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Research Paper

Thiolated Chitosans: Design and *In Vivo* Evaluation of a Mucoadhesive Buccal Peptide Drug Delivery System

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Purpose. Intravenous application of pituitary adenylate cyclase-activating polypeptide (PACAP) has been identified as a promising strategy for the treatment of type 2 diabetes. To generate a more applicable formulation, it was the aim of this study to develop a sustained buccal delivery system for this promising therapeutic peptide.
Methods. 2-Iminothiolane was covalently bound to chitosan to improve the mucoadhesive and permeation-enhancing properties of chitosan used as drug carrier matrix. The resulting chitosan–4-thiobutylamidine conjugate was homogenized with the enzyme inhibitor and permeation mediator glutathione (gamma-Glu-Cys-Gly), Brij 35, and PACAP (formulation A). The mixture was lyophilized and compressed into flat-faced discs (18 mm in diameter). One formulation was additionally coated on one side with palm wax (formulation B). Tablets consisting of unmodified chitosan and PACAP (formulation C) or of unmodified chitosan, Brij 35, and PACAP (formulation D) served as controls. Bioavailability studies were performed in pigs by buccal administration of these test formulations. Blood samples were analyzed via an ELISA method.
Results. Formulations A and B led to an absolute bioavailability of 1%, whereas PACAP did not reach the systemic circulation when administered via formulations C and D. Moreover, in the case of formulations A and B, a continuously raised plasma level of the peptide drug being in the therapeutic range could be maintained over the whole period of application (6 h). Formulations A and B were removed by moderate force from the buccal mucosa after 6 h, whereas formulations C and D detached from the mucosa 4 h after application.
Conclusion. The study reveals this novel mucoadhesive delivery system to be a promising approach for buccal delivery of PACAP.

KEY WORDS: bioavailability; buccal delivery; PACAP; thiolated polymers.

INTRODUCTION

The treatment of type 2 diabetes is limited by numerous problems encountered with therapeutic strategies pursued so far (1). Sulfonylurea therapy, for instance, has the disadvantage of causing hypoglycemia and high primary and secondary failure rates (2). Metformin treatment also has a high primary and secondary failure rate and the potential to cause lactic acidosis (3). Furthermore, thiozolinediones can lead to weight gain and toxic liver damage (4). Ultimately, the majority of diabetics will require insulin treatment, a therapy that produces significant bouts of hypoglycemia when attempting to maintain tight control of plasma glucose levels. Hence, new treatments to retain or enhance normal (glucose-dependent) insulin secretion are needed. Recently, a novel promising peptide drug for treatment of type 2 diabetes, pituitary adenylate cyclase-activating polypeptide (PACAP), has been developed (5). PACAP, a member of the vasoactive intestinal peptide/secretin/glucagon family, stimulates insulin secretion from islets in a glucose-dependent manner at picomolar concentrations (6).

For the use of PACAP in the treatment of type 2 diabetes, a suitable drug delivery system needs to be developed. Because of its relatively short elimination half-life of a few minutes and the need for a permanent blood level of the drug (7,8), implantable pumps or inserted depot formulations are

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required for drug delivery. However, parenteral administration is associated with inconvenience and pain, leading to poor patient compliance.

Buccal administration offers a viable alternative for peptide delivery based on excellent accessibility of the application site, bypass of hepatic first-pass metabolism, and of degradation in the stomach and the intestine. Furthermore, the oral mucosa is less prone to irritation or damage than, e.g., nasal mucosa. One disadvantage of buccal delivery is the limited permeation of peptide drugs across the buccal mucosa (9).

The most successful approach for buccal mucosal delivery of peptides is the use of formulations with mucoadhesive and permeation-enhancing properties. The biopolymer chitosan has been proposed for use in oral mucosal drug delivery (10). Recently, it has been shown that the thiolation of chitosan can lead to an improvement of several properties of unmodified chitosan (11). These thiolated chitosans have numerous advantageous features in comparison to unmodified chitosan, such as significantly improved mucoadhesive and permeation-enhancing properties (12–14). For instance, the mucoadhesive properties of a chitosan–TBA conjugate are improved 250-fold in comparison to unmodified chitosan (15). The strong cohesive properties of thiolated chitosans render them highly appropriate excipients in controlled drug-release dosage forms (15).

The purpose of the present study was to develop a buccal drug delivery system for PACAP based on thiolated chitosan in combination with further auxiliary agents such as enzyme inhibitors and permeation enhancers. The *in vivo* potential of these novel formulations was evaluated in pigs.

MATERIALS AND METHODS

Materials

Glutathione (GSH, gamma-Glu-Cys-Gly), Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)], L-cysteine hydrochloride hydrate, protease-free bovine serum albumin (BSA) and *o*-phenylenediamine dihydrochloride were purchased from Sigma (St. Louis, MO, USA); chitosan (molecular mass 400 kDa) was obtained from Fluka GmbH (Buchs, Switzerland); pituitary adenylate cyclase-activating polypeptide (PACAP) (molecular mass 3741 Da; amino acid structure, HSDAVFTDNYTRLRKQVAAKKYLQSIKNKRY), anti-PACAP Fab (MS-BB-10 MH), and protein-A purified rabbit anti-PACAP antibodies were kindly donated by Bayer AG (Leverkusen, Germany); horseradish peroxidase (HRP)-goat anti-rabbit antibody (PI-1000) was purchased from Vector (Burlingame, CA, USA).

Synthesis of Chitosan–Iminothiolane Conjugates

Five hundred milligrams of chitosan (molecular mass 400 kDa) was dissolved in 50 mL of aqueous 1% acetic acid by stirring the mixture for 1 h. The pH was adjusted to 6 with 5 M NaOH, and 400 mg of 2-iminothiolane HCl (Traut's reagent) was added. The mixture was stirred continuously for 6 h at room temperature. The resulting polymer conjugates were dialyzed against 5 mM HCl, two times against 5 mM HCl containing 1% of NaCl, against 5 mM HCl, and finally against 0.4 mM HCl. Thereafter, samples and controls were freeze-dried at −30°C and 0.01 mbar (Christ Beta 1-8K, Germany) and stored at 4°C until further use.

Determination of the Thiol Group Content

The total amount of sulfhydryl groups immobilized on the polymer is represented by the summation of free thiol groups and of oxidized thiol groups available in the form of disulfide bonds. The amount of free and oxidized thiol groups of the polymer was determined photometrically by using Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] as described previously (16,17).

Preparation of the Buccal Formulations

For formulations A, C, and D, 1 mg of the peptide drug PACAP, the corresponding amounts of polymers, and permeation enhancers, respectively, were homogenized in an aqueous solution. The composition of the different formulations is shown in Table I. The solutions were lyophilized (Christ Beta 1-8K) and compressed into flat-faced discs 18 mm in diameter (Hanseaten Type EI, Hamburg, Germany). Formulation B was composed of an innermost layer consisting of chitosan–TBA (50 mg), GSH (2.5 mg), Brij 35 (2.5 mg), and PACAP (1 mg), whereas the outermost layer consisted of chitosan–TBA (50 mg). First the ingredients of the innermost layer were homogenized in an aqueous solution and poured into a cylindrical mold 18 mm in diameter, after which the solution was frozen. Chitosan–TBA was dissolved in water and poured onto the frozen solution of the innermost layer. After freezing, both layers were lyophilized together. The freeze-dried polymer had a structure like cotton wool. The formulation was compressed into a flat-faced disc (18 mm in diameter) and the outermost layer was additionally covered with palm wax as backing layer. The purpose of the backing layer was to avoid drug loss in the oral cavity. A schematic of formulation B is shown in Fig. 1.

Table I. Composition of the Buccal Formulations Used for *in Vivo* Studies

	Formulation A (mg)	Formulation B (mg)	Formulation C (mg)	Formulation D (mg)
PACAP	1	1	1	1
Chitosan	–	–	74	73.25
Chitosan–TBA	69.5	100	–	–
GHS	3.75	2.5	–	–
Brij 35	0.75	2.5	–	0.75

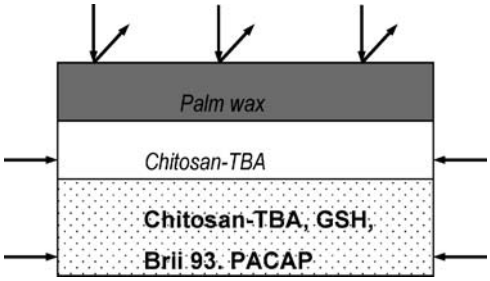

Fig. 1. Schematic of the buccal formulation B. Diffusion of saliva into the polymer matrix is only possible through surface areas, which are not covered by the palm wax film.

Release Studies

In vitro release rate of PACAP from formulations A–D was analyzed as follows: 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 in an oscillating water bath (GFL 1092, 100 rpm) and incubated at 37 ± 0.5°C; sink conditions were maintained throughout all these studies. Aliquots of 150 μL were withdrawn hourly and replaced with an equal volume of release medium preequilibrated at 37°C. Released PACAP was assayed by high-performance liquid chromatography (HPLC) (18).

HPLC Analysis of PACAP

Samples were analyzed by reverse-phase HPLC with a Perkin-Elmer series 200 LC pump (Norwalk, CT, USA), Perkin-Elmer 200 series autosampler with a 20-μL injection loop, and a diode array detector (Perkin-Elmer 235C). PACAP was separated from its degradation products on a precolumn (Nucleosil 100-5C18, 40 mm × 4 mm) and 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% acetonitrile and 10% of 0.1% trifluoroacetic acid in water). The absorbance of the peptide was detected at 220 nm. The amount of PACAP was calculated by interpolation from an appropriate external standard curve.

Determination of the Blood Glucose Level

Blood glucose levels were determined by use of a Medisense Precision Xtra (Abbott, Vienna, Austria) measuring instrument for diabetics.

Determination of PACAP in the Plasma

PACAP concentrations were determined by means of an ELISA. Before analysis, PACAP was extracted from plasma with ethanol (0.35 mL plasma and 1.25 mL 96% ethanol). The samples were thoroughly mixed and centrifuged for 5 min at 24000 × *g*. The supernatant was dried under a stream of air overnight at room temperature. Samples were reconstituted in 220 μL blocking buffer [phosphate buffered saline (PBS)–0.05% Tween 20 + 2% protease-free BSA] and mixed thoroughly. For the ELISA, a Nunc MaxiSorp plate was coated with 5 μg/mL anti-PACAP Fab (MS-BB-10_MH) at 4°C overnight. The plate was dried and blocked with blocking buffer for 1.5 h. The wells were then washed twice with PBS buffer containing 0.05% Tween 20 and subsequently three times with 250 μL of PBS buffer before samples were incubated at room temperature for 1.5 h on a slow shaker. After the washing of the plates as described above, protein-A purified rabbit anti-PACAP antibodies (1 μg/mL) were incubated on the plate for 1 h of incubation at room temperature. The plate was washed and a 1:1000 dilution of HRP-goat anti-rabbit antibody (Fab fragment) was added and incubated for 1 h. After a washing step, *o*-phenylenediamine dihydrochloride was added as substrate. The color development was stopped by addition of 1 M sulfuric acid after 30 min and color intensity was measured at 450 nm.

In Vivo Evaluation of the Delivery System

Eight clinically healthy pigs of both sexes of the crossbreed Edelschwein × Pietrain with an average body weight of 30 kg were used. The animals were kept under standard conditions with free access to drinking water. Twelve hours before anesthesia the animals were deprived of feed. Pigs were premedicated with intramuscular injections of midazolam (0.5 mg/kg), ketamine (10 mg/kg), and butorphanol (0.5 mg/kg). For blood sampling, the arteria saphena was cannulated. Anesthesia was initiated by an intravenous application of 1–2 mg propofol/kg body mass infused via a cannulated ear vein. Pigs were intubated, placed on their right side on a surgery table, and covered with a blanket to maintain stable body temperature. To reduce dehydration of the buccal mucosa, the mouth of each pig was sealed with surgical plastic foil wrapped around the snout region. During the experiment, pigs were mechanically ventilated using an oxygen/air mixture (50% O_2). The cannula in the ear vein was connected to an intravenous infusion device, and propofol (17 mg/kg per hour) and 0.9% sodium chloride solution (10 mL/kg per hour) were administered. Invasive arterial blood pressure, O_2 saturation, ECG, body temperature, arterial blood gases, and total concentration of O_2 and CO_2 were monitored.

Before the application of the delivery system three blood samples were taken at 10-min intervals to determine the baseline of the blood glucose level. The bioavailability studies were performed over a period of 6 h. One of the test formulations (Table I) was placed on the buccal mucosa in the middle region of each cheek. Blood samples of 4 mL were taken hourly six times. The swelling behavior of tablets was monitored three times by endoscopic examination. To avoid further dehydration of the delivery system, approximately 0.75 mL of water was sprayed on the mucosa after 3.5 and 5.5 h. Three hours after the application of the tablets, pigs were turned onto their left side to avoid decubital lesions. After 6 h, the buccal delivery devices were removed and blood samples were taken after 5, 15, 30, 45, and 60 min. Then either an intravenous injection of 0.3 μg PACAP was

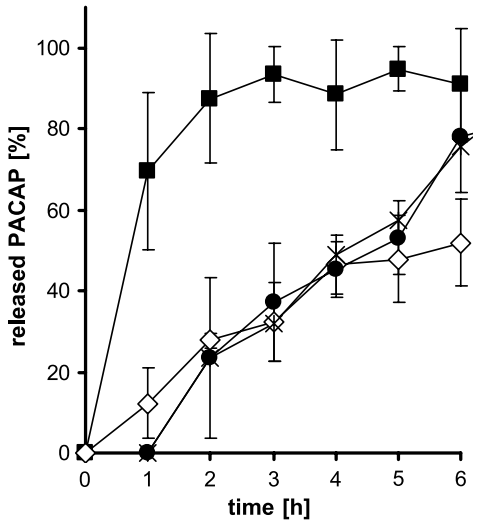

Fig. 2. *In vitro* release profile of PACAP from formulations A (■), B (◊), C (●) and D (×). Studies were carried out in 50 mM phosphate buffer (pH 6.8) at 37°C. Results are given as means ± SD of at least three experiments.

given or 15 μg of PACAP was infused over 45 min. During the infusion, blood samples were taken at 5, 15, 30, and 45 min. After both modes of intravenous administration of PACAP, blood was sampled at 2, 5, 10, 20, and 30 min. The anesthesia was stopped and the phase of recovery controlled. All animal experiments were carried out at the Department of Farm Animals and Herd Management of the University of Veterinary Medicine Vienna, Austria.

The animal experiments were approved by the Bundesministerium für Gesundheit in accordance with the Austrian legislation (Tierseuchengesetz, BGBl. Nr. 501/1998 i.d.F. BGBl. I Nr. 169/1999).

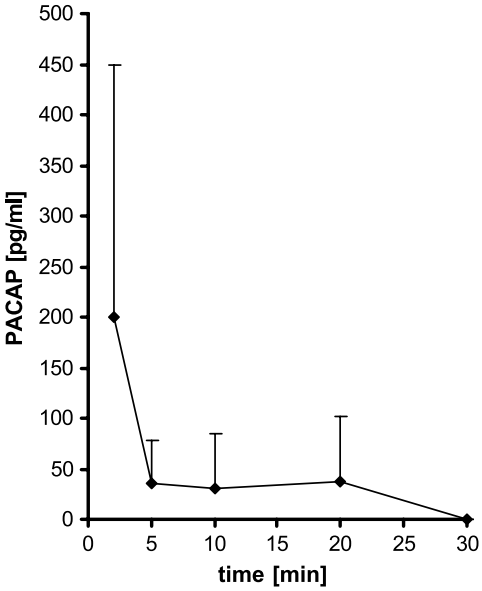

Fig. 3. Concentration of PACAP in plasma obtained after intravenous administration of 0.01 μg PACAP/kg body weight in an aqueous solution. Values are the results (mean ± S.D) from three experiments.

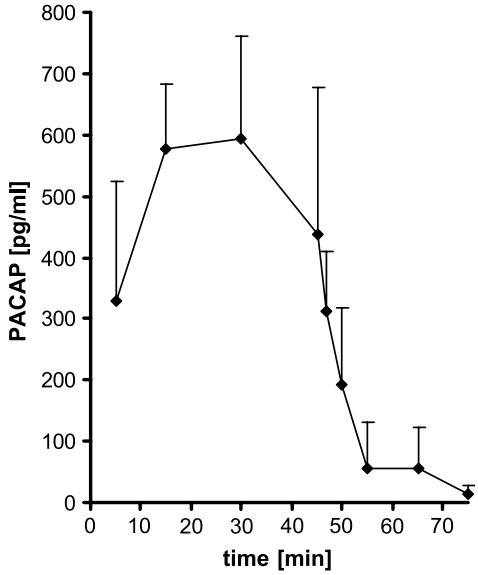

Fig. 4. Plasma concentration of PACAP infused as aqueous solution (0.5 μg/kg body weight) for 45 min in pigs. Results are expressed as means ± SD from three experiments.

Data Analysis

Buccal bioavailabilities were calculated according to the following equation:

$$F(\%) = (\mathrm{AUC}_{\mathrm{buc}} D_{\mathrm{iv}})/(\mathrm{AUC}_{\mathrm{iv}} D_{\mathrm{buc}}) \times 100$$

where F designates the bioavailability and D the administered dose. The areas under the individual plasma concentration time curves (AUC) were calculated by use of the linear trapezoidal rule. Statistical data analysis was performed by t test with $p < 0.05$ as the minimal level of significance.

RESULTS

Characterization of the Chitosan–TBA Conjugate

For the chitosan–TBA conjugate, the amount of covalently attached thiol groups was determined to be 329 ± 28 μM per gram chitosan. During the coupling reaction, thiol groups were oxidized and the resulting amount of free thiol groups was 284 ± 15 μM thiol groups per gram polymer. The preliminary formation of disulfide bonds during the coupling reaction is advantageous because of the resulting crosslinking of the polymer. Consequently, the cohesive properties of thiomer matrix tablets are increased and their stability and water uptake are positively influenced (19). The lyophilized polymer appeared as a white and odorless powder of fibrous structure. It easily swelled in solutions with a pH of 6.8 and formed transparent gels of high viscosity. Mucoadhesive properties were in good correlation with results of recent studies (11).

Table II. Pharmacokinetic Parameters of PACAP in Pigs After Intravenous Injection and Buccal Administration Using the Chitosan–TBA/GSH Delivery System

Parameters	A	B	C	D	Intravenous solution
Administered PACAP (μg/kg)	67	67	67	67	0.5
c_{max} (pg/mL)	227.7	276.8	Not detected	Not detected	594.4
t_{max} (min)	365	300	Not detected	Not detected	30
AUC (pg/mL h)	548.8 ± 167.1	548.8 ± 422.9	Not determined	Not determined	409.9 ± 12.0
Absolute bioavailability (%)	1.02	1.0	Not determined	Not determined	–

In Vitro Characterization of the Delivery Systems

The results of the release studies are shown in Fig. 2. Formulation A showed a rapid drug release. A controlled drug release was achieved by using formulation B. After 6 h, 51.96 ± 10.64% of PACAP was released from this formulation. Using the control formulations C and D, the drug release was decreased during the first 4 h in comparison to formulation A, but after 6 h, nearly the same amount of PACAP as per formulation A was released. All delivery systems did not disintegrate and showed no erosion during the experiment.

In Vivo Evaluation of the Delivery Systems

To calculate the bioavailability and evaluate the sensitivity of the analytical method, PACAP was injected intravenously. In the first trials, 0.3 μg of PACAP was administered as a bolus injection. As shown in Fig. 3, plasma concentrations of PACAP 2 min after injection varied from 5 to 480 pg/mL. For the main study, PACAP was infused via the vena auricularis. The plasma level of PACAP increased up to 329.90 ± 195.32 pg/mL within 5 min after initiation of the infusion and reached almost 600 pg/mL after 30 min (Fig. 4). The infusion was well tolerated by the pigs. For calculation of the absolute bioavailability, the values obtained after the intravenous infusion were used.

Four different buccal drug delivery systems were tested in this study. The absolute bioavailability of PACAP was 1.02 and 1.00% after buccal administration of formulation A and formulation B, respectively. The main pharmacokinetic parameters are listed in Table II and the corresponding plasma profiles are illustrated in Fig. 5. The bioavailability of the control formulations C and D, consisting of unmodified chitosan, could not be calculated, as the plasma concentrations of PACAP were below the detection limit.

Immediately after onset of anesthesia, plasma glucose concentrations were found to increase by approximately 40%, reflecting a common reaction of pigs suffering from stress (20,21). However, within 15 to 30 min, glucose levels gradually returned to normal range, which then remained stable throughout the experiment (Fig. 6). The buccal formulations were administered after the plasma glucose levels were within the normal range again.

A rigid endoscope was used to confirm the adhesion of all formulations on the buccal mucosa and to monitor the

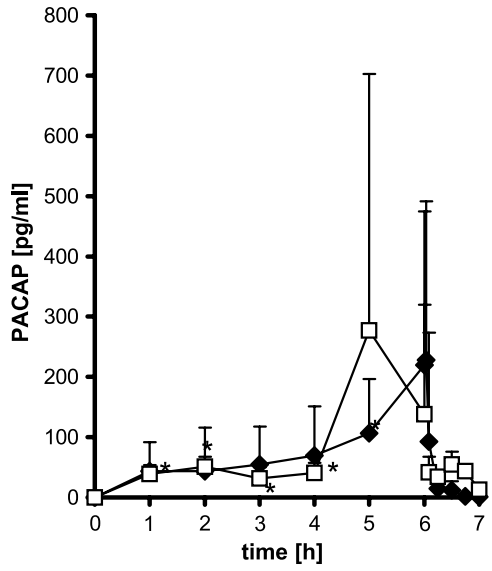

Fig. 5. Plasma concentrations of PACAP after buccal application of formulations A (◆) and B (□). Formulations were applied at time 0 and removed 6 h later. Results of three to four experiments are given as means ± SD. *Significantly different from plasma concentration before application of the respective PACAP formulation (time point 0 min) ($p < 0.05$).

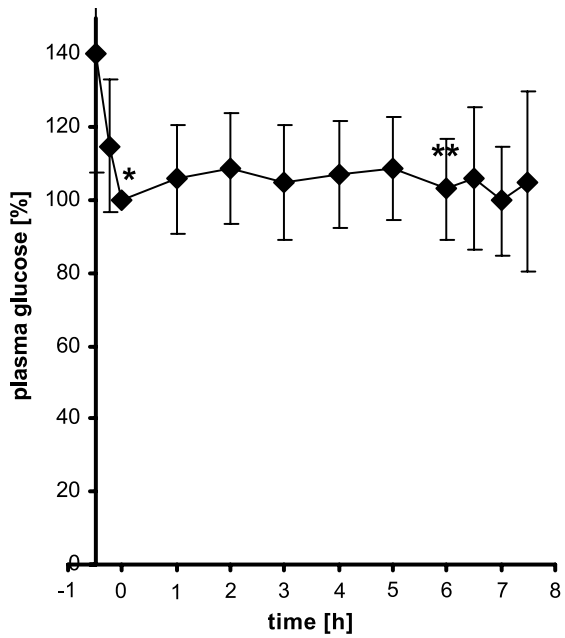

Fig. 6. Plasma glucose levels after onset of anesthesia (time point 0 min) and simultaneous application of the buccal drug delivery system. Results of four experiments are presented as means ± SD. *Attachment of the tablet (formulation A); **detachment of the tablet.

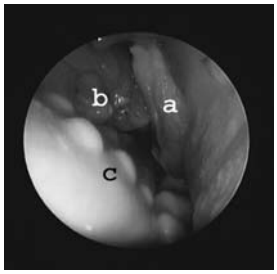

Fig. 7. Endoscopic imaging of a swollen formulation B tablet 5.5 h after application onto the buccal mucosa of a pig; a, tablet; b, premolar tooth; c, mucosal surface of the tongue.

swelling behavior of the delivery system during the time of application. A typical image resulting from an endoscopic examination of formulation B attached to the buccal mucosa is illustrated in Fig. 7. Formulations A and B adhered strongly to the buccal mucosa during the experiment and had to be removed by force after 6 h. A spontaneous detachment of the control formulations C and D could be observed 4 h after application. All formulations showed strong cohesive properties and remained stable during application. The buccal mucous membrane did not show any inflammatory reaction after removal of all formulations tested.

DISCUSSION

PACAP is a novel promising peptide drug designed to behave superior to insulin, as it combines the potency of insulin without risking overdose and hypoglycemia. However, because of its relatively short elimination half-life of a few minutes, implantable pumps or inserted depot formulations are needed (20). Buccal administration offers a viable alternative for peptide delivery based on excellent accessibility, bypass of hepatic first-pass metabolism, and of degradation in the stomach and intestine. In this study, a new buccal drug delivery system for PACAP, based on thiolated chitosan in combination with auxiliary agents such as enzyme inhibitors and permeation enhancers, was applied. The chitosan–TBA conjugate was chosen as polymeric excipient because cationic therapeutic peptides, such as PACAP, need to be embedded in a cationic or nonionic mucoadhesive polymer. Incorporation of peptide drugs exhibiting a cationic net charge in anionic mucoadhesive polymers, however, leads to a strong reduction in the mucoadhesive properties, and the drug release might be hindered by too strong ionic interactions between the therapeutic ingredient and the polymeric network. Apart from these considerations, chitosan–TBA was chosen as drug carrier matrix also for other reasons such as its excellent mucoadhesive and permeation-enhancing properties (11,24). Because of its strong mucoadhesiveness, adhesion of formulations A and B, consisting of chitosan–TBA, on the buccal mucosa was provided, whereas formulations C and D, consisting of unmodified chitosan, detached from the buccal mucosa as early as 4 h after application.

Comparing the release profile of formulations A and B with the control formulations C and D, we found that the drug release from thiolated matrix tablets was not increased in this study compared to delivery systems based on the corresponding unmodified polymer. However, when the improved mucoadhesion and stability of the modified delivery systems is taken into consideration, the potential of these new systems becomes obvious. Formulation B has the advantage of a unidirectional tablet ensuring release of the drug only toward the buccal mucosa without losses in the oral cavity.

The permeation-enhancing properties of chitosan–TBA are significantly increased by the addition of the permeation mediator GSH. This effect of the chitosan–TBA/GSH system was demonstrated *in vitro* by use of preparations of porcine buccal mucosa; these investigations showed that the permeation of PACAP was increased 52-fold by the addition of chitosan–TBA and GSH in comparison with unmodified chitosan (21). The permeation-enhancing effect of chitosan–TBA in oral drug delivery systems was also demonstrated in rats (22). The addition of GSH to the buccal drug delivery system has another advantage, as it provides the stability of the peptide drug. Recently, it was shown that the degradation of PACAP by buccal enzymes is inhibited by adding GSH (23). Without enzyme inhibitor, 20% of PACAP was degraded after 5 h.

The surfactant Brij 35 was additionally added to formulations A, B, and D. Its permeation-enhancing potential has already been shown in buccal applications of insulin *in vivo* (24). A synergistic effect on the permeation of chitosan–TBA/GSH and Brij 35 was expected. However, Brij 35 by itself has shown no effect on the bioavailability of PACAP, as no PACAP was detected in plasma after administration of the control formulation D.

Formulation B was coated with palm wax on one side to avoid the loss of drug in the oral cavity. PACAP, GSH, and Brij 35 were embedded concentrated on the adhesive side of the delivery system to provide high concentrations of the drug and the excipients at the start of the buccal application. Unfortunately, the backing layer of formulation B does not seem to influence the bioavailability. Apparently, only a very small amount of PACAP is absorbed by the buccal mucosa, so that the loss of drug within the oral cavity seems to have no significant influence on the bioavailability.

An explanation for the rapid increase of PACAP in plasma at 5 or 6 h might be that the buccal mucosa acts as a depot for the peptide. Such a depot function was also observed for fluorescence-labeled dextran with a molecular mass of 4 kDa (25). The bilayer structure of formulation B seems to influence the t_{max} value. As PACAP is concentrated in the innermost layer of the formulation, an earlier t_{max} is achieved by this bilayer formulation in comparison to formulation A.

Within this *in vivo* study the proof of concept for thiolated polymer delivery systems could be provided. A bioavailability of about 1% was reached by using drug delivery systems consisting of chitosan–TBA/GSH, whereas no PACAP was detected in plasma with use of unmodified chitosan.

CONCLUSION

A new drug delivery system that might be useful for the delivery of PACAP by the buccal route was tested under *in vitro* and *in vivo* conditions in pigs. The thiolated polymer chitosan–TBA combined with GSH and the surfactant Brij

35 leads to a multifunctional dosage form displaying the following advantages:

1. Strong mucoadhesiveness of the formulation causing prolonged presence at the site of application combined with high cohesive stability of the drug delivery system
2. Significant enhancement of drug permeation by the chitosan–TBA/GSH system in comparison with unmodified chitosan
3. Inhibition of degradation of PACAP by buccal enzymes by addition of GSH

The pharmaceutical usefulness of these features of the chitosan TBA/GSH formulation could be confirmed by experiments in pigs, rendering this dosage form an innovative formulation for further studies on the effective buccal administration of PACAP and probably other peptide drugs.

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