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Evaluation of the inhibition effect of thiolated poly(acrylates) on vaginal membrane bound aminopeptidase N and release of the model drug LH-RH

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Abstract

The purpose of this study was to evaluate the inhibitory effect of thiolated carbopol 974P (carb-cys) on the enzymatic activity of vaginal aminopeptidase N in-vitro. Mediated by a carbodiimide, L-cysteine was covalently linked to carbopol 974P. Depending on the weight ratio of polymer to cysteine during the coupling reaction, resulting conjugates displayed 31.3–54.4 μmol thiol groups per g polymer. The inhibitory effect of carb-cys conjugates was evaluated towards isolated aminopeptidase N and aminopeptidase-N-like activity of excised vaginal mucosa covered with native mucus, respectively. Enzymatic activity was assayed spectrophotometrically using L-leucine-*p*-nitroanilide (L-leu-pNA) as a synthetic substrate. Carb-cys thereby showed a significantly higher inhibitory effect than unmodified polymer towards both isolated enzyme and vaginal mucosa. Moreover, enzyme inhibition was strongly dependent on the amount of thiol groups being immobilised. The more thiol groups available the higher was the inhibitory effect. Due to its additional high cohesive properties and the possibility of a sustained drug release, which could be shown for the model drug LH-RH, carb-cys appears interesting for the development of vaginal peptide drug-delivery systems.

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Introduction

Over the last twenty years, attention has focused on vaginal delivery of therapeutic peptides and proteins as a favourable alternative route to their parenteral administration. Due to the physicochemical characteristics of polypeptides and their susceptibility to enzymatic degradation, however, vaginal absorption of these drugs is normally very low (Richardson & Illum 1992). Beside the absorption barrier, represented by the vaginal epithelium, the enzymatic barrier seems to be the major reason for the low bioavailability of peptides administered via the vaginal route. Therefore, potential new strategies to overcome enzymatic attack are urgently needed. One approach to minimise enzymatic activity might be the use of mucoadhesive drug-delivery systems. Due to their intimate contact with the mucosa, they should hinder proteases from attacking incorporated peptide drugs as they pass from the delivery system to the absorbing membrane. Moreover, it can be demonstrated that mucoadhesive excipients have the potential to inhibit some intestinal proteases (Lueßen et al 1995, 1996). Among the different mucoadhesive polymers, poly(acrylates), such as polycarbophil and carbopol 934P, have received particular attention. They are able to bind divalent cations, such as zinc and calcium, which often represent essential co-factors for proteases. Very recently, a

new class of mucoadhesive excipients has been introduced to pharmaceutical literature (Bernkop-Schnürch et al 1999). Thiolated polymers, so-called thiomers, have been reported to display several advantages over their unmodified basis polymers, including improved mucoadhesive (Bernkop-Schnürch & Steininger 2000) and permeation-enhancing properties (Clausen & Bernkop-Schnürch 2000). Thiolated polycarbophil (PCP-Cys) was even shown to display a significantly higher affinity for zinc ions than unmodified polycarbophil, resulting in higher inhibitory activity towards intestinal exopeptidases (Bernkop-Schnürch & Thaler 2000).

The enzyme pattern and enzyme activity has been found to be very similar at almost all mucosal membranes, including the vaginal tissue (Lee 1988). Moreover, especially aminopeptidase A and N activity is known to be present in various mucosal homogenates of several species (Stratford & Lee 1986). Focusing on vaginal delivery, aminopeptidases are believed to play a major role in the degradation of peptides and proteins. As aminopeptidase N is generally regarded as the most abundant metallo-peptidase in mucosal tissues, the aim of this study was to inhibit aminopeptidase-N-like activity of vaginal tissue.

Hence, a novel thiomer based on carbopol 974P (carb) and cysteine (cys) was generated. Its inhibitory activity towards isolated aminopeptidase N, as well as the protective effect on native vaginal mucosa, was evaluated in-vitro. With regard to the development of a peptide drug-delivery system the disintegration behaviour of carb-cys and the dissolution profile of the model drug LH-RH (luteinising-hormone-releasing hormone) should also be investigated.

Materials and Methods

Materials

LH-RH (luteinising-hormone-releasing hormone) was from Sigma (St Louis, MO); polyacrylic acid (carb; Carbopol 974P, BF Goodrich, Becksville, Ohio) was neutralised with sodium hydroxide as described previously (Bernkop-Schnürch & Krajicek 1998). For the coupling reaction, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (Sigma, St Louis, MO) and L-cysteine hydrochloride monohydrate (Sigma-Aldrich, Steinheim, Germany) were used. Elcema G 250, a microcrystalline cellulose (Degussa, Germany), was used for fast disintegrating control tablets. Aminopeptidase N (E.C. 3.4.11.2) and the substrate L-leucine-*p*-nitroanilide (L-leu-pNA) were from Sigma (St Louis, MO). All other chemicals were of reagent grade.

Synthesis of the carbopol conjugates

First, 250 mg of neutralised carbopol (Nacarb) were hydrated in 62.5 mL of demineralised water. After complete hydration, the carboxylic acid moieties of the polymer were activated for 45 min with EDAC (600 mg; final concentration, 50 mM). Thereafter, L-cysteine hydrochloride monohydrate was added in amounts of 62.5 mg, 125 mg and 250 mg, respectively, to the reaction mixture. After adjusting the pH to 5 by adding 1 M NaOH, the reaction mixture was incubated for 3 h at room temperature under stirring. The resulting conjugates were isolated by dialysing at 10°C in the dark against 0.2 mM HCl at pH 3.0, two-times against the same medium but containing 1% NaCl and finally exhaustively against 1 mM HCl. The pH of the dialysed polymer–cysteine conjugates was then adjusted to pH 4.5 with 1 M NaOH and frozen polymer solutions were lyophilised. A polymer being prepared and isolated in exactly the same way as the carbopol–cysteine conjugates but omitting EDAC during the coupling reaction served as control for all studies. The polymer–cysteine conjugates (carb-cys) and controls were stored at 4°C until further use. An overview of the synthesis of the carbopol conjugates is given in Table 1.

Determination of the thiol group content

The amount of thiol groups on the polymer–cysteine conjugates was determined by iodometric microtitration (1.00 mM iodine; indicator: starch). In brief, 3.0 mg of each conjugate and control, respectively, were hydrated in 1.0 mL of demineralised water. Finally the thiol group content was analysed by microtitration after acidification with 1 M HCl to pH 3.0 (Kast & Bernkop-Schnürch 2001).

Metabolism by aminopeptidase N

The inhibitory activity of carb-cys and unmodified carbopol, respectively, towards the enzymatic activity of aminopeptidase N was evaluated against its synthetic substrate L-leu-pNA. Hydroxyethylcellulose was used as positive control polymer, as it displays neither carboxylic acid groups nor sulfhydryl groups, which are essential for the binding of bivalent cations. Each polymer (1 mg) was hydrated in 50 μL of distilled water and, after addition of 50 μL of acetic acid–sodium acetate (0.2 M) buffer pH 5.0, transferred to the wells of a microtitration plate. Aminopeptidase N (125 μg) was dissolved in 1 mL of 100 mM acetic acid–sodium acetate buffer pH 5.0 and 50 μL of this stock solution was added to each sample followed by an incubation period

Table 1 Protocols of the synthesis of carb-cys conjugates.

Polymer	pH[a]	Carbopol (mg/62.5 mL)	Cysteine (mg mL^{-1})	EDAC (mM)	Thiol-groups (μmol g^{-1})	Average thiol groups (μmol g^{-1})
Carb-cys A1	5	250	4	50	31.3	31.3
Carb-cys B1	5	250	2	50	33.8	
B2					37.9	42.8
B3					45.3	
B4					54.4	
Carb-cys C1	5	250	1	50	28.0	
C2					34.6	
C3					40.4	39.6
C4					43.7	
C5					51.1	
Control	5	250	1–4	—	0.0	

[a]pH during coupling reaction. Carb-cys, carbopol–cysteine conjugate; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; A–C, different batches for synthesis.

of 30 min at 37°C. Thereafter, 50 μL of the substrate medium (0.5 mg L-Leu-pNA per mL of 100 mM acetic acid–sodium acetate buffer pH 5.0) was added. The change in absorbance at 405 nm caused by the enzymatic reaction at room temperature was measured immediately and then every 30 min for 4 h with a microtitration plate reader (Anthos reader 2001, Anthos labtec instruments, Austria).

Preparation of the excised cow vagina

The vagina of a one-year-old cow obtained fresh from the slaughter was washed gently with demineralised water to remove luminal contents. The native mucus-layer was still present on the mucosal surface after preparation. The vaginal section was cut into approximately 10-cm lengths and stored in saline at −20°C until further use.

Substrate-metabolism on native cow vagina

A plastic cylinder with an internal surface area of 1.77 cm^2 was placed vertically on top of the mucosal side of thawed vaginal tissue and clamped, as shown previously (Bernkop-Schnürch et al 1997). Polymers, as listed in Table 2, were hydrated in 0.2 M acetic acid–sodium acetate buffer pH 5.0, containing 2.9% NaCl. Polymer solutions (I–VI, Table 2) were transferred to

Table 2 Polymer preparations for metabolism studies on vaginal mucosa.

Sample	Sodium carbopol	L-Cysteine	Hydroxyethylcellulose	Carb-cys		
				0.49%	0.66%	0.42%
I	10 mg mL^{-1}	2.8 mg mL^{-1}	—	—	—	—
II	10 mg mL^{-1}	—	—	—	—	—
III	—	—	10 mg mL^{-1}	—	—	—
IV	—	—	—	10 mg mL^{-1}	—	—
V	—	—	—	—	10 mg mL^{-1}	—
VI	—	—	—	—	—	20 mg mL^{-1}

Carb-cys, carbopol–cysteine conjugate.

the reaction cylinder. After 30 min of incubation at 37°C, 1 mL substrate solution (0.5 mg L-leu-pNA per mL 0.2 M acetic acid–sodium acetate buffer pH 5.0) was added to each preparation. Samples were incubated for 4 h at 37±0.5°C. At 30-min intervals, samples of 300 μL were withdrawn and replaced with an equal volume of substrate solution pre-equilibrated to temperature. Cervical mucus was removed by mixing withdrawn samples with 300 μL of water–ethanol (1 : 1). After centrifugation (20000 *g*; 4°C, 5 min), 250 μL of the supernatant was vigorously mixed with 50 μL of 50 mM $CaCl_2$. Again, samples were centrifuged and 200 μL supernatant was transferred to the wells of a microtitration plate and the absorbance was measured at 405 nm using a microtitration plate reader (Anthos reader 2001; Anthos labtec instruments, Austria).

Disintegration studies

The disintegration behaviour of matrix-tablets based on carb-cys conjugate displaying 37.9 μmol thiol groups/g polymer (Table 1) was evaluated in comparison with tablets based on the corresponding unmodified polymer and elcema, respectively. The tablets (30 mg; 5 mm i.d.) were compressed directly with an excenter tabletting machine (Hanseaten Type EI, Hamburg, Germany).

The stability of the test tablets was analysed in acetic acid–sodium acetate buffer pH 5.0 (0.2 M) at 37°C with the disintegration apparatus according to the European Pharmacopoeia at an oscillating frequency of 0.5 s^{-1}.

Release studies

Release studies with the model drug LH-RH were performed. For each tablet, 29.5 mg of the polymer–cysteine conjugate displaying 37.9 μmol thiol groups/g polymer (Table 1) were hydrated in 15 mL of demineralised water. After complete hydration of the polymer, 0.5 mg of LH-RH was added and resulting mixtures were homogenised and lyophilised. The 30 mg of polymer–drug lyophilisates were compressed (Hanseaten Type EI, Hamburg, Germany) to flat-faced discs. Control tablets containing LH-RH and unmodified carbopol were prepared in the same way. The in-vitro release rate from these drug-delivery systems was then analysed. The dosage forms were placed in beakers (Schott Duran 25 mL, Germany) containing 10 mL of release medium (0.2 M acetic acid–sodium acetate pH 5.0). The vessels were closed, placed on an oscillating water bath (GFL 1092; 100 rev min^{-1}) and incubated at 37±0.5°C. Sink conditions were maintained during the study. Samples (1 mL) were withdrawn hourly for 6 h. The medium was replaced with an equal volume of release medium pre-equilibrated to temperature. Released LH-RH was assayed by measuring the absorbance photometrically (Lambda 16; Perkin Elmer) at 280 nm. Concentrations were calculated by interpolation from a standard curve. The linearity interval established in the release medium was 6.25–400 μg mL^{-1} (r^2: 0.999971).

Data analysis

Results are expressed as the mean of 3 or 4 (release) experiments±s.d. in Figure 2 and a regression analysis was performed from the relationship of molar concentration of *p*-nitroaniline and time. Afterwards, the slopes of the three treatments were analysed statistically. For statistical data analysis for Figures 2, 3 and 4, a non-parametric Kruskal–Wallis was performed. For Figure 5, the non-parametric Mann–Whitney two-tailed *U*-test has been used. All tests took $P < 0.05$ as a minimal level of significance.

Results and Discussion

Synthesis of the polymer conjugates

The synthesis of the carb-cys conjugates was mediated by a carbodiimide reaction (Figure 1). Cysteine was covalently bound to carbopol (carb) by forming amide bonds between the primary amino group of the amino acid and the carboxylic acid moieties of the polymer. The molar ratio of polymer to cysteine varied (1 : 1, 2 : 1 and 4 : 1, respectively). Corresponding coupling rates are listed in Table 1. Results demonstrated no correlation between the polymer-to-cysteine ratio at the coupling reaction and the amount of covalently attached cysteine. Moreover, the highest amount of cysteine resulted in the lowest coupling rate. This observation is opposite to a previously performed study, in which cysteine had been linked to polycarbophil, another representative poly(acrylate) (Bernkop-Schnürch et al 1999). One possible explanation might be the differences in the three-dimensional structure of the two poly (acrylates), due to different cross-linkers. Polycarbophil is an acrylic-acid derivative cross-linked by divinylglycol, whereas carbopol 974P is cross-linked via allyl ethers of sucrose and pentaerythrol.

Thiol groups are known to form very stable intermediate products with EDAC (Aslam & Dent 1998). As

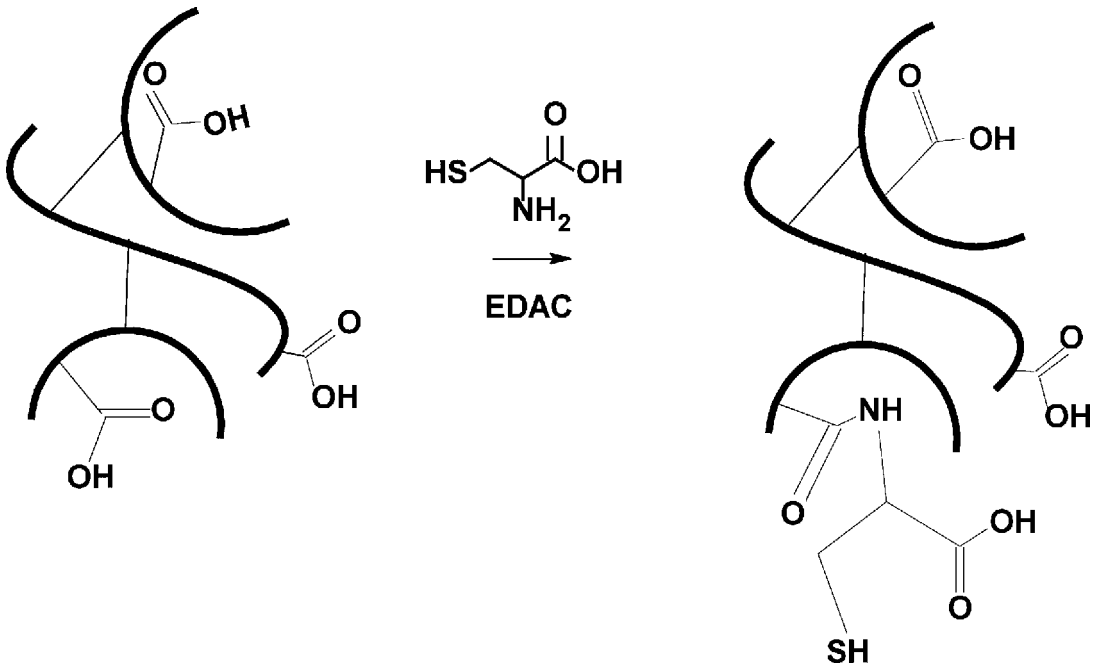

Figure 1 Synthesis of carb-cys conjugates. EDAC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

they can only, with difficulty, be attacked by a nucleophile such as a carboxylic acid group, they are no longer available for the coupling reaction. Especially, higher concentrations of thiol groups might disturb carbodiimide-mediated coupling reactions in this manner.

Polymer inhibition of aminopeptidase N

Figure 2 shows the inhibitory effect of carb-cys, unmodified carbopol and hydroxyethylcellulose towards isolated aminopeptidase N.

Enzymatic activity of aminopeptidase N is strongly dependent on the presence of zinc ions, representing an essential co-factor within the enzyme structure. Recently, it has been demonstrated that the binding affinity of polycarbophil towards zinc was significantly increased by the covalent attachment of cysteine on the polymer (Bernkop-Schnürch & Thaler 2000). Results of our study are in good accordance with these findings, as the inhibitory effect of carb-cys towards aminopeptidase N was approximately two-fold higher than that of corresponding unmodified polymer. Hydroxyethylcellulose, on the other hand, had no effect as, in general, binding properties for metal ions are negligible for non-ionic cellulose ethers (Bernkop-Schnürch & Krajicek 1998; Valenta et al 1998). The occurring enzymes are able to attack the hydrated polymers more freely than compacted polymers (tablets). Furthermore, it has been shown that tablets consisting of polycarbophil–cysteine conjugates are able to protect insulin from enzymatic degradation (Marschütz et al 2000).

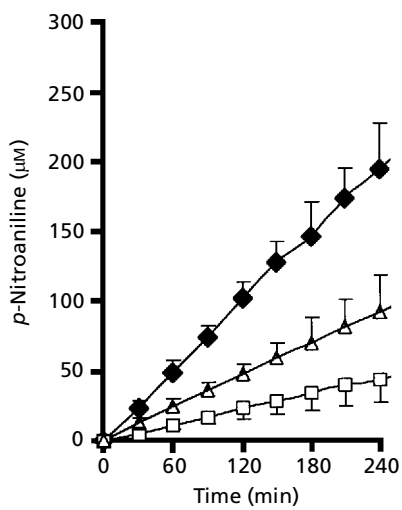

Figure 2 Hydrolysis of L-leucine-*p*-nitroanilide to L-leucine and *p*-nitroaniline by aminopeptidase N in the presence of hydroxyethylcellulose (◆), unmodified carbopol (△, control) or carb-cys conjugate with 43.7 μmol thiol groups per g polymer (□). Indicated values are the means±s.d. of 3 experiments.

Polymer inhibition on native vaginal mucosa

The inhibitory effect of the polymers was also tested on native cow vaginal mucosa. Microscopic investigations of the mucosa showed that a mucus gel layer still covered the tissue after it had been rinsed with buffer solution. Because the mucus layer was present on the mucosa this experimental set-up came much closer to in-vivo conditions.

A comparison of the inhibitory effect of hydroxyethylcellulose, carbopol, carbopol mixed with unbound cysteine, and carb-cys conjugate was performed. Only the enzyme inhibition of carb-cys conjugate was significantly different to the other polymer samples. Hydroxyethylcellulose, neutralised carbopol and the mixture of carbopol and cysteine were not significantly different. Because of the higher mobility of cysteine molecules it was expected that the carbopol–free cysteine mixture should exhibit a higher inhibitory activity than the immobilised cysteine on the polymer. This mixture, however, showed an inhibitory activity in the range of carbopol, whereas the thiolated polymer carb-cys was significantly more effective in protecting L-leu-pNA from enzymatic hydrolysis. Results confirmed the studies with isolated aminopeptidase N described above. Nevertheless, the inhibitory effects of both polymers carbopol and carb-cys appeared to be less pronounced on vaginal mucosa than towards isolated aminopeptidase N. This might be because of the presence of the additional mucus layer separating the polymer from the enzyme.

Furthermore, it could be shown that the inhibitory effect of thiolated carbopol is strongly dependent on the amount of thiol groups available on the polymer. Figure 3 clearly indicates that the more thiol moieties are available, the higher is the inhibitory effect. Dodda Kashi & Lee (1986) found that the enzymatic activity of nasal, buccal, rectal and vaginal mucosa did not vary substantially from that of ileal mucosa. It was suggested that aminopeptidases were responsible for the degradation of peptides and that the activity of these enzymes was similar in each mucosal homogenate.

Disintegration studies

Disintegration studies revealed a much higher stability of tablets based on thiolated carbopol compared with unmodified polymer (Figure 4). The disintegration time of tablets containing unmodified carbopol was thereby in good correlation with earlier investigations, demonstrating almost the same disintegration behaviour for tablets containing neutralised freeze-dried polycarbophil, which represents a very similar poly(acrylic acid) derivative, as already mentioned above (Bernkop-Schnürch et al 2000). The higher stability of tablets based on carb-cys can be explained by a cross-linking process due to the formation of disulfide bonds which

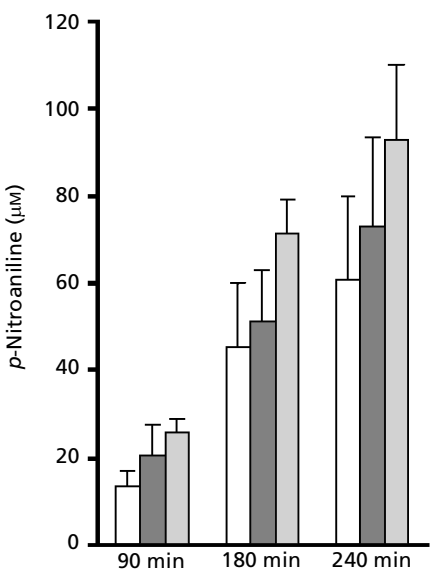

Figure 3 Influence on the formation of *p*-nitroaniline from L-leu-*p*-nitroanilide by intact vaginal cow mucosa of different carb-cys polymers in control buffer. Blank bars, 2% carb-cys with 34.6 μmol thiol groups per g polymer; dark grey bars, 1% carb-cys with 54.4 μmol thiol groups per g polymer; light grey bars, 1% carb-cys with 40.4 μmol thiol groups per g polymer.

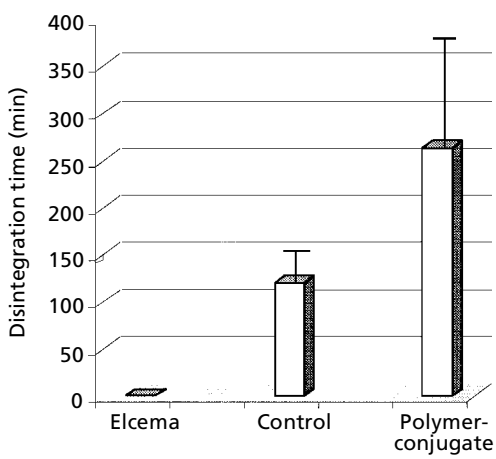

Figure 4 Comparison of the disintegration behaviour of matrix tablets (30 mg) containing carb-cys with 37.9 μmol thiol groups per g polymer, unmodified carbopol (control) or microcrystalline cellulose (elcema). Indicated values are the means$\pm$s.d. of 3 experiments.

takes place during the swelling process within the thiolated polymer. This simple oxidation process provides an improved cohesiveness of the matrix system. This improved stability of matrix tablets based on thiolated polymers seems to be of high practical relevance offering various advantages over well-established polymeric carrier systems, as already described for thiolated polycarbophil tablets (Bernkop-Schnürch et al 2000) and thiolated-polymer films (Valenta et al 2001a). Focusing on vaginal (poly)peptide drug delivery, the high stability of the carb-cys carrier matrix should strongly reduce pre-systemic metabolism in the vagina. In addition, by adsorbing, swelling and capillary effects, mucoadhesive polymers are supposed to take water from the underlying mucosal tissue leading to a strong adhesion (Duchene & Ponchel 1992). Disulfide exchange reactions between the sulfur-containing glycoproteins of the vaginal mucus layer and the thiolated polymer are feasible, thereby leading to a prolonged residence time of the delivery system in the vagina. Hence, improved uptake of embedded (poly)peptide drugs should be possible.

Release studies with LH-RH

Release rates of LH-RH from tablets based on the control polymer and carb-cys conjugate, respectively, are depicted in Figure 5. The retardation in drug release was much more pronounced in the tablets consisting of thiolated polymer. Recently performed release studies of progesterone from carb-cys tablets confirm the results with LH-RH (Valenta et al 2001b). One reason for the slow release may be the possibility of disulfide bond formation during the swelling within the tablet so that consequently a better cohesiveness is guaranteed. Statistical analysis shows that during the first 3 h there is no significant difference in release, whereas the release rates are significantly different from the fourth to the sixth hour. Bioadhesive polymers are hydrophilic macromolecules with substituents capable of forming hydrogen bonds, in particular carboxyl groups, and in our case additional thiol groups are available which stabilise the polymeric network by forming disulfide bonds. The experimental set-up was not very close to in-vivo conditions and should be changed for further investigations.

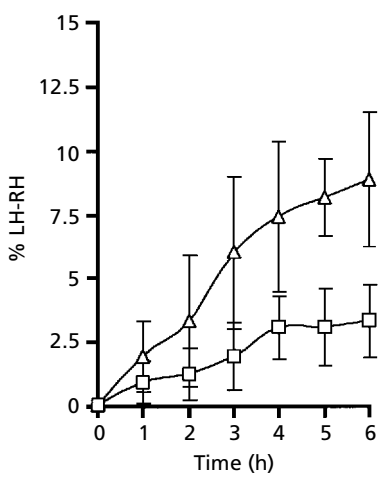

Figure 5 Release profiles of LH-RH from tablets based on unmodified carbopol (△, control) and carb-cys (□, 43.7 μmol thiol groups per g polymer). Studies were carried out in 0.2 M acetic acid–sodium acetate buffer pH 5.0; (n = 4).

Conclusions

Results of this study proved carbopol–cysteine conjugates to be interesting as a tool for the vaginal administration of (poly)peptide drugs. By covalent attachment of cysteine to carbopol 974P, an improved inhibitory activity towards vaginal membrane-bound aminopeptidase N could be achieved. Moreover, an inhibitory effect towards other metalloproteases present on vaginal tissue seems to be feasible. Because of these inhibitory features, high cohesiveness of tablets and the possibility of a controlled drug release these carb-cys conjugates are interesting matrices for vaginal drug-delivery systems.

References

Aslam, M., Dent, A. (eds) (1998) *Bioconjugation*. London, McMillan Reference Ltd, pp. 1–50

Bernkop-Schnürch, A., Krajicek, M. (1998) Mucoadhesive polymers for peroral peptide delivery: synthesis and evaluation of chitosan-EDTA conjugates. *J. Control. Release*. **52**: 1–16

Bernkop-Schnürch, A., Steininger, S. (2000) Synthesis and characterisation of mucoadhesive thiolated polymers. *Int. J. Pharmaceutics* **194**: 239–247

Bernkop-Schnürch, A., Thaler, S. C. (2000) Polycarbophil–cysteine conjugates as platforms for oral polypeptide delivery systems. *J. Pharm. Sci*. **89**: 901–909

Bernkop-Schnürch, A., Paikl, C., Valenta, C. (1997) Novel bioadhesive chitosan–EDTA conjugate protects leucine enkephalin from degradation by aminopeptidase N. *Pharm. Res*. **14**: 917–922

Bernkop-Schnürch, A., Schwarz, V., Steininger, S. (1999) Polymers with thiol groups: a new generation of mucoadhesive polymers? *Pharm. Res*. **16**: 876–881

Bernkop-Schnürch, A., Scholler, S., Biebel, R. (2000) Development of controlled drug release systems based on thiolated polymers. *J. Control. Release*. **66**: 39–48

Clausen, A. E., Bernkop-Schnürch, A. (2000) In vitro evaluation of the permeation-enhancing effect of thiolated polycarbophil. *J. Pharm. Sci.* **89**: 1253–1261

Dodda Kashi, S., Lee, V. H. L. (1986) Enkephalin hydrolysis in homogenates of various absorptive mucosae in the albino rabbit: similarities in rates and involvement of aminopeptidases. *Life Sci.* **38**: 2019–2028

Duchene, D., Ponchel, P. (1992) Principle and investigation of the bioadhesion mechanism of solid dosage forms. *Biomaterials* **10**: 709–714

Kast, C. E., Bernkop-Schnürch, A. (2001) Thiolated polymers–thiomers: development and in vitro evaluation of chitosan–thioglycolic acid conjugates. *Biomaterials* **22**: 2345–2353

Lee, V. H. (1988) Enzymatic barriers to peptide and protein absorption, *Crit. Rev. Ther. Drug Carrier Syst.* **5**: 69–98

Lueßen, H. L., Verhoef, J., Borchard, G., Lehr, C.-M., de Boer, A. G., Junginger, H. E. (1995) Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic enzyme trypsin. *Pharm. Res.* **12**: 1293–1298

Lueßen, H. L., de Leeuw, B. J., Perard, D., Lehr, C.-M., de Boer, A. G., Verhoef, J. C., Junginger H. E. (1996) Influence of mucoadhesive excipients on the proteolytic activity of intestinal enzymes. *Eur. J. Pharm. Sci.* **4**: 117–128

Marschütz, M. K., Caliceti, P., Bernkop-Schnürch, A. (2000) Design and in vivo evaluation of an oral delivery system for insulin. *Pharm. Res.* **17**: 1468–1474

Richardson, J. L., Illum, L. (1992) The vaginal route of peptide and protein drug delivery. *Adv. Drug Delivery Rev.* **8**: 341–366

Stratford, R. E., Lee, V. H. (1986) Aminopeptidase activity in homogenates of various absorptive mucosae in the albino rabbit: implications for peptide delivery. *Int. J. Pharmaceutics* **30**: 73–82

Valenta, C., Christen, B., Bernkop-Schnürch, A. (1998) Chitosan–EDTA conjugate: a novel polymer for topical used gels. *J. Pharm. Pharmacol.* **50**: 1–8

Valenta, C., Walzer, A., Clausen, A. E., Bernkop-Schnürch, A. (2001a) Thiolated polymers: development and evaluation of transdermal delivery systems for progesterone. *Pharm. Res.* **18**: 211–216

Valenta, C., Kast, C. E., Harich, I., Bernkop-Schnürch, A. (2001b) Development and in vitro evaluation of a mucoadhesive vaginal delivery system for progesterone. *J. Control. Release* **77**: 323–332