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Research paper

Degradation of raw or film-incorporated β-cyclodextrin by enzymes and colonic bacteria

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Abstract

β-cyclodextrin (β-CD) is a suitable excipient for peroral use, which improves the solubility of lipophilic drugs, as well as for colon-specific drug release when it is mixed with coating polymers. The first aim of this work was to examine the suitability of various enzymes as a simple in vitro model for the glycolytic activity in the human colon. α-Amylase (source *Aspergillus oryzae*) and taka diastase (source *A. oryzae*) showed remarkable degradation capacity of free β-CD, whereas other α-amylases (sources *Bacillus subtilis* or *Hog pancreas*) were found to be unsuitable. The next aim was to find out if film-incorporated β-CD is also degraded by these enzymes. Therefore, diffusion studies of 5-aminosalicylic acid (5-ASA) through Eudragit® RS or Eudragit® NE films containing β-CD were performed with taka diastase present in the buffer medium. Pronounced diffusion of the drug through the Eudragit® RS film was found only when swelling excipients like crosslinked sodium carboxymethylcellulose (CMC-CL sodium) or polyvinylpyrrolidone (PVP 25) were present in the film, indicating enhanced accessibility of β-CD by the enzyme. Films containing CMC-CL without β-CD showed even higher permeability, which also points to enzymatic degradation of CMC-CL. Permeabilization by taka diastase of Eudragit® NE films without swelling agents correlated with the β-CD content, whereas control films containing talcum remained impermeable upon enzyme action. Furthermore, the β-CD degradation capacity of colonic bacteria like *Escherichia fergusonii*, *Serratia odorifera* or *Proteus mirabilis* was examined with β-CD coatings on tablets, which contained bisoprolol as a model drug. Tablets with β-CD-containing Eudragit® RS coatings showed the highest drug release upon incubation with *P. mirabilis*. The moderate drug release by *E. fergusonii* could be increased almost to the same level when the bacteria were pre-incubated for 24 h in medium containing 2.5 mg/ml β-CD, indicating the induction of glycolytic enzymes by β-CD in this colonic bacteria strain.

Keywords: β-cyclodextrin; Colonic bacteria; Colon-specific drug delivery; Enzymatic degradation; Film coating; Eudragit®

1. Introduction

Colon-specific drug release has become an important field of research within recent years due to biotechnical advances producing therapeutic peptides at a reasonable cost. Peptide drugs are usually degraded in the upper gastrointestinal tract, whereas in the colon there is less proteolytic activity, leaving the latter as a potential absorption site [1,2]. In addition, the treatment of colonic diseases like colitis ulcerosa as well as once-a-day sustained release formulations require a better understanding of absorption characteristics in the colon [3,4]. Colon targeting has been approached by considering different physiological parameters in several parts of the gastrointestinal tract such as pH differences, transit times, luminal pressure or the bacterial flora in the colon. A range of different delivery systems are in development regarding these parameters for their release design [5]. pH-triggered delivery systems exploiting the changes of pH within the gastrointestinal tract use for example

Abbreviations: 5-ASA, 5-aminosalicylic acid; BP, British pharmacopeia; CMC-CL, sodium, sodium salt of crosslinked carboxymethylcellulose; DEP, diethylphthalate; PVP, 25, polyvinylpyrrolidon 25; rpm, rotations per minute; β-CD, β-cyclodextrin; U, unit: glycosidic enzyme activity (referring to the liberation of 1 μmol maltose per minute at pH 6.0 and 25 °C); USP, United States pharmacopeia.

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Eudragit® L or Eudragit® S [6]. Time-triggered delivery systems considering the transit times have also been described [7]. Recently, pressure-controlled delivery systems using the strong peristalsis in the colon were published [8]. However, the bacterial flora of the colon is unique and the involvement of the enzymatic activity of bacterial strains for colon-specific drug release is thought to be very effective [9–11]. There are several polymers monitored intensively for degradability by colonic bacteria like dextran esters and galactomannans [12,13], pectins [14,15] and azopolymers [16,17]. Prodrugs such as steroids linked to drug carriers were investigated and showed promising degradation characteristics [18,19]. The degradation of raw β-cyclodextrin (β-CD) by colonic bacteria or colonic flora has been reported [20,21]. β-CD is a cyclic oligosaccharide of seven α-(1,4) linked glucose units, which is less soluble in water (18 mg/ml) compared to the other cyclodextrins due to cluster formation. Numerous drugs have been described which form complexes with β-CD enhancing drug stability and/or absorption performance [22,23]. Drug formulations coated by suitable polymers containing β-CD are thought to reach the colon intact, where colonic bacteria then degrade β-CD resulting in increased solubility or porosity of the coating and enhanced drug release. Tablets containing 5-aminosalicylic acid and coated with β-CD dispersed in Eudragit® RS, which has a solubility independent of pH, were shown to be degraded in a colonic environment using the colonic microflora test [24]. This medium consists of pigs caecum content, ileostomy effluent and buffer and was widely used to examine the degradation of several polymers for colon targeting [25]. Eudragit® RS films with incorporated inulin also were described for their successful degradation in human faecal medium [26]. However, the high variability of enzymatic activity within caecal or colonic contents reduces the possibility of comparing degradation characteristics at an early stage of formulation development.

The aim of the present study is to evaluate raw as well as film-incorporated β-CD for their in vitro biodegradation by commercially available or isolated glycolytic enzymes or by particular colonic bacteria strains which produce a variety of polysaccharide degrading enzymes of the colon. The enzymatic degradation of β-CD by taka diastase or by different α-amylases was monitored. Drug diffusion rates through Eudragit® RS or Eudragit® NE films containing β-CD were determined after exposure with these enzymes. Additionally, an in vitro assay based on the colonic bacteria *Escherichia fergusonii*, *Serratia odorifera* or *Proteus mirabilis*, which were all isolated from the porcine colon, was established in order to mimic the colonic release of bisoprolol containing tablets coated with Eudragit® RS containing β-CD or talcum as a control.

2. Materials and methods

2.1. Materials

β-CD was obtained from Syntapharm (Germany). Taka diastase (*Aspergillus oryzae*), 1.5 U/mg; α-amylase (*A. oryzae*), 30 U/mg, α-amylase (*Bacillus subtilis*), 50 U/mg and hemin were from Fluka Chemie (Switzerland), α-Amylase (*Hog pancreas*), 29 U/mg was from Sigma (USA). The enzyme activities are referring to the degradation of starch according to the manufacturer's declaration for the particular batches. Sodium salt of crosslinked carboxymethylcellulose (CMC-CL sodium) was purchased from FMC (USA). Schaedler broth and tryptic soy broth from Difco (USA). 5-Aminosalicylic acid (5-ASA) was from Euroresearch S.R.L (Italy). Eudragit® RS 30D and Eudragit® NE 30D were from Röhm GmbH. Proteose peptone, L-cysteinium chloride, trishydroxymethylaminomethane, granulated yeast, bisoprolol hemifumarate, diethylphthalate (DEP), Tween® 80, sodium carbonate and phenolphthalein were from Merck KGaA, polyvinylpyrrolidone (PVP) 25 from BASF AG, talcum from Luzenac, calcium dihydrogenphosphate from Chemische Fabrik Budenheim, magnesium stearate from Bärlocher, Avicel PH 101 from Nordmann–Rassmann (all Germany). Phosphate buffer pH 6.8 was prepared according to BP 1988.

2.2. Methods

2.2.1. Enzyme assay

β-CD was determined with a colorimetric assay using phenolphthalein described in detail elsewhere [27]. The degradation of β-CD by taka-amylase A (identical with taka diastase) is already discussed in earlier studies [28,29]. Samples were incubated with taka diastase or α-amylase (*A. oryzae*, *B. subtilis* or *H. pancreas*) at 37 °C and pH 6.0 using phosphate buffer according to BP 1988. The enzyme activity was 10 U/ml, substrate concentration was 0.1 mmol/l and incubation times varied between 10 and 60 min. After incubation the contents were boiled for 10 min which was sufficient for enzyme inactivation and then cooled to room temperature. Phenolphthalein was then added and the pH was adjusted to 10.5 using sodium carbonate. The volume was adjusted as well. Samples were immediately measured spectrometrically at 550 nm, where no interference by components of enzymatic origin was found.

2.2.2. Film preparation

All excipients used for film preparation were calculated as percentages (w/w) referring to dry polymer weight. 50 or 100% β-CD or 100% talcum were dispersed in (333%) purified water by magnetic stirring and then poured into dispersions of either Eudragit® RS 30D containing 20% DEP or Eudragit® NE 30D containing 5% Tween® 80.

Eudragit® RS and NE are both diffusion coatings, insoluble in water and show pH-independent permeation properties. In addition, some preparations contained 25% CMC-CL sodium or 25% PVP 25, which were added to the aqueous β-CD or talcum containing dispersions. Total solid contents are different in the films according to their composition the percentage is equal in any formulation. Films were cast with an Erichsen apparatus 509/1 (Erichsen and Co. KG, Germany) on low adhesion foil (Scotchpak® type 1006) and dried at 35 °C. The integrity of the films was checked visually and the film thickness was measured conductometrically (Minitest 3000, Erichsen and Co. KG, Germany).

2.2.3. Diffusion studies

Diffusion studies were performed using specially designed diffusion cells. These are made of high-grade steel and have a rounded bottom that fits in USP dissolution vessels. The donor compartment (2.5 ml) was filled with 5-ASA (5 mg/ml) as a model drug in phosphate buffer pH 6.8 BP 1988 and mounted with different film preparations with a diffusion area of 1.5 cm^2. The diffusion cells were then transferred into dissolution vessels according to USP, which were either filled with 500 ml phosphate buffer pH 6.8 BP 1988 alone or with the enzyme preparation. The stirring speed was adjusted to 50 rpm using USP paddles and the temperature was kept constant at 37 °C. Samples were taken from the dissolution vessels after 0.5, 1, 2, 4 and 6 h, filtered through Anatop® 25 (0.2 μm pore size, Merck KGaA, Germany) and the absorbance was measured spectrometrically at 331 nm.

2.2.4. Film tablet preparation

Bisoprolol hemifumarate 5 mg, calcium dihydrogenphosphate 49 mg, magnesium stearate 1 mg, and Avicel PH 101 5 mg (amounts per tablet) were mixed (Turbula System Schatz, Typ T20P), sieved and pressed to tablets 5 mm in diameter (Fette excenter press, Germany). Dispersions of Eudragit® RS, DEP 20% and β-CD 20% or talcum 20% were prepared and sieved. Tablets were coated using a Hi Coater (Loedige, Type HCT 30 mini, Germany).

2.2.5. Tablet incubation and HPLC monitoring of drug release

E. fergusonii, *S.odorifera* or *P. mirabilis* were isolated from the pig colon, pre-cultivated in Schaedler broth for 24 h and then inoculated into an organic nutrient medium (pH 7.5), consisting of tryptic soy broth 1 g, proteose peptone 0.5 g, granulated yeast 0.5 g, trishydroxymethylaminomethane 0.3 g, L-cysteinium chloride 0.04 g and hemin 1 mg (referring to 100 g medium). Tablets with β-CD or talcum containing coatings were incubated in 10 ml of each bacterial suspension for 8 h at 37 °C. In addition, β-CD containing tablets were incubated in each bacterial suspension where β-CD 2.5 mg/ml had been added prior to the bacterial 24 h incubation. All work was carried out under laminar air flow conditions. After incubation the medium was diluted to 2% with water. The HPLC analyses of bisoprolol release were performed using a LiChroSpher 100, RP-18 pre-column and a LiChroCART 250-4 Superspher 60 RP select B column (Merck KGaA). The injected volume was 100 μl. Elution was carried out with components A (17.25 g ammonium dihydrogen phosphate and 5 ml *o*-phosphoric acid in 5 l distilled water) and B (65% acetonitril and 35% component A) in a 1:1 ratio. The flow rate was 0.8 ml/min, and the temperature was 50 °C.

3. Results and discussion

3.1. Enzymatic studies

An assay was developed to quantify the degradation of free β-CD or β-CD-containing film preparations by suitable glycolytic enzymes. The assay should give information on the film integrity and quality after optimum enzymatic exposure in the colon, rather than characterising the degradation process within the complex colonic environment. In Fig. 1 the amount of free β-CD (initial β-CD concentration was 100 μmol/l) cleaved by taka diastase or α-amylases (*A. oryzae*, *B. subtilis* or *H. pancreas*), each at a concentration of 10 U/ml, is plotted versus incubation time. The degradation of β-CD was found to vary for the different enzymes. α-Amylase (*A. oryzae*) and taka diastase, which are both of fungal origin, showed the highest degradation capacities, whereas no degrading ability was found for the α-amylases from *B. subtilis* or from *H. pancreas*. The latter result is in agreement with data reported elsewhere [30], showing the resistance of β- and α-CD against human saliva

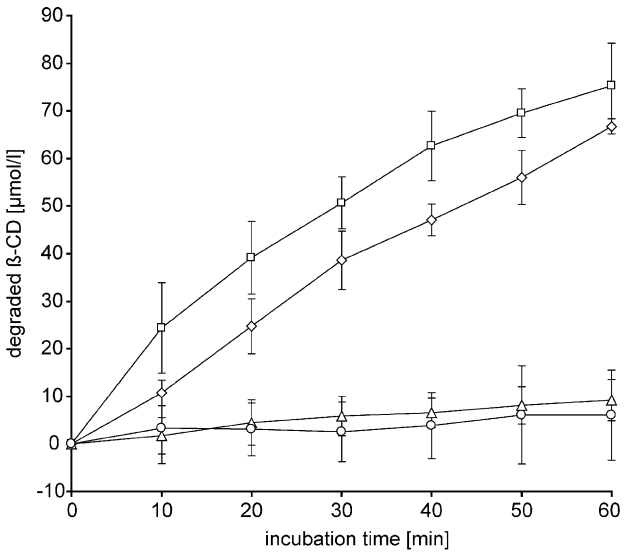

Fig. 1. Enzymatic degradation of free β-cyclodextrin (β-CD, initially 100 μmol/l) by different α-amylases and taka diastase, monitored by phenolphthalein release; (○) α-amylase (*hog pancreas*), (△) α-amylase (*Bacillus subtilis*), (◇) taka diastase, (□) α-amylase (*Aspergillus oryzae*), each 10 U/ml. Error bars are means ± range ($n = 3$).

and pancreas α-amylases. β-CD is not degraded by amylases in the upper part of the gastrointestinal tract and can, therefore, reach the colon where it can be degraded in principle by other bacterial enzymes [31,32].

3.2. Diffusion studies of free films

In previous studies on the degradation of β-CD in polyacrylic films taka diastase was found to be more effective than α-amylase from *A. oryzae* and was, therefore, chosen as the model enzyme in the following studies (data not shown). The diffusion rates of 5-ASA in buffer with or without taka diastase 10 U/ml through Eudragit® RS films were monitored. The films contained 20% (referring to the dry weight of the polymer) DEP as plasticizer in addition to 100% β-CD, and 25% of either CMC-CL sodium or PVP 25 as swelling excipients to improve the accessibility of β-CD for the enzymes. As controls, films containing 25% of either CMC-CL sodium or PVP 25 without β-CD were studied. As a further control, the diffusion behavior of Eudragit® RS films loaded with 100% talcum and 25% of either CMC-CL sodium or PVP 25 was monitored. Fig. 2 shows the diffusion characteristics focusing on CMC-CL sodium effects. The diffusion of 5-ASA through films containing β-CD without CMC-CL sodium was low and was not altered by taka diastase. For β-CD and CMC-CL sodium loaded preparations the diffusion rate was increased by approx. 50% after enzyme incubation. To a lesser extent this effect was also observed for talcum and CMC-CL sodium loaded films. Eudragit® RS films additionally containing only CMC-CL sodium showed a pronounced, i.e. approx. four-fold increase in the diffusion rate of 5-ASA after enzymatic treatment. In Fig. 3 the diffusion characteristics of films were similarly studied for PVP 25 effects. Films loaded with talcum and PVP 25 showed negligible permeability with or without enzyme incubation. Films loaded exclusively with PVP 25 showed a moderate increase in diffusion rates after enzymatic treatment, films containing β-CD and PVP 25 moderate diffusion rates in buffer which were more than doubled upon incubation with taka diastase.

Our data suggest that due to the low permeability properties of Eudragit® RS, incorporated β-CD is almost inaccessible to enzymatic degradation, which was measured as either unincreased or only moderately increased diffusion rates in enzyme-containing medium. The incorporation of components with swelling capacities such as CMC-CL sodium or PVP 25 into the polymer structure increases the permeability of the material, making it more accessible for enzymatic cleavage of incorporated β-CD. Interestingly enough, CMC-CL sodium and PVP 25 were found to also increase the diffusion characteristics after enzymatic incubation in films without β-CD, and in the case of CMC-CL sodium this effect was found to be extensive. This might be explained by the nature of taka diastase, which is a multienzyme representing more than 30 different enzymatic functions. Cellulose derivatives including CMC-CL sodium are reported to be degraded to some extent by amylolytic enzymes and bacteria [33,34].

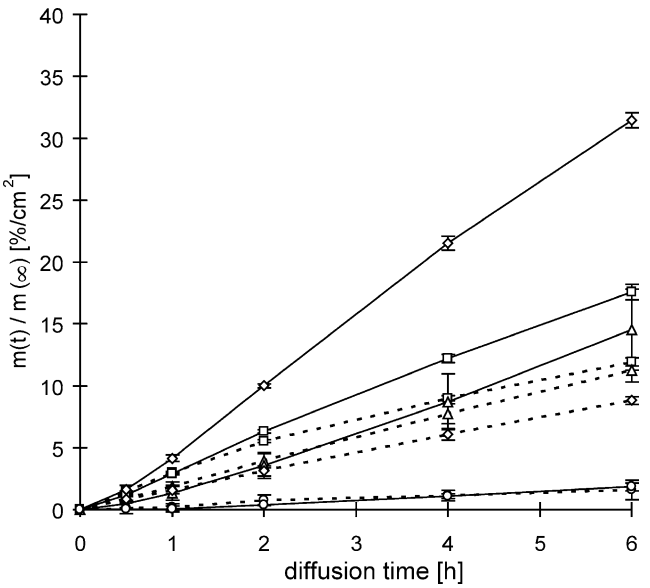

Fig. 2. Influence of CMC-CL sodium on the diffusion of 5-ASA through Eudragit® RS films containing 20% DEP and (○) 100% β-CD [78.7 μm average film thickness], (□) 100% β-CD and 25% CMC-CL sodium [76.1 μm], (△) 100% talkum and 25% CMC-CL sodium [62.9 μm], or (◇) 25% CMC-CL sodium [43.6 μm]; percentage of excipients referring to dry weight of film polymer; drug diffusion is given as m(t)/m(∞) [%/cm^2], i.e. mass of drug diffused per cm^2 related to diffused mass of drug in the final steady state; dotted lines: in phosphate buffer pH 6.8; solid lines: additionally containing taka diastase 10 U/ml; error bars are means ± range ($n = 3$).

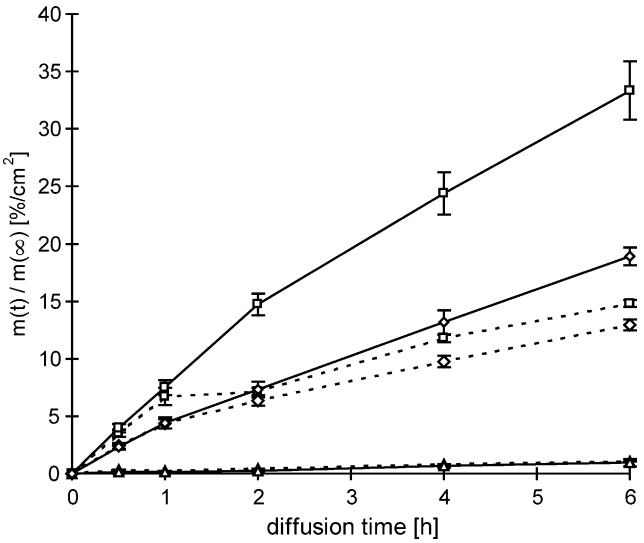

Fig. 3. Influence of PVP 25 on the diffusion of 5-ASA through Eudragit® RS films containing 20% DEP and (□) 100% β-CD and 25% PVP 25 [83.9 μm average film thickness], (△) 100% talkum and 25% PVP 25 [80.3 μm], or (◇) 25% PVP 25 [71.3 μm]; dotted lines: in phosphate buffer pH 6.8; solid lines: additionally containing taka diastase 10 U/ml; error bars are means ± range ($n = 3$).

In addition, the incubation with taka diastase might result in partial cleaving of glycosidic bonds of CMC-CL sodium and in formation of more soluble cellulose components, explaining the pronounced increase of the diffusion rates of the drug. This will be investigated in further studies. Talcum or β-CD in combination with CMC-CL sodium showed lower diffusion rates of the model drug 5-ASA compared to CMC-CL sodium alone, which could be explained by a limited swelling ability of the polymeric material in the presence of incorporated talcum or β-CD. The enzymatic incubation of films containing PVP 25 as well as β-CD resulted in considerably increased diffusion rates, assuming that the degradation of β-CD is the predominant process. A moderate increase in diffusion rates was observed in PVP 25 containing films with no β-CD after enzymatic incubation which could be due to enzymatic interactions with film components.

In Fig. 4, drug diffusion is monitored for films consisting of Eudragit® NE. For films loaded with 50% β-CD, diffusion rates increase after 1 h for samples incubated with taka diastase compared with buffer alone. The diffusion rate is approximately doubled for films containing 100% β-CD. This clearly opens the possibility of controlling the diffusion rate by varying the β-CD content in the film coating. Films loaded with 100% talcum showed negligible diffusion rates after incubation in medium with or without taka diastase.

The diffusion rates of Eudragit® NE films loaded with 100% β-CD and 5% Tween® 80 and incubated with two different α-amylases (*A. oryzae* or *B. subtilis*) are shown in Fig. 5. The diffusion rates for film incubated with α-amylase from *A. oryzae* showed an increase in diffusion rates with

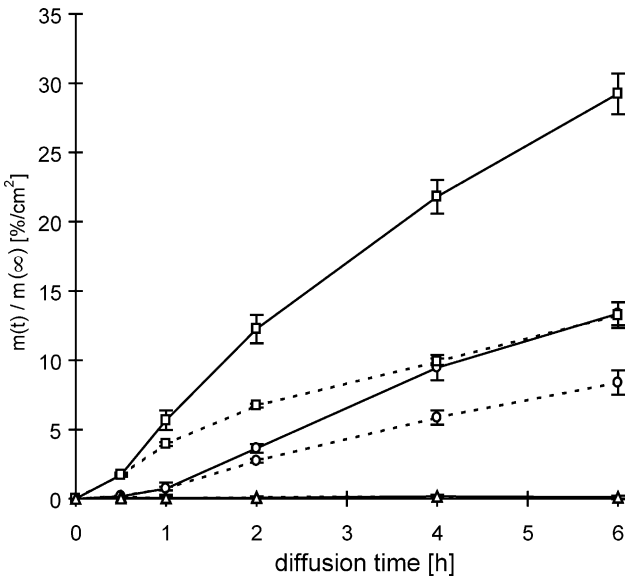

Fig. 4. Diffusion of 5-ASA through Eudragit® NE films containing 5% Tween® 80 in addition to (○) 50% β-CD [101.3 μm average film thickness], (□) 100% β-CD [80.7 μm] or (△) 100% talkum [49.6 μm]; dotted lines: in phosphate buffer pH 6.8; solid lines: films additionally containing taka diastase 10 U/ml; error bars are means ± range ($n = 3$).

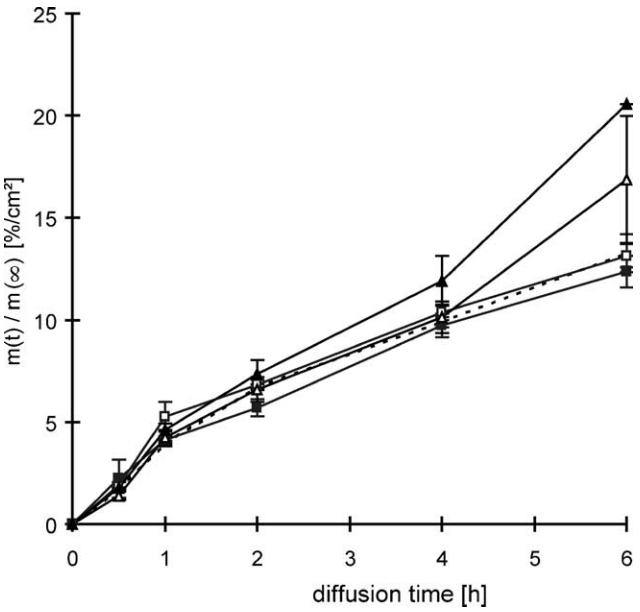

Fig. 5. Diffusion of 5-ASA through Eudragit® NE films containing 5% Tween® 80 and 100% β-CD [79.9 μm average film thickness]; dotted line: in phosphate buffer pH 6.8; solid lines: buffer containing one of the following enzymes: (□) α-amylase (*Bacillus subtilis*) 10 U/ml, (■) α-amylase (*Bacillus subtilis*) 40 U/ml, (△) α-amylase (*Aspergillus oryzae*) 10 U/ml, (▲) α-amylase (*Aspergillus oryzae*) 40 U/ml; error bars are means ± range ($n = 3$).

increasing enzyme activities, whereas the diffusion rates of films incubated with α-amylase from *B. subtilis* remained unchanged. Eudragit® NE is characterized by higher basic permeability properties as compared to Eudragit® RS, which might be the reason for better enzymatic accessibility of β-CD within the polymer structure and in turn the increased degradation of β-CD.

3.3. Influence of bisoprolol release by bacteria isolated from the pig colon

Bacteria isolated from the pig colon were monitored for their growth characteristics in organic nutrient medium which additionally contained 2.5 or 5 mg/ml β-CD. From 10 isolated species of bacteria from the pig colon, *E. fergusonii*, *S. odorifera* and *P. mirabilis* were found to show the highest growth rates (data not shown) and were consequently selected in order to study their influence on the drug release properties of bisoprolol containing tablets. Both β-CD and talcum containing Eudragit® RS film tablets containing bisoprolol as a model drug were incubated for 8 h at 37 °C in nutrient medium containing one of the bacterial strains, which were pre-incubated for 24 h. In addition, the β-CD containing formulation was incubated with each of the described bacteria in the same nutrient medium, where β-CD 2.5 mg/ml has been added prior to bacterial pre-incubation. Fig. 6 shows bisoprolol release patterns of both β-CD and talcum containing film tablets after 8 h incubation in nutrient medium containing *E. fergusonii*,

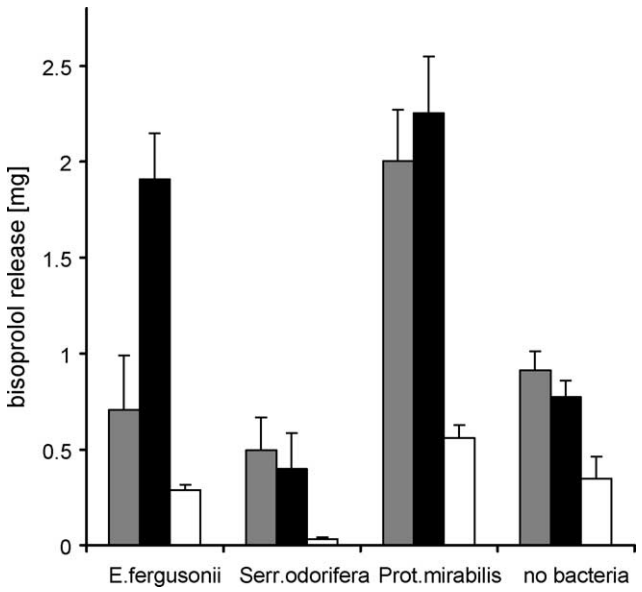

Fig. 6. Bisoprolol release [in mg] from tablets with Eudragit® RS films containing β-CD (grey bars) or talkum (white bars), after incubation for 8 h at 37 °C with bacteria from pig colon. Organic nutrient medium contained suspensions of *Escherichia fergusonii*, *Serratia odorifera* or *Proteus mirabilis*, or was free of bacteria. Additionally, bacteria were incubated for 24 h with β-CD 2.5 mg/ml before incubation with β-CD containing film tablets (black bars). Error bars are means ± range ($n = 3$).

S. odorifera or *P. mirabilis*, as well as in bacteria-free medium. Incubation in medium containing *E. fergusonii* or *S. odorifera* did not increase the release of bisoprolol, whereas upon incubation in medium containing *P. mirabilis* the release of the model drug was more than doubled. It was also shown that 2.5 mg/ml β-CD added to the nutrient medium prior to the incubation with *E. fergusonii* or *P. mirabilis* resulted in an increased bisoprolol release of β-CD. This effect was not observed for tablets containing talkum. It is thought that *P. mirabilis* partially degrades β-CD within the coating material, resulting in increased release of the model drug. *P. mirabilis* is described as very versatile in its ability to use various sources of carbon and energy for cell growth [35]. *E. fergusonii* increases the release of bisoprolol only after pre-incubation for 24 h in β-CD containing medium. This is explained by a lower grade of versatility compared to *P. mirabilis*, and where an adaption time is needed to use β-CD as a carbon and energy source. Once adapted to β-CD during the pre-incubation process, *E. fergusonii* is also capable of producing or activating β-CD degrading enzymes resulting in increased bisoprolol release. The higher grade of versatility of *P. mirabilis* is also obvious by the insignificant activation after pre-incubation. Both bacteria are of an aerobic nature and consequently are easy to cultivate and could be used as test strains for colon-selective in vitro assays. The ratio of aerobic to anaerobic bacteria in the colon is small. However, this fact does not automatically imply that degradation capacities of aerobic bacteria in the colon are low. The easy culturing property of these bacteria renders them ideal candidates to monitor degradation characteristics in a colonic environment.

4. Conclusions

A simple in vitro assay was developed to estimate the cleavage of β-CD incorporated into Eudragit® RS or NE films for colon targeting. Among several α-glycolytic enzymes, taka diastase was found to be the most powerful in evaluating both quality and integrity of these coating materials. For Eudragit® RS films with β-CD and CMC-CL sodium or PVP 25 it was shown that incubation with taka diastase increased the drug diffusion rate. Both CMC-CL sodium and PVP 25 induce swelling of the polymer and make it more accessible for the taka diastase to degrade the incorporated β-CD. In addition, CMC-CL sodium is also probably cleaved by taka diastase. In Eudragit® NE films containing β-CD the permeability by taka diastase or α-amylase (*A. oryzae*) was increased. This polymer is more permeable than Eudragit® RS and therefore no additional CMC-CL sodium or PVP 25 was needed. Several aerobic bacteria isolated from the pig colon were shown to increase drug release through film tablet formulations consisting of Eudragit® RS and β-CD. Both *P. mirabilis* and *E. fergusonii* were found to increase the release of bisoprolol, the latter only after pre-incubation in β-CD containing nutrient medium. In summary, our studies suggest some suitable in vitro assays of colonic degradation by glycolytic enzymes of materials containing β-CD for site-specific drug delivery. These assays are easily to perform and ideal for monitoring at an early stage of development of the application form. For the assays presented in this paper it was not necessary to mimic the conditions of the colon (anaerobic, redox potential), because only the principal degradation of β-CD should be proved. But these conditions will be taken in consideration in further research.

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