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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