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Stabilization of somatropin by heparin

Camellia Zamiri and Michael J. Groves

Abstract

Somatropin, human growth hormone (hGH), is an unstable protein, posing challenging problems for its formulation and long-term stability. Since hGH formed insoluble adducts with heparin our aim was to evaluate heparin as a stabilizing agent for the drug. These adducts were characterized by particle diameter, tertiary structure variations and release studies. Studies were also carried out to determine the stability of hGH in the presence and absence of heparin by an interfacial denaturation method and real-time stability studies by measuring hGH activity and particle diameter. Moreover, biological activity of hGH and hGH/UH (unfractionated heparin) adducts was identified by daily subcutaneous injections to hypophysectomized rats. There was a decrease in mean hydrodynamic particle diameter of hGH/UH adducts with increased pH (54.4 to 12.2 nm from pH 3 to pH 7) indicating that the adducts were either dissociating or dissolving at high pH. Furthermore, second-derivative spectroscopy indicated that complexation of hGH with heparin did not cause a major disruption in the tertiary structure of hGH but decreased the hydrophilic environment around the tyrosine residues. Release of hGH from hGH/UH adducts was pH and ionic strength dependent with the highest release at pH 8 (93%) and lowest release at pH 3 (0%) over the first hour. Interfacial denaturation methods indicated that vortex agitation over 120 s resulted in no change in the optical density of hGH/UH adducts compared with a substantial increase for hGH alone at pH 6.8. Real-time stability studies over 93 days demonstrated that hGH/UH adducts at both pH 3 and 7 with an excess of heparin produced the highest percent of active hGH remaining in the solution at 4°C and 37°C. The higher stability of hGH/UH adducts with excess heparin compared with the stoichiometric ratio was also confirmed by particle size measurements during storage. The biological activity of these adducts was comparable with hGH alone by weight-gain studies in hypophysectomized rats. The findings suggest the value of using hGH/heparin adducts to stabilize the protein.

Introduction

Somatropin, human growth hormone (hGH), is a protein with a molecular weight of approximately 22 kDa and has been approved by the US Food and Drug Administration (FDA) for the treatment of growth hormone deficiency in children and adults with a history of hypothalamic pituitary disease, short stature associated with chronic renal insufficiency before renal transplantation or short stature in patients with Turner's syndrome (American Association of Clinical Endocrinologists 2003).

Like most large proteins, hGH possesses unique chemical and physical properties, which result in difficult stability issues. hGH is susceptible to chemical and physical degradation. Major chemical degradation pathways for hGH are deamidation, oxidation, reduction or interchange of disulfide bonds and hydrolysis. Physical instability refers to changes in higher-order structures (secondary and above). These include denaturation, adsorption to surfaces, aggregation and precipitation (Pearlman & Bewley 1993). Aggregation is a significant problem in the pharmaceutical development of most proteins. Small amounts of insoluble aggregates may affect the pharmaceutical elegance of the product, producing a parenteral product unsuitable for use. Aggregates may also result in reduced bioactivity as has been shown to be the case for hGH (Becker et al 1987). A more serious consequence is the implication that aggregated growth hormone promotes the formation of an antibody response to the protein (Rougeot et al 1991). Efforts have been made to stabilize proteins by additives, excipients, chemical modification and the use of site-directed mutagenesis to produce a more stable protein species (Manning et al 1989).

Department of Biopharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, USA

Camellia Zamiri*, Michael J. Groves†

Correspondence: M. J. Groves, 980 C Ivy Lane, Deerfield, IL 60015, USA.
E-mail: Mikegroves58@aol.com

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Current addresses: *Protein Design Labs, Inc., 34801 Campus Drive, Fremont, CA 94555, USA; †980 C Ivy Lane, Deerfield, IL 60015, USA.

Glycosaminoglycans (GAGs), including heparin, are known to bind to proteins such as growth factors, plasma lipoproteins, fibronectin and antithrombin III to regulate metabolic processes in both normal and disease states (Jackson et al 1991). Electrostatic binding occurs between the negatively charged GAGs and positively charged macromolecules, such as proteins (Comper & Laurent 1978). The heparin-binding regions of these proteins are characterized by clusters of basic amino acid residues such as arginine, lysine and histidine. The use of heparin as a stabilizing agent for growth factors, such as acidic fibroblast growth factor (aFGF), keratinocyte growth factor (KGF) and transforming growth factor-beta 2 (TGF-β2), has been reported (Chen et al 1994; Volkin & Middaugh 1996; Schroeder-Tefft et al 1997). TGF-β2 loses biological activity under physiological conditions as measured by loss of activity in phosphate-buffered saline (PBS), pH 7.4, at 37°C. In-vitro studies showed that heparin/TGF-β2 remained active and TGF-β2 alone lost activity, when stored over two months in PBS at 37°C (Schroeder-Tefft et al 1997).

hGH has eight lysine, ten arginine and three histidine residues (Bewley & Li 1975). Therefore, it is reasonable to assume that hGH has sufficient basic amino acid residues to interact with heparin. The objective of this study was to improve the stability of hGH by forming an adduct with heparin. Therefore hGH/heparin adducts were prepared and characterized. The physical stability of hGH under stress conditions and real-time stability (3 months) in the presence and absence of heparin were investigated. Finally, biological activity of hGH in the presence and absence of heparin was evaluated in hypophysectomized rats.

Materials and Methods

Materials

Norditropin (somatropin, recombinant human growth hormone, rhGH) from Novo Nordisk (Bagsvaerd, Denmark) was obtained from the UIC hospital pharmacy (Chicago, IL). Each vial of Norditropin contains 4 or 8 mg somatropin, 8.8 mg glycine, 1.3 mg disodium phosphate dihydrate, 1.1 mg sodium dihydrogen phosphate dihydrate and 44 mg mannitol. Heparin Sodium USP (porcine intestinal mucosa) (179 USP units/mg) was purchased from Sigma (St Louis, MO). Ion-exchange resin (Bio-Rex 70 resin, 100–200 mesh, Sodium form) was obtained from Bio-Rad Laboratories (Richmond, CA). MICRO-ELISA hGH kit was purchased from Bio-Clin, Inc. (St Louis, MO). The remainder of the materials were purchased from Fisher Scientific (Itasca, IL).

Preparation of human growth hormone/heparin adducts

Sodium heparin or unfractionated heparin (UH) was passed through an acidic ion-exchange resin column and converted to its acid form (heparinic acid). Then heparinic acid, pH 3.0–4.0, was added to the acidified (with HCl 0.1 M) solution of hGH, pH 3, at the stoichiometric ratio (the condition in which neither excess hGH nor UH was remaining in the filtrate) of 1.8:1 (molar ratio), hGH and UH respectively (Zamiri & Dadey 2000). A suspension of the hGH/UH adduct was obtained (pH $\cong$ 3).

Particle size measurement

The effect of pH on the mean hydrodynamic particle diameter, $\bar{d}$, of hGH/UH adducts was determined by photon correlation spectroscopy (PCS). hGH/UH adducts were prepared at the stoichiometric ratio (pH 3). Following preparation, pH was adjusted by adding NaOH (0.01 M) to different pH values (pH 4–7). The volume weighted mean hydrodynamic particle diameter, $\bar{d}_v$, of the insoluble adducts was determined by PCS using a Nicomp 380 Submicron Particle Sizer (Pacific Scientific Instrument Division, Silver Spring, MD) at 23°C. The instrument was equipped with a 5 mW helium–neon laser light source (632.8 nm) operated at 90° to the detector.

Second-derivative spectroscopy

Near-UV absorption spectroscopy (240–320 nm) has been shown to provide useful information about the aromatic amino acids that absorb in the spectral range and provide a method for evaluating the tertiary structure variations (Havel et al 1989). Second-derivative spectroscopy, because of its ability to reduce overlap of the spectral contributions of aromatic chromophores, has been successfully applied in the determination of conformationally dependent tyrosine and tryptophan exposures in proteins (Terada et al 1984; Havel et al 1986).

UV absorption spectroscopy studies in the near UV region (250–320 nm) were performed by a Beckman DU 640 Spectrophotometer (Fullerton, CA) using a sampling interval of 1 nm with four scans per sample at ambient temperature (23 $\pm$ 2°C). The spectra of hGH (1.2 mg mL^{-1}) in aqueous solutions of hGH/UH adducts at the stoichiometric ratio at pH 3 and 6 were obtained. hGH aqueous solutions (1.2 mg mL^{-1}) at pH 3 and 7 were used as controls, with PBS (pH 7.4) as the blank.

Scatter correction in the spectrophotometer software program was applied to eliminate the effect of light scattering in the turbid samples, such as hGH/UH adducts at pH 3. Derivative analysis of a given spectrum was performed directly on the unsmoothed data by translocation of the spectrum, first 1 nm towards higher wavelengths to generate a red spectrum, then 1 nm towards the lower wavelength to produce a blue spectrum. Subtraction of the red spectrum from the blue spectrum generated the corresponding derivative spectrum of $\lambda = 2$ nm. Repeating this process provided second-derivative spectra (Ackland et al 1991).

In-vitro release studies

The release studies were performed in a shaker bath (Precision Scientific, Winchester, VA) at 37°C in buffers at different pH (pH 3 and 5, citrate-phosphate buffer; pH

7.4 and 8, PBS) and different ionic strengths of PBS (pH 7.4) ($\mu = 0.04$, 0.174 and 0.7, by adding NaCl to adjust the ionic strength) in closed test tubes with occasional shaking for 48 h. The ionic strength of buffer media at pH 5 was slightly higher than pH 3, 7.4 and 8 ($\mu \cong 0.17$). Lyophilized hGH/UH adducts at the stoichiometric ratio (143.7 μg mL^{-1} hGH/61.6 μg mL^{-1} UH) were prepared and suspended in buffers in individual test tubes for each time interval. Three test tubes for each time interval were prepared. At predetermined time intervals the samples were filtered through 20-nm filter membranes (Whatman Anodisc low protein binding filter membranes; Fisher Scientific, Itasca, IL) and the filtrate assayed for hGH by ELISA and the absorbance of the samples was determined by a plate reader (Lab systems Multiskan Plus manufactured for Fisher Scientific, Itasca, IL) at 450 nm. The amount of hGH lost during filtration by 20-nm filter membranes in different buffer media was calculated and accounted for in interpreting the results.

Statistical analysis
Statistical significance was assessed by analysis of variance and the Student–Newman–Keuls method, with $P < 0.05$ taken as being statistically significant.

Aggregation method: interfacial denaturation

A high air–water interface was introduced into the samples by vortex agitation, as a comparative denaturing technique, to induce aggregation. hGH aqueous solutions and hGH/UH adducts at the stoichiometric ratio and with excess heparin (four times the amount required at the stoichiometric ratio) and different pH values (adjusting pH by adding NaOH 0.01 M) were prepared. Each sample (0.5 mg mL^{-1} hGH, 1.3 mL) was vortexed (Vortex Genie; Fisher Scientific, Itasca, IL) for 0, 20, 40, 60 and 120 s. Following vortexing, precipitation was allowed to proceed at room temperature for 30 min, after which the optical density due to light scattering was assessed at 450 nm using a Beckman UV spectrophotometer. Comparison of formulations was made by plotting optical density changes (ΔOD) versus agitation time. The increase in optical density (OD) was an indication of aggregation of the protein.

Real-time stability studies

Preparation of hGH formulations
hGH aqueous solutions, hGH/UH adducts at the stoichiometric ratio and with an excess heparin at different pH values were prepared (150 μg mL^{-1} hGH). For each day, temperature (4 and 37°C) and analysis method, individual vials of hGH formulations were prepared. Samples for stability studies at 4°C were placed in the refrigerator and at 37°C in an incubator.

Determination of activity of hGH
hGH formulations were passed through a 0.45-μm filter membrane (Whatman polysulfone low protein binding 13 mm syringe filter; Fisher Scientific, Itasca, IL) to remove the insoluble aggregates. The filtrate was dissolved in 300 μL of NaOH (0.1 M) to dissociate the hGH/UH adducts and analysed by ELISA.

Particle size determination
hGH formulations were passed through a 1-μm filter membrane (Whatman polysulfone low protein binding 13 mm syringe filter; Fisher Scientific, Itasca, IL) to remove protein aggregates bigger than 1 μm produced over time. The filtrate was analysed using a Nicomp 380 Submicron Particle Sizer to measure the volume weighted mean hydrodynamic particle diameter, $\bar{d}_v$, of hGH formulations.

hGH in-vivo bioactivity

Preparation of hGH formulations
hGH/UH adducts at the stoichiometric ratio were prepared as previously described and lyophilized individually for each day's experiment and kept in the refrigerator until the day of administration. A stock solution of hGH alone was also prepared and lyophilized in small volumes for each day's experiment. On the day of each experiment hGH (0.32 mg kg^{-1}), hGH/UH adducts (0.32 mg hGH/kg and 0.13 mg heparin/kg) were reconstituted in PBS (pH 7.4). The PBS was filtered through a 0.22-μm filter membrane. All samples kept at room temperature for 0.5–1 h before administration.

Animal studies
The ACC protocol (no. A-02-033) for animal use was approved by the Animal Care Committee (ACC) of the University of Illinois at Chicago. Female hypophysectomized Sprague-Dawley rats, 85–99 g, were obtained from Taconic (Germantown, NY). The rats had been hypophysectomized one week before shipping and were acclimatized for one week before experimentation. As an assurance of hypophysectomy, no rat was included in the study if it gained more than 5 g in weight during this pre-experimental period. The rats were group-housed 3 or 4 per cage on bedding, with a 12-h light–dark cycle, in a humidity- and temperature-controlled room. The rats were provided with water containing 5% sucrose for 7 days after shipping (recommended by the supplier) and thereafter allowed free access to tap water and a commercial rat diet (over all days). Immediately after receiving the rats, a daily substitution treatment with 500 μg kg^{-1} subcutaneous hydrocortisone (Solu Cortef; Pharmacia Upjohn, Kalamazoo, MI) and 10 μg kg^{-1} subcutaneous levothyroxine (Bedford Laboratories, Bedford, OH) was begun. This was necessary to prevent complications arising from hypox-associated hypothyroidism and cortisone deficiency (Kidder et al 1997). Rats were randomly assigned to treatment groups, which were balanced to give approximately equal mean initial body weights. Using a similar procedure, the rats were also assigned at random to cages. The rats were individually weighed every day on a Sartorius balance (Goettingen, Germany) at 0900 h each morning, and daily injections (0.09–0.12 mL/injection, in the lateral flank, s.c.) were administered at 1400 h for 11 days.

The bioassay response for each day, ΔBWG (body weight gain), was computed by the formula:

$$\Delta BWG = BW_d - BW_i \quad (1)$$

where BW_i is the body weight on the initial day of injection (bioassay day 1) and BW_d is the body weight on any subsequent day, including the last day, of the bioassay period (Groesbeck & Parlow 1987).

Statistical analysis

Statistical comparisons were assessed by the Kruskal–Wallis test (the non-parametric analysis of variance) with $P < 0.05$ taken as being statistically significant. Individual differences between groups were determined by Dunn's method.

Results and Discussion

Preparation of hGH/UH adducts

Upon addition of UH to hGH, a turbid solution or precipitate was formed immediately. This phenomenon has been observed previously in the complexation of GAGs with basic proteins (Scott 1968). To ensure that there was no interaction between UH and hGH excipients, including mannitol and glycine, each excipient was mixed separately with UH at different amounts by exactly the same procedure as described before. The addition of UH to excipients resulted in no turbidity, indicating absence of interaction.

Particle size measurement

The mean hydrodynamic particle diameter of hGH/UH adducts, as a function of media pH, is shown in Table 1. There was a poor linear relationship ($r^2 = 0.845$) (diameter $= -10.0\,pH + 81.1$) between $\bar{d}_v$ and pH. By increasing the pH, $\bar{d}_v$ was decreased from 54.4 ± 8.0 nm to 12.2 ± 1.9 nm (pH 3 to pH 7). This explains the changes in visual appearance of hGH/UH adducts from turbid in acidic pH to clear at neutral pH. It is likely that by increasing the pH, hGH/UH adducts are either dissociating or dissolving. Since the size of the hGH monomer is about 4 nm (DeFelippis et al 1992; Maa & Hsu 1996), at neutral pH, it is evident that hGH/UH adducts are not dissociating completely.

Second-derivative spectroscopy

Tryptophan 86, located on helix II in the hydrophobic interior of the protein, is the only tryptophan residue in hGH and has been shown to be sensitive to the folded state of the protein (Brems et al 1990; DeFelippis et al 1995). In solution tryptophan possesses two vibronic bands, ^1L_a centered at 292 nm and ^1L_b at 291 nm (Bewley & Li 1984). The tryptophan absorption bands in hGH were shifted to around 303 and 292 nm, respectively. It has been shown that a large part of this shift is due to hydrogen-bond formation between Trp 86 on helix II and Asp169 located on helix IV (Bewley & Li 1984). In the hGH/UH adducts, perturbations typical of tryptophan absorption are observed in the 290–310 nm region (data not shown). The most dramatic shift toward shorter wavelengths (blue shift) relative to native somatropin at pH 7 was exhibited by the wavelength maximum of the ^1L_b absorption band. Thus, only the ^1L_b bands are presented in Table 2. hGH/UH adducts at pH 3 did exhibit minor blue shifts compared with somatropin at pH 7. The blue shift may be partially due to acidification of hGH before making hGH/UH adducts (DeFelippis et al 1995). The limited blue shift observed indicates that the hydrogen bond distance or angle has been altered due to partial unfolding of the protein, although 1.2 nm shifts suggest that the indole in tryptophan is undoubtedly still hydrogen bonded. Therefore, helices II and IV are likely to be still within hydrogen bonding distance of each other. Second-derivative spectra of hGH/UH adducts at pH 6 did not exhibit spectral perturbations, indicating no disruption of the hGH tertiary structure under these conditions.

Tryptophan side chains are usually less exposed than the more polar tyrosine residues and their spectral features are less sensitive to solvent polarity. Therefore, peak-to-trough values of 283–289 nm (a) and 290.5–295 nm (b), and a/b ratio, were calculated from the second-derivative spectra to evaluate the average polarity of microenvironment of tyrosine residues (Ragone et al 1984; Ackland et al 1991). Furthermore, unlike tyrosine, the relative exposure of tryptophan does not influence the a/b ratio (Ragone

Table 1 Mean hydrodynamic particle diameter of hGH/UH adducts at different pH by photon correlation spectroscopy

pH	Mean hydrodynamic particle diameter (nm)
3.2	54.4 ± 8.0
4.0	31.5 ± 3.8
4.7	38.5 ± 8.7
5.4	27.5 ± 4.9
6.9	12.2 ± 1.9

Data are means ± s.d., n = 3.

Table 2 Wavelength maxima of the ^1L_b absorption band of Trp 86 and the shift of these maxima in hGH/UH adducts relative to that of the native type at pH 7

Protein at different conditions	^1L_b (nm)[a]	Δ^1L_b (nm)
hGH (pH 7) (control)	292.2	
hGH (pH 3)	291.4	0.8
hGH/UH adducts (pH 3)	291.0	1.2
hGH/UH adducts (pH 6)	292.2	0.0

[a]Values are the analysis of the mean of the number of independent determinations of second-derivative spectra.

Table 3 The a/b ratio from the second-derivative absorption analysis in hGH and hGH/UH adducts

Protein at different conditions	a/b ratio
hGH (pH 7) (control)	5.06
hGH (pH 3)	5.16
hGH/UH adducts (pH 3)	3.57
hGH/UH adducts (pH 6)	5.49

a, peak-to-trough values of 283–289 nm from the second-derivative absorption analysis; b, peak-to-trough values of 290.5–295 nm from the second-derivative absorption analysis. Values are the analysis of the mean of the number of independent determinations of second-derivative spectra.

et al 1984). The results are tabulated in Table 3. The data obtained suggest that the decrease in the a/b ratio between native hGH (pH 7) and hGH/UH adducts at pH 3 is related to the changes occurring in the tyrosyl microenvironment, which becomes more non-polar and hydrophobic following protein complexation. However, no significant changes were observed in hGH/UH adducts at pH 6.

In general, comparison of tryptophan results and a/b ratio indicated that the acid conditions (pH 3) and complexation with UH used in this study did not cause extensive unfolding of the protein or a major disruption in tertiary structure of hGH but a decrease in the hydrophilic environment around the tyrosine residues was detected. However, secondary structure variations of hGH in the presence of heparin could not be determined due to the turbidity of the solution. Copeland et al (1991) reported that neither the fluorescence nor the CD spectrum of aFGF changed upon the addition of heparin, implying that no major conformational changes occurred in the overall secondary structure when interacted with heparin.

In-vitro release studies

Release of hGH from hGH/UH adducts in different pH of buffer media at 37°C resulted in a pH-dependent initial burst effect during the first hour followed by no release over 48 h. However, hGH was released in PBS (pH 7.4, $\mu = 0.174$, isotonic) from 36.7% during the first hour to 56.8% over 24 h. The percentage of hGH release during the first hour at different pH of buffer media is shown in Table 4. The percentage was reduced by decreasing the pH of the buffer until it reached zero at pH 3. It seems likely that hGH/UH adducts in PBS buffer at pH 8 are dissociating faster because of repulsion due to negative charges on the hGH ($P < 0.01$ compared with pH 3, 5 and 7.4). On the other hand, at pH 3, there are more attractive forces between the positively charged hGH and heparin, resulting in reduced dissociation and increased aggregation of hGH (Davio & Hageman 1993) and, therefore, zero release ($P < 0.01$ compared with pH 5, 7.4 and 8). At pH 5, close to the isoelectric point of hGH, the net charge of attractive and repulsive forces between hGH and UH would be intermediate between the low and high pH and therefore allows an intermediate release pattern of hGH. At pH 7.4, although hGH is negatively charged, it follows approximately the same percentage of release as pH 5 with no statistical difference ($P > 0.05$). Probably other types of interactions besides electrostatic interaction, such as van der Waals interaction and formation of intermolecular hydrogen bonds, may be involved (Cardin & Weintraub 1989).

Table 4 The effect of pH of buffer media on the release of hGH from hGH/UH adducts at the stoichiometric ratio (pH 3) at 37°C

pH of buffer media	Percent of hGH released
3.0	0.0 ± 0.0
5.0	48.4 ± 7.9
7.4	36.7 ± 8.7
8.0	93.0 ± 4.3

Data represent mean ± s.d., n = 3.

Table 5 The effect of ionic strength (μ) on the hGH release from hGH/UH adducts at the stoichiometric ratio (pH 3) in PBS (pH 7.4) at 37°C

Ionic strength	Percent of hGH released
0.04	66.7 ± 7.0
0.17	36.7 ± 8.7
0.70	60.9 ± 5.4

Data represent mean ± s.d., n = 3.

Release of hGH from hGH/UH adducts in different ionic strengths of PBS buffer is shown in Table 5. Release of hGH in PBS buffer at $\mu = 0.04$ and 0.7 showed no statistical difference ($P > 0.1$) but a significant increase compared with $\mu = 0.170$ ($P < 0.01$). Electrostatic binding of GAGs and proteins has been characterized by sensitivity to pH and ionic strength. The bonds are formed at low ionic strengths and dissolved by increasing the salt concentration. The highest release of hGH (66.7%) at the lowest ionic strength of PBS buffer ($\mu = 0.04$) therefore suggests the possibility of involving other types of interactions between hGH and heparin.

Release dependence of hGH on the pH of the buffer media was a strong indication that the main driving force for hGH and heparin interaction was electrostatic. This observation was confirmed by other reports that electrostatic binding between GAGs and some endogenous proteins occurred (Comper & Laurent 1978).

Aggregation method: interfacial denaturation

The physical stability of hGH under stress conditions was investigated. These or similar situations may be commonly encountered during formulation and processing of proteins. For example, air–water interfaces are generated

during manufacturing (e.g., mixing in tanks), shipping, handling (e.g., reconstitution), aerosolization and spray drying (Mumunthaler et al 1994). Three different methods have been reported in the literature, including thermal denaturation, interfacial denaturation and guanidine dilution, and these induced aggregation under stress conditions (Katakam et al 1995; Katakam & Banga 1997). However, in this investigation thermal denaturation and guanidine dilution did not result in a linear relationship between hGH absorbance and the stress variables (temperature and guanidine concentration) and they were associated with high variation in results (data not shown). Consequently, interfacial denaturation was used as a means of inducing hGH denaturation and aggregation (Charman et al 1993; Katakam & Banga 1997).

The influence of agitation time and pH on the extent of precipitation using an initial hGH concentration of $0.5\,mg\,mL^{-1}$ is presented in Figure 1. Precipitation of hGH at different pH values, over time, followed the descending order of pH 4.7 > 7.2 > 3 > 8.6. The highest precipitation of hGH at pH 4.7 is probably due to the proximity to the isoelectric point, 5.3, resulting in minimum solubility and precipitation of protein (Pearlman & Bewley 1993).

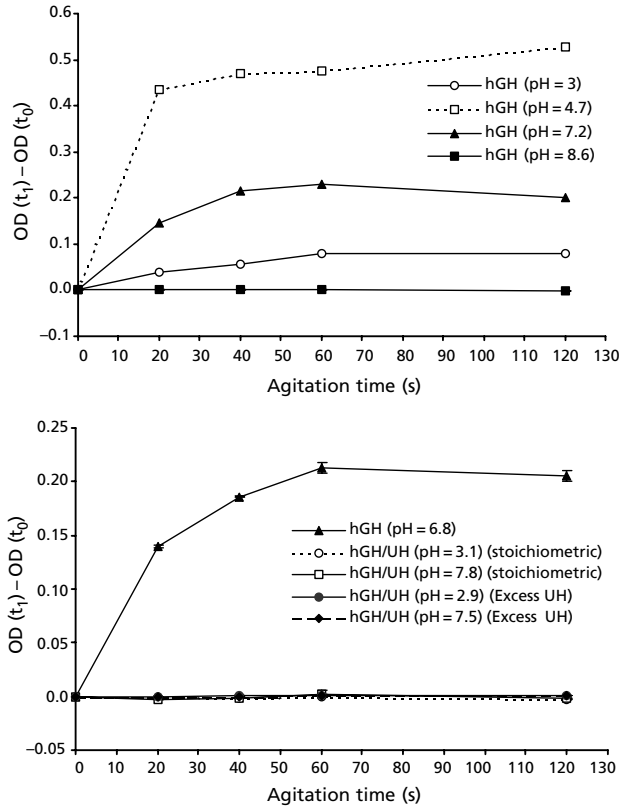

Figure 1 Effect of agitation time on the precipitation of hGH alone ($0.5\,mg\,mL^{-1}$) at different pH values (top) and hGH ($0.5\,mg\,mL^{-1}$) formulations at different pH values (bottom) following vortex at a constant speed. Data represent (mean ± s.d., n = 3) changes in the optical density at 450 nm.

This technique was also applied to hGH and hGH/UH adducts at different pH and optical densities were assessed by UV spectrophotometry (Figure 1). The results indicated vortex agitation over 120 s produced no changes in the optical density of hGH/UH adducts at different pH, with or without an excess heparin, compared with a substantial increase for hGH alone at pH 6.8. The substantially reduced precipitation in the hGH/UH adducts is most likely due to stabilization of hGH by complexation with heparin, because none of the hGH/UH adducts were prepared at pH 8.6 (minimum precipitation of hGH alone).

Real-time stability studies

Effect of pH and heparin on hGH stability

The percent of immunoactive hGH remaining in the solution was measured by ELISA during storage at 4°C and 37°C (Figure 2). During the stability studies, if the active hGH was reduced to less than $2\,ng\,mL^{-1}$, the sample was discontinued and the remaining analysis terminated. By storing at 4°C and pH 3, hGH/UH adducts with excess heparin produced the most stable formulation resulting in 71.4% and 35.7% active hGH compared with 24.9% and 16.7% for hGH alone after 8 and 93 days storage, respectively. hGH/UH adducts with excess heparin resulted in a persistently active hGH during the first month compared with a substantial drop for the other two formulations. However, by the end of 93 days, hGH/UH adducts without excess heparin resulted in a slightly higher active hGH compared with those with an excess of heparin.

Storing at 4°C and pH 7, hGH/UH adducts with excess heparin created the most stable formulation resulting in 78.4% and 48.8% active hGH compared with 40.2% and 25.6% for hGH alone after 8 and 93 days storage, respectively.

Storage at 37°C and pH 3 resulted in the most unstable condition for hGH formulations, producing an immediate loss of activity during the first week for hGH (3.3% day 8) and hGH/UH adducts at the stoichiometric ratio (8.7% day 8) followed by the second week for hGH/UH adducts with excess heparin (83.8% day 8).

By storing at 37°C and pH 7, hGH/UH adducts with excess heparin produced the most stable formulation resulting in 97.5% and 20.6% active hGH compared with 36.2% and 0.2% for hGH alone after 8 and 36 days storage, respectively.

By combining results at pH 3 and pH 7, the percentage of hGH remaining in the solutions at 4°C over 3 months was according to the following descending order: hGH/UH (excess heparin) (pH 7), 48.8% > hGH/UH (pH 3), 45.3% > hGH/UH (pH 7), 39.6% > hGH/UH (excess heparin) (pH 3) 35.7% > hGH (pH 7), 25.6% > hGH (pH 3), 16.7%. However, excluding month 3, hGH/UH adducts (excess heparin) (pH 3) produced the most stable formulation (over 65 days) with persistently active hGH during the first month. By storing at 37°C, the percentage of hGH remaining in the solutions over 3 months was according to the following descending order: hGH/UH (excess heparin) (pH 7), 0.54% (93 days) > hGH/UH (pH 7), 0.20% (65 days) > hGH (pH 7), 0.22%

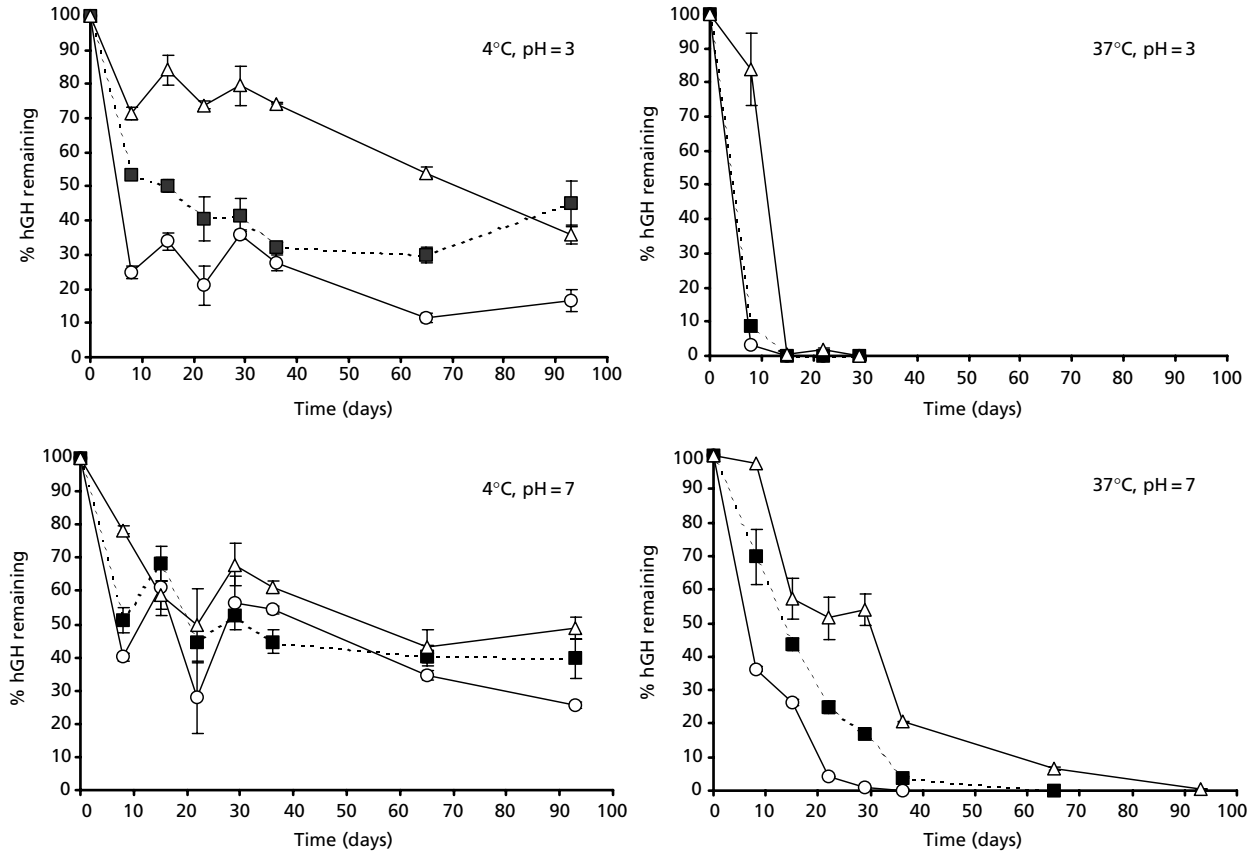

Figure 2 Percentage of hGH remaining in the filtrate determined by ELISA over 93 days storage at 4°C (left) and 30 days storage at 37°C (right) in the presence of (■, hGH/UH (stoichiometric ratio, pH = 3 (top) and pH = 7 (bottom); △, hGH/UH (excess heparin, pH = 3 (top) and pH = 7 (bottom)) and in the absence of heparin (○, hGH, pH = 3 (top) and pH = 7 (bottom)); data represent mean ± s.d., n = 3

(36 days) > hGH/UH (excess heparin) (pH 3), 1.8% (22 days) > hGH/UH (pH 3), 8.6% (8 days) > hGH (pH 3), 3.3% (8 days).

In general, by comparing activity of hGH formulations at different temperatures, various conclusions may be drawn from these results. Firstly, hGH was more stable at pH 7 than pH 3. Increasing the acidity of the protein solution results in an increase in the number of charged groups on a protein, which produces an increase in non-specific charge repulsion within the protein. Therefore the folded protein conformation destabilizes because the charge density on the folded protein state is greater than unfolded protein (Dill 1990; Chi et al 2003). Secondly, heparin has the potential to stabilize hGH at both pH 3 and pH 7. Moreover, an excess heparin considerably improved the stability of hGH formulations. By the Wyman linkage function, differential binding of a ligand into a two-state equilibrium (native ↔ unfolded) will shift the equilibrium toward the state with greater binding capacity (Timasheff 1998). Thus, electrostatic interaction or binding of polyanions to the native state of aFGF (Tsai et al 1993) or recombinant KGF (Chen et al 1994) greatly shifted the equilibrium between native and unfolded states to favour the formation of the native state, preventing aggregation. Once again, hGH formulations with heparin showed greater stability at both pH 3 and pH 7, which was improved in the presence of an excess of heparin. Electrostatic interaction or binding of hGH to heparin and the possibility of stabilization of the native state or partially unfolded state of the hGH by the heparin, therefore, may not be the only factor involved. Another feasible mechanism could be prevention of intermolecular protein interactions by heparin. The highly negative charges of heparin (especially in excess amounts) around the hGH surface may act as a shielding effect to produce repulsive interactions between hGH molecules and consequently stabilize hGH solutions, making assembly processes, such as aggregation, unfavourable (Mulkerrin & Wetzel 1989; Tsai et al 1998). However, more studies need to be performed to find out the exact mechanism of action.

Particle size

The mean hydrodynamic particle diameter of hGH/UH adducts at pH 3, with or without an excess heparin, was monitored over time at 4°C and 37°C. Analysis of the remainder of the hGH formulations could not be carried out due to a low intensity or photopulse rate (< 70 kHz). Samples were filtered though 1-μm filter membranes to

remove the large hGH aggregates that appear over time and otherwise generate inaccuracies in the Nicomp performance by light scattering.

Analysis of hGH/UH adducts (pH 3) over time at 4°C and 37°C resulted in three particle populations ($\bar{d}_v$), including species A (major population, 78.2–98.4% by volume) in the range 5.9–38.1 nm, species B (1–63.8%) in the range 112.8–173.5 nm and species C (0.6–45.7%) in the range 403.3–751.9 nm. Likewise, analysis of hGH/UH adducts with excess heparin (pH 3) over time at 4°C and 37°C resulted in three particle populations ($\bar{d}_v$), including species A (major population, 65.8–94.3% by volume) in the range 16.2–38.8 nm, species B (5.7–34.2%) in the range 131.2–183.3 nm and species C (5.1–10.5 %) in the range 467.9–804.3 nm. The first species (A) was likely to be hGH/UH adducts with or without an excess heparin. This was due to the proximity of species A particle diameters to previously measured particle diameters of hGH/UH adducts and major contribution (highest percentage) in particle size distribution. The mean hydrodynamic particle diameter of hGH/UH adducts at pH 3 and day 0 (35.3 ± 1.8 nm) was slightly different from previously measured hGH/UH adducts (54.4 ± 8.0 nm). The difference may be due to the passage of samples through the 1-μm filter membrane. The other two species (B and C) were likely to be aggregates of hGH. However, species C did not appear on all days and only constituted a small percentage of the population.

Comparison of hGH/UH adducts with and without excess heparin at 4°C indicated the higher stability of adducts in the presence of excess heparin over time (Tables 6 and 7). hGH/UH adducts without excess heparin were dissociating and associating over time with no consistency. On the contrary, hGH/UH adducts with excess heparin were consistent over time and an increase in particle diameter from 16.2 nm to 38.8 nm was observed between days zero and 94, respectively. Furthermore, an increase in hGH aggregate (species B) was also detected from 139.1 nm to 177.9 nm on days zero and 94, respectively. Analysis of hGH/UH adducts with or without excess heparin at 37°C produced the same results (data not shown). hGH/UH adducts with an excess heparin were consistent over 30 days and an increase in particle size from 16.2 nm to 33.8 nm was observed on days zero and 30, respectively. Furthermore, an increase in hGH aggregates (species B) was also detected from 139.0 nm to 158.7 nm on days zero and 30, respectively. The increase in particle size is probably due to denaturation of protein over time and production of aggregates. However, at different temperatures a divergent mechanism of degradation for the production of aggregates may also have been involved (Brange 2000).

Table 6 Mean hydrodynamic particle diameter ($\bar{d}_v$) of hGH/UH adducts (stoichiometric ratio, pH 3) over 94 days storage at 4°C. The particles are divided into two different populations: hGH/UH adducts (species A) and hGH aggregates (species B)

Days	Species A		Percent	Species B		Percent
	$\bar{d}_v$ (nm)	s.d.		$\bar{d}_v$ (nm)	s.d.	
0	35.3	1.8	93.3	138.4	8.8	6.7
2	8.6	0.8	96.3	129.3	0.5	2.1
9[a]	—	—	—	153.3	0.6	63.8
16	5.9	0.5	98.4	115.7	1.4	1
23[a]	—	—	—	115.2	1.0	54.3
30	15.2	1.7	86.2	133.1	4.5	7.9
38	10.7	0.8	94.6	131.9	0.9	3.4
66[a]	—	—	—	129.9	1.3	60.8
94	15.8	0.8	82.9	141.6	6.5	10.9

Data represents mean of triplicates. [a]36.2–45.7% of species C (470.6–751.9 nm) was observed in these days.

Table 7 Mean hydrodynamic particle diameter ($\bar{d}_v$) of hGH/UH adducts (excess heparin, pH 3) over 94 days storage at 4°C. The particles are divided into two different populations: hGH/UH adducts (species A) and hGH aggregates (species B)

Days	Species A		Percent	Species B		Percent
	$\bar{d}_v$ (nm)	s.d. (nm)		$\bar{d}_v$ (nm)	s.d. (nm)	
0	16.2	7.4	94.3	139.1	12.0	5.7
2	17.8	1.2	76.7	131.2	4.8	16.6
9	19.8	0.6	78.7	148.6	1.2	16.2
16	21.7	1.4	73.6	135.8	0.5	19.8
23	26.7	0.6	78.6	162.2	1.0	21.4
30	28.4	4.7	70.0	160.0	15.1	23.1
38	28.7	0.4	67.9	163.1	9.4	21.6
66	30.9	0.3	77.1	183.3	0.6	22.9
94	38.8	10.6	65.8	177.9	20.9	34.2

Data represents mean of triplicates.

hGH in-vivo bioactivity

Comparisons of mean net BWGs of female hypox rats receiving equivalent doses of hGH in two different formulations and the control group (glycine and mannitol) are presented in Figure 3. The excipient-treated rats showed the anticipated minimal weight gain over the 11-day period compared with the hGH and hGH/UH groups. Statistical comparison of BWGs of different formulations of hGH, hGH/UH and control revealed significant differences on almost all days (except day 1) between treatment groups ($P < 0.05$). Individual group comparisons indicated significant differences between hGH treatments and control ($P < 0.05$). hGH/UH groups showed insignificant differences to both hGH and control groups ($P > 0.05$), indicating that the complexation of hGH with UH did not affect the growth-promoting activity of hGH. However, due to insignificant differences of hGH/UH and control groups a definite conclusion could not be drawn.

In-vitro release studies indicated that hGH was not released completely from hGH/UH adducts at pH 7.4 (56.8% during 24 h) and this was confirmed by size analysis.

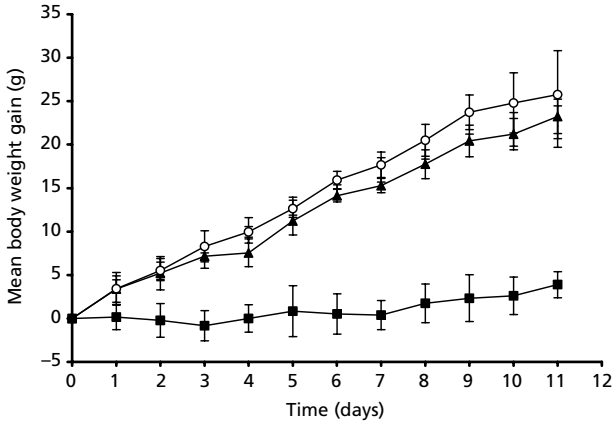

Figure 3 The cumulative body weight gains for female hypox rats injected subcutaneously with approximately equivalent doses of hGH and excipient for 11 days. Points ± s.d. represents mean cumulative net BWG in n = 4 rats for different formulations of hGH (hGH alone (○, 0.32 mg kg^{-1}), hGH/UH adducts (▲, 0.32 mg hGH/kg–0.13 mg heparin/kg) and n = 3 for excipients (■).

However, in-vivo studies demonstrated equivalent biological activity of hGH and hGH/UH adducts after subcutaneous administration to hypophysectomized rats. An incomplete in-vitro release of hGH (40% during 192 h) has also been reported (Garcia et al 2002) from polylactic glycolic acid laminar implants, although higher biological activity in hypophysectomized rats was found. While the exact mechanism is unknown, probably other in-vivo factors may be involved in the dissociation and release of hGH from hGH/UH adducts.

Conclusions

hGH interacted with heparin to form insoluble adducts. These adducts formed at low pH, dissociated or dissolved at high pH, producing a decrease in particle diameter. The hGH tertiary structure did not change extensively upon complexation with heparin. The release of hGH from hGH/UH adducts was dependent on pH and ionic strength. Heparin stabilized the hGH by preventing physical degradation during vortex agitation. Moreover, real-time stability studies demonstrated hGH/UH adducts with an excess heparin at both pH 3 and 7 produced a higher stability over hGH alone. Furthermore, the biological activity of hGH/UH adducts was comparable with hGH treatments, by weight gain through daily subcutaneous administration to hypophysectomized rats. The formulation studies with hGH described are of particular interest because of the ability of a polyanion such as heparin to have the potential to enhance the stability of this inherently labile protein. Nevertheless, further studies are necessary for these formulations to be fully characterized and to elucidate, in detail, the mechanism of action of the heparin in stabilization of hGH.

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