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Development of buccal drug delivery systems based on a thiolated polymer

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Abstract

The purpose of the present study was to investigate the benefit of thiolated polymers (thiomers) for the development of buccal drug delivery systems. L-Cysteine was thereby covalently attached to polycarbophil (PCP) mediated by a carbodiimide. The resulting conjugate displayed 140.5 ± 8.4 μM thiol groups per gram polymer. Disintegration studies were carried out with tablets based on unmodified polymer and conjugated polymer, respectively. Due to the formation of disulfide bonds within the thiolated polymer, the stability of matrix-tablets based on this polymer was strongly improved. Additionally tensile studies were carried out, which were in good correlation with further results obtained by mucoadhesion studies, using the rotating cylinder method. These results showed that tablets based on thiolated PCP remained attached on freshly excised porcine mucosa 1.8 times longer than the corresponding control. Moreover, the enzyme inhibitory properties of polymers were evaluated as well. Thiolated PCP increased the stability of the synthetic substrate for aminopeptidase *N*-leu-*p*-nitroanilide (*N*-leu-*p*NA) and the model drug leucin-enkephalin (leu-enkephalin) against enzymatic degradation on buccal mucosa. Due to the use of thiolated polymers also a controlled drug release for leu-enkephalin was guaranteed over a time period for more than 24 h. Results of the present studies suggest that thiolated polymers represent a very useful tool for buccal delivery of peptide drugs.

Keywords: Buccal drug delivery; Mucoadhesion; Enzyme inhibition; Leu-enkephalin; Leu-*p*-nitroanilide

1. Introduction

Recently, considerable attention has been focused on the development of alternative drug delivery systems for proteins and peptide drugs. As the peroral administration has disadvantages such as the hepatic first pass metabolism and enzymatic degradation within the gastrointestinal tract, proteins and peptides are usually not suitable for peroral administration and are mostly delivered by parenteral administration (Harris and Robinson, 1990). Nasal, ocular, vaginal, rectal and buccal mucosal membranes have been evaluated as potential alternative routes for peptide absorption. Buccal administration of drugs provides a convenient route of administration for both systemic and local drug actions (Kurosaki and Kimura, 2000). A limitation of buccal delivery is the lack of adhesion of the delivery system at the site of absorption. It could be shown that mucoadhesive polymers, modified by the introduction of

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thiol groups provide much higher adhesive properties than polymers generally considered to be mucoadhesive (Bernkop-Schnürch et al., 1999). Another obstacle for delivering peptides across the buccal mucosa is the proteolytic hydrolysis of peptidic molecules. However, there are few proteolytic enzymes as compared to oral, nasal, vaginal and rectal administration (Lee and Yamamoto, 1990). Indeed, the buccal mucosa seems to be deficient in proteinases such as pepsin, trypsin and chymotrypsin present in gastric and intestinal secretion which are known to contribute to peptide hydrolysis (Veuillez et al., 2001). Aminopeptidases appear to be the only peptidases active on the buccal mucosa, therefore representing a major metabolic barrier to the buccal delivery of peptide drugs (Stratford and Lee, 1986). The absence of endopeptidase and carboxypeptidase activities will be advantageous for the buccal delivery of peptides which are susceptible to these activities. Recently, it could be observed that thiolation of polycarbophil (PCP) enhances the inhibitory potency of PCP towards aminopeptidase *N* and membrane bound peptidases involved in the digestion of leu-*p*-nitroanilide (leu-*p*NA) and leucin-enkephalin (leu-enkephalin). Aminopeptidases appeared to play a major role in the digestion of enkephalins, and to a smaller extend by dipeptidyl-carboxypeptidase and dipeptidyl-peptidase also known as enkephalinase A and B, respectively. These enzymes involved in the digestion of enkephalins are all metallopeptidases and therefore, they can be inhibited potentially by the chelator activity of thiolated polymers such as PCP-cysteine conjugates (Walker et al., 2001).

In our investigation we chose a model peptide, leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), which is a naturally occurring pentapeptide that has been shown to have pain modulating properties (Bilsky et al., 2000).

The aim of the study was to develop a buccal mucoadhesive drug delivery system based on thiolated PCP. Mucoadhesive properties, cohesiveness, enzyme inhibiting properties, and release profiles of a buccal delivery system consisting of PCP-cysteine have been evaluated. A combination of different properties within a unique system, for instance mucoadhesive and enzyme inhibiting properties, could be obtained by the use of thiolated PCP in a buccal drug delivery system.

2. Materials and methods

2.1. Synthesis of PCP-cysteine conjugates

The PCP-cysteine conjugate was synthesized according to a method described previously by our research group (Bernkop-Schnürch and Steininger, 2000). In brief, the covalent attachment of cysteine to neutralized PCP was achieved by the formation of amide bonds between the primary amino group of cysteine and the carboxylic acid group of the polymer. PCP ($M_w = 3.5 \times 10^9$ g/mol; Noveon AA1, BF Goodrich, Brecksville, OH) was hydrated in 1 l of demineralized water. To activate the carboxylic acid moieties of the polymer for conjugation 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO) was added to the hydrated polymer in a final concentration of 50 mM. After 45 min incubation under stirring at room temperature the pH of the reaction mixture was adjusted to 5 by the addition of 1 M HCl and L-cysteine (Sigma) was added to give a weight-ratio of 2:1 (polymer:cysteine). The pH of the reaction mixture was maintained at 5 over the 3 h incubation period, under constant mixing at room temperature. The resulting polymer-cysteine conjugate was dialyzed in the dark at 10 °C to avoid oxidation of the cysteine moieties. Polymers were dialyzed once against 1 mM HCl, two times against the same medium but also containing 1% NaCl and then exhaustively against 0.5 mM HCl. Polymer prepared and isolated in the same way as the polymer-cysteine conjugate, but omitting EDAC during the coupling reaction served as control. Samples were lyophilized by drying frozen aqueous polymer solutions at −30° at 0.01 mbar (Christ Beta 1–8 K; Osterode am Harz, Germany). The polymer-cysteine conjugate and control were stored at 4 °C until further use.

2.2. Determination of the thiol group content

The degree of modification was determined by quantifying the amount of thiol groups on the PCP-cysteine conjugates and controls. Initially, 3.00 mg each of the conjugate and the control were hydrated in 1 ml of demineralized water. The pH value was then adjusted with 1 M HCl to 2–3 and 500 μl of aqueous starch solution (1%) was added.

The samples were titrated with an aqueous iodine solution (1 mM) until a permanent light-blue colour was maintained (Bernkop-Schnürch et al., 2000).

2.3. Disintegration studies

The disintegration behavior of matrix-tablets based on the lyophilized PCP-cysteine conjugate was evaluated in comparison with tablets based on the unmodified polymer.

A total of 160 mg each of the lyophilized polymer-cysteine and control were compressed (Korsch Type EK O, Berlin, Germany) into flat-faced discs (8 mm diameter, thickness of 2 mm). The compaction pressure was kept constant during the preparation of all discs. The hardness of the tablets was determined (Pharma Test PTB 311, Hainburg, Germany). The stability of polymer tablets in 50 mM phosphate buffer pH 6.8 at 37 °C was analyzed with a disintegration test apparatus according to the European Pharmacopeia with an oscillating frequency of $0.5\,s^{-1}$.

2.4. Tensile studies

First, 160 mg matrix-tablets as described before were attached to the porcine intestinal mucosa in the following manner. The tablet was attached to a stainless steel flat disc (8 mm in diameter, 0.3 g of weight in the system), which was hung by a nylon thread (15 cm) from a laboratory stand. The porcine mucosa was fixed on a glass mount using a cyanoacrylate adhesive. The tissue mount and fixed mucosa were placed in a beaker and 100 mM phosphate-buffer saline pH 6.0 was added sufficiently to immerse mount and tissue. The beaker was placed on a balance, then carefully raised by a mobile platform until the mucosa came in contact with the tablet. The contact was determined when the nylon thread holding the tablet became bent. After 60 min incubation at 25 °C, the mucosa was pulled down from the tablet at a rate of 0.1 mm/s. Data points were collected every second by a personal computer (WINWEDGE software; TAL Technologies Inc., Philadelphia, PA) linked to the balance. Data were transferred to EXCEL 97 (Microsoft, USA) and the force/distance curve, i.e. the total work of adhesion (TWA) and the maximum detachment force (MDF) was calculated. The experimental set-up for tensile studies has been illustrated elsewhere (Bernkop-Schnürch, 2000).

2.5. In vitro mucoadhesion studies

A previously described method was used to evaluate the binding period of PCP-cysteine tablets on porcine mucosa (Kast and Bernkop-Schnürch, 2001). Tablets as described before were thereby attached to freshly excised intestinal porcine mucosa, which has been fixed on a stainless steel cylinder (diameter: 4.4 cm; height: 5.1 cm; apparatus 4-cylinder, USP XXII). Thereafter, the cylinder was placed in a dissolution apparatus according to the USP containing 100 mM phosphate buffer pH 6.0 at 37 ± 0.5 °C. The fully immersed cylinder was agitated at 125 rpm. The detachment of tablets was observed.

2.6. Degradation of leu-enkephalin and leu-p-nitroanilide on intact porcine buccal mucosa

A plastic cylinder with an internal surface area of $1.77\,cm^2$ was placed vertically on top of the mucosal side of thawed buccal tissue and clamped, as shown by Bernkop-Schnürch et al. (1997). PCP-cysteine conjugate and unmodified PCP were hydrated in 50 mM sodium phosphate buffer pH 6.8, containing 2% NaCl, in a final concentration of 0.25% (m/v). A total of 1 ml of buffer either with or without polymer equilibrated at 37 °C was added into the reaction cylinder. After 30 min incubation 1 ml either of the leu-*p*NA (2 mM) or the leu-enkephalin (1 mM) prepared in control buffer at 37 °C was added to the incubate and mixed. At various times after the start of the reaction samples of 300 μl were taken. Leu-*p*NA samples were placed on ice, centrifuged at 20,000 × *g* at 4 °C for 5 min and a 200 μl sample of the supernatant was transferred to a microtitre plate and the absorbance was measured at 405 nm using an Anthos reader 2001 (Anthos Labtec Instruments, Austria). Leu-enkephalin samples were added to 20 μl of 20% (v/v) triflouroacetic acid (TFA) to stop the reaction. The resulting mixture was centrifuged (20,000 × *g* for 5 min) and the supernatants were analyzed by HPLC as described in the following section.

2.7. HPLC analysis of leucine-enkephalin

Analysis of leu-enkephalin reaction samples by reversed phase HPLC was conducted using a Perkin–Elmer series 200 LC pump (Norwalk, CT), Perkin–Elmer 200 series auto sampler with a 20 μl injection loop and a diode array detector (Perkin–Elmer 235C). Remaining traces of polymer were held back on a precolumn (Nucleosil 100-5C18, 40 mm × 4 mm). Leu-enkephalin and its degradation products were separated on a C_{18}-column (Nucleosil 100-5C18, 250 mm × 4 mm) at 40 °C. Gradient elution was performed as follows: flow rate 1 ml/min; 0–22 min; linear gradient from 90% A/10% B to 10% A/90% B (eluent A: 0.1% trifluoroacetic acid in water; eluent B: 90% acetonitrilie, 0.1% trifluoroacetic acid in water). Peptide was detected by absorbance at 220 nm. Degradation of leu-enkephalin was evaluated by following the disappearance of leu-enkephalin from the reaction mixture by HPLC. Peak areas were directly proportional to the mass of standards injected and peptide hydrolysis was quantified from integrated peak areas and molar absorbance values calculated from standards for leu-enkephalin.

2.8. Release studies

In order to homogenize the polymer and the polymer-conjugate, respectively, with the model drug, the polymers were hydrated in a sufficient amount of demineralized water. Leu-enkephalin was added and the mixtures were frozen and lyophilized. Tablets containing 1 mg of leu-enkephalin as model drug and 29 mg of the PCP-cysteine and of the unmodified polymer, respectively, were compressed to tablets as described before. The in vitro release rate from these drug delivery systems was then analyzed. The dosage forms were placed in 25-ml beakers (Schott, Duran 25 ml, G) containing 10 ml of release medium (50 mM phosphate buffer, pH 6.8). The vessels were closed, placed on an oscillating water bath (GFL 1092; 100 revolution/min) and incubated at 37 ± 0.5 °C; sink conditions were maintained throughout all studies. Aliquots of 150 μl were withdrawn in 1-h intervals and replaced with an equal volume of release medium pre-equilibrated at 37 °C. Released leu-enkephalin was assayed by HPLC as described before.

2.9. Statistical data analysis

Statistical data analysis was performed using the *t*-test with $P < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Synthesis of PCP-cysteine conjugates

The purified PCP-cysteine conjugate displayed 140.5 ± 8.4 μM (mean ± S.D. of three experiments) sulfhydryl groups per gram polymer. Omitting the coupling reagent EDAC during the reaction led to a polymer exhibiting a negligible amount of sulfhydryl groups. The stability, swelling behaviour, and mucoadhesive properties of various PCP-cysteine conjugates have already been evaluated (Bernkop-Schnürch et al., 1999; Bernkop-Schnürch et al., 2000). The features of the polymer-cysteine conjugate described here were in good agreement with them. The polymer was easy compressable and no other excipient was necessary. However, the situation might be different in large scale production.

3.2. Disintegration studies

The hardness of the tablets was 203 ± 19 N (n = 10). Results of the disintegration studies are shown in Fig. 1. The disintegration studies revealed a much higher stability of tablets based on thiolated polymers in comparison to the tablets based on unmodified polymer. The matrix-tablets containing PCP-cysteine conjugate were stable for more than 1 day. Moreover, in contrast to the unmodified PCP, no erosion could be observed for thiolated PCP tablets during this study. The disintegration time of tablets containing PCP was thereby in good correlation with earlier investigations, demonstrating almost the same disintegration behavior for tablets containing neutralized freeze dried carbomer which represents a very similar poly(acrylic acid) derivative (Bernkop-Schnürch et al., 2000). The high stability of tablets based on thiomers can be explained by the formation of disulfide bonds within the thiolated polymers, providing an improved cohesiveness of the matrix system. The prolonged stability of matrix-tablets based on thiomers

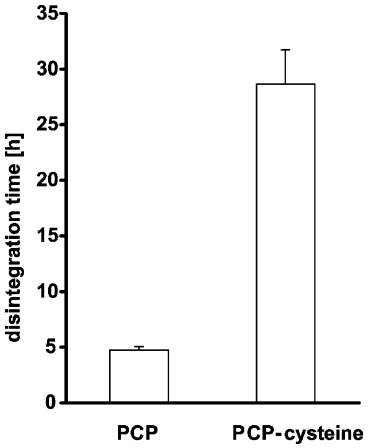

Fig. 1. Comparison of the disintegration behaviour of matrix-tablets containing either PCP-cysteine conjugate or unmodified polymer. Indicated values are means (±S.D.) of at least three experiments.

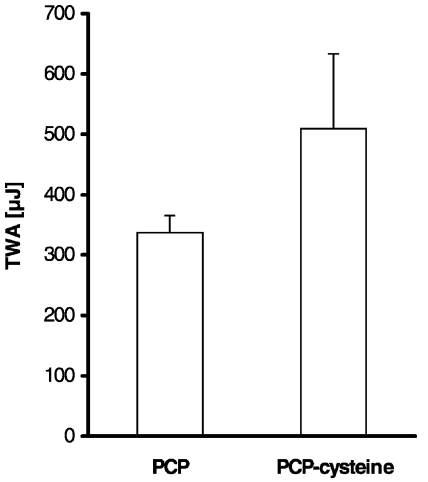

Fig. 2. Comparison of the mucoadhesive properties of PCP-cysteine conjugate and unmodified polymer, respectively. Represented values are means (±S.D.; $n = 3$–5) of the TWA determined in tensile studies.

seems to be of high practical relevance compared to well-established polymeric carrier systems. In case of buccal (poly)peptide drug delivery systems based on mucoadhesive polymers, for instance, a high stability of the carrier-matrix would reduce the disintegration and dissolution of the drug delivery system in the oral cavity (Rathbone et al., 1994). A rapid disintegration of the delivery system would lead to drug release to non-target sites. Thereby released drug will not only be distributed to less permeable regions of the oral cavity but also localized drug concentrations in the mouth can occur which may or may not be of a sufficiently high concentration for effective plasma concentrations. The cohesiveness of the buccal tablet has also a great influence on the mucoadhesion. High adhesive properties are worthless, if binding fails within the delivery system itself. However, in vivo the tablet consisting of PCP-cysteine will not adhere for 25 h, because the delivery system itself will erode by mechanical load much earlier. Therefore, the increased cohesion is substantial. In addition, due to a high stability of the carrier-matrix, a sustained drug release can be controlled even over a time period of several hours, which will be impossible if the delivery system disintegrates too early. Thiomers seem, therefore, to represent useful novel excipients in order to increase the stability of matrix-tablets in controlled buccal drug release.

3.3. Mucoadhesion studies

Tensile studies were carried out with the PCP-cysteine conjugate and the corresponding unmodified polymer. Porcine small intestinal mucosa was used, because a significant correlation coefficient value between in vitro TWA and in vivo mucoadhesion was obtained, when small intestine mucosa was employed as a model mucus membrane for buccal tissue (Sampath Kumar et al., 2001). Further advantages of the small intestine mucosa are the large number of samples that can be obtained from a single animal and that no preparation of the tissue is necessary, whereas the buccal tissue must be prepared carefully before use. Results of the adhesion studies are shown in Fig. 2. The TWA of the PCP-cysteine conjugate increased thereby 1.5-fold compared to the unmodified polymer. For all samples the MDF (data not shown) was in good correlation with the corresponding TWA.

In order to confirm the results of tensile studies by another mucoadhesion test system, mucoadhesion studies were also carried out with the dissolution apparatus according to the USP in combination with a standard steel cylinder and freshly excised porcine small intestinal mucosa. Thereby obtained results which are shown in Fig. 3 were in good accordance with the results of tensile studies described before. In particular,

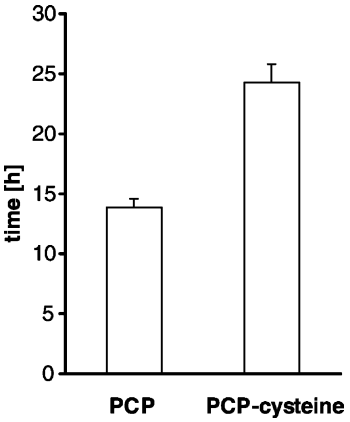

Fig. 3. Comparison of the adhesion time of PCP-cysteine and unmodified polymer. The indicated time of adhesion represents the mean (±S.D.) of at least three experiments.

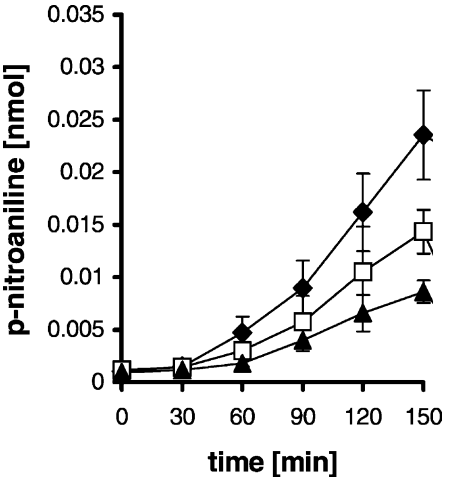

Fig. 4. Time-course of the formation of *p*-nitroaniline from leu-*p*NA by intact buccal mucosa during incubation without polymer (◆); with 0.25% (w/v) PCP (□); with 0.25% (w/v) PCP-cysteine conjugate (▲). Each point represents the mean (±S.D.; $n = 3$).

tablets based on the PCP-cysteine conjugate remained even after 25 h of incubation very stable attached to the mucosa. The corresponding control detached the mucosa after 13.8 h. Within this study, we could confirm published results which suggested the improvement of mucoadhesive properties of polymers due to the immobilization of thiol groups (Bernkop-Schnürch and Steininger, 2000). In contrast to former studies the tablets had a contact area of 50.27 mm^2 instead of 19.64 mm^2. The expectation that the buccal tablets, with the major area of attachment, would show higher TWA values and a longer mucoadhesion time could be confirmed.

In the oral cavity the interaction of salivary mucus glycoproteins with oral epithelium involves a specific cell surface receptor (Slomiany et al., 1993). The salivary mucins are forming a protective coating known as oral mucosal coat. The mucoadhesive properties of the polymer can be explained by the interaction with the glycoproteins of the mucus mainly based on non-covalent bonds such as ionic interactions, hydrogen bonds and van der Waal's forces (Peppas and Mikos, 1990). The mucoadhesive improvement of the thiolated PCP can be explained by the formation of additional covalent bonds between the thiol groups of the polymer and the mucus layer being stronger than non-covalent bonds. These thiolated polymers are supposed to interact with cysteine-rich subdomains of mucus glycoproteins via disulfide exchange reactions (Snyder et al., 1983). Mucoadhesive drug delivery systems promise several advantages that arise from the localization at a given target site (I), a prolonged residence time at the site of drug absorption (II), and an intensified contact with the mucosa increasing the drug concentration gradient and subsequently uptake (III) (Kast and Bernkop-Schnürch, 2001).

3.4. Enzyme inhibition studies

The inhibitory effect of the polymers was tested on intact buccal mucosa. As shown in Fig. 4 both thiolated and unmodified polymer significantly inhibited the hydrolysis of leu-*p*NA by aminopeptidases present on the intact buccal mucosa. Thiolated PCP was thereby significantly more effective than unmodified polymer in protecting leu-*p*NA from enzymatic hydrolysis. Fig. 5 shows the degradation of leu-enkephalin by intact buccal mucosa and in the presence of thiolated and unmodified PCP. The polymers inhibited the hydrolysis of leu-enkephalin dramatically. Hydrolysis of leu-enkephalin was very rapid, after 300 min merely 13.1% of the peptide remained stable. In the presence of unmodified polymer and thiolated polymer, respectively, after 300 min the peptide was still present to more than 79%. The results of Fig. 4 demonstrate that thiolation of PCP

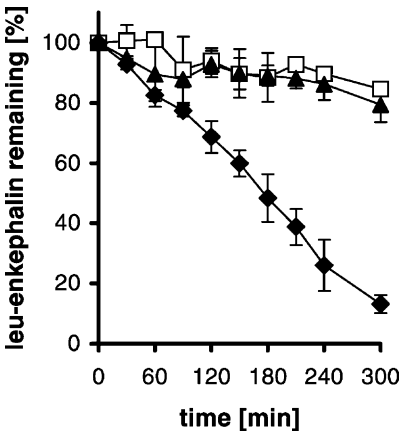

Fig. 5. Time-course of the percentage remaining of leu-enkephalin on intact buccal mucosa during incubation with 50 mM phosphate buffer pH 6.8 containing 2% NaCl; without polymer (◆); with 0.25% (w/v) PCP (□); with 0.25% (w/v) PCP-cysteine conjugate (▲). Each point represents the mean (±S.D.; $n = 3$).

enhances the inhibitory potency of PCP towards aminopeptidase *N* and other membrane bound peptidase(s) involved in the digestion of leu-*p*NA. The enzyme assay using the leu-enkephalin seems to be not as sensitive as the assay using leu-*p*NA since the inhibitory activity of the unmodified PCP was sufficient to stabilize the model peptide. The improvement of the enzyme inhibiting properties of PCP by thiolation towards leu-enkephalin on the intestinal mucosa could be demonstrated by Walker et al. (2001).

It is well-established for aminopeptidase *N* that the complexation of the divalent ion Zn^{2+} within the enzyme structure is essential for its activity (Garner and Behal, 1974). It is likely that the mechanism of enzyme inhibition by the conjugated polymer is by chelation of the Zn^{2+} from the protein structure. This mechanism is supported by the observation that the thiolated PCP has a greater affinity for Zn^{2+} and a corresponding greater inhibitory potency towards aminopeptidase *N* activity in comparison to the control polymer. The stabilization of leu-*p*NA and leu-enkephalin on the buccal mucosa by PCP and PCP-cysteine suggests that the use of these polymers in buccal mucoadhesive systems will improve the bioavailability of peptides. Protection against enzymatic attack by aminopeptidases may also be of value for other peptides susceptible to this activity, for instance calcitonin analogues (Ogiso et al., 1997).

3.5. Release studies

Studies carried out with tablets based on PCP and thiolated PCP demonstrated only a slight difference in the release profile of the model drug leu-enkephalin. Because of the small quantities of PCP-cysteine conjugates available and the fact, that a preliminary study showed no difference in the release behavior of tablets with a weight of 160 and 30 mg, respectively, all release studies were carried out with tablets with a weight of 30 mg. The results of release studies are shown in Fig. 6. In this study the drug release of the delivery system was not unidirectional. For practical use the buccal drug delivery system should be covered on one side, but further studies are required to develop an appropriate coating. It was observed in preliminary studies that the PCP-cysteine conjugate is not compatible with cationic charged drugs. Due to ionic interactions between the cationic charged drug and the anionic character of the PCP-cysteine conjugate no drug release could be observed (data not shown).

Another possible limitation might be thiol and disulfide exchange reactions between the thiolated polymer and peptide drugs bearing thiol and/or disulfide substructures. Whether the peptide is bound to the polymer-conjugate or not has to be evaluated from case to case (Bernkop-Schnürch and Thaler, 2000). At first sight, the drug release from thiolated matrix-tablets does not seem to offer any advantages compared to delivery systems based on the

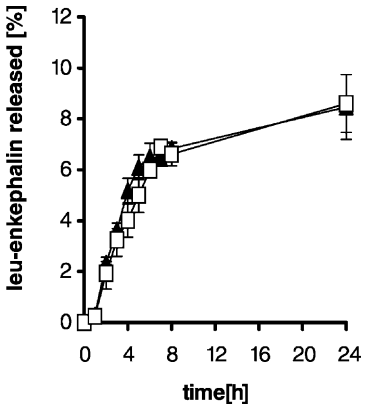

Fig. 6. Release profile of leu-enkephalin from tablets based on the PCP-cysteine conjugate (▲) and PCP (□). Studies were carried out in 50 mM phosphate buffer pH 6.8 at 37 °C. Indicated values are means (±S.D.) of at least three experiments.

corresponding unmodified polymer. However, taking also results of disintegration and mucoadhesion studies into consideration, the great potential of these new systems becomes obvious. Disintegration studies revealed that the stability of matrix-tablets based on PCP-cysteine can be guaranteed for 28 h. The mucoadhesion time for buccal tablets consisting of PCP-cysteine has been determined to be 24 h. A controlled drug release for such an extended time period might, therefore, become very important and can only be provided by a buccal delivery system based on thiolated polymers, which can guarantee a high stability of the carrier-matrix and a prolonged time of mucoadhesion.

4. Conclusion

The covalent attachment of cysteine to the anionic polymer PCP leads to an improvement of the stability of matrix-tablets consisting of thiolated polymer. The mucoadhesive properties are also enhanced, which is confirmed by two different in vitro test systems. In addition, thiolation increases the inhibitory potency of PCP towards buccal enzymes, and thereby the stability of leu-enekphalin and leu-*p*NA is raised. Due to these features matrix-tablets based on thiolated PCP represent a promising type of buccal drug delivery systems.

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