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Research paper

Thiolated chitosan: Development and in vivo evaluation of an oral delivery system for leuprolide

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ABSTRACT

The aim of the present study was to develop an oral delivery system for the peptide drug leuprolide. Gel formulations based on unmodified chitosan/reduced glutathione (GSH) and chitosan–thioglycolic acid (chitosan–TGA)/GSH were prepared, and their effect on the absorption of leuprolide was evaluated in vitro and in vivo in male Sprague Dawley rats. Transport studies were performed with freshly excised rat intestinal mucosa mounted in Ussing-type chambers. Due to the addition of gel formulations comprising 0.5% (m/v) unmodified chitosan/0.5% (m/v) GSH and 0.5% (m/v) chitosan–TGA/0.5% (m/v) GSH, the transport of leuprolide across excised mucosa was improved up to 2.06-fold and 3.79-fold, respectively, in comparison with leuprolide applied in buffer ($P_{app} = 2.87 \pm 0.77 \times 10^{-6}$ cm/s).

In vivo, the addition of oral gel formulation comprising 8 mg of unmodified chitosan, 1 mg of GSH and 1 mg of leuprolide increased the area under the plasma concentration–time curve (AUC_{0-8}) of leuprolide 1.39-fold in comparison with leuprolide having been administered just in saline. Moreover, the administration of oral gel formulation comprising 8 mg of chitosan–TGA, 1 mg of GSH and 1 mg of leuprolide resulted in a further enhanced leuprolide plasma concentration, and the area under the plasma concentration–time curve (AUC_{0-8}) of leuprolide was increased 3.72-fold in comparison with the control. With the oral gel formulation comprising 8 mg of chitosan–TGA, a relative bioavailability (versus s.c. injection) of 4.5% was achieved in contrast to the control displaying a relative bioavailability of 1.2%. Thus, according to the achieved results, it is suggested that chitosan–TGA in combination with GSH is a valuable tool for improving the oral bioavailability of the peptide drug leuprolide.

1. Introduction

Administration of therapeutic peptide and protein drugs still remains a challenge to the pharmaceutical industry. Due to their poor oral bioavailability most protein and peptide drugs are currently administered via parenteral routes, which is often painful and inconvenient. Thus, development of non-invasive delivery systems for these hydrophilic macromolecules is strongly on demand.

Leuprolide acetate is a synthetic nanopeptide and a potent agonist of the luteinizing hormone-releasing hormone (GnRH or LH–RH) receptor. It is one of the most widely used agents for the non-surgical hormonal treatment of advanced prostate cancer and also found to be effective in the treatment of non-cancer indications and hormone-dependent diseases such as endometriosis, uterine fibroids and central precocious puberty in children [1,2]. Like most other peptide and protein drugs, leuprolide has a poor permeability across intestinal epithelia which results in a very low oral bioavailability of around 1–2% depending on the animal species. Rapid degradation of leuprolide by chymotrypsin and rat intestinal mucosa homogenates has already been reported [3,4]. Due to low oral bioavailability, leuprolide is currently administered via subcutaneous, intramuscular and intranasal routes [5]. Undoubtedly, oral route is the most convenient and efforts are under way to identify and rectify the absorption problems associated with the GI-tract. Ping et al., for instance, investigated the transport properties, mechanisms and causes of low bioavailability of leuprolide and demonstrated that the peptide followed the passive diffusion (paracellular) pathway, and transport of leuprolide was improved by the addition of chitosan, EDTA and trypsin inhibitor [6]. These results suggest that the opening of tight junctions and the use of enzyme inhibitory agents would be the key factors to improve the intestinal absorption of leuprolide.

Over the past few years, thiolated polymers or so-called thiomers have appeared as a promising excipient for the delivery of drugs including peptides [7–9]. Due to the formation of inter- and intramolecular disulphide bonds within thiomers or with mucus glycoproteins, they display mucoadhesive properties, prolonged disintegration time and a comparatively more controlled release of incorporated drugs. Furthermore, thiolated

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polymers particularly in combination with reduced glutathione (GSH) were shown to improve the uptake of hydrophilic macromolecules from the GI-tract [10]. The mechanism responsible for the permeation-enhancing effect of thiomers seems to be based on the inhibition of protein tyrosine phosphatase, being involved in the closing process of tight junctions, via a GSH-mediated mechanism [11]. Because of their high molecular mass, thiomers are not absorbed from the GI-tract and remain therefore located in the GI-tract at the site of drug absorption. Thus, systemic side effects can be excluded by the use of these thiolated polymeric excipients as drug carriers. To the best of our knowledge, to date, no data exist about the gel formulations based on chitosan derivatives (chitosan–TGA) for oral peptide delivery system. The use of mucoadhesive oral gel was thought to offer advantage of prolonged residence time of the dosage form at the site of absorption. Therefore, an enhanced oral bioavailability could be achieved resulting in many cases in a reduced dosing frequency and patient compliance. Consequently, thiolated chitosan/GSH gel formulation could be a valuable tool for oral delivery of various therapeutic peptides. It was therefore the aim of the present study to evaluate the effect of thiolated chitosan/GSH gel formulation for oral delivery of the poorly permeable peptide drug leuprolide.

2. Materials and methods

2.1. Materials

Leuprolide (H-4060) and internal standard (IS) (H-4070) were purchased from Bachem AG, Switzerland. Chitosan highly viscous ($\geqslant$400 Pa s, 500–700 kDa, degree of deacetylation 75–85%) was obtained from Fluka, Buchs, Switzerland. Sodium borohydride, L-glutathione reduced form (GSH), L-cysteine hydrochloride (anhydrous, minimum 98%), Ellman's reagent (DTNB, 5,5′-dithiobis (2-nitrobenzoic acid)), thioglycolic acid (TGA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and *N*-(2-hxdroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were obtained from Sigma-Aldrich, St. Louis, MO.

2.2. Synthesis of the chitosan–TGA

First, 500 mg of chitosan was hydrated in 4 mL of 1 M HCl and dissolved by the addition of demineralised water to obtain a 1% solution of chitosan hydrochloride. Thereafter, 500 mg of TGA was added. After TGA was completely dissolved in the chitosan hydrochloride solution, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) was added in a final concentration of 125 mM in order to activate the carboxylic acid moieties of TGA. The reaction mixture was incubated at pH 5 for 3 h at room temperature under stirring. Sample prepared in exactly the same way but omitting EDAC during the coupling reaction served as a control for the analytical studies [12].

In order to eliminate unreacted TGA and to isolate the polymer conjugates, the reaction mixtures were dialysed five times in tubings (molecular weight cut-off 12 kDa; dialysis tubings, cellulose membrane; Sigma, St. Louis, MO) for 3 days in total at 10 °C in the dark. In detail, the polymer conjugates were dialysed one time against 5 mM HCl, then two times against the same medium but containing 1% NaCl to quench ionic interactions between the cationic polymer and the anionic sulfhydryl compound. Finally, the polymer conjugates were dialysed exhaustively two times against 1 mM HCl to adjust the pH of the polymer solutions to 4. Thereafter, chitosan–TGA conjugate and control were lyophilised by drying frozen aqueous polymer solutions at −70 °C and 0.01 mbar (VirTis benchtop 6 K freeze dryer; NY, USA) and stored at 4 °C until further use.

2.3. Determination of thiol group content

The amount of thiol groups immobilized on chitosan was spectrophotometrically determined with Ellman's reagent. First, 0.5 mg of each of the modified and unmodified polymer conjugates was hydrated in 250 μL of demineralised water separately. Then, 250 μL of 0.5 M phosphate buffer (pH 8.0) and 500 μL of Ellman's reagent (3 mg in 10 mL of 0.5 M phosphate buffer pH 8.0) were added. The samples were incubated for 3 h at room temperature. The supernatant was separated from the precipitated polymers by centrifugation (24,000*g*, 5 min). Thereafter, 300 μL of the supernatant was transferred to a microtitration plate, and the absorbance was measured at a wavelength of 450/620 nm with a microtitration-plate reader (Tecan infinite M200 spectrophotometer, Grödig, Austria). TGA standards were used to calculate the amount of thiol groups immobilised on the polymer [7].

2.4. Formation of disulphide bonds

Disulphide content was measured after the reduction with $NaBH_4$ and addition of 5,5′-dithiobis(2-nitrobenzoic acid). To determine the total amount of bound thiol functions, 0.5 mg of the polymer conjugates was hydrated in 350 μL of demineralized water and then 650 μL of 0.05 M phosphate buffer (pH 6.8) was added. After a swelling process of 30 min, 1 mL of a freshly prepared 4% (m/v) solution of sodium borohydride was added to the polymer suspensions. The mixtures were incubated for 1 h in an oscillating water bath at 37 ± 0.5 °C. Thereafter, 200 μL of 5 M HCl was added, and the reaction mixtures were agitated for 10 min in order to destroy the remaining sodium-borohydride. The solutions were neutralised by the addition of 1 mL 1 M phosphate buffer (pH 8.5) and 100 μL of 0.4% (m/v) Ellman's reagent dissolved in 0.5 M phosphate buffer (pH 8.0) were immediately added. After incubation for 1 h at room temperature, aliquots of 250 μL were transferred to a microtitration plate and the absorbance was measured at 450 nm with a microtitration-plate reader (Tecan infinite M200 spectrophotometer, Grödig, Austria). The quantity of bound TGA was calculated using a standard curve obtained by the thiol group determination of a series of solutions containing increasing concentrations of L-cysteine hydrochloride. The amount of disulphide bonds was calculated by subtracting the quantity of free thiol groups from the total thiol moieties present on the polymer conjugates [7].

2.5. Tensile studies

The adhesive strength of gel formulations based on (0.5% m/v) chitosan–TGA and (0.5% m/v) unmodified chitosan was evaluated using excised porcine intestinal mucosa by applying some modifications in a previously described method [13]. Briefly, porcine mucosa was glued to a glass slide (thickness 1.0 mm, dimensions of 26 × 76 mm) using a cyanoacrylate adhesive and hung from a laboratory stand with a nylon thread (15 cm). Then 10 mg of the gel was uniformly spread over another segment of porcine mucosa, already glued to a glass platform and placed on a balance. Mobile platform was then carefully raised until the gel layer came in contact with the hanging mucosal segment. The contact was determined when the nylon thread holding the glass slide became slightly bent. After a contact time of 20 min, the mobile platform was pulled down from upper mucosal segment at a rate of 0.1 mm/s. Data points were collected every second by computer software (SartoCollect V 1.0; Satorius AG, Germany) linked to the balance with integrated interface. Data were transferred to EXCEL 2007 (Microsoft, USA), and the force versus displacement curves were analysed to calculate the maximum force of detachment

(MDF) and the total work of adhesion as the area under the curve (AUC) in accordance with the trapezoidal rule.

2.6. Rheological measurements

The viscoelastic properties of gels based on chitosan–TGA and unmodified chitosan were determined with a cone-plate rheometer (RotoVisco RT20, Haake, Karlsruhe, Germany). Briefly, chitosan–TGA conjugate and the corresponding unmodified chitosan were hydrated in 1% (v/v) acetic acid at 37 °C to give final concentration of 0.5% (m/v). Aliquots of 1000 μL were transferred to a cone-plate viscometer. Rheological measurements were performed within the linear viscoelasticity region of the gels, and viscoelastic properties such as elastic modulus (G'), the viscous modulus (G'') and the dynamic viscosity (η) were determined at a frequency of 1.0 Hz [14].

2.7. In vitro transport studies

In vitro transport studies were carried out in low volume vertical Ussing/Diffusion chambers (Hugo Sachs Elektronik – Harvard Apparatus GmbH). The volume capacity of each side of the Ussing chamber (apical or basolateral) was 1 mL with a permeation area of 0.64 cm^2. To mimic the intestinal fluid, an incubation medium was prepared containing 250 mM NaC1, 2.6 mM $MgSO_4$, 10 mM KC1, 40 mM glucose and 50 mM $NaHCO_3$ buffered with 40 mM HEPES, pH 7.2.

Non-fasting male Sprague Dawley rats weighing 200–250 g were used for transport studies. After sacrificing by cervical dislocation, the distal ileum (20 cm) was removed immediately and preserved in 200 mL of 0.9% (m/v) solution of NaCl. The tissue was cut into strips of 1.5–2 cm, rinsed free of luminal contents and mounted in Ussing chambers without stripping off the underlying muscle layer. All experiments were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C [15]. After 30 min of pre-incubation with the artificial intestinal fluid, the media of the donor compartment was substituted either by the permeation-enhancing mediator GSH (0.5% m/v; 16.27 μmol/mL), chitosan–TGA conjugate (0.5% m/v; 0.01 μmol/mL) with GSH (0.5% m/v; 16.27 μmol/mL) or by the corresponding unmodified polymer (0.5% m/v; 0.01 μmol/mL) with GSH (0.5% m/v; 16.27 μmol/mL). Leuprolide was used in a final concentration of 13.46 μmol/mL (0.05% m/v). Aliquots of 100 μL were withdrawn from the acceptor compartment every 60 min over a period of 3 h. Samples were immediately replaced by 100 μL of artificial intestinal fluid pre-equilibrated at 37 °C. The amount of permeated leuprolide was determined using HPLC. The HPLC system was consisted of a HPLC pump L-2130 (Merck–Hitachi, Darmstadt, Germany), a Merck–Hitachi autosampler L-2200, a column thermostat (25 °C), a Vydac protein and peptide C18 5 μm, 4.6 d 50 mm column (Grace Davison Discovery Sciences, USA) with a pre-column and a diode array detector (DAD) L-2450 from Merck–Hitachi at 220 nm. The composition of the mobile phase was acetonitrile and 0.1% trifuroacetic acid (30:70) delivered isocratically at a flow rate of 1 mL/min [6]. The injection volume was 80 μL. The lower limit of quantification (LLOQ) was identifiable and reproducible at 1 μg/mL for leuprolide. Cumulative corrections were made for previously removed samples. The apparent permeability coefficients (P_{app}) for leuprolide were calculated in accordance with the following equation:

$$P_{app} = Q/(Act)$$

where P_{app} is the apparent permeability coefficient (cm/s), Q is the total amount of leuprolide permeated within the incubation time (μg), A is the diffusion area of the Ussing chamber (cm^2), c is the initial concentration of leuprolide in the donor compartment ($\mu g/cm^3$), and t is the total time of the experiment (s). Transport enhancement ratios (R) were calculated from P_{app} values according to the following equation:

$$R = P_{app}(\text{chitosan or chitosan} - \text{TGA/GSH})/P_{app}(\text{buffer control})$$

2.8. Measurement of the transepithelial electrical resistance (TEER)

A Millicell® ERS meter (Millipore Corp., Bedford, MA) connected to a pair of side-by-side electrodes was used to monitor the effect on the TEER of the intestinal mucosa. Measurements were performed every 5 min before applying the test compounds and then every 30 min within 3 h.

2.9. Histological study

The viability of the rat intestinal mucosa by trypan blue treatment was evaluated as previously described by our research group [16].

2.10. In vivo evaluation of the delivery system with rats

The protocol for the in vivo studies on animals was approved by the Animal Ethical Committee of Vienna, Austria and adheres to the Principles of Laboratory Animal Care. In vivo studies were performed on male Sprague Dawley rats weighing 200–250 g. For the in vivo evaluation, 25 rats were used. The rats were divided into 5 groups of 5 animals in each group. Detail about the composition of different dosage forms is illustrated in Table 2.

Rats were fasted 2 h before the administration of all dosage forms and then for additional 2 h during the experiments but had free access to water. Oral gel formulations were administered through a stomach tube with a blunt and round tip. Intravenous solutions were injected into one of the tail veins. About 150 μL of blood samples was taken via the tail vein at 0.25, 0.5, 1, 1.5, 2, 4, 6 and 8 h after the administration of the dosage forms.

2.11. Analytical evaluation

Fresh blood samples from rats were spiked with 3.8% sodium citrate. After the centrifugation, plasma was collected and stored at −80 °C till further analysis. The concentration of leuprolide was determined using LC–MS as described below.

2.11.1. Preparation of standards and calibration curve

Stock solution of both leuprolide (1 ng/mL – 100 μg/mL) and IS (75 ng/mL) was prepared in 50% acetonitrile and 50% water with 0.1% formic acid. Calibration curves (0.5 ng/mL – 2000 ng/mL) were prepared freshly in rat plasma by adding 20 μL of leuprolide and 20 μL of IS to 100 μL of plasma.

2.11.2. Sample preparation

IS was added to 100 μL of rat plasma before the extraction procedure. Plasma proteins were precipitated by the stepwise addition of 400 μL of ice-cold acetonitrile, followed by a brief vortexing and centrifugation at 12,000 g for 8 min. The supernatant was transferred into glass vials and evaporated in SC210A SpeedVac® Plus – Thermo Savant coupled with RVT400 Refrigerated Vapor Trap – Thermo Savant for 60 min at 45 °C. An aliquot of 125 μL of mobile phase (95% A: 10% acetonitrile, 90% water, 2 mM ammonium acetate, 0.1% formic acid and 5% B: 90% acetonitrile, 10% water, 2 mM ammonium acetate, 0.1% formic acid) was added to each sample, vortexed and transferred to glass vials for the analysis by LC–MS [17].

2.11.3. LC–MS analysis and quantification

HPLC was performed with an Agilent 1200 Series system (Agilent Technologies, Waldbronn, Germany) equipped with a

Table 1
Comparison of elastic modulus (G'), the viscous modulus (G''), dynamic viscosity (η'), total work of adhesion (TWA) and maximum detachment force (MDF of 0.5% (m/v) solutions of chitosan–TGA conjugate and unmodified chitosan (means ± SD, $n = 3$).

Tested polymers	G' (Pa)	G'' (Pa)	η'(Pa s)	TWA (μJ)	MDF (mN)
Unmodified chitosan (0.5%; m/v)	77 ± 28	39 ± 8	14 ± 5	130 ± 13	87 ± 10
Chitosan–TGA (0.5%; m/v)	1931 ± 208	142 ± 76	308 ± 33	225 ± 25	208 ± 12

G1312B SL binary pump, G1329B autosampler, vacuum degasser and G1316B temperature-controlled column compartment. The mobile phase consisted of solvents A: 10% acetonitrile, 90% water, 2 mM ammonium acetate, 0.1% formic acid and B: 90% acetonitrile, 10% water, 2 mM ammonium acetate, 0.1% formic acid. A Vydac protein and peptide C18 5 μm, 4.6 d 50 mm column (Grace Davison Discovery Sciences, USA) was used at a flow rate of 1.0 mL/min (with a split to give 0.3 mL/min to the mass spectrometer). A gradient (run time 25 min) from 95% A – 5% B to 50% A – 50% B over 12 min, and 50% A – 50% B up to 15 min was used for the separation of leuprolide and IS. Typical injection volume was 40 μL. Mass spectrometry was performed with Bruker MicoOTOF-Q II system. The instrument was operated in positive ion mode under the following conditions: end plate offset, −500 V; capillary voltage, −4500 V; nebulizer pressure, 29 psi; dry gas (nitrogen) flow rate, 6.0 L/min; dry temperature, 200 °C; funnel 1 RF, 300 Vpp; funnel 2 RF, 400 Vpp; ISCID energy, 0 eV; hexapole RF, 500 Vpp; ion energy, 6.0 eV; collision energy, 10.0 eV; collision RF, 600 Vpp; transfer time, 85 μs; pre pulse storage, 12.0 μs; mass range, 50–1500 m/z. Quantification was performed in the positive mode and the extracted ion chromatogram with m/z 584.5 ± 0.5 at 13.0–13.1 min and 605.4 ± 0.5 at 14.8–14.9 min was detected and integrated after background subtraction of IS and leuprolide for the quantification, respectively. The lower limit of quantification was established as 0.08 ng/mL for leuprolide.

2.12. Pharmacokinetic and statistical data analysis

The pharmacokinetic parameters of leuprolide after intravenous, subcutaneous or oral administration were obtained by applying a non-compartmental pharmacokinetic analysis to the plasma concentration–time data using the software, OriginPro 7G SR4 version 7.0552. The area under the concentration versus time curve up to the last measured time (AUC_{0-last}) was calculated according to the linear trapezoidal rule, using kinetic data collected from individual values. The absolute as well as relative bioavailability was calculated from the dose corrected areas under the curves for oral versus intravenous or subcutaneous administration.

Statistical data analyses were performed using the Student's t-test with $p < 0.05$ as the minimal level of significance. All values were expressed as the means ± SD.

3. Results and discussions

3.1. Synthesis and characterisation of chitosan–TGA conjugate

Determination of the thiol groups attached to the polymer by the Ellman's test demonstrated that on average 650.75 ± 123.41 μmol of thiol groups were immobilized per gram of chitosan–TGA conjugate. The amount of inter and/or intra molecular disulphide bonds was 19.63 μmol per gram of chitosan–TGA conjugate. The efficacy of the purification method for the resulting chitosan–TGA conjugate was verified by control which was prepared in exactly the same way as the chitosan–TGA conjugate but omitting EDAC during the coupling reaction, exhibiting a 28.55 ± 7.64 μmol of thiol groups per gram of the control polymer. The obtained polymers appeared as white, odourless powder of fibrous structure. For all experiments, the fibrous structured lyophilizates were used and no pulverisation of the products was carried out. The modified polymer was easily swellable in aqueous solutions below pH 5 and formed transparent gel of high viscosity in 1% (v/v) acetic acid solution. The lyophilised conjugates were stored at 4 °C and found stable towards air oxidation during the course of the study.

3.2. Tensile studies

The results of the tensile studies of gels are shown in Table 1. Results showed that the thiolation process greatly improves the mucoadhesive properties of chitosan. In comparison with unmodified chitosan, gels based on chitosan–TGA exhibit 2.7- and 3.0-fold higher TWA and MDF, respectively. It is believed that thiolated polymers interact with cysteine-rich sub-domains of mucus glycoproteins either via thiol/disulphide exchange reactions or a simple oxidation of free thiol groups thereby resulting in improved mucoadhesion and prolonged residence time.

Table 2
HPLC profile read-outs (mAU), actual calculated concentrations (μg/mL) and the in vitro apparent permeability coefficients (P_{app}) of leuprolide in presence of indicated test compounds and improvement ratios (means ± SD, $n = 3$).

Test compound (concentration)	HPLC profile read-outs (mAU)	Actual calculated concentrations of leuprolide (μg/mL)	P_{app} (cm/s) × 10^{-6}	Improvement ratio
Buffer	112,62,117 98,11,395 67,05,758	9.91 ± 1.69	2.87 ± 0.77	–
GSH (0.5% m/v)	141,81,216 158,97,394 124,31,216	14.17 ± 1.33	4.10 ± 0.50	1.43
Unmodified chitosan (0.5% m/v)	193,75,756 207,89,685 177,96,242	20.47 ± 1.36	5.92 ± 0.51	2.06
Chitosan–TGA conjugate[a] (0.5% m/v)	258,56,937 331,90,365 414,49,481	37.58 ± 6.46	10.89 ± 1.81	3.79

[a] Difference from buffer control $p < 0.0001$.

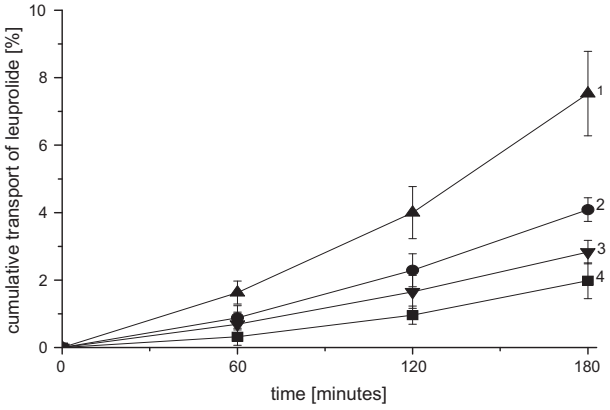

Fig. 1. In vitro transport data of leuprolide across rat intestinal mucosa in the absorptive direction in absence of polymer (■), with GSH (0.5% m/v) (▼), with unmodified chitosan (0.5% m/v) (●) and with chitosan–TGA conjugate (▲). Transport data (means ± SD, $n = 3$) are expressed as percentage of the total dose of leuprolide applied to the apical side of mucosa. 1 differs from 2, 3 and 4, $p < 0.0001$.

3.3. Rheological measurements

Oscillatory measurements are believed to provide useful information about the dynamic properties, the elastic modulus (G') and the viscous modulus (G'') of the investigated gels. Oscillatory measurements were performed within the linear viscoelasticity region. Results are mentioned in Table 1. It is clearly seen from the results, thiolation process greatly improves the viscoelastic properties of chitosan. Consequently, in comparison with unmodified chitosan, gel based on chitosan–TGA exhibits 25-, 3.6- and 22-fold improved G', G'' and η', respectively.

3.4. In vitro transport studies

The influence of 0.5% (m/v) unmodified chitosan in combination with 0.5% (m/v) permeation-enhancing mediator reduced glutathione (GSH) and 0.5% (m/v) chitosan–TGA along with 0.5% (m/v) GSH on the transport of leuprolide was evaluated across freshly excised rat intestinal mucosa mounted in Ussing-type chambers. The tissue was not muscle-stripped in order to avoid the time delay that might affect the tissue viability and to compare results with previous studies [10,11,14]. Leuprolide was used in a final concentration of 0.05% (m/v) to perform transport studies. Results of the in vitro transport studies are shown in Fig. 1 and Table 2. Within this study, it could be demonstrated that due to the addition of 0.5% (m/v) GSH to the buffer, transport of leuprolide was 1.43-fold (14.17 ± 1.33 μg/mL) improved, in comparison with leuprolide (9.91 ± 1.69 μg/mL) applied just in buffer. Moreover, due the addition of 0.5% (m/v) unmodified chitosan and 0.5% (m/v) GSH to the buffer, transport of leuprolide was 2.06-fold (20.47 ± 1.36 μg/mL) improved, in comparison with leuprolide applied just in buffer. Furthermore, the gel formulation comprising 0.5% (m/v) chitosan–TGA and 0.5% (m/v) GSH improved the transport of leuprolide 3.79-fold (37.58 ± 6.46 μg/mL) in comparison with the buffer control.

Former permeation studies demonstrated that GSH alone showed only a slight enhancement in the permeation of hydrophilic compounds. In contrast, use of GSH in combination with thiomers was attributed to a significantly improved permeation-enhancing effect. For instance, Clausen et al. demonstrated that a combination of 0.4% (m/v) GSH with 0.5% (m/v) polycarbiphil-cysteine (PCP-cys) leads to an improved paracellular transport of sodium fluorescein (NaFlu) up to 2.93-fold [11]. These observations could be confirmed by the results achieved during this study where due to the addition of 0.5% (m/v) GSH to the buffer; transport of leuprolide was only 1.43-fold improved, whereas the gel formulation comprising 0.5% (m/v) chitosan–TGA and 0.5% (m/v) GSH improved the transport of leuprolide 3.79-fold in comparison with the buffer control.

Previous studies reported that chitosan enhances epithelial permeability through the opening of tight junctions between epithelial cells [18]. Moreover, the covalent attachment of thiol groups to polymers greatly improves their mucoadhesive and permeation properties without affecting biodegradability [19,20]. The proposed mechanism of permeation enhancement by thiomers seems to be based on inhibition of the enzyme protein tyrosine phosphatase (PTP) via formation of disulphide bonds among the cysteine residues of PTP and intestinal GSH [21]. This results in an increased number of phosphorylated tyrosine groups on the extracellular loops of the membrane protein occludin, leading to the opening of the tight junctions. Glutathione is known to be present in its reduced (GSH) and oxidised (GSSG) form at the apical side of mucosa and it is believed

Table 3
Composition of the dosage forms used for in vivo studies in male Sprague Dawley rats weighing 200–250 g.

	Test formulation A (oral gel)	Test formulation B (oral gel)	Control A (oral solution)	Control B (i.v. injection)	Control C (s.c. injection)
Leuprolide	1.0 mg	1.0 mg	1.0 mg	1.0 mg	1.0 mg
Unmodified chitosan	8.0 mg	–	–	–	–
Chitosan–TGA	–	8.0 mg	–	–	–
GSH	1.0 mg	1.0 mg	–	–	–
Medium	1% (v/v) Acetic acid	1% (v/v) Acetic acid	0.9% (m/v) NaCl	0.9% (m/v) NaCl	0.9% (m/v) NaCl
Volume	1000 μL	1000 μL	500 μL	500 μL	500 μL

Table 4
Main pharmacokinetic parameters calculated after oral administration of the solution, the unmodified chitosan/GSH (oral gel), the chitosan–TGA/GSH (oral gel), the intravenous administration and the subcutaneous administration of leuprolide to rats (means ± SD, $n = 5$). Absolute and relative bioavailability were calculated with reference to intravenous injection (control B) and subcutaneous injection (control C), respectively. a, b and c differs from d, e and f $p = 0.0001$.

	Test formulation A (oral gel)	Test formulation B (oral gel)	Control A (oral solution)	Control B[a] (i.v. injection)	Control C[b] (s.c. injection)
AUC_{0-8} (h μg/mL)	0.025 ± 0.012	0.067 ± 0.034	0.018 ± 0.004	5.798 ± 3.073	1.481 ± 0.125
c_{max} (μg/mL)	0.023	0.09[a]	0.009[d]	18.91	0.87
t_{max} (h)	0.5–1	1	1	–	0.5–1
Absolute bioavailability[a] (%)	0.43	1.15[b]	0.3[e]	–	25.54
Relative bioavailability[b] (%)	1.688	4.517[c]	1.215[f]	–	–

*Absolute bioavailability was calculated versus i.v. injection (control B).
**Relative bioavailability was calculated versus s.c. injection (control C).

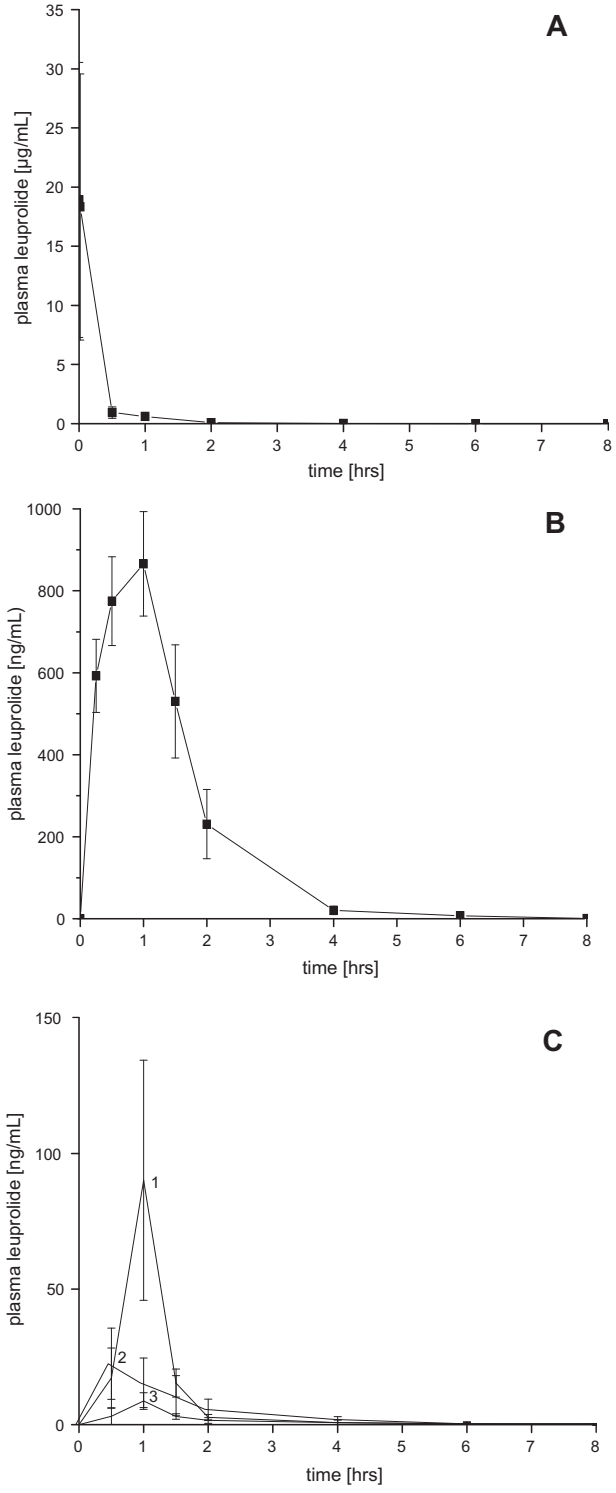

Fig. 2. (A) Plasma concentration of leuprolide after intravenous administration to rats (IV dose = 4 mg/kg). (B) Plasma concentration of leuprolide after subcutaneous administration to rats (SC dose = 4 mg/kg). (C) Plasma curves of leuprolide after oral administration of 1.0 mg leuprolide in 0.9% (m/v) solution of NaCl (■), in unmodified chitosan/GSH (oral gel) (●) and chitosan–TGA/GSH (oral gel) (▲). Blood samples were taken after 0.016, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 h. Indicated values are means (±SD, $n = 5$).

that immobilized thiol groups present on the surface of thiomers reduce the GSSG thereby raising the amount of GSH at the absorption area for PTP inhibition. The mechanism of permeation enhancement with thiomers via GSH is supported by a previous study in which (0.5% m/v) polycarbophil-cysteine in combination with (0.5% m/v) GSH significantly enhanced the transmucosal transport of sodium fluorescein and fluorescence labelled bacitracin across guinea pig duodenum 2.93-fold and 2.06-fold, respectively [11]. Furthermore, another previous study showed a 3-fold higher permeation-enhancing effect of the chitosan–TBA conjugate/GSH system in comparison with unmodified chitosan [22]. The transepithelial electrical resistance (TEER) of the intestinal tissue was determined before and after the transport studies to be 151 ± 17 and 143 ± 12 Ω cm^2, respectively. Results of TEER studies suggested that the effect of the thiolated polymer/GSH system on the intercellular junctions is reversible and does not lead to permanent cell damages. Histological studies performed before and after transport studies showed that the mucosa is not damaged and remains viable during transport studies (data not shown).

Taking all these aspects in mind, the combination of 0.5% (m/v) chitosan–TGA conjugate and 0.5% (m/v) GSH may be considered as potent permeation-enhancing excipient for paracellular permeation enhancement.

3.5. In vivo studies

In vivo studies were performed on male Sprague Dawley rats weighing 200–250 g. The details about different formulations that were tested in the present study are summarised in Table 3. Main pharmacokinetic parameters were calculated after intravenous, subcutaneous and oral administration of 1 mg of leuprolide in solution and as oral gel formulations comprising 1 mg of GSH along with either 8 mg of unmodified chitosan or with 8 mg of chitosan–TGA conjugate. These pharmacokinetic parameters are reported in Table 4. The plasma concentration–time profiles of leuprolide administered as subcutaneous injection or intravenously into the tail vein are shown in Fig. 2A and B, respectively. Leuprolide was found to be completely eliminated within 5 h following intravenous injection and within 8 h after subcutaneous injection. Plasma concentration–time curves of leuprolide given orally as solution or with the test formulations composed of oral gels containing GSH along with either unmodified chitosan or chitosan–TGA conjugate are presented in Fig. 2C. The peptide drug leuprolide was poorly absorbed after administration in the form of an oral solution with absolute bioavailability of 0.3% and an AUC_{0-8} of 0.018 ± 0.004 h µg/mL. The relative bioavailability of the oral solution of leuprolide was 1.2%. Administration of oral gel (test formulation A) containing 1 mg of leuprolide, 1 mg of GSH and 8 mg of unmodified chitosan resulted in a significant increase in the plasma concentration of leuprolide, and the AUC_{0-8} and c_{max} were 1.39-fold and 2.56 times improved, respectively. On the other hand, increases in absolute and relative bioavailability were 43% and 39%, respectively. The oral administration of gel formulation (test formulation B) containing 1 mg of leuprolide, 1 mg of GSH and 8 mg of chitosan–TGA conjugate resulted in a further enhanced leuprolide plasma concentration and improved bioavailability. The AUC_{0-8} and c_{max} were improved 3.72-fold and even 10-fold in comparison with the buffer control, respectively. Thus, absolute and relative bioavailability was improved by 283% and 272% in comparison with the buffer control, respectively.

Delivery of peptide drugs such as leuprolide still remains a challenge in pharmaceutical technology. Currently, leuprolide is administered via parenteral routes such as intravenous and subcutaneous injections. As this drug is intended for long-term treatment, easier routes of administration are strongly on demand in order to reduce inconvenience, pain and risks of complications for patients, such as severe inflammation on the application site. Patient compliance would clearly benefit from an oral application form as the oral route is usually preferred by the patients. Therefore, high requirements have to be met by an oral drug delivery system for the peptide drug. The peptide must be protected from denaturation in the harsh acidic environment of the stomach and digestion by proteolytic enzymes.

In order to reach systemic circulation, an intimate contact with the absorption membrane is advantageous for uptake driven by a steep concentration gradient and longer residence time. Taking all that in consideration, a suitable carrier system based on chitosan–TGA and GSH was established.

The major hurdle for orally administered peptide drugs is the enzymatic barrier before reaching the systemic circulation. It was therefore the aim to develop a formulation that can on the one hand protect the peptide drug from enzymatic degradation and on the other hand enhance its absorption. In order to deliver the drug to the absorption site in intact form and in high concentration, the use of oral gel formulations based on multifunctional mucoadhesive polymers, such as thiolated chitosan and thiolated polyacrylates, is an interesting approach as these multifunctional thiolated polymers offer advantages, such as mucoadhesive and cohesive properties, providing an intimate contact with the area of drug absorption and additionally, systemic adverse effects might be excluded, as thiolated polymers will remain in the gut due to their high molecular mass [7,9]. Apart from all that thiolated chitosan offers the advantages of long-term storage stability and being safe according to the toxicological point of view. As a permeation enhancer, thiolated chitosans were previously reported to use at least in a final concentration of 0.5% (m/v). This was confirmed by in vitro transport studies that showed a 3.79-fold improved transport of leuprolide in the presence of 0.5% (m/v) chitosan–TGA. On the basis of encouraging in vitro results, chitosan–TGA was used in a final concentration of 0.8% (m/v) for in vivo studies [23,24]. Furthermore, similar concentrations of thiolated polymers were already used in the previous in vivo studies. Moreover, several drug/excipients combinations were tested for in vivo studies, and a mixture of chitosan–TGA:GSH:leuproide (8:1:1) was chosen because of better viscoelastic and mucoadhesive properties and quantification on LCMS.

Results from in vivo pharmacokinetic studies indicate a 10-fold improved oral bioavailability of leuprolide when administered in the form of oral gel in comparison with leuprolide having been administered just in saline. In the presence of chitosan–TGA/GSH system, the bioavailability with 1.15% in comparison with most other orally given drugs is certainly low. It should be considered that the oral administration of peptide and protein drugs represents probably the most challenging route of application for this class of therapeutic agents because of their unfavourable physicochemical properties such as rapid enzymatic degradation, poor membrane permeability and large molecular size. Moreover, other peptides such as desmopressin are already on the market for more than 20 years with an oral bioavailability of even less than 0.5, Moreover, it was not enviable to use the oral delivery system based on thiolated chitosan/GSH for cancer treatment where constant release is vital. Rather, it was desirable to use the gel formulation based on chitosan–TGA/GSH for the treatment of endometriosis where pulsitile delivery of leuprolide (recommended intramuscular dose = 3.75 mg/month) corresponding to 1% oral bioavailability is sufficient to reach the effective plasma concentration (4.6–10.2 ng/mL) of leuprolide [25].

4. Conclusion

In the present study, an oral delivery system based on the combination of chitosan–TGA and GSH was evaluated in vitro as well as in vivo for the transport of leuprolide in rats. The oral delivery system based on chitosan–TGA and GSH demonstrate a promising potential and led to significantly enhanced the plasma level and increased bioavailability of leuprolide up to 10-fold. This indicates the therapeutic efficacy of the oral delivery system. It is suggested that improved uptake was most likely due to the permeation-enhancing and mucoadhesive properties of the thiolated chitosan. Thus, this study suggests that the oral delivery system based on gel formulation comprising chitosan–TGA and GSH might turn out to be a valuable tool in order to improve the intestinal uptake of peptides such as leuprolide.

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