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Characterisation of the thiol–disulphide chemistry of desmopressin by LC, μ-LC, LC-ESI-MS and Maldi-Tof

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Summary. To date, the majority of therapeutic peptides and proteins have to be administered via parenteral routes, which are painful and inconvenient. In order to gain sufficient high blood concentrations after oral application, various barriers in the gastrointestinal tract have to be overcome. Apart from a poor membrane uptake and intense enzymatic degradation, this study will demonstrate that thiol–disulphide reactions are an underestimated essential part of the presystemic metabolism. Glutathione, integrative part of the antioxidant defence system in the gastrointestinal tract, may play an important role in the inactivation of orally given peptides and proteins. In order to verify this hypothesis, desmopressin which bears a single disulphide bond was used as model peptide drug. Desmopressin was incubated with GSH in various concentrations, and the extent of thiol/disulphide exchange reactions between the peptide drug and GSH was investigated in dependence on pH and ratio of reactants determined as a function of time via HPLC, LC-MS and Maldi-Tof-MS analyses.

Results showed that desmopressin is degraded by 1% reduced glutathione at pH 4 and pH 5.5. In presence of 0.01%, 0.1% and 1% of reduced glutathione 6.1%, 19.4% and 52.1% of desmopressin, respectively, were degraded. The masses of the conjugates after deconvolution measured by liquid chromatography and electrospray ionisation mass spectrometric detection were m/z 1069.67, m/z 1376.50, m/z 1683.40 and m/z 2138. These molecular masses, confirmed by Maldi-Tof-MS analysis, correspond with the masses of conjugates expected in theory. Under defined conditions, these results reveal that thiol–disulphide exchange reactions have a considerable impact on the alteration of peptide drugs and proteins.

Keywords: Glutathione – Desmopressin – Thiol-disulphide exchange – Drug delivery – Peptides

1 Introduction

The oral bioavailability of most therapeutic peptides and proteins is comparatively very poor. For this reason the majority of these drugs have to be administered via parenteral routes, which is often inconvenient, painful and occasionally dangerous. According to this, there is both a great scientific interest and a medical need for the development of non-invasive delivery systems for the multitude of potential therapeutic peptides and proteins emerging from the pharmaceutical arena (Shah et al., 2002). However, various barriers have to be overcome in order to gain sufficient high blood concentrations after oral application. These barriers include the enzymatic barrier (Bernkop-Schnürch, 1998), the absorption barrier (Pusztai, 1989) and the barrier function of the mucus covering gastrointestinal epithelia (Bernkop-Schnürch and Fragner, 1996).

Desmopressin, a commonly used drug against nocturia, is well-investigated concerning its behaviour face to each of the above mentioned barriers. However a substantial additional aspect of the presystemic metabolism encountered by orally applied peptide drugs bearing a disulphide bond is posed by thiol–disulphide exchange reactions. Although this concept is well-known from its theoretical approach, very few data concerning the thiol–disulphide chemistry of desmopressin is found in literature.

Reduced glutathione (GSH), a thiol-bearing tripeptide plays a significant role in the antioxidant defense system of most mucosal tissues. Its thiol group can react as nucleophile with exogenous and endogenous electrophilic species. As one of the most important intracellular antioxidants, glutathione serves in the detoxification of chemical toxins, procarcinogens and reactive oxygen species (ROS). The concentrations of both the reduced and oxidized form of glutathione in the intestinal fluid and mucosa are derived from a combination of endogenous synthesis (Meister, 1988), interorgan transport and

absorption from the diet (Flagg, 1994) and the biliary secretions. This glutathione concentration varies in a range between the mean value of 150 mg GSH absorbed per day from the diet (Flagg et al., 1994) and 10 mM of GSH determined intracellularly (Loguercio et al., 1991), representing a range from 0.3%–0.01%.

Concerning this significant concentration of reduced GSH in the intestine and being aware of the glutathione concentrations found in diet, the following study focuses on possible thiol–disulphide exchange reactions between desmopressin as peptide model drug on the one hand and reduced glutathione as sulfhydryl bearing reactant on the other hand. The possible binding motif of this peptide to its receptors is based on a positive charge, an apolar aromatic side chain and a β-methylene group. The integrity of the molecular conformation, necessary for the activity of this hormone, is maintained by the only disulphide bond between positions 1 and 6, which is not involved in binding (Wang et al., 1996). However, this bond is an apparent electrophilic target susceptible for oxidation.

Escalating this theory, each orally given peptide and protein drug, where a disulphide bond within the over all structure is required for the activity of the drug, might be susceptible for inactivation by thiol–disulphide exchange reaction.

Therefore, in order to elucidate this specific aspect of the presystemic metabolism, expected thiol/disulphide exchange reactions between desmopressin and reduced glutathione were investigated in dependence on pH and increasing concentrations of reduced glutathione. Latest analytical methods, including high performance liquid chromatography, μ-high performance liquid chromatography, LC-ESI-MS/MS and Maldi-Tof-MS, were applied for the detection of desmopressin-glutathione conjugates.

2 Materials and methods

2.1 Stability studies

2.1.1 pH-dependant stability

The stability of desmopressin in face of reduced glutathione was tested in various incubation media with pH values of pH = 3, pH = 4 and pH = 5.5. All buffers were prepared at 25°C and the pH was checked with an electronic pH meter before each use. The test solutions were formed freshly before each use by dissolving desmopressin (Bachem, Switzerland) in the appropriate buffer in a final concentration of 0.02% (m/v).

The experiment started immediately after the addition of reduced glutathione in a final concentration of 1% to the desmopressin solutions. Samples were incubated in closed vials at 37°C for 3 h under slight shaking (300 rpm). Samples of 100 μl were withdrawn at time points 0, 15, 30, 45, 60, 90, 120, 150 and 180 minutes. Samples were either stored at −20°C or immediately analysed as described below.

2.1.2 Concentration-dependant stability

Desmopressin was freshly dissolved in 50 mM acetate buffer pH 5.5 at a final concentration of 0.02% (m/v). Three different test solutions were prepared by adding reduced glutathione to the desmopressin solution in a final concentration of 0.01%, 0.1% and 1%, respectively. All samples were incubated in closed vials for 3 hours at 37°C under shaking (300 rpm) and stored at −20°C until analysis as described above.

2.2 HPLC analysis

The desmopressin concentration was determined by two different HPLC systems. On the one hand a standard reversed-phase (RP) HPLC system was used formed of a Hitachi LaChromeElite series L-2130 pump, Hitachi LaChromeElite series L-2200 auto sampler and a Hitachi LaChromeElite L-2450 diode array detector. Samples were eluted from a Merck C18 RP (250 mm × 4 mm) column at 40°C at a flow rate of 1 ml/min. The mobile phase was composed of acetonitrile (ACN) and a tetramethylammonium-hydroxid buffer pH 2.7 in a ratio 23/77 (v/v). The injection volume was 20 μl. Detection wavelength was 210 nm. The compounds were identified according to peak retention times.

The second system used for RP-HPLC consisted of a low-pressure gradient pump (Model 616, Waters), a controller (Model 600S, Waters), a column heater (Model TC 1900, ICI, Welshpool, Australia), a helium degassing system, an autosampler (Model 717 plus, Waters) and a photo diode array detector (DAD, Model 996, Waters) with a 10 mm pathlength flow-cell. Data were recorded on a computer-based data system (Millenium^{32}, Version 3.05.01, Waters). A Phenomenex Luna C-18 column (250 × 4.6 mm I.D., 5 μm, 120 Å, Torrance, CA, USA) was used. The mobile phase consisted of A: 0.1% trifluoroacetic acid (TFA) in bidistillated water; B: ACN; linear gradient: 9–61% B in 22 min. The flow rate was 1.0 ml/min at a temperature of 40°C. The volume injected was 20 μl. Acetonitrile (analytical reagent grade), trifluoroacetic acid (TFA, analytical reagent grade) were purchased from Merck (Darmstadt, Germany). Tetramethylammoniumhydroxid (TMAH) was purchased from Sigma (OH, USA).

2.3 μ-High performance liquid chromatography (HPLC) with UV-detection

The μ-HPLC-UV system consisted of a low pressure gradient micropump (model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (ERC-3415α, Ercatech, Alteglofsheim, Germany), a microinjector (model 7520, Rheodyne, Cotati, CA, USA) with a 0.5 μl rotor connected to a UV-vis detector (model 345, Varian, Darmstadt, Germany) with a fused silica bubble cell. The stationary phase used was a ProntoSIL C18 3 μm, 120 Å encapsulated by poly(styrene/divinylbenzene) (PS/DVB) copolymer: 200 mm of silanized fused silica capillary (200 μm I.D.) was packed with stationary phase (Huang and Horvath, 1997; Stecher et al., 2003). For encapsulation, the packed capillary was filled with the polymerization mixture comprising 90 μl of styrene, 90 μl of divinylbenzene, 300 μl of decanol and 5 mg of azoisobutyronitril (AIBN) and polymerized at 70°C for 24 h. Finally the capillary was washed with acetonitrile for 2 hours and cut into the desired length of 8 cm.

The mobile phase consisted of A: 0.1% TFA in water and B: ACN and was pumped at a constant flow rate of 300 μl/min (actual flow rate 4.0 μl/min). Separation was carried out isocratically at 50% B at 40°C. The volume injected was 0.5 μl. Detection was performed at 220 nm.

2.4 High-performance liquid chromatography coupled to electrospray ionization quadrupole ion trap mass spectrometry (LC-ESI-MS/MS)

For LC-ESI-MS experiments a low-pressure gradient micropump (Model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (Model DG-301, Phenomenex, Torrance, CA, USA), a microinjector (Model CC00030,

Valco, Houston, TX, USA) with a 20 μl internal loop connected to a quadrupole ion trap mass spectrometer (Model LCQ, Finnigan, San Jose, CA, USA) were used. The following parameters were applied in all experiments: positive ion mode; source voltage, 4.52 kV; source current, 2.24 μA; sheath gas flow rate, 59.63 (Finnigan units; nitrogen); capillary voltage, 46.84 V; temperature of the heated capillary, 199.2°C; tube lens offset, −7.32 V; first octapole offset, −16.29 V; second octapole offset, −7.32 V. For RP-LC a Phenomenex Luna C-18 column (125 × 2 mm I.D., 3 μm, 100 Å, Torrance, CA, USA) was used. The mobile phase consisted of A: 0.1% TFA in water; B: ACN; linear gradient: from 9% B to 61% B in 22 min. The flow rate was 0.2 ml/min at a temperature of 40°C. The volume injected was 10 μl.

2.5 Maldi-Tof-MS analysis

Investigations were carried out by Maldi-Tof-MS (Ultraflex MALDI-TOF-TOF, Bruker Daltonics, Bremen, Germany). All samples were measured in reflector mode. Data were collected by averaging 150 laser shots and analyzing mass region from 500 to 2000 Da. The validation of all data obtained, external calibration using Protein Standard I (Bruker Daltonics, Bremen, Germany) and all further data processing, was carried out by using Flex analysis 2.0 post analysis software and for data acquisition by Flex control 2.0.

2.6 Statistical data analysis

The student t test with $p < 0.05$ as the minimal level of significance was used to perform statistical data analysis. Calculations were done using the software OriginPro 7G SR4 version 7.0552.

3 Results

The possible conjugates, which might origin from a thiol/disulphide exchange reaction between the model peptide desmopressin and the reduced form of glutathione are shown in Fig. 1.

3.1 Influence of the pH

The influence of reduced glutathione on the stability of desmopressin was measured at various pH values (Fig. 2). In the formiate buffer pH 3, desmopressin is not degraded by reduced glutathione (data not shown). By shifting the pH from pH 3 to a value of pH 4, the concentration of the reactive thiolate anion of glutathione bearing a net charge of −1, is increased. These net charges on the reactants favour a thiol/disulphide exchange reaction. At pH 4, the nonapeptide is continuously degraded from the beginning to reach a maximum level after 1 hour. In an acetate buffer adjusted to pH 5.5, desmopressin was also degraded by reduced glutathione. In the first hour of the test procedure, a similar amount of desmopressin as observed for the pH 4 buffer was degraded, but this decomposition continued during the following two hours to reach a maximum where half of the amount used was degraded. As expected from former studies, desmopressin was not degraded in any of these buffers by omitting reduced glutathione (data not shown).

3.2 Influence of the concentration of reduced glutathione

The luminal pH gradient is steepest in the proximal 10 cm of the duodenum where the acidity is reduced from pH = 2 as in the stomach to a pH varying between pH = 4 and pH = 7 (Watson et al., 1972). A pH value of

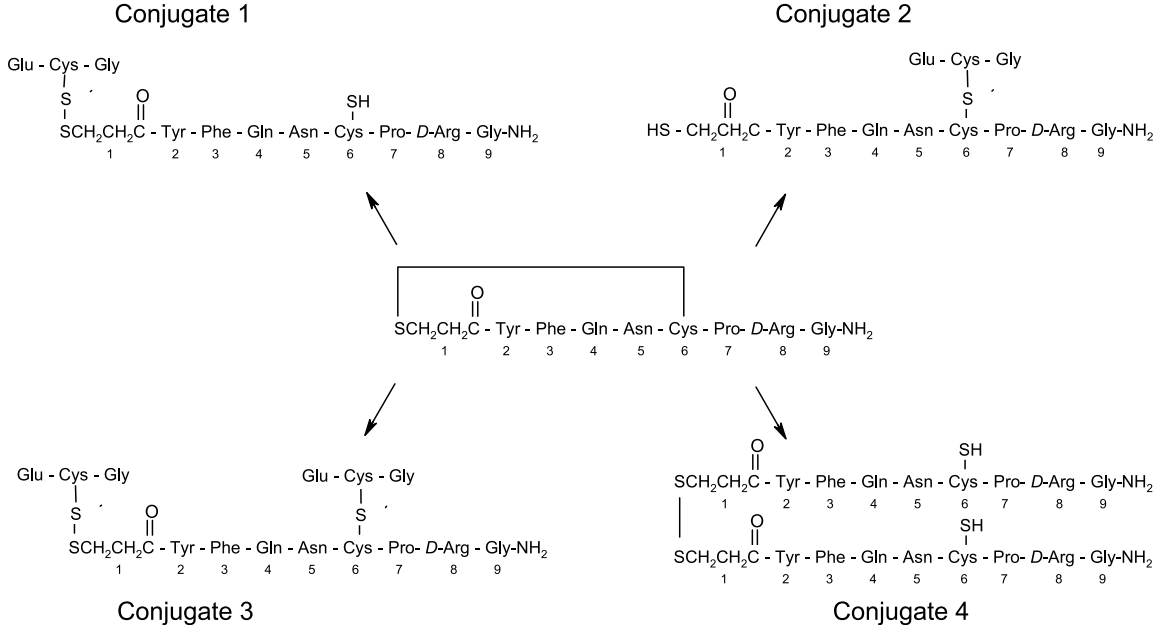

Fig. 1. Possible thiol–disulphide exchange reactions between desmopressin and glutathione

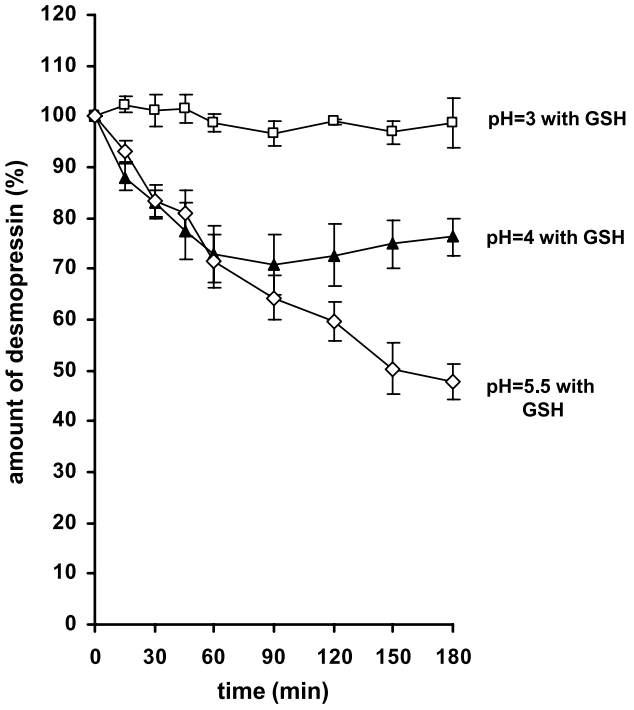

Fig. 2. pH-Dependant degradation of desmopressin in presence of 1% reduced glutathione. The amount of remaining desmopressin was tested at pH 3, pH 4 and pH 5.5. Individual values, expressed in percent are means ± SD (n = 3)

pH = 5.5 was chosen in order to simulate physiological conditions in the first part of the upper small intestine characterised by frequent wide and rapid pH fluctuations.

In an acetate buffer pH 5.5, desmopressin is degraded in presence of any of the used concentrations of reduced glutathione (Fig. 3). After 3 hours of incubation, 6.1%, 19.4% and 52.1% of desmopressin were degraded in presence of 0.01%, 0.1% and 1% of reduced glutathione, respectively. This degradation follows the course of a polynom second order.

Two different HPLC methods were used in order to exclude any influence from the analytical setups. Separation was carried out applying two different stationary phases. The first phase was the conventional used Silica-C18 (250×4 mm, 5 μm, 120 Å) at a flow rate of 1 ml/min. The second phase was a 200 μm I.D. capillary column packed with ProntoSIL C18 3 μm, 120 Å encapsulated by poly(styrene/divinylbenzene) (PS/DVB) copolymer. Figure 4 shows the chromatograms of desmopressin after incubation with reduced glutathione for 3 hours in acetate buffer at pH 5.5 applying the conventional silica phase (Fig. 4a) and the encapsulated capillary column (Fig. 4b). Both chromatograms show two small peaks at lower retention times than desmopressin corresponding to several conjugates after possible thiol–disulphide exchange reactions.

The utility of LC separation for the identification of these conjugates was greatly enhanced by mass spectrometric (MS) detection. The LC system was coupled to a quadrupole ion trap mass spectrometer by using an electrospray ionisation (ESI) interface, which allowed one to confidently identify the peaks. LC-ESI-MS analysis was carried out employing a 125×2 mm I.D. column packed with 3 μm/100 Å octadecylsilica stationary phase comprising a linear gradient from 9% ACN in 0.1% TFA up to 61% ACN within 22 minutes. Under the electrospray ionisation conditions all compounds could be transformed into protonated molecules $[M+H]^+$. Tracking of selected ion traces from the ion current depicted in Fig. 5a showed a peak for desmopressin at 10.41 minutes (m/z 1069–1071; Fig. 5b). Deconvolution of m/z 1069.67 showed an additional mass of 2138.00 which corresponds with conjugate 4 (Fig. 1). Conjugate 3 showed a peak at 7.98 minutes (m/z 841–842; Fig. 5c), conjugates 1 and 2 at 10.36 minutes (m/z 687–691; Fig. 5d). Convolution of parent ion masses of conjugate 3 (m/z 1683.48) gave a double charged daughter ion at m/z 841.72, of conjugate 1 and 2 (m/z 1376.78) a double charged ion at m/z 687.5. Colission induced dissociation (CID) of m/z 1683.4 and m/z 1376.78 both showed the presence of desmopressin (m/z 1069.67) after loss of one or two molecules glutathione, respectively. Finally, the deconvoluted molecular masses of the conjugates 1, 2, 3 and 4 determined by LC-ESI-MS corresponded with their theoretical masses (Table 2).

Table 1. Physiological concentration of reduced glutathione on mucosal membranes

Tissue	Quantity	Units	Reference
Antrum	23.0 ± 0.7	nmol/mg protein	(Hoensch et al., 2002)
Duodenum	35.1 ± 1.0	nmol/mg protein	(Hoensch et al., 2002)
Buccal mucosa	4.8	nmol/mg protein	(Wong et al., 1994)
Nasal mucosa	0.6 ± 0.2	μmol/g wet weight	(Westerveld et al., 1997)
Conjunctiva	1.7 ± 0.3	μmol/g wet weight	(Ingram et al., 2003)
Colorectal	28.2 ± 9.2	nmol/mg protein	(Özdemirler et al., 1998)

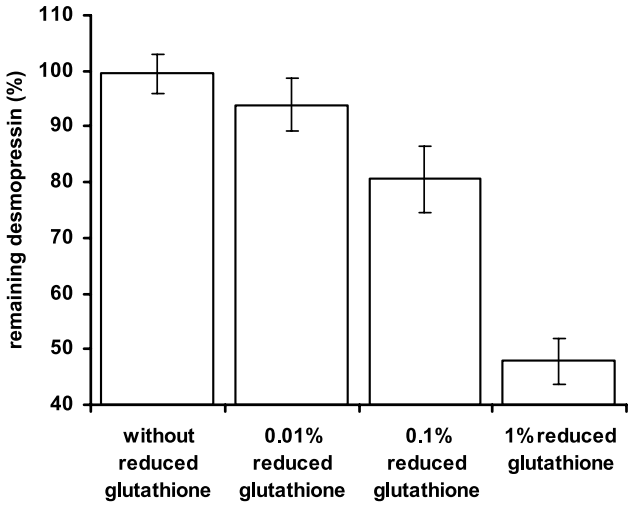

Fig. 3. Remaining amount of desmopressin after 3 hours of incubation at 37°C with increasing concentrations of reduced glutathione at pH 5.5. The tested concentrations of glutathione were 0.01%, 0.1% and 1%. Remaining desmopressin is expressed in percent. Indicated values are means ± SD (n = 3)

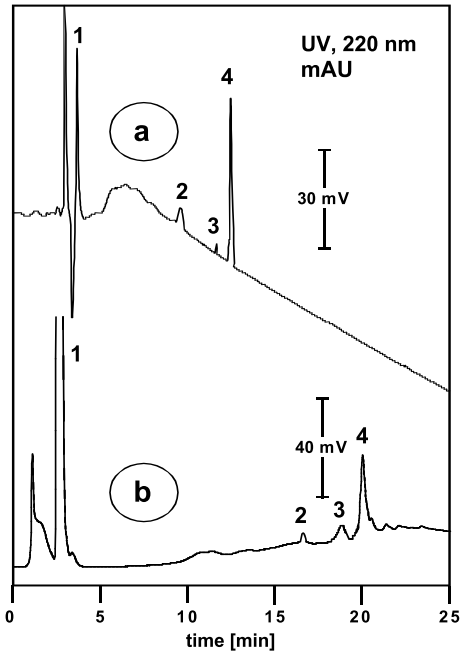

Fig. 4. RP-LC of desmopressin after incubation with 1% glutathione in acetate buffer at pH 5.5 for 3 hours. Conditions: (**a**), stationary phase, Phenomenex Luna C18 (250 × 4 mm, 5 μm, 120 Å); mobile phase, A: 0.1% TFA, B: ACN; flow rate, 1 ml/min; linear gradient, 9–61% B in 22 min; temperature, 40°C; detection, UV, 220 nm; injection volume 20 μl; (**b**) stationary phase, ProntoSIL C18 (3 μm, 120 Å) encapsulated by poly(styrene/divinylbenzene) (PS/DVB) (8 cm × 200 μm I.D.); mobile phase, 50% ACN in 0.1% TFA; flow rate, 4 μl/min after split; temperature, 40°C; detection, UV, 220 nm; injection volume, 0.5 μl. Peak identification: ***1*** Glutathione, ***2*** Desmopressin +2 GSH, ***3*** Desmopressin +1 GSH, ***4*** Desmopressin

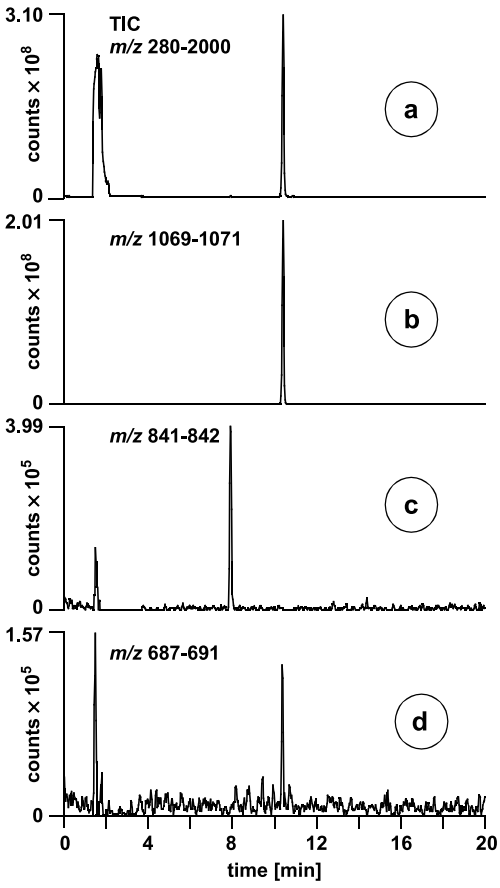

Fig. 5. LC-ESI-MS analysis of desmopressin after incubation with 1% glutathione for 3 hours. Conditions: stationary phase, Phenomenex Luna C18 (125 × 2 mm, 3 μm, 100 Å); mobile phase, A: 0.1% TFA, B: ACN; linear gradient, 9–61% B in 22 min; flow rate, 0.2 ml/min; temperature, 40°C; scan (**a**), selected ion monitoring at m/z 1069–1071 (**b**), m/z 841–842 (**c**), m/z 687–691 (**d**); injection volume, 10 μl

Table 2. Masses of desmopressin, conjugate 1, 2, 3 and 4 after deconvolution measured by liquid chromatography and electrospray ionisation mass spectrometric detection

Compound	Theoretical mass	Measured mass LC-ESI-MS
Desmopressin	1069.22	1069.67
Conjugate 1 and 2	1376.44	1376.50
Conjugate 3	1683.44	1683.40
Conjugate 4	2138.44	2138.00

Maldi-Tof-MS analysis of desmopressin after incubation with 1% reduced glutathione for 3 hours in acetate buffer pH 5.5 further confirmed the results obtained from LC-ESI-MS. In Fig. 6 two peaks at m/z 560.33 and m/z 655.69 are present beside the desmopressin corresponding m/z 1069.76 and m/z 1091.80 ($M + Na^+$). m/z 655.69 corresponds with the double charged peak of conjugate

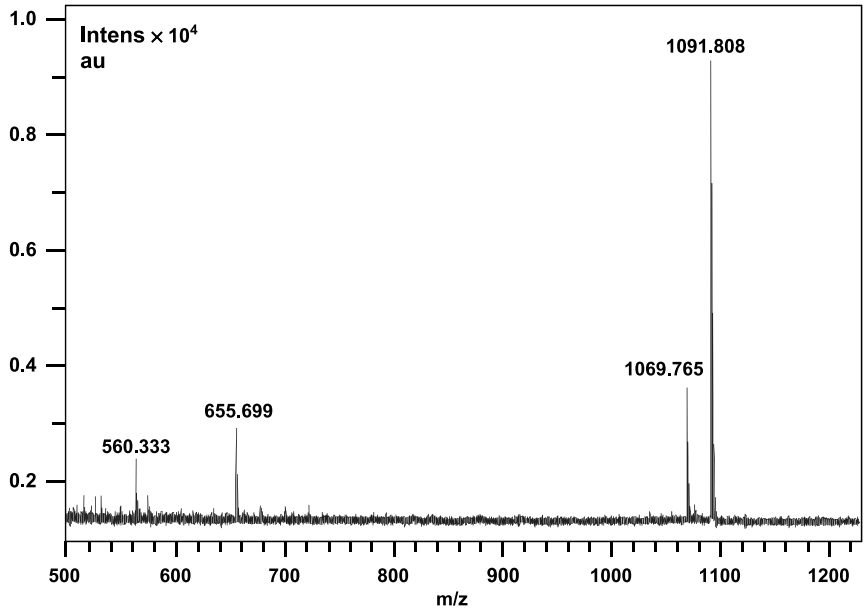

Fig. 6. Maldi-Tof mass spectrum of desmopressin after incubation with 1% glutathione in acetate buffer (pH 5.5) for 3 hours. Peak identification: m/z 560.333 corresponds with the threefold charged peak of conjugate 3; m/z 655.59 corresponds with the double charged peak of conjugate 1 and 2 after loss of sulphur; m/z 1069.765 corresponds with desmopressin and m/z 1091.80 corresponds with desmopressin ($M + Na^+$)

1 and 2 after loss of sulphur and m/z 560.333 corresponds with the threefold charged peak of conjugate 3.

4 Discussion

Glutathione, a naturally occurring tripeptide composed of the amino acids glutamine, cysteine and glycine, is present in many food sources and in almost all mammalian cells (Anderson and Luo, 1998). The sulfhydryl group of cysteine renders glutathione to act as a biological redox agent, as coenzyme, cofactor, and as substrate in certain reactions catalysed by the glutathione S-transferase.

In view of these physiological functions it is not surprising that most mucosal membranes contain considerable amounts of glutathione necessary to prevent oxidative damage and noxious effects from the external environment. The average physiological glutathione content of selected mucosal tissues was listed in Table 1; (Wong et al., 1994; Westerveld et al., 1997; Özdemirler et al., 1998; Hoensch et al., 2002; Ingram et al., 2003). In contrast to these comparatively low concentrations, high amounts of glutathione are ingested daily with regular diet (Meister, 1988; Wierzbicka et al., 1989). Consequently a high interindividual variability has to be expected. In order to cover the whole range of possible glutathione concentrations in the human gastrointestinal juice, three different concentrations of glutathione were used to measure the effect on a model peptide. The activity of the glutathione system in the gastrointestinal tract is influenced by variables like chemical structure of the drug, localisation and food intake. An imbalance of this system perturbs the cellular redox status and is observed in a wide range of pathologies including gastrointestinal abnormalities, cancer and aging (Townsend et al., 2003). Though, under normal healthy conditions a glutathione homeostasis can be observed.

To a certain extent, disulphide exchange reactions are influenced by the electrostatic environment of the reacting partners (Snyder et al., 1983). Factors such as the relative position and the presence of aromatic or electrostatically charged neighbours in the local environment may influence the conjugation processes. The research work of Snyder et al. showed that aromatic neighbours stabilize disulphides by forming S-π complexes and neighbouring histidines may enhance a cysteine's participation in disulphide exchange reactions (Snyder et al., 1981). In order to differentiate local environment effects from general attraction or repulsion the charge of the neighbouring residues should be different from the net charge of the total peptide.

Charged groups exert their effect both by Coulombic forces acting through space and by inductive effects through the bonds which alter the electron density of the sulphur atom. The deprotonated high pH thiolate form of cysteines favours the initiation of the disulphide exchange reaction, whereas lowering the pH slows exchange by several orders of magnitude. These results are in accordance with our findings where an increased degradation of desmopressin was observed at higher pH. The consideration that the electrostatic conformation of desmopressin would influence the nucleophilic attack of reduced glutathione could be demonstrated by the detection of all the conjugates

expected in theory. It can be concluded that thiol–disulphide exchange reactions function as limiting factor, decreasing the amount of unmodified desmopressin. These facts may explain the reduced and delayed bioavailability of desmopressin 1–5 hours after meal caused by degradation through the considerable amounts of glutathione found in fresh fruits and vegetables, which are in the range of 40–150 mg/kg wet weight (Wierzbicka et al., 1989).

It will be the aim of further studies to set up a guideline which allows anticipating a probable degradation of orally administered peptide drugs. For this purpose various peptides should be screened in order to see if the uptake observed on different areas of absorption correlates with parameters influencing the degradation like the local concentration of reduced glutathione or pH. Strategies to overcome the mentioned problems may include the use of multifunctional polymeric matrices of high buffer capacity, which would be highly beneficial in protecting the drug from presystemic degradation (Bernkop-Schnürch and Gilge, 2000). According to results shown in Fig. 2, incorporating disulphide bond bearing peptides such as vasopressin, oxytocin or octreotide in a carrier matrix should provide protection towards an attack by GSH at those pH values where degradation is favoured. In order to avoid thiol/disulphide exchange reaction of the therapeutic peptide on the way between the delivery system and the absorption membrane, the carrier matrix should provide an intimate contact with the mucosa. Such an effect might be achieved by the use of mucoadhesive polymers.

An alternative approach to protect a peptide drug from thiol/disulphide exchange reaction may lie in the stabilization of its disulphide bonds by chemical derivatisation. This strategy is reasonable when disulphide bonds are essential for the biological activity of the molecule.

In conclusion, the present study showed that desmopressin as model drug for peptides bearing disulphide bonds is susceptible for a thiol/disulphide exchange reaction. Results proved that desmopressin forms various expected conjugates with reduced glutathione, an essential part of the antioxidant system. This reaction depends on different parameters: the local pH, the concentration of reduced glutathione on the site of reaction and on the electrostatic environment of the applied drug, which characterizes its reactivity. At defined concentrations more than half of the deployed drug has been degraded by reaction with reduced glutathione. Considering that many peptides with variable reactivity may enter thiol–disulphide reactions and given that reduced glutathione is found ubiquitary in diet and in almost all mammalian cells, this study reveals that the aspect of thiol–disulphide reactions as integrative part of the presystemic metabolism of orally applied peptide drugs needs further investigation in the future.

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