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Effect of protamine on the solubility and deamidation of human growth hormone

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ABSTRACT

The effect of protamine on the solubility and deamidation of human growth hormone (hGH) was investigated. Protamine is an extremely basic peptide. It is isolated from sperm cells of salmon where it can build a complex with DNA due to electrostatic interactions. We hypothesize a similar electrostatic effect between negatively charged hGH and positively charged protamine residues. Arising cationic complexes are stabilized by electrostatic repulsion. This stabilizing complexation allows solubilization of hGH down to a pH of 5.4 (pI 5.3) at concentrations of 3.4 mg/ml. The minimal solubilizing molar ratio between hGH and protamine was found to be at least 1:23 (hGH:protamine) by turbidity and dynamic light scattering (DLS) measurements. Complexation was characterized by small-angle X-ray scattering (SAXS) and isothermal titration calorimetry (ITC). Electrostatic binding of protamine to hGH was also observed by a reversal in surface charge, as shown by zeta potential measurements. The presence of protamine did not alter the conformational structure of hGH which was determined by circular dichroism (CD) spectroscopy. A pH of 5.4 is known to slow deamidation of hGH and consequently retardation of hGH deamidation could be detected after 3 months storing by reversed-phase high-performance liquid chromatography (RP-HPLC).

1. Introduction

Development of liquid therapeutic protein formulations requires careful consideration of solubility and stability of the protein. Once a formulation is designed, solubility and stability must be maintained during its shelf life (Banga, 2009). Protein solubility depends on several parameters, such as the polarity of individual amino acids, protein structure and environmental conditions, like temperature, pH and the presence of cosolvents (Banga, 2009; Friess et al., 2010). At their isoelectric point proteins have a lower solubility because neutralization of surface charges takes place and thus charge–charge repulsion does not occur. Human growth hormone (hGH) has its isoelectric point at approximately pH 5.3 with a minimal solubility of about 1 mg/ml hGH (Cleland et al., 1993; Pearlman and Bewley, 1993). hGH belongs to the group of cytokines and plays an essential role in therapy of human growth disorders. It comprises 191 amino acid residues with a molecular weight of 22,125 Da (Marian, 2002; Pearlman and Bewley, 1993).

It was shown that some excipients are able to increase solubility of proteins by affecting protein solvation. Therefore they are also called cosolvents. There are three proposed hypotheses how cosolvents can affect physical stability of proteins: Either by preferential hydration, where the cosolvent is excluded from the protein surface, by preferential interaction, where the cosolvent binds to the protein surface or by the "gap effect" proposed by Baynes and Trout, where the cosolvent is not strongly bound nor excluded from the protein surface (Baynes and Trout, 2004; Cleland et al., 1993; Schneider and Trout, 2009). In the current study the solubilizing effect of the polyamine protamine on hGH was investigated. Protamine is a FDA approved polycationic peptide and is used as complexing agent in intermediate-acting insulin formulations, such as the widely used NPH (Neutral Protamine Hagedorn) depot insulin (Brange et al., 1987). It consists of about 32 amino acid residues and has a molecular weight of about 4200 Da (Hoffmann et al., 1990; http://www.accessdata.fda.gov/scripts/cder/iig/getiigWEB.cfm, 03-10-2011.). Protamine is isolated from sperm cells of salmon where it naturally builds a complex with DNA during spermatogenesis due to electrostatic interactions. Because of its high arginine content (approximately 68%), it is an extremely basic peptide (Ando et al., 1973). Polyamines are known as solubility enhancer

Abbreviations: hGH, human growth hormone; pI, isoelectric point; NTU, nephelometric turbidity unit; DLS, dynamic light scattering; SAXS, small-angle X-ray scattering; ITC, isothermal titration calorimetry; CD, circular dichroism; RP-HPLC, reversed-phase high-performance liquid chromatography.

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of several proteins, but the exact mechanism is still not clear (Hamada et al., 2009; Yasui et al., 2010). The positive charge of these molecules has the potential to interact with the negatively charged protein residues and arising charge repulsion is expected to lead to a reduction of intermolecular interactions (Kudou et al., 2003). Due to its large size, protamine could also crowd out the protein–protein interactions as described by the "gap effect". The gap effect leads to an increase in the free energy of protein–protein encounter complexes and an increase in the barrier to association (Baynes and Trout, 2004; Shukla and Trout, 2010).

Deamidation is one of the major routes of chemical degradation of human growth hormone (hGH). hGH tends to undergo deamidation as a function of pH. The reaction rate for deamidation occurs typically faster at neutral and alkaline pH. Deamidation is also sequence, structure and time dependent and is favored by elevated temperature (Manning et al., 2010). 9 asparaginyl (Asn) and 13 glutaminyl (Gln) residues in hGH are potential deamidation sites. Gln residues generally deamidate much slower than Asn residues (Cholewinski et al., 1996; Jenkins et al., 2008). At acidic pH (pH 1–2) deamidation is proposed to proceed by direct hydrolysis of asparagine to aspartate. At physiological pHs, deamidation proceeds primarily through a two-step process. The first step involves five-membered cyclic imide formation, in which either the backbone NH transfers its H to the Asn side chain NH2 or vice versa. The succinimide ring then spontaneously hydrolyzes to yield either an aspartyl or isoaspartyl residue, usually in a 3:1 ratio (Kirchhoff, 2010; Patel and Borchardt, 1990; Robinson et al., 2004). Asn149 was determined as major deamidation site of hGH by Becker et al. (1988). The biological activity of deamidated hGH is known to be equal to that of the native protein (Riggin and Farid, 1990). However, the formation of the not naturally occurring amino acid iso-Asp, which implies an alteration in the backbone of hGH, can present a potential immunogenic risk (Cleland et al., 1993; Jenkins et al., 2008).

In this study we investigated the effect of protamine on the solubility of hGH at pH 5.4, close to the isoelectric point of hGH. In general the solubility of hGH would be approximately 1 mg/ml at such acidic conditions. However, pharmaceutical formulation require at least a concentration of 3.4 mg/ml or higher which should be reached in our study. In addition, the deamidation of hGH was studied at pH 5.4 to test whether the solubilized hGH showed a decreased deamidation due to the low pH or not.

2. Materials and methods

2.1. Materials

The r-hGH was supplied by Sandoz GmbH (Kundl, Austria) in a 10 mM sodium phosphate buffer at pH 7.0. Protamine from salmon (grade IV) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium phosphate mono- and di-basic, ortho-phosphoric acid, acetonitrile, trifluoracetic acid (TFA), glycine and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Ethylenediaminetetraacid (EDTA) and ammonium bicarbonate were purchased from Fluka GmbH (Buchs, Switzerland). Sterile water for injection from GalenicaSenese (Monteroni d'Arbia, Italy) was used throughout the experiments. Formulations were filtered using 0.22 μm PVDF filters from Millipore (Carrigtwohill, CO, Ireland).

2.2. Methods

2.2.1. Preparation and characterization of formulations

Formulations with protamine were prepared by titrating a protamine solution with hGH in a 6R vial under orbital shaking. Additionally to orbital shaking (230 rpm) a rotating glass spherule was used to achieve fast rearrangement. For the titration process hGH was added dropwise in 100 μl steps every 10 s to protamine in 10 mM sodium phosphate buffer, pH 5.9. hGH was titrated into protamine solution and not vice versa because titrating protamine into hGH led immediately to precipitation of hGH. After titration the pH of the formulations was adjusted to 5.4 with phosphoric acid. The final hGH concentration was 3.4 mg/ml at molar ratios of hGH-to-protamine from 1:1 to 1:39. 3.4 mg/ml hGH were chosen because this concentration is commonly used in marketed products (e.g. Omnitrope®, Norditropin®). Formulations with and without protamine at a pH of 7.0 were prepared as control samples. Sample preparation was carried out at 2–8 °C. Finally the formulations were filtered through a 0.22 μm filter. High concentrations of protamine were required for solubility of hGH at pH 5.4. Samples with a hGH-to-protamine ratio of 1:23 (mol/mol) were prepared for further characterization.

2.2.2. Turbidimetry

The turbidity measurements were performed as outlined in the Ph.Eur. 6.0 method 2.2.1 (Ph.Eur. 2.21, 2008). A NanoPhotometer™ (Implen GmbH, Munich, Germany) was used for these experiments, measuring the transmission of the formulations at 400 nm. The instrument was calibrated against formazin reference suspensions. A calibration line between transmission and formazin concentration was plotted where a correlation coefficient of 0.999 was achieved. NTU (nephelometric turbidity unit) values of the hGH formulations were obtained by the calibration equation (Ph.Eur. 2.21, 2008). The measurements were performed in triplicate at 25 °C.

2.2.3. UV spectroscopy

The protein concentration was determined on a NanoPhotometer™ (Implen GmbH, Munich, Germany). An extinction coefficient for hGH of 17,420 M^{-1} cm^{-1} at 280 nm was identified by using the ProtParam tool of ExPASy (Swiss Institute of Bioinformatics, Basel, Switzerland; http://www.expasy.ch/tools/protparam.html) based on the hGH sequence of Ph.Eur. 6.0/0951 (Ph.Eur. 6.0/0951, 2008). All samples were shortly centrifuged to remove insoluble protein and the concentration in the supernatant was detected.

2.2.4. Dynamic light scattering (DLS) and zeta potential measurements

DLS and zeta potential measurements were carried out on a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) with a 532 nm green laser. For DLS analysis, each sample was measured in single-use UV-plastic cuvettes (Brand GmbH and Co KG, Wertheim, Germany), first equilibrated for 2 min at 25 °C after which a time scale of the scattered light intensity fluctuations was measured. Autocorrelation analysis was carried out with the use of the software Zetasizer V6.20 (Malvern Instruments Ltd., UK). The particle size was expressed by the hydrodynamic diameter (d_h) and the width of the size distribution was expressed by the polydispersity index (PdI). The zeta potential was measured by applying an electric field across the sample solutions using the technique of laser Doppler anemometry. All measurements were carried out at 25 °C in three replicate measurements.

2.2.5. Small angle X-ray scattering (SAXS)

Small angle X-ray scattering (SAXS) curves were measured with a SWAXS camera (System 3, Hecus X-ray Systems, Graz, Austria) mounted on a sealed X-ray tube generator from Seifert (Ahrensburg, Germany), which was operated at 50 kV and 40 mA. The X-ray beam was filtered for CuKa radiation ($\lambda = 0.1542$ nm) using a Ni-foil and a pulse height discriminator. SAXS patterns were recorded using a linear, one-dimensional, position-sensitive detector (PSD

50, Hecus X-ray Systems, Graz, Austria). Measurements were performed in a scattering vector range (q-range) between 0.088 nm^{-1} and 3.0 nm^{-1}, where $q = (4\pi \sin\theta)/\lambda$, with 2θ being the scattering angle. Calibration of the small-angle region was performed with silver stearate. All experiments were performed at 20 °C using a quartz capillary with a volume of 50 μl and an inner diameter of 1.0 mm. Formulations in the absence and presence of protamine were measured in 10 mM sodium phosphate buffer at pH 7.0 and pH 5.4, respectively. The hGH concentration was 3.4 mg/ml at an hGH-to-protamine ratio of 1:23 (mol/mol). The exposure time was 1 h. Raw data processing (buffer background subtraction and normalisation to intensity) was done with the program EasySWAXS (Hecus X-ray Systems, Graz, Austria). The radius of gyration (R_g), a value which relates to particle size, was determined by Guinier approximation from the low q-range of the scattering profiles. The processed data were subsequently analyzed with the program package ATSAS 2.3. Indirect Fourier transformation was performed with the program GNOM (Svergun, 1992) to evaluate the pair-distance distribution function $p(r)$ after desmearing according to slit width and slit length profiles of the primary beam. The $p(r)$-function gives information about particle size, shape and additionally yields the maximum dimension of the particle (d_{max}) as distance when the $p(r)$ function approaches zero. The theoretical scattering curve for hGH in aqueous solution was derived from the crystal structure (PDB ID: 3HHR, using the protein coordinates without receptor) using the program CRYSOL (Svergun et al., 1995).

2.2.6. Isothermal titration calorimetry (ITC)

Microcalorimetry experiments were performed on a VP-ITC MicroCalorimeter (MicroCal LLC, Northampton, MA, USA). Both protamine and hGH were in the same buffer (10 mM sodium phosphate pH 7.0) and degassed for at least 15 min prior to the measurements using a magnetically stirred vacuum apparatus. A pH of 7.0 was chosen for the buffer since the control experiment without protamine could not have been performed at pH 5.4 due to aggregation of pure hGH. 466 μM hGH solution was titrated in 3 μl steps into a 1.421 ml calorimeter cell, which contained 49 μM protamine in 10 mM phosphate buffer. The cell was stirred at 270 rpm and was thermally equilibrated at 25 °C until a flat baseline was obtained. The time between the injections was set to 300 s. To correct for the heat of dilution of the titrant, control experiments were performed by titrating hGH into buffer and the resulting heat of dilution was substracted from the actual binding isotherm prior to data analysis. The resulting data were integrated using Origin 7.0 (Origin Lab, Northampton, MA, USA) to obtain the enthalpy of binding.

2.2.7. Far-UV circular dichroism (CD)

Samples were prepared by diluting the formulations to 0.05 mg/ml hGH with sodium phosphate buffer of pH 5.4 or pH 7.0 for formulations with or without protamine, respectively. Circular dichroism spectra were measured on a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using a 0.1 cm quartz cuvette equilibrated at 25 °C. Spectra were recorded at 25 °C between 190 and 250 nm. The CD spectrometer was set to operate with a spectral bandwidth of 1 nm and a response time of 1 s. The background spectrum of the buffer with or without protamine was recorded and subtracted. Six spectra were averaged and then converted to mean residue ellipticity using a mean residue molecular weight of 115.838. The secondary structure contents were estimated using the CDSSTR program on the online server DICHROWEB (Whitmore and Wallace, 2004).

2.2.8. Chromatographic analysis of deamidation

The deamidation products of hGH were detected with a Reversed-Phase high performance liquid chromatography (RP-HPLC) system. A gradient HPLC system from Merck Hitachi Ltd. (Tokyo, Japan) was used. Separation was achieved using a Zorbax 300 Extend C18 column (4.6 mm × 100 mm length, 3.5 μm bead diameter and 300 Å pore size) (Agilent Technologies) operating at 50 °C. Two HPLC assays were established. One to analyze protamine-containing and a second one to analyze protamine-free formulations. For the two elution conditions four solvents were required: solvent A (0.007% TFA in Milli-Q water, pH 3.0), solvent B (70% acetonitrile, 30% solvent A, pH 3.0), solvent C (10% acetonitrile containing 10.5 mM $(NH_4)HCO_3$ and 0.105 mM EDTA, pH 7.0) and solvent D (70% acetonitrile containing 6.0 mM $(NH_4)HCO_3$ and 0.060 mM EDTA, pH 7.0). To achieve separation of protamine from hGH, a gradient of 2–40% solvent B with solvent A for 6.5 min at pH 3.0 was utilized. For analysis of hGH a second assay at neutral pH was linked, using a gradient elution from 60% to 95% solvent D with solvent C in 24.5 min, followed by a 5.5 min equilibration of 98% A and 2% B. Protamine-free samples were analyzed with a binary solvent system consisting of solvent C and solvent D. Absorbance at 210 nm was recorded for 30 min and 31.5 min in the absence and presence of protamine, respectively. The flow rate was 1.0 ml/min. Before analysis protamine-containing samples were diluted with solvent A and protamine-free samples were diluted with 266 mM glycine pH 7.0, according to an existing validated method for pure hGH. The final hGH concentration was 1 mg/ml in each case. The sample injection volume was 10 μl. The samples were kept at 4 °C in the autosampler until injection. Data was collected, integrated and analyzed using the Hitachi Ltd. Software. According to an existing validated method, deamidation products were identified by comparing their retention time with a suitable reference sample, acquired in the same sequence. Relative content of main deamidated and succinimide variants were evaluated by comparing the peak area of each component in the sample to the sum of all peak areas in the chromatogram. Peak area percentages of each component were calculated according to the following equation:

$$\frac{\text{Area component}}{\text{Area sum}} \times 100 \quad (1)$$

3. Results and discussion

3.1. Influence of protamine on hGH solubility

3.1.1. Turbidity studies

Solubility enhancing effect of protamine on hGH at a pH close to the isoelectric point of hGH was investigated by turbidity measurements. Turbidity is described as the cloudiness of a solution caused by sub-visible individual particles of various sizes. They scatter and absorb light and give the optical property of a liquid (Eckhardt et al., 1994). Ascending molar ratios of hGH-to-protamine from 1:1 to 1:39 at a pH of 5.4 were studied by monitoring the turbidity of the formulations (Table 1). Data of hGH-to-protamine ratios higher than 1:23 are not shown, as these samples remained free from visible particles and hGH concentration stayed constant. At an equimolar ratio of hGH-to-protamine, turbidity significantly reached values far exceeding hundred NTU units due to precipitation. However, the turbidity values decreased in accordance to the increase in amount of protamine. From a hGH-to-protamine ratio larger than 1:23, samples remained free from visible particles and were clear to slightly opalescent solutions, according to the Ph.Eur. 6.0 method 2.2.1 (Ph.Eur. 2.21, 2008).

The control samples without protamine at pH 5.4 were highly turbid, whereas pure hGH at pH 7.0 showed similar solubility as samples with a molar ratio of hGH-to-protamine of 1:23 or higher at pH 5.4. The investigated hGH–protamine complex (1:23), however, was not soluble at pH 7.0. We suppose the isoelectric point of the complex in this pH range. Pure hGH shows the highest

Table 1
Turbidity measurements and concentration detection of hGH formulations with ascending amounts of protamine. Samples at a hGH-to-protamine ratio of 1:23 (mol/mol) were chosen for further investigations.

hGH:protamine mol/mol	pH	Transmission [%]	Opalescence [NTU]	hGH concentration in the supernatant [mg/ml]
1:0	7.0	95.3 ± 0.45	4.2 ± 0.34	3.4 ± 0.06
1:0	5.4	0.4 ± 0.10	103.3 ± 0.44	1.5 ± 0.10
1:1	5.4	<0.3	–	0.8 ± 0.05
1:5	5.4	0.6 ± 0.07	102.5 ± 0.07	1.8 ± 0.05
1:10	5.4	52.3 ± 0.85	48.8 ± 0.15	2.9 ± 0.10
1:13	5.4	70.5 ± 0.69	30.0 ± 0.12	2.9 ± 0.10
1:16	5.4	79.3 ± 0.68	20.8 ± 0.72	3.1 ± 0.11
1:23	5.4	94.6 ± 0.26	5.0 ± 0.15	3.4 ± 0.02

Values represent mean ± standard deviation, $n = 3$.

solubility at pH 7.0, whereas increased aggregation tendency appears around the isoelectric point at approximately 5.3 (Pearlman and Bewley, 1993). This is because the net charge on the protein is zero at its isoelectric point, causing maximum interaction between the protein molecules (Banga, 2009). In this study it was achieved to increase the solubility minimum of hGH from about 1.5 mg/ml to 3.4 mg/ml at a pH of 5.4 by the addition of protamine. We could see clear to slightly opalescent solutions of 3.4 mg/ml hGH at a pH of 5.4 by the presence of an excess of protamine. Recently we could further increase the solubility of hGH up to 6.8 mg/ml at a pH of 5.4 by keeping the ratio between hGH and protamine constant (data not shown). It seems that protamine prevents hGH from a pH dependent aggregation by a surplus of protamine molecules. The results of the turbidity measurements were consistent with the data obtained by the determination of the hGH concentration in the supernatant (Table 1) where only soluble protein is present.

3.1.2. Dynamic light scattering

DLS was used as complementary analytical method additionally to turbidity measurements to detect soluble particles additionally to insoluble particle inspection via turbidity determination. DLS is suited to detect and characterize soluble particles on a length scale of ca. 1 nm to 10 μm (Mahler et al., 2009). The particle size and the width of the size distribution of hGH formulations with protamine at a molar ratio of 1:23 and without protamine are presented in Table 2. The d_h of the individual components was found to be about 6.0 nm for hGH and 3.8 nm for protamine. The PdI of both measurements was <0.25, indicating a narrow particle size distribution (Müller and Schuhmann, 1996). In the presence of protamine, a clear increase in size to 7.4 nm was detected. DLS measurements confirmed the results of the solubility study by turbidity measurements. No larger particles attributed to non-dissolved protein were detected for the hGH–protamine complex at pH 5.4.

3.2. Interaction of hGH and protamine

3.2.1. Small angle X-ray scattering

To get morphological information about the effects of protamine on the structure of hGH, we have performed SAXS experiments. The evaluation data are summarized in Table 3. The desmeared SAXS profiles are shown in Fig. 1A. First, from the Guinier plot we have determined the radii of gyration $R_g = 2.6\ nm^{-1}$ and $R_g = 2.11\ nm^{-1}$ for the protamine–hGH and pure hGH, respectively. Second, the decay in the scattering curves for $q > 1\ nm^{-1}$ was recorded, indicating a change in the protein shape upon addition of protamine. Both the increase in size and the change in shape for the protamine–hGH are also visible in the distance distribution function $p(r)$ shown in Fig. 1B. The $p(r)$ functions from pure protamine solution ($d_{max} \sim 4$ nm), protamine-free hGH solution ($d_{max} \sim 6.5$ nm) and the theoretical data derived from the crystal structure of hGH

Table 2
Size and polydispersity index (PdI) of hGH, the hGH–protamine complex (1:23, mol/mol) and protamine.

	Size [d_h, nm]	PdI
hGH	6.0 ± 0.2	0.162 ± 0.031
hGH–protamine complex	7.4 ± 0.1	0.210 ± 0.001
protamine	3.8 ± 0.1	0.089 ± 0.016

Values represent mean ± standard deviation, $n = 3$.

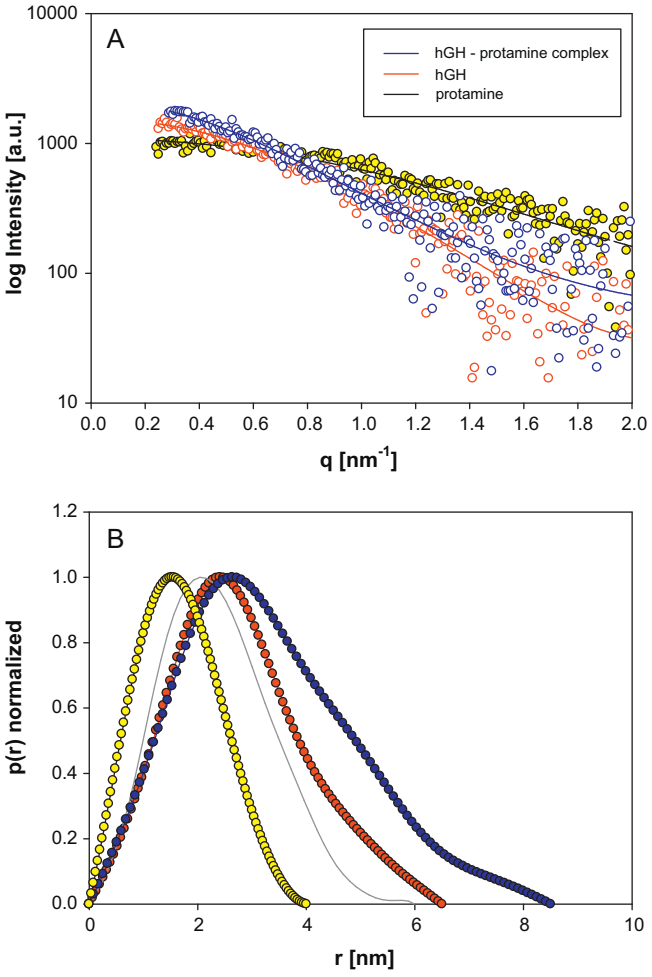

Fig. 1. Small angle X-ray scattering (SAXS) measurements: (A) Desmeared experimental scattering curves for the hGH–protamine complex (1:23, mol/mol, blue circles), hGH (red circles) and protamine (yellow circles) and the reciprocal space fits of scattering (lines) are shown. (B) Corresponding distance distribution functions $p(r)$ (same color code as before). As reference the theoretical scattering data for hGH calculated from the crystal structure (PDB ID: 3HHR, protein coordinates without receptors) are shown in gray.

Table 3
SAXS-data evaluation. The crystal structure of wild type human growth factor was used as reference (PDB ID: 3HHR, protein coordinates without receptor) (de Vos et al., 1992). Lysozyme (Lys) was used as standard to calibrate the molecular mass. The term $I(0)/c$ represents the concentration normalized absolute scattering intensity at $q = 0$ (Mylonas and Svergun, 2007).

Sample	R_g [nm]	d_{max} [nm]	$I(0)/c$ [10^{-2} cm^2 mg^{-1}]	Molecular mass [kDa]
hGH	2.11	6.5	5.40	26.5
hGH–protamine complex	2.60	8.5	9.11	45.1
protamine	1.23	4.0	1.12	5.7
hGH-crystal structure	1.85	6.0	–	22.1
SAXS standard (Lys)	1.58	5.0	2.95	14.3

($d_{max} \sim 6$ nm) are shown for comparison. Pure protamine reveals a compact globular shape in solution, although the highly positively charged polypeptide chain is more likely to show an elongated structure. Pure hGH exhibits curve characteristics indicative for spherical particles, however, a slight tailing observed at larger distances suggests that hGH is somewhat extended in solution. This feature is in good accordance with Synchrotron SAXS data presented recently by Chen et al. (2010). The authors also found a somewhat larger dimension for hGH in solution compared to the crystal structure. For hGH in presence of protamine a shift of the maximum in the $p(r)$ function to larger distances and a pronounced increase in maximum particle diameter ($d_{max} \sim 8.5$ nm) were observed (see Fig. 1B). The shape of the distance distribution function indicates that the overall structure of hGH–protamine is more elongated and that a conformational change has occurred upon addition of protamine. The most likely interpretation of these data would be a complex formation of hGH with several protamine molecules, although an indirect alteration of protein structure induced by protamine cannot be ruled out.

3.2.2. Determination of zeta potential

Zeta potential measurements give information about the surface electrical properties of proteins. The magnitude of the surface charge of proteins depends on the acidic or basic strengths of the surface groups. It also depends on the pH of the solution (Bowen et al., 1998) therefore we measured the zeta potential of pure hGH and of the hGH–protamine complex at both pH values, pH 5.4 and pH 7.0 (Table 4). In spite of partial insolubility of 3.4 mg/ml pure hGH at pH 5.4 and of the hGH–protamine complex at pH 7.0, zeta potential could be detected because they stayed stable colloidal systems during the measurement. The zeta potential of 0.8 ± 0.5 mV of pure hGH at pH 5.4 reflects the closeness to its isoelectric point. Also the zeta potential of 3.5 ± 1.6 mV of the hGH–protamine complex at pH 7.0 indicates the closeness to the isoelectric point of the complex. At pH 7.0 pure hGH was measured to carry a negative surface charge in 10 mM sodium phosphate buffer. The zeta potential of pure protamine could not be detected, as its particle size (molecular weight ~4200 Da) is too small for zeta potential measurements. Protamine is expected to have a positive surface charge due to its high arginine content. By adding protamine to hGH, the charge changes to a positively charged surface at pH 5.4 (Table 4). Changing zeta potential from negative to positive charge at the presence of protamine indicates electrostatic adherence between one negatively charged hGH molecule and more strongly positively charged protamine molecules.

Table 4
Zeta potential measurements of hGH and the hGH–protamine complex (1:23, mol/mol) at pH 5.4 and pH 7.0.

	pH	Zeta potential [mV]	pH	Zeta potential [mV]
hGH	5.4	0.8 ± 0.5	7.0	-8.5 ± 1.5
hGH–protamine complex	5.4	7.4 ± 1.9	7.0	3.5 ± 1.6

Values represent mean ± standard deviation, $n = 3$.

3.2.3. ITC binding studies

ITC is the method of choice for determining the thermodynamic changes in the course of ligand binding to a protein. ITC measures the heat associated with binding of a cosolvent to a protein (Schön and Velazquez-Campoy, 2005). It can also give information about nonspecific binding, like electrostatic interactions (Ball and Maechling, 2009). ITC experiments with hGH and protamine were performed to supplement SAXS and zeta potential binding data. As shown in Fig. 2A the binding of protamine to hGH resulted in an endothermic reaction. The observed endothermic reaction could result from the displacement of water from hGH hydration shell by protamine binding. This effect has been described for other cosolvents like guanidine which binds nonspecifically to proteins (Schneider and Trout, 2009). The obtained thermodynamic parameters, enthalpy and entropy were positive. Due to aggregation during titration at a molar ratio of hGH-to-protamine < 1:23, it was not possible to distinguish between energy influx of protamine binding and heat capacity changes associated with precipitation. Therefore the binding behavior between protamine and hGH could only be qualitatively analyzed and a determination of the accurate binding stoichiometry between hGH and protamine was not

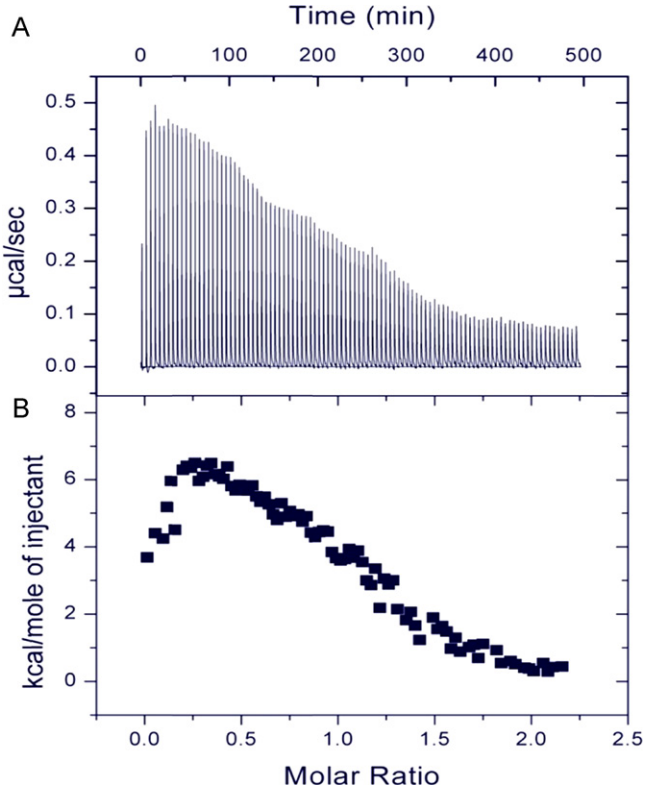

Fig. 2. (A) Raw isothermal titration calorimetry (ITC) data for protamine titration with hGH. (B) ITC isotherm for the binding of protamine to hGH. After integration of the raw signal, enthalpy of binding of each injection was obtained and plotted versus molar ratio of hGH:protamine.

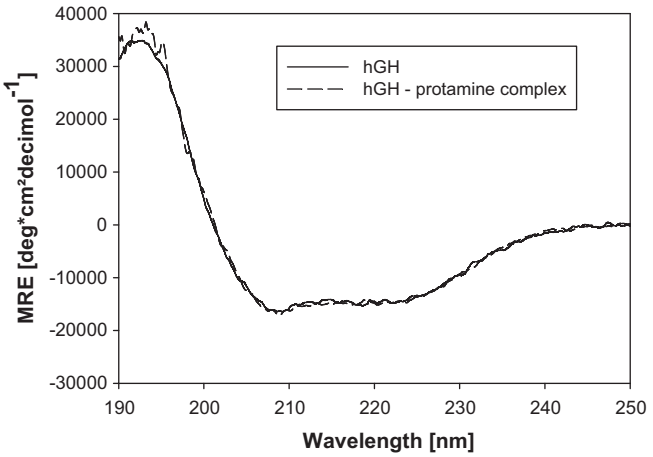

Fig. 3. Circular dichroism spectra of pure hGH (pH 7.0) and the hGH–protamine complex (1:23, mol/mol; pH 5.4).

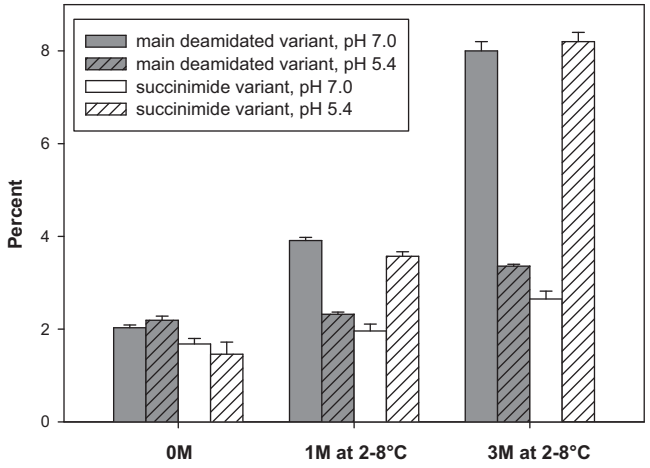

Fig. 4. Deamidation products of hGH measured by RP-HPLC after preparation, after 1 and 3 months storing at 2–8 °C. The main deamidated variant is represented by gray columns and the succinimide variant is represented by white columns. The columns without diagonal patterns indicate the absence of protamine and a pH of 7.0 whereas the columns with diagonals indicate the presence of protamine and a pH of 5.4.

possible. By plotting the integrated areas of the enthalpy curve against hGH-to-protamine molar ratios (Fig. 2B) and performing a nonlinear least-squares fit, the binding constants were evaluated. The dissociation constant calculated from the binding constants for estimated ten possible binding sites of hGH for protamine was in the range of 3.2–10 μM. Due to the expected high number of binding sites, we assumed a nonspecific binding between hGH and protamine molecules.

3.3. Structural characterization by CD spectroscopy

Secondary structure of hGH in the presence and absence of protamine at pH 5.4 and pH 7.0 respectively, was investigated to evaluate the influence of protamine on the structural integrity of hGH. Both spectra show a negative peak with two maxima at 222 nm and 208 nm and a positive peak at about 195 nm, indicative of an alpha-helical structure (Fig. 3). hGH without protamine at pH 7.0 as well as hGH with protamine at pH 5.4 were found to have an alpha-helical content of 54% (Table 5). This value agrees with previously reported data about the alpha-helical content of hGH, as determined by circular dichroism (Bewley and Li, 1972). In the presence of protamine at pH 5.4 minor alterations appeared in β-sheet and turns content. Therefore, our findings indicate integrity of hGH in the presence of protamine at pH 5.4.

3.4. Stability study

3.4.1. Solubility study

Turbidity of protamine-free and protamine-containing (1:23, mol/mol) hGH formulations was investigated in a storage stability study at 2–8 °C. Samples were sterile filtered after preparation and turbidity was investigated on the same day. Further turbidity measurements were performed after 1 and 3 months of incubation at 2–8 °C. The sterile filtration did not influence the concentration of hGH in the formulations. As can be seen in Table 6, both formulations, protamine-free and protamine-containing, showed similar transmission values and corresponding NTU values at all three time points. They can be described as clear to slightly opalescent solutions. Standard deviations of the transmission values are not statistically significant. Both protamine-containing samples and pure hGH samples were up to the requirements of the European pharmacopeia with a turbidity of well below 30 NTU over the whole storage time.

Table 5
Secondary structure of hGH formulations with and without protamine at pH 5.4 and pH 7.0 respectively, determined by CDSSTR analysis.

	α-Helix	β-Sheet	Turns	Unordered
hGH	0.54 ± 0.02	0.20 ± 0.03	0.11 ± 0.02	0.15 ± 0.02
hGH–protamine complex	0.54 ± 0.01	0.22 ± 0.02	0.09 ± 0.03	0.15 ± 0.03

Values represent mean ± standard deviation, $n = 6$.

3.4.2. Deamidation study

It is known that deamidation proceeds through a cyclic imide intermediate at neutral and alkaline pH values. Furthermore deamidation is accelerated at neutral or alkaline conditions. From pH 5 to pH 12, the rate constant of deamidation increases with hydroxide ion concentration (Kirchhoff, 2010; Manning et al., 2010, Patel and Borchardt, 1990). Therefore lowering the pH to 5.4 should result in a reduced rate of deamidation. By retarding deamidation, the formation of the racemic mixture of aspartyl and isoaspartyl residues is reduced. When isoasparagine forms, the peptide backbone is extended by one carbon atom. This alteration in the backbone of hGH can be potentially immunogenic (Cleland et al., 1993; Jenkins et al., 2008).

Deamidation products of hGH in the absence and presence of protamine at pH 7.0 and pH 5.4 respectively, were detected by RP-HPLC. Samples were measured directly after preparation to determine if any deamidation occurred during the sample preparation steps. Deamidation products were also detected after 1 and 3 months storing at 2–8 °C. The proportion of succinimide and the main deamidated variant were assessed as described in Section 2.28, from peak area data, and plotted over 3 months. The main deamidated variant eluted at approximately 1.3 min in front of the peak of native hGH because the side chain carboxylic acid of isoaspartate and aspartate bears a negative charge. It is less hydrophobic than the side chain amide of asparagine and therefore elutes earlier. The cyclic imide variant contains one less negative charge than the native protein and thus elutes after the main peak. As shown in Fig. 4, addition of protamine and a pH of 5.4 had no effect on the deamidation level of hGH immediately after sample preparation. Both formulations, with and without protamine, contained about 1.6% succinimide and 2.1% main deamidated variant after preparation. In the absence of protamine at pH 7.0, the level of succinimide variant increased slightly and the proportion of main deamidated variant increased significantly from 2.1% to 8.0% during 3 months

Table 6
Turbidity measurements of protamine-free and protamine-containing hGH formulations (1:23, mol/mol) after preparation, after 1 month and 3 months storing at 2–8 °C. Formulations were sterile filtered before initial time point.

	pH	Transmission [%]			Opalescence [NTU]		
		0 M	1 M	3 M	0 M	1 M	3 M
hGH	7.0	98.4 ± 1.60	95.8 ± 1.38	94.3 ± 0.57	1.1	3.7	5.3
hGH–protamine complex	5.4	96.0 ± 3.32	94.0 ± 3.75	93.7 ± 0.96	3.5	5.6	5.9

Values represent mean ± standard deviation, $n = 3$.

storing at 2–8 °C. On the contrary, by addition of protamine at pH 5.4, the level of succinimide variant increased significantly but the main deamidated variant increased only marginally during 3 months storing. It seems that the succinimide variant is almost stable at pH 5.4 and deamidation is retarded at this stage. Compared to pH 7.0, there can be seen a notedly retardation of deamidation at the succinimide intermediate with protamine at pH 5.4. A reduction of the deamidated racemic mixture of aspartyl and isoaspartyl residues could be achieved.

4. Conclusions

In this study, a solubility enhancing effect of protamine on hGH close to its isoelectric point at pH 5.4 was demonstrated. Binding of protamine molecules to hGH was postulated and could be verified by SAXS and ITC measurements. Inversion of surface charge indicated electrostatic interactions causing complexation between hGH and protamine molecules. Integrity of secondary structure of hGH in the presence of protamine was confirmed by CD spectroscopy. Storage studies at 2–8 °C showed stability related to solubility. Deamidation could be retarded at succinimide intermediate at pH 5.4. Protamine presents a promising cosolvent by providing sufficient benefit on hGH solubility. It enables the production of higher concentrated hGH formulations at pH values close to the isoelectric point of hGH. Solubilization of hGH at pH 5.4 leads to a decrease of the deamidation rate.

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References

Ando, T., Yamasaki, M., Suzuki, K., 1973. Protamines: Isolation, Characterization, Structure and Function, vol. 1. Springer Verlag, Berlin.
Ball, V., Maechling, C., 2009. Isothermal microcalorimetry to investigate non specific interactions in biophysical chemistry. Int. J. Mol. Sci. 10, 3283–3315.
Banga, A.K., 2009. Formulation of therapeutic peptides and proteins. In: Banga, A.K. (Ed.), Therapeutic peptides and proteins Formulation, Processing and Delivery Systems. Technomic Publishing Company Inc., Lancaster, pp. 81–129.
Baynes, B.M., Trout, B.L., 2004. Rational design of solution additives for the prevention of protein aggregation. Biophys. J. 87, 1631–1639.
Becker, G.W., Tackitt, P.M., Bromer, W.W., Lefeber, D.S., Riggin, R.M., 1988. Isolation and characterization of a sulfoxide and a desamido derivative of biosynthetic human growth hormone. Biotechnol. Appl. Biochem. 10, 326–337.
Bewley, T.A., Li, C.H., 1972. Circular dichroism studies on human pituitary growth hormone and ovine pituitary lactogenic hormone. Biochemistry 11, 884–888.
Bowen, W.R., Hall, N.J., Pan, L., Sharif, A.O., Williams, P.M., 1998. The relevance of particle size and zeta-potential in protein processing. Nat. Biotechnol. 16, 785–787.
Brange, J., Skelbaek-Pedersen, B., Langkjaer, L., Damgaard, U., Ege, H., Hayelund, S., Heding, L.G., Jorgensen, K.H., Lykkeberg, J., Markussen, J., Pingel, M., Rasmussen, E., 1987. Galenics of Insulin – The Physico-Chemical and Pharmaceutical Aspects of Insulin and Insulin Preparations, first ed. Springer Verlag, Berlin.
Chen, W.Q., Salmazo, A., Myllykoski, M., Sjoblom, B., Bidlingmaier, M., Pollak, A., Baumgaertel, P., Djionovic-Carugo, K., Kursula, P., Lubec, G., 2010. Purification of recombinant growth hormone by clear native gels for conformational analyses: preservation of conformation and receptor binding. Amino Acids 39, 859–869.
Cholewinski, M., Lückel, B., Horn, H., 1996. Degradation pathways, analytical characterization and formulation strategies of a peptide and a protein calcitonine and human growth hormone in comparison. Pharm. Acta Helv. 71, 405–419.
Cleland, J.L., Powell, M.F., Shire, S.J., 1993. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. Crit. Rev. Ther. Drug Carrier Syst. 10, 307–377.
de Vos, A.M., Ultsch, M., Kossiakoff, A.A., 1992. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255, 306–312.
Eckhardt, B.M., Oeswein, J.Q., Yeung, D.A., Milby, T.D., Bewley, T.A., 1994. A turbidimetric method to determine visual appearance of protein solutions. PDA J. Pharm. Sci. Technol. 48, 64–70.
Friess, W., Mahler, H.C., Jörg, S., 2010. Highly concentrated protein formulations. In: Mahler, H.C., Borchard, G., Luessen, H.L. (Eds.), Protein Pharmaceuticals – Formulation, Analytics and Delivery. Editio Cantor Verlag, Aulendorf, pp. 192–220.
Hamada, H., Arakawa, T., Shiraki, K., 2009. Effect of additives on protein aggregation. Curr. Pharm. Biotechnol. 10, 400–407.
Hoffmann, J.A., Chance, R.E., Johnson, M.G., 1990. Purification and analysis of the major components of chum salmon protamine contained in insulin formulations using high-performance liquid chromatography. Protein Expr. Purif. 1, 127–133.
Jenkins, N., Murphy, L., Tyther, R., 2008. Post-translational modifications of recombinant proteins: significance for biopharmaceuticals. Mol. Biotechnol. 39, 113–118.
Kirchhoff, C., 2010. Introduction to protein chemistry. In: Mahler, H.C., Borchard, G., Luessen, H.L. (Eds.), Protein Pharmaceuticals – Formulation, Analytics and Delivery. Editio Cantor Verlag, Aulendorf, pp. 18–43.
Kudou, M., Shiraki, K., Fujiwara, S., Imanaka, T., Takagi, M., 2003. Prevention of thermal inactivation and aggregation of lysozyme by polyamines. Eur. J. Biochem. 270, 4547–4554.
Mahler, H.C., Friess, W., Grauschopf, U., Kiese, S., 2009. Protein aggregation: pathways, induction factors and analysis. J. Pharm. Sci. 98, 2909–2934.
Manning, M.C., Chou, D.K., Murphy, B.M., Payne, R.W., Katayama, D.S., 2010. Stability of protein pharmaceuticals: an update. Pharm. Res. 27, 544–575.
Marian, M., 2002. Growth hormones. In: Crommelin, D.J.A., Sindelar, R.D. (Eds.), Pharmaceutical Biotechnology – An Introduction for Pharmacists and Pharmaceutical Scientists. Taylor & Francis, Abingdon, pp. 245–258.
Müller, R.H., Schuhmann, R., 1996. Teilchengrößenmessung in der Laborpraxis, first ed. Wissenschaftliche Verlagsgesellschaft mbh, Stuttgart.
Mylonas, E., Svergun, D.I., 2007. Accuracy of molecular mass determination of proteins in solution by small-angle X-ray scattering. J. Appl. Crystallogr. 40, S245–S249.
Patel, K., Borchardt, R.T., 1990. Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. Pharm. Res. 7, 703–711.
Pearlman, R., Bewley, T.A., 1993. Stability and characterization of human growth hormone. In: Wang, Y.J., Pearlman, R. (Eds.), Stability and Characterization of Protein and Peptide Drugs: Case Histories. Plenum Press, New York, pp. 1–58.
Ph.Eur. 2.21, 2008. Clarity and Degree of Opalescense of Liquids, 6.2 edition. European Directorate for the Quality of Medicine, p. 23.
Ph.Eur. 6.0/0951, 2008. Somatropin, 6.2 edition. European Directorate for the Quality of Medicine, pp. 3937–3940.
Riggin, R.M., Farid, N.A., 1990. Analytical chemistry of therapeutic proteins. In: Horvath, C., Nikelly, J.G. (Eds.), Analytical Biotechnology: Capillary Electrophoresis and Chromatography. American Chemical Society, Washington DC, pp. 113–126.
Robinson, N.E., et al., 2004. Structure-dependent nonenzymatic deamidation of glutaminyl and asparaginyl pentapeptides. J. Peptide Res. 63, 426–436.
Schneider, C.P., Trout, B.L., 2009. Investigation of cosolute-protein preferential interaction coefficients: new insight into the mechanism by which arginine inhibits aggregation. J. Phys. Chem. B 113, 2050–2058.
Schön, A., Velazquez-Campoy, A., 2005. Calorimetry. In: Jiskoot, W., Crommelin, D.J.A. (Eds.), Methods for Structural Analysis of Protein Pharmaceuticals, vol. 3. AAPS, Arlington, VA, pp. 573–589.
Shukla, D., Trout, B.L., 2010. Interaction of arginine with proteins and the mechanism by which it inhibits aggregation. J. Phys. Chem. B 114, 13426–13438.

Svergun, D.I., 1992. Determination of the regularization parameter in indirect-transform methods using perceptual criteria. J. Appl. Crystallogr. 25, 495–503.
Svergun, D.I., Barberato, C., Koch, M.H.J., 1995. CRYSOL – a program to evaluate X-ray solution scattering of biological macromolecules from atomic coordinates. J. Appl. Crystallogr. 28, 768–773.
Whitmore, L., Wallace, B.A., 2004. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res. 32, W668–W673.
Yasui, K., Uegaki, M., Shiraki, K., Ishimizu, T., 2010. Enhanced solubilization of membrane proteins by alkylamines and polyamines. Protein Sci. 19, 486–493.