the chondroitin sulfate did occur. The IR spectra exhibit the following results: (a) The appearance of the $-CH_2-$ band (2840–2900 cm^{-1}) for which the increase correlated with the amount of the diamino reactant used in the cross-linking treatment. While chondroitin sulfate has no peak at this region, ChS_{70} typically has a peak which is even greater for the ChS_{55} product. This result was expected, as the ChS_{55} product was treated with the greatest amount of diamino reagent. (b) The reaction of chondroitin sulfate with the diamino reagent resulted in an increase in the typical peak of –CONH– at 1600–1620 cm^{-1}. It should be noted that the amide group at position 2 of the galac-

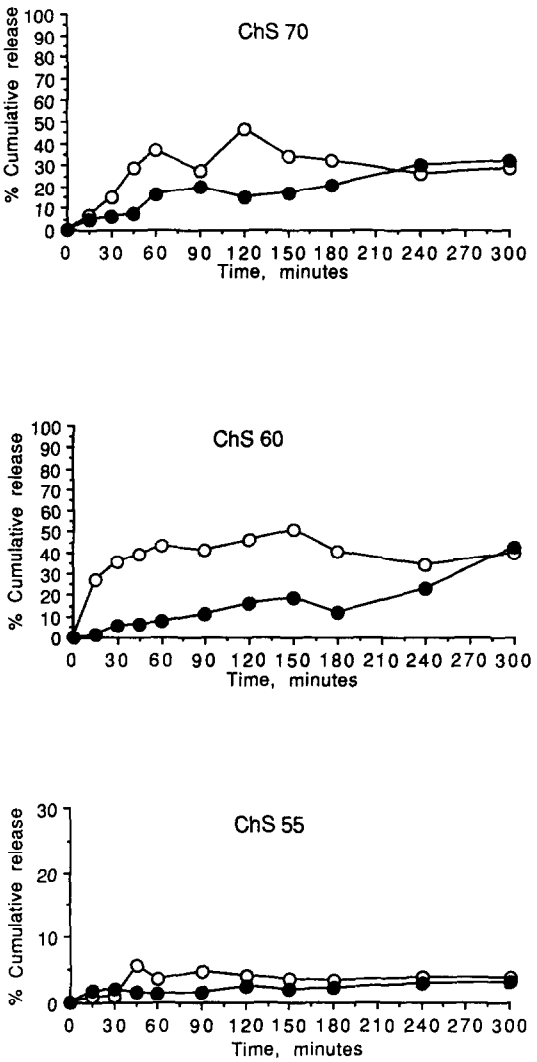

Fig. 5. Percent cumulative amounts of indomethacin released from three chondroitin sulfate formulations, ChS_{70}, ChS_{60}, and ChS_{55}, in sonicated rat caecal content in PBS (●———●) and in PBS (control) (○———○).

tosamide moiety of untreated chondroitin is also observed in Fig. 3 (top spectrum).

Fig. 5 summarizes the drug release experiments performed in the presence of rat caecal sonicates, compared to PBS controls. Since the matrices of the cross-linked products were insoluble in water, the initial release of indomethacin into the PBS control medium can be related to a diffusion from the surface of the dosage forms. Compared to the drug release profiles in the control experiments, less drug was released in the presence of sonicated caecal content in the beginning of each experiment. However, while the indomethacin release profiles in the PBS control medium reached a maximum level after 1–2 h, the release profile of the drug in the sonicated medium rose constantly, until reaching similar drug levels to those in the control experiments. This happened at 240–300 min. The percent cumulative release of indomethacin at the end of the experiments was 29.0, 40.0 and 3.8 for ChS_{70}, ChS_{60} and ChS_{55}, respectively, in the PBS control medium, and 32.0, 43.0 and 3.3 for ChS_{70}, ChS_{60} and ChS_{55}, respectively, in the sonicate medium. When untreated chondroitin was used as a carrier in these drug release experiments, 100% release of the indomethacin was seen within 1 h, as a result of the rapid dissolution of the ChS. In these experiments with chondroitin, the addition of sonicated caecal content to the PBS release medium had no significant effect (data not shown).

The second set of experiments, with intact caecal contents lasted 28 h. This value is close to the reported transit time of solid dosage forms in the colon (McLean et al., 1990). Fig. 6 demonstrates the difference between indomethacin release profiles from ChS_{70} in PBS medium with

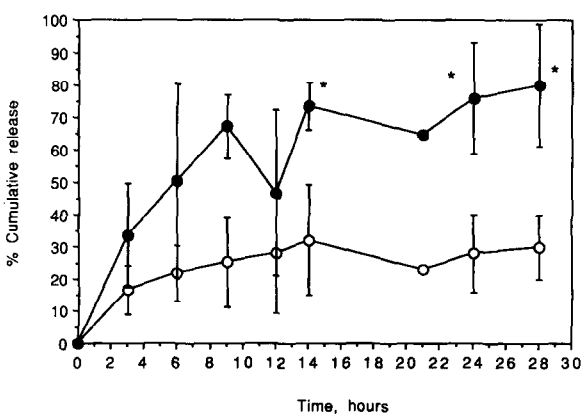

Fig. 6. Percent cumulative amounts of indomethacin released from ChS_{70} as analyzed in rat caecal content medium (●———●) and in PBS (○———○). Data shown are the mean of three sets of experiments.

and without caecal content. In the caecal medium the amounts of indomethacin released were higher than in the PBS control medium over the entire experiment, and significantly higher from 14 h on ($p < 0.05$). The release profiles of indomethacin from ChS_{60} and ChS_{55} into PBS with rat caecal content are summarized in Fig. 7. In both formulations the release in rat caecal content medium was greater than in the PBS control media (data not shown), and was significantly higher after 28 h for ChS_{60} and after 13 h for ChS_{55}. Fig. 7 shows a typical erosion-dependent release pattern with apparent zero-order kinetics. Such linear release kinetics was observed in neither the control experiments nor when ChS_{70} formulation was tested in PBS with rat caecal content. The ChS_{55} product, which was cross-linked to a higher extent than ChS_{60} product, released its drug load at a slower rate than the latter (Fig. 7).

Fig. 8 shows the differences between the total amounts of indomethacin released in the PBS control medium and in the rat caecal content medium at the end of each experiment (i.e., after 28 h). The values for the PBS controls were: 30.1 ± 10.0, 19.7 ± 15.0 and $9.0 \pm 4.1\%$ of indomethacin released for ChS_{70}, ChS_{60} and ChS_{55}, respectively. The corresponding indomethacin values for the caecal content medium were: 71.0 ± 19.0, 48.7 ± 35.0 and $22.5 \pm 7.9\%$ for ChS_{70}, ChS_{60} and ChS_{55}, respectively. There is a linear

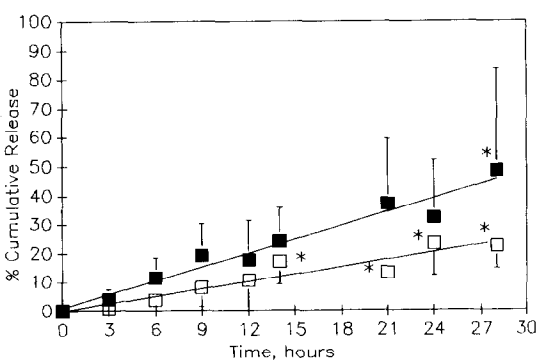

Fig. 7. Percent cumulative amounts of indomethacin released from ChS_{60} (■ ——— ■) and ChS_{55} (□ ——— □) as analyzed in rat caecal content medium. Data shown are the mean of three sets of experiments. * Significantly higher than the value obtained in the PBS control ($p < 0.05$).

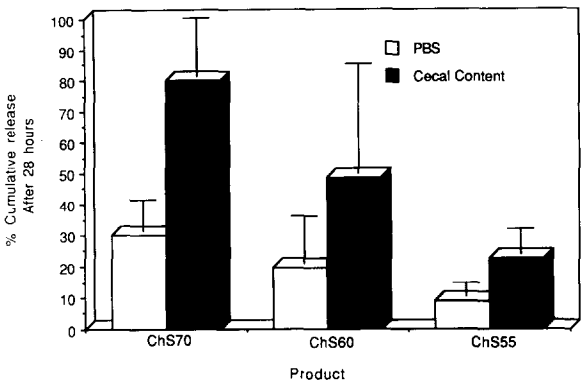

Fig. 8. Maximum amounts of indomethacin released after 28 h from the three cross-linked chondroitin sulfate formulations, ChS_{70}, ChS_{60}, and ChS_{55}, in rat caecal content and PBS control. Data shown are the mean of three different sets of experiments.

correlation between the measured indomethacin levels at 28 h and the extent of cross-linkage of the carriers as expressed by their measured RMN values: 68.6 for ChS_{70}, 60.5 for ChS_{60}, and 54.4 for ChS_{55}. Linear regression analysis for the correlation between the extent of cross-linkage and the total drug released yielded the following equations:

$$y = 4.04x - 196.62, \; r = 0.999 \qquad (2)$$

for the caecal content

$$y = 1.47x - 70.48, \; r = 0.992 \qquad (3)$$

for the PBS control.

Discussion

Cross-linked chondroitin sulfate matrices are biodegradable by bacterial enzymes in the rat caecum. The differences in the drug release profiles in the PBS controls and in the rat caecal contents media can be explained by the effect of rat caecal bacteria and bacterial enzymes on the cross-linked chondroitin carriers. When the release of indomethacin was analyzed in sonicated rat caecal content over 5 h, the release profiles

differed from the profiles found in PBS media that did not contain sonicates (controls). When the dissolution media did not contain sonicated rat caecal content, maximum drug levels were achieved within 60–120 min (Fig. 5). When the dissolution media contained sonicates, gradual elevation in the indomethacin levels was observed until attainment of similar levels to those in the control experiments (Fig. 5). The exponential-like release profiles together with the low drug recoveries suggest that the release of indomethacin in the control studies was driven by diffusion, presumably occurring at the surface of the insoluble matrices. When the PBS contained sonicated rat caecal content, the diffusion was apparently suppressed. This could be caused by adhesion or sedimentation of bacterial debris and other high molecular weight impurities originating in the rat caecum. Bacterial biofilm formation was reported previously to affect the performance of solid drug carriers in physiologic environments containing microorganisms (Costerton et al., 1987; Rubinstein et al., 1992b). In our case, the possible formation of bacterial films may interfere with drug release into the medium. Since diffusion was suppressed, the drug release resulted mostly from the biodegradation of the matrices. Fig. 5 shows the alteration in the drug release pattern from a burst diffusion, in the control experiments, to a constant elevation in those experiments that contained caecal sonicates. This type of release pattern is typical of an erosion mechanism (Heller, 1987).

In the non-sonicated media, where the release experiments lasted for 28 h, the indomethacin levels exceeded those that were attained in the PBS controls. This is demonstrated in Fig. 6, which summarizes the release of ChS_{70} formulation. Similar observations (i.e., the release profile in PBS medium which contains rat caecal content is higher than in the control experiment) were noted with the ChS_{60} and ChS_{55} formulations. However, it took 6–9 h until the released drug levels in the rat caecal media exceeded those in the control media (data not shown). Statistically significant difference was achieved after 13 h for ChS_{55} and only after 28 h for ChS_{60}. The drug release experiments involving ChS_{55} and ChS_{60} formulations in the presence of rat caecal contents, were characterized by apparent zero-order kinetics (Fig. 7). This observation is indicative of biodegradation of the chondroitin formulations which resulted in the release of the indomethacin into the dissolution medium. This is in contrast to what has been found in the release experiments with ChS_{70} formulation, which was more hydrophilic, and therefore diffusion together with biodegradation were probably the rate-controlling mechanisms in its drug release into rat caecal content medium (Fig. 6).

Salyers (1979) and Salyers and O'Brien (1980) have already shown that human colonic anaerobic bacteroides can use ChS as a substrate. We have shown here that chondroitin-induced enzymes in the rat caecum, probably of bacterial origin, can also degrade ChS even after it has been cross-linked to various extents. The results of the reported study suggest that caecal enzymes degrade the matrices, and that from the beginning of the process, bacterial adhesion and sedimentation on the surface of the delivery system interfere with the free diffusion of the drug, and initiate bioerosion-controlled drug release.

Note that in order to analyze the specific degradation of a drug carrier it was necessary to use a water insoluble drug model like indomethacin. Using a highly soluble drug in such a matrix system would make it hard to distinguish between simple diffusion of the drug and erosion of the drug carrier. However, the use of insoluble drug complicates the sampling procedure and increases the variability between the replicates (e.g., 12 h time point in Fig. 6).

After 28 h, the total amount of indomethacin released in the caecal medium is proportional to the RMN of the cross-linked ChS: it is inversely proportional to the degree of cross-linkage (Fig. 8). Of course, increased cross-linkage is also necessary to protect the drug until it arrives in the lower bowel; thus control of drug release may be optimized by either using mixtures of the variously cross-linked ChS carrier in the matrices, or balancing the cross-linking procedure.

We have shown that matrices of cross-linked ChS can be used as specific colonic delivery systems. Such matrices can retain most of their drug

content for over 10 h at pH 7 (Figs 6 and 8) which is close to the physiologic pH of the small intestine. Since the colon contains relatively low amounts of fluid, it is postulated that the mechanism of the enzymatic biodegradation of the chondroitin carriers is influenced by adsorption of liquid having a pH value of 7, while moving in the small intestine. The formulation technique presented here permits the incorporation of a variety of drugs for the treatment of large bowel diseases. These include drugs like steroids or salicylate derivatives such as 5-aminosalicylic acid, used for the treatment of inflammatory bowel diseases (IBD). Irritable bowel syndrome (IBS) is a frequent functional disorder which may require local treatment of drug such as pinaverium bromide (Passaretti et al., 1989). Over the past decade, there have been reports of carriers used to specifically target drugs to the colon (Brown et al., 1983; Friend and Chang, 1985; Harboe et al., 1989, Larsen et al., 1990; Tozer et al., 1991). However, most of those carriers were prodrugs used to carry 'only' a single drug to the colon. The studies of Saffran et al. (1986, 1988, 1990) were the first attempts to develop a microbially controlled drug delivery system to the colon, which is not a prodrug. These studies employed coating techniques for insulin and lysine-vasopressin. If in fact protein drugs are less susceptible to proteolytic degradation in the colon than in the stomach (Longer et al., 1989; Ikesue et al., 1991), then cross-linked chondroitin sulfate could serve as a carrier for these drugs as well. Drugs that are well absorbed through the colonic wall (Fara, 1989) are clearly the best candidates for use with such a specific colonic carrier.

The degree of similarity between the microbial populations of the rat caecum and of the human colon is as yet unclear. We are presently studying the usability of cross-linked chondroitin sulfate as a drug carrier in cannulated dogs. In addition, we are investigating the effect of the concentration of chondroitin sulfate used to induce the postulated enzymes in the rat caecum. More broadly, these questions relate to the interesting problem of the effect of diet on bacterial enzymes in the gut. We shall report our results in a subsequent paper.

Acknowledgments

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