The Relation Between Swelling Properties and Enzymatic Degradation of Azo Polymers Designed for Colon-Specific Drug Delivery

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Copolymers of 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA), and terpolymers of HEMA, MMA, and methacrylic acid (MA) were synthesized in the presence of N,N′-bis(methacryloyloxyethyloxycarbonylamino)azobenzene (B(MOEOCA)AB) and evaluated as coating materials for colonic targeting. The release of ibuprofen, a model drug, from capsules coated with the azo polymers was investigated in vitro. The release medium was made up of sonicated rat cecal content, benzyl viologen, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nicotine amide dinucleotide phosphate (NADP) in phosphate buffer (pH 6.8, 0.05M). The drug-release profiles indicate that the degradation of the azo polymer coatings depends on their degree of swelling, due to a higher accessibility of the azo bonds for the bacterial azo reductase. The best results were obtained with azo polymers containing MA: 98.7 (±1.1) % of ibuprofen was released after 19 hours residence in the release medium, while only 26.2 (± 4.9) % in the control experiment. These findings demonstrate that azo polymers are promising materials for delivering drugs selectivity to the colon.

KEY WORDS: colon-specific drug delivery; azo reductase; azo polymers; bacterial degradable polymers; coating.

INTRODUCTION

There is need for pharmaceutical delivery systems that enable selective release of drugs in the colon. Applications for colon-specific drug delivery include the local treatment of disorders of the large intestine, and the oral administration of protein and peptide drugs. The large intestine may be optimal for peptide delivery because of high residence time and low digestive enzymatic activity (1,2).

Targeting drugs to the large intestine can be achieved by coating drugs with pH-sensitive polymers (3,4), coating drugs with bacterial degradable polymers (5), delivery of drugs through bacterially degradable matrices and hydrogels (6–9), and delivery of drugs as prodrugs (10–13). Recently, different types of copolymers of methacrylic acid derivates, containing covalently bound azo compounds, were synthesized and evaluated as coating materials for colon-specific drug delivery (14,15). As a result of bacterial reduction of the azo bonds, the azo polymers are biodegradable, and therefore promising materials for colon-targeting.

The aim of the present study was to investigate the release of ibuprofen, a model drug, from capsules coated with the azo polymers in sonicated rat cecal contents. Rats were chosen since their colonic bacterial population is comparable to that of humans (16). Different types of azo polymers were investigated: copolymers of 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA), and terpolymers of HEMA, MMA, and methacrylic acid (MA). The azo agent N,N′-bis(methacryloyloxyethyloxycarbonylamino)azobenzene (B(MOEOCA)AB) was incorporated in the polymers. Ibuprofen was quantified using high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Chemicals

Ibuprofen (BP 88) was obtained from Alpha Pharma (Vichte, Belgium). Naproxen, used as the internal standard, was kindly provided by Syntex (Brussels, Belgium). Nicotinamide adenine dinucleotide phosphate (NADP), benzyl viologen, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma (Bornem, Belgium). All other reagents and organic solvents were of analytical or HPLC grade. The water used for HPLC was purified with a Milli-Q system (Millipore, Brussels, Belgium). Azo polymers were prepared as described previously (14,15). The composition of the azo polymers as well as their degree of swelling is given in Table 1.

Preparation of Coated Capsules

Gelatin capsules, containing approximately 20 mg of ibuprofen, were coated with the different azo polymers by dipping the capsules in an ethanolic solution of the azo polymers (20% w/w). Polyethyleneglycol 400 (20% w/w, calculated on the polymer content) was added to the coating solution as plasticizer. The amount of polymer coating on the capsules was 9.7 (±0.4) % w/w.

Preparation of the Rat Cecal Content Release Medium (RCCRM)

Male, wistar rats (±300 g) were killed, ligatures were made before and after the cecum, and the cecum was removed. Subsequently, under anaerobic conditions, the cecum was opened, and its content suspended in a phosphate buffer (pH 6.8, 0.05 M), which was previously bubbled with nitrogen to remove the oxygen. The suspension of rat cecal content (10 g/100 ml of phosphate buffer) was filtered through glass wool and sonicated (50 Watt) for 20 min. at 4°C to disrupt the bacterial cells, and to increase the azo reductase activity.

After sonication, the mixture was centrifugated at 20000 rpm (4°C) for 30 min., and the following compounds were added to the release medium: benzyl viologen (1.4×10^{-4} M), NADP (2.5×10^{-4} M), glucose-6-phosphate (8.5×10^{-4} M), and glucose-6-phosphate dehydrogenase (1 U/ml) (13).

The different cofactors were added to the release medium in order to create an electron generating system. In the experimental set up, glucose-6-phosphate is converted to glucono-δ-lacton-6-phosphate in the presence of glucose-6-

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phosphate dehydrogenase, and simultaneously, NADP is reduced to NADPH. Subsequently, NADPH is oxidized, and the electron mediator, flavins or benzyl viologen, is reduced and acts as an electron shuttle from the NADPH-dependent azo reductase to the azo compound, which is then reduced. Simultaneously, the redox mediator is reoxidized.

The potential of the rat cecal content release medium was −360 mV (combined Pt/Ag/AgCl electrode), pointing out its reductive capacity.

In order to characterize the release medium for its reductive capacity, the degradation kinetics of tartrazine, a water-soluble azo dye, was studied in RCCRM.

A stock solution of the dye (1.4×10^{-4} M) was prepared in 0.05 M phosphate buffer (pH 6.8) and sterilized by autoclaving. One ml of the sterile dye solution was added to 3 ml of RCCRM. The samples were incubated at 37°C in the anaerobic workstation. Azo dye concentrations were calculated at a given incubation time under anaerobic conditions from the absorbance at 425 nm. The relationship between absorbance (Y) and chromophore concentration (X) is: $Y = 1.96 * 10^{-3} + 2.18 * 10^{4}X$ ($r = 0.999$; $n = 5$). The standard deviation of the residuals ($S_{y,x}$) was 1.71×10^{-3}. The reference solution consisted of RCCRM diluted with phosphate buffer to correct for addition of the dye solution to the sample.

Analysis of Ibuprofen

Determination of ibuprofen in RCCRM was based upon a method described by Geisslinger et al. (17). Naproxen (40.9 µg/ml) was used as internal standard.

Isocratic HPLC was performed using a LiChroGraph L-6000 HPLC pump (Merck-Hitachi, Darmstadt, Germany); a Rheodyne Model 7125 Syringe Loading Sample Injector (Rheodyne Inc., Cotati, CA, USA) equipped with a 20 µl loop; a LiChroGraph L-4000 UV detector (Merck-Hitachi, Darmstadt, Germany), set at 220 nm; and a Merck-Hitachi Model D-2500 Chromato-Integrator (Darmstadt, Germany). The 24.4 × 0.4 cm column was packed with LiChrospher 60 RP-select B (5 µm) (Merck, Darmstadt, Germany). The mobile phase, which consisted of methanol:water:phosphoric acid (70:30:0.1; v/v/v), was filtered through a nylon membrane filter (0.45 µm) and degassed by ultrasonication before use. The flow rate was 1.0 ml/min. (a typical chromatogram is shown in Figure 1).

The relationship between the peak area ratio of ibuprofen to naproxen (Y) and ibuprofen concentration (X) is: $Y = 7.42 * 10^{-2} + 2.42 * 10^{-2}X$ ($r = 0.999$; $n = 7$). The standard deviation of the residuals ($S_{y,x}$) was 2.32×10^{-2}. The detection limit for ibuprofen was 10.2 ng. The relative standard deviation of both the intra- and interday variability was less than 4%. The recovery of ibuprofen from RCCRM was between 94 and 99% in the investigated concentration range (2.90–93.00 µg/ml).

Ibuprofen Release Experiments

The release of ibuprofen from the coated capsules was performed in 200 ml of freshly prepared RCCRM and in 200 ml of phosphate buffer (pH 6.8, 0.05 M) as control study. The release study was carried out in the Compact Anaerobic Workstation (DW Scientific, West Yorks, U.K.) at 37°C. The

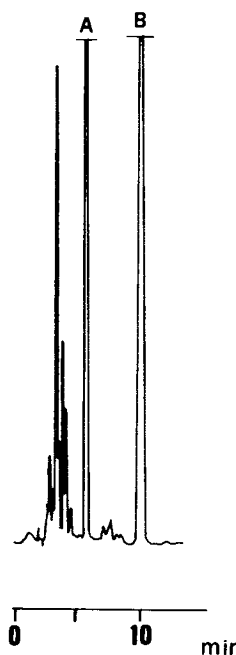

Fig. 1. Representative chromatogram of the analysis of ibuprofen in RCCRM. A = naproxen (I.S), B = ibuprofen.

significance of the differences between the amount of ibuprofen released in RCCRM and the control was established using a two-tailed, unpaired T-test. A difference was considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Reductive Capacity of the RCCRM

The reduction of tartrazine appeared to start immediately after contact with RCCRM (Figure 2). The linear dependence of the chromophore concentration on time ($r > 0.997$) indicates a zero order reduction rate with respect to tartrazine concentration, and corresponds with other obser-

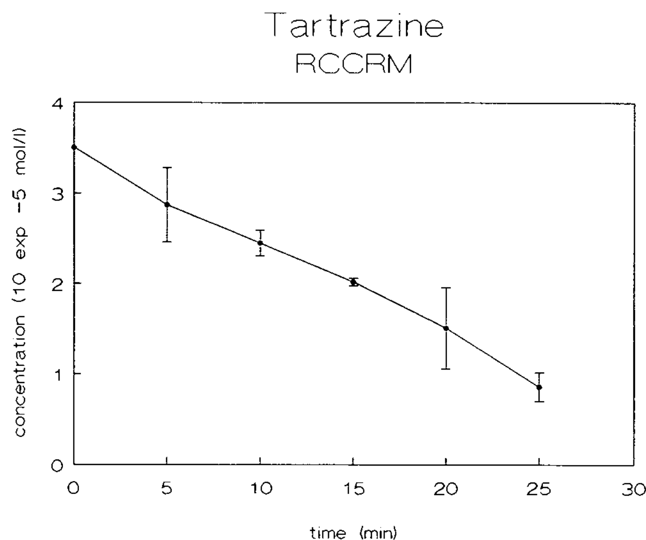

Fig. 2. Degradation plot of tartrazine in RCCRM. Error bars indicate the standard deviation.

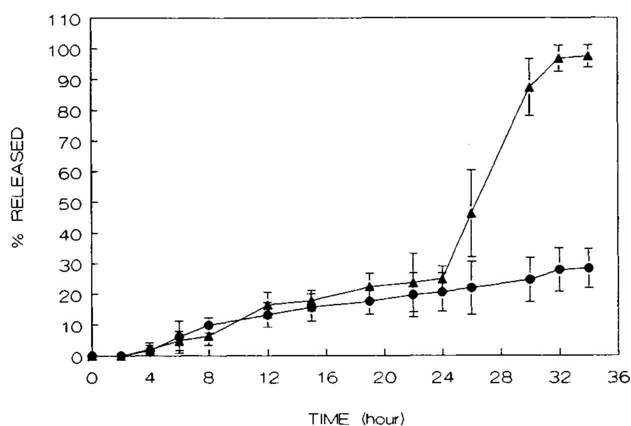

Fig. 3. Release of ibuprofen from capsules coated with azo polymer S1 in RCCRM (▲) and phosphate buffer (●). N = 6; error bars indicate the standard deviation.

vations in the literature (18,19). The calculated zero order rate constant was 9.85×10^{-7} mol 1^{-1}min^{-1}.

In previous studies (14,15), we used a degradation medium that contained a high concentration of human feces (>50% w/w), but this is not suited for release experiments due to its high viscosity. Therefore, the use of a sonicated rat cecal content medium was preferred. One must take into account that disruption of bacterial cells as well as the addition of supplementary cofactors (NADP, redox mediator) significantly increases the reduction of azo bonds as compared to suspensions of whole bacterial cells without the addition of cofactors (20,21). However, the reduction rate of tartrazine in RCCRM was similar to the reduction rate of the dye in the previously used degradation medium, because of the high concentration of human feces in the medium, and to the high amount of dead bacteria in feces. Indeed, it has been shown previously that the rate of azo reduction increases with cell mortality (18).

Polymer Degradation and Ibuprofen Release

A highly water-soluble drug would be released by diffusion through the polymer coating before bacterial degradation of the coating starts. Because of its low water-solubility, ibuprofen was selected as the model drug.

The amount of drug that was released after 34 hours is 14.6 (±2.3) and 25.4 (±4.4) % from capsules coated with S3 and S2, respectively. These values were not significantly different from the control studies (13.7 (±2.8) % for S3 and 21.1 (±2.8) % for S2) and the release of ibuprofen in both media is only based upon simple diffusion of the drug through the coating.

The release of ibuprofen in RCCRM from capsules coated with polymer S1 is not attributable to diffusion alone. After 26 hours, a significant difference ($p = 0.005$) was found between the two release media, and after approximately 32 hours, 96.7 (± 4.3) % of the drug is released, while only 28.4 (±4.3) % is released in the control experiment (Figure 3). This can be explained by the effect of the rat cecal azo reductase on the azo polymer coatings. It appears that reduction of azo bonds is a general reaction rather than a reaction that is carried out by only a few bacteria (22). The mechanism of anaerobic azo bond cleavage probably represents a non-enzymatic reduction by enzymatically generated reduced flavins. The reduced flavins or other redox mediators such as benzyl viologen, act as an electron carrier from the NADPH-linked azo reductase to the azo compound. The protein responsible for flavin reduction and thus for azo reduction in Streptococcus faecalis is thought to be a flavoprotein (23).

It has been shown previously (14,15) that the azo bonds in azo polymers can be reduced by the colonic microflora, on the condition that the polymers have a high degree of hydrophilicity. However, a balance must be found in the ratio between the amount of hydrophilic component (HEMA), which ensures good availability of the azo bonds for bacterial reduction, and the more hydrophobic component (MMA), which impedes the swelling of the polymers (24), thereby preventing premature drug release in the stomach and small intestine, but also reducing the accessibility of the azo bonds for bacterial reductase. The absence of an ionizable group in these polymers explains that their swelling behaviour is independent of pH (Table 1).

Taking into account that capsules and tablets generally pass through the colon in approximately 20–30 hours, and that the viscosity of the colonic content increases dramatically after the hepatic flexure, thereby reducing the possibility for a drug to diffuse from the colonic lumen to the site of absorption, one can conclude that neither S1, S2, or S3 meet the requirement of being degraded within an acceptable period of time.

In order to create pH-sensitive azo polymers, we developed terpolymers, two of which were selected to be evaluated as materials for colonic targeting: P11 and P12 (Table 1).

Table I. Composition of Azo Polymers and Their Swelling Index (Is%) as a Function of pH

Polymer	HEMA:MMA:MA (w:w:w)	Azo Agent	Is%[1]				
			pH1	pH5	pH6	pH7	pH8
S1	6:1:0	B(MOEOCA)AB	36.2	35.7	35.9	35.9	35.4
S2	5:1:0	B(MOEOCA)AB	30.4	30.6	30.1	30.8	30.8
S3	4:1:0	B(MOEOCA)AB	26.4	26.8	25.9	26.7	27.0
P11	4:1:0.05	B(MOEOCA)AB	25.7	25.7	26.5	47.5	67.3
P12	9:2:0.10	B(MOEOCA)AB	26.0	26.4	29.4	63.8	99.9

[1] The swelling index was calculated as follows (24): Is% = 100 * (Ws − Wd)/Wd; where Wd is the weight of the dry polymer, and Ws is the weight of the polymer after swelling. 0.7 mol% of B(MOEOCA)AB was added to the monomer mixture.

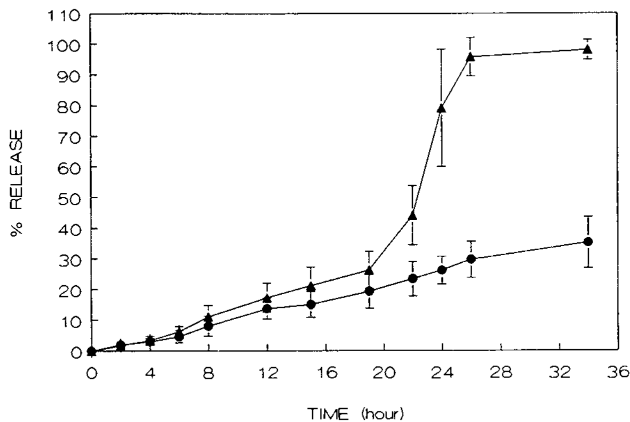

Fig. 4. Release of ibuprofen from capsules coated with azo polymer P11 in RCCRM (▲) and phosphate buffer (●). N = 6; error bars indicate the standard deviation.

Since only a small amount of MA is incorporated, the polymers are still water-insoluble. The incorporation of MA results in a degree of swelling that increases when passing from acidic to neutral or slightly alkaline pH due to neutralization of the carboxylic acid groups of MA. Compared to polymers of the S-type, premature release of the drug in the stomach and the small intestine can thus be diminished, and the azo bonds are now better accessible for the bacterial azo reductase at the end of the ileum and the large intestine.

Figures 4 and 5 depict the difference between ibuprofen release profiles from capsules coated with polymer P11 and P12 in RCCRM and phosphate buffer. The release of ibuprofen is always higher in RCCRM, and becomes significantly higher after 22 hours for P11 and after 12 hours for P12. After 26 hours, 95.8 (±6.2) % of the ibuprofen is released in RCCRM, while only 29.7 (±5.9) % in phosphate buffer, when using P11. When polymer P12 is used, 98.7 (±1.1) % is released in RCCRM, while 26.2 (±4.9) % in phosphate buffer, after 19 hours.

Due to the high degree of swelling of the pH-sensitive azo polymers at pH 6.8, a certain amount of the drug will be released by simple diffusion through the highly swollen polymer membrane. However, taking into account that the stomach to cecum transit is approximately 4–6 hours (16), the amount of drug released before reaching the colon will be low. The results obtained with the pH-sensitive azo polymers are promising, especially that obtained with polymer P12. A colonic residence time of 19 hours to release approximately 100% of an active agent is an acceptable value, and opens perspectives for further research in this field.

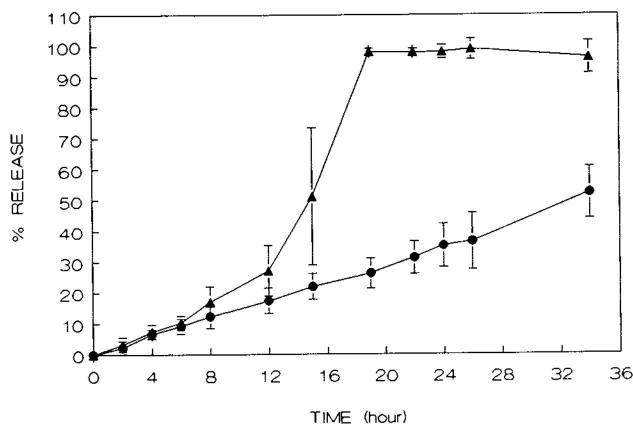

Fig. 5. Release of ibuprofen from capsules coated with azo polymer P12 in RCCRM (▲) and phosphate buffer (●). N = 6; error bars indicate the standard deviation.

In conclusion, the results reported in this paper indicate that azo polymers are potential candidates to be used in colon-specific release formulations. The in vitro release experiments show that the film coatings of pH-sensitive azo polymers are able to release a drug in vitro within an acceptable period of time.

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