In Vivo Evaluation of an Oral Salmon Calcitonin-Delivery System Based on a Thiolated Chitosan Carrier Matrix

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Purpose. To develop and evaluate an oral delivery system for salmon calcitonin.

Methods. 2-Iminothiolane was covalently bound to chitosan in order to improve the mucoadhesive and cohesive properties of the polymer. The resulting chitosan-TBA conjugate (chitosan-4-**t**hio**b**utyl**a**midine conjugate) was homogenized with salmon calcitonin, mannitol, and a chitosan-Bowman-Birk inhibitor conjugate and a chitosan-elastatinal conjugate (6.75 + 0.25 + 1 + 1 + 1). Optionally 0.5% (m/m) reduced glutathione, used as permeation mediator, was added. Each mixture was compressed to 2 mg microtablets and enteric coated with a polymethacrylate. Biofeedback studies were performed in rats by oral administration of the delivery system and determination of the decrease in plasma calcium level as a function of time.

Results. Test formulations led to a significant ($p < 0.005$) decrease in the plasma calcium level of the dosed animals in comparison to control tablets being based on unmodified chitosan. The addition of glutathione in the tablets led to a further improvement in the oral bioavailability of salmon calcitonin with an earlier onset of action and a decrease in the calcium level of about 10% for at least 10 h.

Conclusions. The combination of mucoadhesive thiolated chitosan, chitosan-enzyme-inhibitor conjugates and the permeation mediator glutathione seems to represent a promising strategy for the oral delivery of salmon calcitonin.

KEY WORDS: controlled release; thiolated polymer; enzyme inhibitors; reduced glutathione; salmon calcitonin; oral delivery system.

INTRODUCTION

Salmon calcitonin is a peptide drug composed of 32 amino acids currently marketed in injectable and nasal spray forms to treat bone diseases such as osteoporosis, Paget's disease and hypercalcemia. Its unique structure protects it against sequestration in the liver, muscle, and bone, and makes it 20 to 30 times more potent than human calcitonin (1,2). Due to inconvenience and pain associated with injectable forms and a low patient acceptance of the nasal formulation it is believed that currently commercially available salmon calcitonin products have not reached their full market potential. An oral delivery system for the drug would allow full clinical exploration of the role of salmon calcitonin in the mentioned diseases and would lead to a higher patient compliance. However, there are several difficulties associated with delivering peptide drugs in an oral formulation.

To gain sufficient blood concentrations after oral dosing of peptides and proteins, different physiologic barriers encountered with the peroral route of application have to be overcome. The most important of these barriers are the absorption barrier (I), the diffusion barrier (II), and the enzymatic barrier (III) (3). Attempts to overcome these barriers include the use of liposomes (4), of nano- and microparticles (5), of colon targeted delivery systems (6), of peptide analoga, of enzyme inhibitors, and of mucoadhesive polymers (3). In recent years some new strategies protecting peptides from the enzymatic digestion in the intestine have gained considerable attention, like the immobilization of protease inhibitors on mucoadhesive polymers used as drug carrier matrices (7) and also the utilization of thiolated polymers, or so called thiomers (8,9). Thiomers are characterized by the immobilization of thiol moieties on the backbone of hydrophilic macromolecules and are believed to be able to form covalent bonds with cysteine-rich subdomains of glycoproteins, thus leading to strongly improved mucoadhesion. In addition, cohesiveness of polymeric drug carrier systems based on thiomers can be provided due to the formation of intra- and intermolecular disulfide bonds within the polymeric network (10,11).

The aim of this study is to develop a suitable oral formulation for salmon calcitonin with features proper to overcome the already mentioned barriers. For this purpose a promising thiomer—the chitosan-TBA conjugate (Fig. 1)—was used as main excipient for the development of the delivery system. The addition of two already characterized chitosan-protease inhibitor conjugates—namely a chitosan-BBI conjugate (Bowman–Birk inhibitor) and a chitosan-elastatinal conjugate—should provide stability of salmon calcitonin toward enzymatic degradation by luminally secreted proteases, being mainly responsible for the digestion of the peptide in the intestine (12). Based on these excipients tablets were compressed and characterized with regard to their release profile and efficacy *in vivo* in rats. Furthermore, the increase in the oral bioavailability of salmon calcitonin by addition of the permeation mediator glutathione (GSH) (13) was evaluated as well.

MATERIALS AND METHODS

Synthesis and Purification of Chitosan-TBA Conjugate

Initially, 500 mg of chitosan (medium molecular mass: 400 kDa; degree of deacetylation: 80.2%; Fluka GmbH, Buchs, Switzerland) were dissolved in 50 mL of 1% acetic acid and the mixture was stirred for 1 h to obtain a 1% (m/v) solution. After adjusting the pH to 6.5 with 5 M NaOH 200 mg of 2-iminothiolane HCl (Traut's reagent; Pierce, Oud Beijerland, NL) were added. The reaction mixture was then incubated for 24 h at room temperature under continuous stirring. The resulting polymer conjugate was dialyzed against 5 mM HCl, two times against 5 mM HCl containing 1% NaCl, against 5 mM HCl and finally against 0.4 mM HCl. Thereafter, the polymer was freeze-dried at –30°C and 0.01 mbar (Christ Beta 1-8K; Germany) and stored at 4°C until further use (11).

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ABBREVIATIONS: chitosan-TBA conjugate, chitosan-4-**t**hio**b**utyl-**a**midine conjugate; chitosan-BBI conjugate, chitosan-Bowman–Birk inhibitor conjugate; GSH, reduced glutathione.

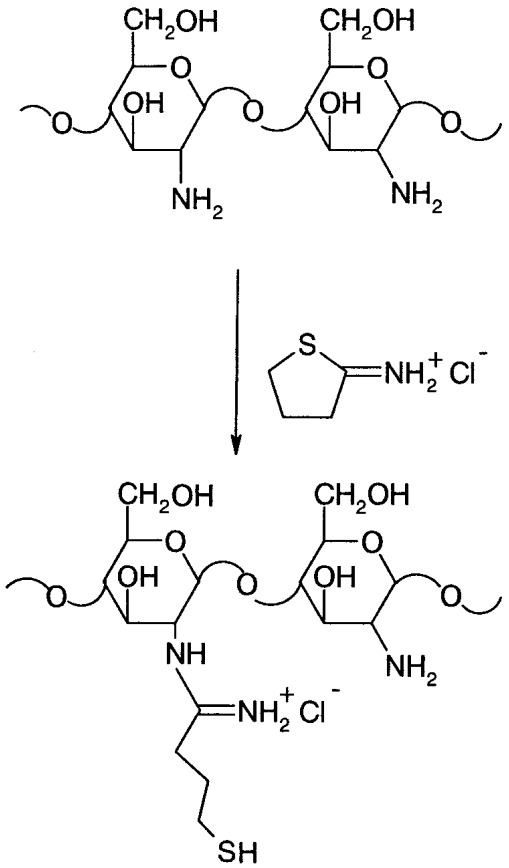

Fig. 1. Synthesis scheme for generation of chitosan-TBA conjugate. Covalent attachment of 2-iminothiolane was achieved by the constitution of an amidine bond between the carboxylic C-atom of the reagent and a free amino group of the polymer.

Synthesis of the Polymer-Inhibitor Conjugates

The polymer-inhibitor conjugates chitosan-BBI and chitosan-elastatinal were synthesized according to a method described previously by our research group (12). Bowman–Birk inhibitor (BBI; Sigma, St. Louis, MO) and elastatinal (Sigma, St. Louis, MO) were thereby directly linked to the polymer (chitosan medium molecular mass: 400 kDa; degree of deacetylation: 80.2%; Fluka GmbH, Buchs, Switzerland) via a condensation reaction mediated by 1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO).

Determination of the Degree of Modification of Chitosan-TBA Conjugate

5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, Sigma, St. Louis, MO) was used to quantify the amount of thiol groups on modified chitosan. First, 0.5 mg conjugate were dissolved in 250 μl of demineralized water, then 250 μl of 0.5 M phosphate buffer pH 8.0 and 500 μl of an Ellman's reagent solution (3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) dissolved in 10 ml of 0.5 M phosphate buffer pH 8) were added. The reaction was allowed to proceed for 2 hours at room temperature. The precipitated polymer was removed by centrifugation (24000*g*; 5 min, Hermle Z 323K). Thereafter, 300 μl of the supernatant fluid were transferred to a microtitration plate and the absorbance was immediately measured at 450 nm (microtitration-plate reader, Anthos Reader, Salzburg, Austria). The amount of thiol groups was calculated from an according standard curve obtained by the sulfhydryl group determination of a series of solutions containing increasing concentrations of L-cysteine hydrochloride hydrate (Sigma-Aldrich, Steinheim, Germany).

Preparation of Salmon Calcitonin Dosage Forms

The composition of test formulation A tablets, of test formulation B tablets, of control A solution, and of control B tablets is shown in Table 1.

Preparation of Test Formulation A and Test Formulation B Tablets

In order to be able to homogenize the initially lyophilized chitosan conjugates with the other components, the polymer derivatives were hydrated in 2 mL of demineralized water and then adjusted to pH 2 with 1 M HCl. After the addition of 250 μg of salmon calcitonin (Bachem AG, Bubendorf, Switzerland; activity of about 5200 international units/mg) in form of a trituration in mannitol (1:5) each solution was stirred for 20 min. For the preparation of test formulation A 10 μg of reduced glutathione (GSH; Sigma, St. Louis, MO) were added. The solution was then frozen at –80°C, dried by lyophilization and the dried mixture was compressed to 2 mg microtablets (diameter: 1.5 mm; depth: 1.0 mm). The compaction pressure was kept constant during the preparation of all tablets. Thereafter, all tablets were enteric coated with Eudragit L 100-55 (Röhm GmbH, Darmstadt, Germany). Because of the small size and quantity, tablets were not spray-coated in a fluidized-bed apparatus. Instead, the enteric coat-

Table I. Composition of the Dosage Forms Used for *in Vivo* Studies

	Test formulation A* (2 mg tablet)	Test formulation B* (2 mg tablet)	Control A (0.2 ml solution)	Control B* (2 mg tablet)
Salmon calcitonin	50 μg	50 μg	50 μg	50 μg
Chitosan-TBA conjugate	1340 μg	1350 μg	—	—
Unmodified chitosan	—	—	—	1950 μg
Chitosan-BBI conjugate	200 μg	200 μg	—	—
Chitosan-elastatinal conjugate	200 μg	200 μg	—	—
Reduced glutathione	10 μg	—	—	—
Mannitol	200 μg	200 μg	—	—
0.1 M Ascorbic acid sol.	—	—	200 μl	—

* Tablets were coated with Eudragit® 100-55 (Röhm GmbH).

ing was achieved by dipping the microtablets in a 3% (m/v) acetonic Eudragit L 100-55 solution and air drying. This coating procedure was repeated four times. The tablets were stored at –20°C until administration.

Preparation of Control A Solution

As control A for oral administration a salmon calcitonin ascorbic acid solution was used. Fifty micrograms of salmon calcitonin were dissolved in 200 μl of a 0.1 M aqueous ascorbic acid solution. The solution was frozen and stored at –20°C until administration.

Preparation of Control B Tablets

Control B microtablets were obtained by pulverizing unmodified chitosan (medium molecular mass: 400 kDa; degree of deacetylation: 80.2%; Fluka GmbH, Buchs, Switzerland) in a mortar, homogenizing the polymer with salmon calcitonin, compressing tablets out of it, enteric coating, and storing in the same way as test formulation tablets.

Preparation of Salmon Calcitonin Solution for Intraveinous Injection

An intravenous injection of a salmon calcitonin solution served as positive control. Because of the very low stability of salmon calcitonin at physiologic pH and because of its high thermolability the following proceeding was necessary: 1 μg of salmon calcitonin was dissolved in 200 μl of a sterile 0.9% NaCl solution, filtered through a cellulose acetate filter unit (pore size: 0.22 μm, Millipore S.A., Molsheim, France) and then immediately frozen and stored at –20°C until use.

In Vitro Release Studies from Test Formulations

As the efficacy of the enteric coating described here has already been demonstrated elsewhere (8), the drug release studies were focused on the dissolution behavior at intestinal pH conditions. Preliminary studies showed no difference in the drug release from tablets with and without 0.5% glutathione, therefore, the release behavior was investigated with non-coated test formulation A microtablets. The *in vitro* release rate of salmon calcitonin from the oral delivery system was determined by a method which is not conform to United States Pharmacopeia (USP) due to the small size of the tablets. The dosage forms were each placed into 1.5 ml eppendorf tubes containing 0.5 ml release medium (50 mM phosphate buffer pH 6.8 containing 1.5 mMol guanidine hydrochloride). Guanidine hydrochloride was added to the release medium as stabilizing agent in order to reduce the aggregation and degradation of salmon calcitonin during the experiment. The closed tubes were then placed on an oscillating waterbath (GFL 1092; 60 rpm) and incubated at 37 ± 0.5°C. Aliquots of 100 μl were withdrawn at 1 h intervals and replaced with an equal volume of release medium equilibrated at 37°C. Sink conditions were maintained throughout the study. The amount of salmon calcitonin released was evaluated by HPLC analysis according to a method previously described by our research group (14). Concentrations were quantified from integrated peak areas and calculated by interpolation from an according standard curve. Cumulative corrections were made for previously removed samples.

In Vivo Evaluation of the Delivery System

The protocol for the studies on animals was approved by the Animal Ethical Committee of Vienna, Austria and adhered to the Principles of Laboratory Animal Care. For *in vivo* studies male Wistar rats SPF (200–250 g body weight) obtained from the Institut für Labortierkunde und Genetik, University of Vienna were used. Before dosing the animals 100 μl of blood samples were taken from the tail vein and the blood calcium level was determined in the plasma utilizing a chromogenic assay (calcium-cresolphthalein kit) of SIGMA-Diagnostics®. This first determination was used as reference level (time point zero). The rats were divided in five groups and treated separately with the different dosage forms. On the one hand test formulation A tablets, test formulation B tablets, or control B tablets were administered by placing the tablets deeply into the throat in order to initiate the swallow-reflex. Additionally, 50–70 μl of a 0.1 M aqueous ascorbic acid solution were administered. This application technique seemed to be closer to conditions suitable for humans than the use of a stomach tube. On the other hand, 200 μl control A solution were given orally. Another cohort of rats was dosed with 1 μg of salmon calcitonin by I.V. injection in the tail vein. The dosed rats were fasted for the time of the trial and kept in restraining cages with free access to water. Blood samples of 100 μl were taken in 120 min intervals for 8 h and then after 12 and 24 h from the tail vein. Blood samples were centrifuged (4000 *g*, 5 min, 4°C, Hermle Z 323K) and plasma samples were collected and stored at –20°C until analysis. The calcium level in plasma was determined at least three times from each sample as a biologic response for the salmon calcitonin bioavailability using the chromogenic assay mentioned earlier.

Statistical Data Analysis

Statistical data analysis was performed using the student *t* test with $p < 0.05$ as the minimal level of significance. Calculations were done using the software Xlstat version 5.0 (b8.3).

RESULTS

Characterization of the Chitosan-TBA Conjugate

As shown in Fig. 1, 2-iminothiolane was attached to chitosan via an amidine bond formation between the carboxylic C-atom of the reagent and a free primary amino group of the polymer. The purified chitosan-TBA conjugate displayed 118.5 ± 14.3 μM (mean ± SD n = 3) thiol groups per gram polymer. The obtained polymer was slightly yellowish, odorless, and showed a fibrous structure. The stability, swelling behavior, and mucoadhesive properties of several chitosan-TBA conjugates have already been evaluated (11). The features of the polymer derivative described here were in good accordance with them.

Characterization of the Chitosan-Inhibitor Conjugates

In the case of the trypsin/chymotrypsin inhibitor conjugate the amount of covalently bound BBI on the chitosan backbone was 3.5 ± 0.1% (m/m; n = 4), whereas on the elastase inhibiting conjugate the amount of immobilized elastatinal was 0.5 ± 0.03% (m/m; n = 4).

The inhibitory efficacy of the chitosan-BBI and chitosan-elastatinal conjugates as well as their protective effect for salmon calcitonin toward the luminally secreted serine proteases trypsin, α-chymotrypsin, and elastase have already been evaluated (12). The properties of the polymer-inhibitor conjugates described within this study were in good agreement with them.

In Vitro Release of Salmon Calcitonin

The release rate of salmon calcitonin from test formulation A tablets is displayed in Fig. 2. Within the first 8 h 7.93 ± 0.38 nMol (mean ± SD n = 3) salmon calcitonin representing 53% of the total dose were liberated showing a pseudo zero order release profile. Due to the high cohesiveness within the matrix-system, tablets remained stable during the whole experiment and no disintegration could be observed. The presence of 0.5% glutathione in tablets mainly based on chitosan-TBA conjugate influenced neither their disintegration behavior nor the release profile, as no difference regarding these characteristic was observed between test formulation A and test formulation B (data not shown). Since the release profile was not altered by the addition of glutathione, an interaction of salmon calcitonin with glutathione can be excluded. This finding could be confirmed, as such interactions would lead to the appearance of additional peaks in the HPLC spectrum, which was not the case.

In Vivo Study

In order to obtain a positive control and to evaluate also the sensibility of the analytical method used, salmon calcitonin was injected intravenously into the tail vein of the rats. As shown in Fig. 3, the maximal decrease in the plasma calcium level was of 20.5 ± 5.3% (mean ± SD n = 4) and was observed

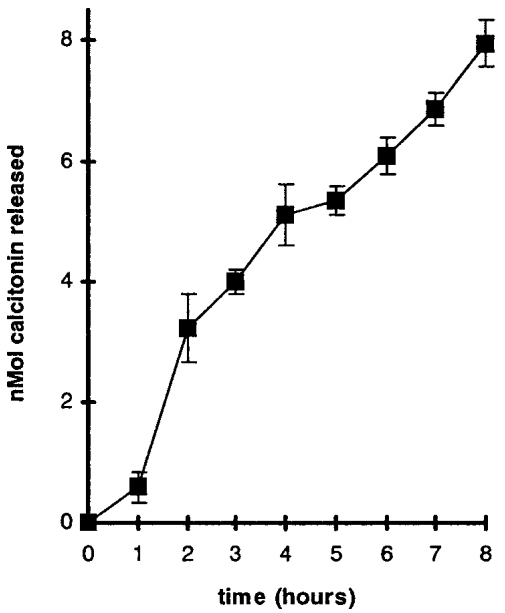

Fig. 2. Release profile of salmon calcitonin from test formulation A. The dosage form was incubated with 0.5 ml release medium (50 mM phosphate buffer pH 6.8 containing 1.5 mMol guanidine hydrochloride) at 37 ± 0.5°C. Each point represents the mean ± SD of three experiments.

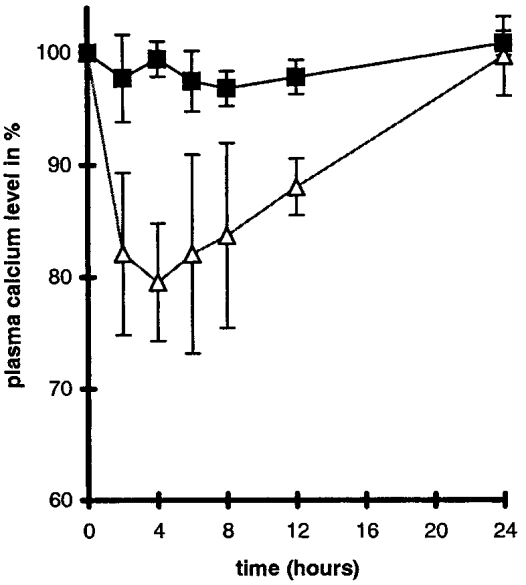

Fig. 3. Decrease in Ca^{2+} level in plasma as a biologic response for the salmon calcitonin bioavailability in fasted rats after i.v. injection (△) and after oral administration of control A (ascorbic acid solution of salmon calcitonin) (■). Indicated values are the mean results from 4 rats ± S.D.

4 h after injection. These results were in good agreement with the outcome of earlier studies confirming the validity of the analytical method used (15,16). The different oral formulations tested in this study are listed in Table 1. As shown in Fig. 3, an orally given ascorbic acid solution containing salmon calcitonin (control A) did not lead to a significant decrease in the plasma calcium level. In contrast the oral administration of salmon calcitonin with a carrier matrix consisting mainly of chitosan-TBA conjugate combined with minor shares of chitosan-BBI and chitosan-elastatinal conjugates (test formulation A), resulted in a significant decrease of the plasma calcium level. This effect was also of significance in comparison to control tablets made of unmodified chitosan (control B). The results of this study are shown in Fig. 4. An important role for the absorption of salmon calcitonin seemed thereby to have the addition of the permeation mediator glutathione. Figure 4 shows clearly that a stronger and longer enduring decrease of the plasma calcium level was obtained by the administration of test formulation A containing glutathione compared to test formulation B being prepared without the permeation mediator. Glutathione seems primarily to be responsible for an earlier onset of action. Accordingly, the best results within this study were obtained by dosing the animals with the more elaborated test formulation A, leading to a decrease of the plasma calcium level of about 10% for at least 10 h, whereat the maximal decrease in calcium level in each animal was between 12% and 16%.

DISCUSSION

In this study we examined the possibility of increasing the bioavailability of salmon calcitonin after oral administration by incorporating the drug in a matrix system based on the mucoadhesive polymer chitosan-TBA. This novel mucoadhesive excipient should have, similarly to other thiomers, the capability of forming inter- as well as intramolecular disulfide

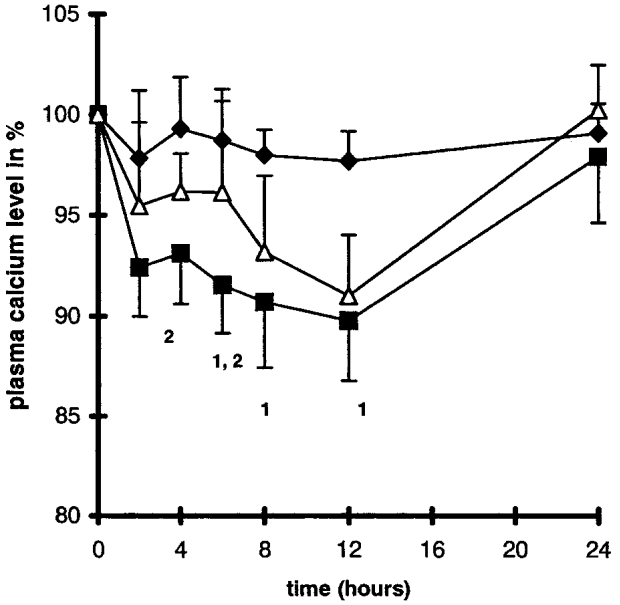

Fig. 4. Decrease in Ca^{2+} level in plasma as a biologic response for the salmon calcitonin bioavailability in fasted rats after oral administration of test formulation A (containing 0.5% glutathione) (■), test formulation B (without glutathione) (△) and control B (◆). Indicated values are the mean results from 6 rats (test formulation A) and from 4 rats (test formulation B and control B) ± S.D.; [1], differs from control $p < 0.002$; [2], differs from test formulation B $p < 0.05$.

bonds within the polymeric network (17), providing a sufficient stability of the dosage form. The sustained drug release, described within this study, can be controlled over a time period of several hours because of the high cohesiveness and stability of the matrix system, and would otherwise be impossible if the delivery system disintegrated too early. Furthermore, strong unintended interactions between the polymeric matrix system and the peptide drug could be excluded according to this controlled and sustained release profile. Consequently, also chemical reactions between the thiol moieties of the chitosan-TBA conjugate and the disulfide bond present in the structure of salmon calcitonin could be excluded. Moreover, incompatibilities based on ionic interactions were excluded, as the peptide drug exhibiting an isoelectric point above pH 9 (18) is positively charged within the cationic carrier matrix of pH 2. In contrast, ionic interactions would have to be expected between anionic polymers, such as polyacrylic acid derivatives, and the positively charged salmon calcitonin. The utilization of this kind of polymers for the oral delivery of the named peptide appeared therefore not to be appropriate. Consequently, deploying the anionic thiomer polycarbophil-cysteine conjugate, with which the oral bioavailability of insulin was significantly increased (8), was not possible.

Apart from these considerations, however, chitosan was chosen as drug carrier matrix also for other reasons such as its mucoadhesive and permeation enhancing properties. Kashima *et al.* (19), for instance, achieved a significantly increased oral bioavailability of elcatonin incorporated in nanospheres by coating them with chitosan in order to improve the mucoadhesive properties of the delivery system. Similar results were obtained by Takeuchi *et al.* (4) with orally administered liposomes, containing a model peptide drug, after coating them with chitosan. As the novel mucoadhesive excipient chitosan-TBA conjugate is characterized by more than 10-fold higher mucoadhesive properties compared to unmodified chitosan (11), even a more pronounced effect could be expected by the use of this novel chitosan derivative. Because of its mucoadhesiveness the chitosan-TBA conjugate should be able to provide an intimate contact between the intestinal mucosa and the polymeric matrix system. This on its part should avoid a degradation of the embedded drug on the way between the carrier system and the absorption membrane. The potential of such systems, however, has a "natural" limitation by the mucus turn over determined to be between 47–270 min in rats (20) and 12–24 h in humans (21,22).

To guarantee a protective effect for salmon calcitonin also within the polymeric carrier matrix toward penetrating luminally secreted proteases (12), chitosan-enzyme inhibitor conjugates were added. The efficacy of the combination of thiolated polymers with polymer-enzyme inhibitor conjugates has already been demonstrated in another *in vivo* study (8).

The permeation enhancing properties of chitosan and its derivatives (23,24), on the other hand, should also contribute to an improved oral bioavailability of peptide drugs (25). Trimethylchitosan, for instance, is an excipient with permeation enhancing features of practical relevance for oral delivery of peptides (26,27). Recently, it could be shown that the permeation enhancing effect of chitosan can be significantly improved by the covalent attachment of thiol groups on the polymer (28).

In one of the following studies, glutathione was shown to mediate this permeation enhancing effect of thiomers, leading to an even more pronounced drug uptake through excised intestinal tissue (13). The further increase in the efficacy of the salmon calcitonin delivery system by the addition of the permeation mediator glutathione provides additional evidence for this theory. The usefulness of the thiomer/GSH system for permeation enhancement has been demonstrated also in other *in vivo* studies. Kast *et al.*, for instance, achieved a relative oral bioavailability of low molecular weight heparin of 19% using a carrier system based on thiolated polycarbophil containing GSH, whereas using unmodified polycarbophil without GSH did not lead to a significant amount of heparin in the plasma (9).

CONCLUSION

Within the present study a mucoadhesive delivery system has been generated, which might be useful for salmon calcitonin delivery by the oral route. The thiolated polymer chitosan-TBA was combined with minor amounts of polymer-inhibitor conjugates and the permeation mediator GSH. This combination has led to a multifunctional dosage form displaying following advantages:

(a) Because of the high stability and of the cohesive properties of thiolated chitosan, a sustained drug release can be achieved. This result and the mucoadhesiveness of the thiomer, which should lead to a prolonged residence time of the formulation on the mucosa, explain to some extent the increased absorption rate of the incorporated drug.

(b) The chitosan-enzyme-inhibitor conjugates can guarantee a protective effect for salmon calcitonin against the most abundant intestinal proteases. The enzymatic digestion of the peptide within the carrier system should be strongly limited because of the addition of these excipients.

(c) The strong permeation enhancing effect of the thiolated polymer/GSH system for hydrophilic compounds leads to an improved drug uptake from the intestine.

The combination of these mentioned effects and advantages has led to the encouraging *in vivo* results shown here, making the dosage form described within this study a promising formulation for oral salmon calcitonin delivery.

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REFERENCES

1. Y.–H. Lee and P. J. Sinko. Oral delivery of salmon calcitonin. *Adv. Drug Deliv. Rev.* **42**:225–238 (2000).
2. M. Torres–Lugo and N. A. Peppas. Transmucosal delivery systems for calcitonin: a review. *Biomaterials* **21**:1191–1196 (2000).
3. A. Bernkop–Schnürch. The use of inhibitory agents to overcome the enzymatic barrier to perorally administered therapeutic peptides and proteins. *J. Cont. Rel.* **52**:1–16 (1998).
4. H. Takeuchi, H. Yamamoto, T. Niwa, T. Hino, and Y. Kawashima. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. *Pharm. Res.* **13**:896–901 (1996).
5. E. Mathiowitz, J. S. Jacob, Y. S. Jong, G. P. Carino, D. E. Chickering, P. Chaturvedi, C. A. Santos, K. Vijayaraghavan, S. Montgomery, M. Bassett, and C. Morrell. Biologically erodable microspheres as potential oral drug delivery systems. *Nature* **386**:410–414 (1997).
6. A. Rubinstein, B. Tirosh, M. Baluom, T. Nassar, A. David, I. Gliko–Kabir, R. Radai, and M. Friedman. The rationale for peptide drugs delivery to the colon and the potential of polymeric carriers as effective tools. *J. Cont. Rel.* **46**:59–73 (1997).
7. A. Bernkop–Schnürch, G. H. Schwarz, and M. Kratzel. Modified mucoadhesive polymers for the peroral administration of mainly elastase degradable therapeutic (poly)peptides. *J. Cont. Rel.* **47**: 113–121 (1997).
8. M. K. Marschütz, P. Caliceti, and A. Bernkop–Schnürch. Design and in vivo evaluation of an oral delivery system for insulin. *Pharm. Res.* **17**:1468–1474 (2000).
9. C. E. Kast, D. Guggi, N. Langoth, and A. Bernkop–Schnürch. Development and in vivo evaluation of an oral delivery system for low molecular weight heparin based on thiolated polycarbophil. *Pharm. Res.* **20**:931–936 (2003).
10. A. Bernkop–Schnürch, V. Schwarz, and S. Steininger. Polymers with thiol groups: a new generation of mucoadhesive polymers? *Pharm. Res.* **16**:876–881 (1999).
11. A. Bernkop–Schnürch, M. Hornof, and T. Zoidl. Thiolated polymers–Thiomers: modification of chitosan with 2-iminothiolane. *Int. J. Pharm.* **260**:229–237 (2003).
12. D. Guggi and A. Bernkop–Schnürch. In vitro evaluation of polymeric excipients protecting calcitonin against degradation by intestinal serine proteases. *Int. J. Pharm.* **252**:187–196 (2003).
13. A. E. Clausen, C. E. Kast, and A. Bernkop–Schnürch. The role of glutathione in the permeation enhancing effect of thiolated polymers. *Pharm. Res.* **19**:602–608 (2002).
14. M. K. Marschütz and A. Bernkop–Schnürch. Oral peptide drug delivery: polymer-inhibitor conjugates protecting insulin from enzymatic degradation in vitro. *Biomaterials* **21**:1499–1507 (2000).
15. M. Baluom, D. I. Friedman, and A. Rubinstein. Absorption enhancement of calcitonin in the rat intestine by carbopol-containing submicron emulsions. *Int. J. Pharm.* **154**:235–243 (1997).
16. Y. Watanabe, M. Y., A. Kubomura, M. Kiriyama, N. Utoguchi, and M. Matsumoto. Studies of drug delivery systems for therapeutic agent used in osteoporosis. I. Pharmacodynamic (hypocalcemic effect) of elcatonin in rabbits following rectal administration of hollow-type suppositories containing elcatonin. *Biol. Pharm. Bull.* **21**:1187–1190 (1998).
17. A. Bernkop–Schnürch, S. Scholler, and R. G. Biebel. Development of controlled drug release systems based on thiolated polymers. *J. Cont. Rel.* **66**:39–48 (2000).
18. J. F. Sigurjónsdóttir, T. Loftsson, and M. Másson. Influence of cyclodextrins on the stability of the peptide salmon calcitonin in aqueous solution. *Int. J. Pharm.* **186**:205–213 (1999).
19. Y. Kawashima, H. Yamamoto, H. Takeuchi, and Y. Kuno. Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin. *Pharm. Dev. Technol.* **5**:77–85 (2000).
20. C. M. Lehr, F. G. J. Poelma, H. E. Junginger, and J. J. Tukker. An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop. *Int. J. Pharm.* **70**:235–240 (1991).
21. J. F. Forstner. Intestinal mucins in health and disease. *Digestion* **17**:234–263 (1978).
22. A. Allen, D. A. Hutton, J. P. Pearson, and L. A. Sellers. Mucus glycoprotein structure, gel formation and gastrointestinal mucus function. *Ciba Found. Symp.* **109**:137–156 (1984).
23. N. G. M. Schipper, K. M. Varum, P. Stenberg, G. Ocklind, H. Lennernäs, and P. Artursson. Chitosans as absorption enhancers for poorly absorbable drugs. 3: Influence of mucus on absorption enhancement. *Eur. J. Pharm. Sci.* **8**:335–343 (1999).
24. G. Ranaldi, I. Marigliano, I. Vespignani, G. Perozzi, and Y. Sambuy. The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line(1). *J. Nutr. Biochem.* **13**:157–167 (2002).
25. O. Felt, P. Buri, and R. Gurny. Chitosan: a unique polysaccharide for drug delivery. *Drug Dev. Ind. Pharm.* **24**:979–993 (1998).
26. H. L. Luessen, B. J. de Leeuw, M. W. Langemeyer, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. Mucoadhesive polymers in peroral peptide drug delivery. VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in vivo. *Pharm. Res.* **13**:1668–1672 (1996).
27. M. Thanou, J. C. Verhoef, J. H. Verheijden, and H. E. Junginger. Intestinal absorption of octreotide using trimethy chitosan chloride: studies in pigs. *Pharm. Res.* **18**:823–828 (2001).
28. A. Bernkop–Schnürch, U. M. Brandt, and A. E. Clausen. Synthesis and in vitro evaluation of chitosan-cysteine conjugates. *Sci. Pharm.* **67**:196–208 (1999).