Quality Evaluation of Commercial Lyophilized Human Growth Hormone Preparations

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The quality of three commercial injections (Genotropin, Humatrope and Norditropin) of lyophilized recombinant human growth hormone (r-hGH) was evaluated in tests by visual inspection, high-performance gel permeation chromatography, polyacrylamide gel electrophoresis, scanning electron microscopy and energy dispersion X-ray microanalysis. The influence of the reconstitution method on gel formation was examined as follows: rapid injection of the diluting solution into a vial against the wall, slow injection onto the surface of the content, and rapid injection onto the surface of the content. The degree of gel formation differed among reconstitution methods. Moreover, fibrous particulate matter in addition to degradation products of r-hGH were evident in all preparations. The quality of r-hGH injection differed among commercial products. Norditropin included the least particulate matter when examined immediately after reconstitution, but it was easily denatured after storage in solution. We advise medical specialists to reconstitute a preparation by the optimal method.

Key words recombinant human growth hormone; gel formation; particulate matter; degradation

Human growth hormone (hGH) has 191 amino acid residues and a molecular weight of about 22000 daltons. Most peptides are unstable in solution, therefore denaturation by fragmentation, aggregation, polymerization, oxidation and desamido formation is easily induced. Several reports on the lack of efficacy and safety of peptides due to their denaturation in solution have brought the attention to medical specialists and patients. Commercial recombinant hGH (r-hGH) injection is now supplied as a lyophilized preparation with an accompanying diluting solution. However, opalescence occurs after reconstitution in some of these preparations.

In this study, we evaluated the quality of three commercial r-hGH preparations after reconstitution by various means.

MATERIALS AND METHODS

Materials We examined the following commercial, lyophilized r-hGH preparations packaged with accompanying diluting solutions: Genotropin (GEN; 4 IU, 1 ml water, Lot SC028, Sumitomo, Osaka, Japan), Humatrope (HUM; 4 IU, 2 ml saline, Lot F99304A-25, Japan Eli Lilly, Kobe, Japan) and Norditropin (NOR; 4 IU, 1 ml water, Lot KYK40Y, Yamanouchi, Tokyo, Japan). These were all from the same batch. Standard hGH obtained from pituitaries (pit-hGH, 2.4 IU/mg) was purchased from Sigma (St.Louis, MO, U.S.A.). A Gel Filtration Standard kit (components: thyroglobulin, gamma globulin, ovalbumin, myoglobin, and cyanocobalamin) was purchased from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of analytical grade. Distilled water was passed through a Millipore Milli Q water purification system equipped with a 0.22 μm final membrane filter (Millipore, Tokyo).

Reconstitution of Commercial Lyophilized r-hGH Preparations The commercial r-hGH preparations were stored at 4°C in a light-protected package until use. After standing at room temperature for 30 min, the accompanying diluting solution was injected into the r-hGH vial using a silicon-free disposable syringe (Top UC 2.5 ml; Top, Tokyo, Japan) equipped with a needle (22G; Terumo, Tokyo). The reconstitution procedures were: method #1, injecting diluting solution rapidly into the vial against the wall; method #2, injecting slowly onto the surface of the content over 20 s, and method #3, injecting rapidly onto the surface of the content. The mixtures were then rotated for 10 s.

Visual Inspection The vials were visually inspected against a white light, and evaluated arbitrarily as clear (−), opalescent (+) and markedly opalescent (++. The vials were stored at 4°C in a light-protected package and examined 0, 1 and 7 days after reconstitution.

High-Performance Gel Permeation Chromatography (HPGPC) The HPGPC system consisted of an LKB 2150 pump (Japan Pharmacia, Tokyo), a Rheodyne 7125 injector with a 20 μl sample loop (Cotati, CA, U.S.A.), an RI-8 differential refractometer (Tosoh, Tokyo), an SPD-2A UV detector (Shimadzu, Kyoto, Japan) operating at 280 nm and a CP 8000 integrator (Tosoh) together with a TSK gel G2000SWXL column (7.8 × 300 mm) and a TSK gel SWXL guard column (6.0 × 40 mm, Tosoh). The elution buffer, 50 mM phosphate (pH 6.8) containing 0.3 M sodium chloride and 0.05% (w/v) of sodium azide, was filtered (0.45 μm) and degassed prior to use. The flow rate was 0.8 ml/min and the HPGPC system was operated at ambient temperature (25–30°C). The column void volume (Vv) and the total bed volume (Vb) were determined using blue dextran and glycy1-tyrosine dissolved in the elution buffer. One vial of Gel Filtration Standard was dissolved in 1 ml of the buffer. The pit-hGH was dissolved in 12.5 mM ammonium bicarbonate at a concentration of 2 mg/2 ml. These reagent solutions and HPGPC injection samples were passed through a SJH1004NS filter (0.45 μm, Nihon Millipore Kogyo, Yonezawa, Japan) before injection into the system. r-hGH was identified by having the same elution time as pit-hGH and the amount of protein in the peak fraction was measured by the Folin–Wu

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method. The additives in the preparations were also identified on the chromatogram by comparison with the standards. There was no apparent adsorption of hGH to the syringe or filter.

**Electrophoresis** The Phastsystem (Japan Pharmacia, Tokyo) was used for native-polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-PAGE and isoelectric focusing analysis (IEF). We used the following materials and kits: PhastGel Homogeneous 20 gel, IEF 4–6.5 gel, PhastGel Silver Kit, Pharmacia Low pl and LKB LMW standard (Japan Pharmacia). One vial of Low pl and LMW standard was dissolved in 1 and 0.2 ml of water, respectively. Samples of r-hGH for PAGE were prepared as described for electrophoresis. PAGE and IEF were performed as described in the Pharmacia technical manual for the Phastsystem.

**Scanning Electron Microscopy (SEM) and Energy Dispersion X-Ray Microanalysis (EDXM)** Samples were observed under a Hitachi S-510 scanning electron microscope (Hitachi, Tokyo) equipped with an EMAX-1770 X-ray energy dispersion microanalyzer (Horiba, Tokyo). The samples were passed through an HA type filter (0.45 μm, diameter 4 mm, Japan Millipore) attached to a glass funnel (Hamada Rika, Osaka), and the filter was rinsed with water (10 ml) twice. After drying at ambient temperature, the filter was fixed on an aluminum stub (diameter 10 mm) and carbon-coated using a Hitachi SGB vacuum evaporator for SEM and EDXM.

**RESULTS AND DISCUSSION**

**Visual Inspection** Figure 1 shows opalescence in r-hGH solutions immediately after reconstitution by methods #1, #2 and #3, and the opalescence is evaluated in Table 1. Opalescence occurred in GEN and HUM reconstituted by all three methods, but not in any solutions of NOR. The opalescence in GEN was more severe when reconstituted by method #2 than by #1 or #3. Factors affecting the formation of particulate matter in solutions of peptide and protein include pH values, mechanical stress, temperature, metal ions, concentration and additives. The pH values of GEN, HUM and NOR were 6.7, 6.9 and 8.4, respectively, and remained constant for 7 days after reconstitution. The opalescence was confirmed even when the lyophilized preparation was reconstituted by the mechanically mild method #1. These results suggest that GEN or HUM itself contains particulate matter prior to reconstitution. The weak alkaline pH of NOR may be involved in the absence of opalescence.

**SEM and EDXM** We clarified the cause of the opalescence in solution by SEM and EDXM. Figure 2 shows SEM photographs of particulate matter trapped on the filter (0.45 μm) immediately after the reconstitution of commercial preparations by methods #1, #2 and #3. Fibrous particulate matter and gel formation alternated among the reconstitution methods. The SEM of GEN reconstituted by method #2 could not be achieved because the gel formation was too thick to pass through the filter. The size of the particulate matter in GEN induced by method #3 was larger than that by method #1. In HUM, small particulates were trapped on the filter regardless of reconstitution method. Method #1 induced the most fibrous particulate matter, as only half the volume (1 ml) could be filtered. Gels also formed in the samples after reconstitution by methods #2 and #3. In NOR, slight particulate matter was also observed by SEM, although the solution was usually clear.

EDXM showed that the particulate matter in sample solutions contained several multivalent metal ions and other elements. The elements detected in translucent gel-like matter included Cr, Cu, Al, Si and S in GEN, Cr, Cu, Al, Si and S in HUM, and Cr, Cu, Al, Si, Ti, Mg and S in NOR, which were almost exogenous for r-hGH preparations. In NOR, Ca, Fe, Al and Si were also detected in solid-like matter, which was opaque and had sharp edges and a distinct margin. It is suggested that contaminants from the glass vial and gum stopper as well as unknown compounds that arise during manufacture are present in the commercial lyophilized r-hGH preparations prior to reconstitution.

**HPGPC** Under our experimental conditions, the correlation between the concentration of r-hGH and the
peak area was satisfactory at the range of 1—4IU/ml in GEN and NOR and 0.5—2IU/ml in HUM. The correlation coefficient of the regression line in any preparation was above 0.999. The elution time of column void volume ($V_0$) and total bed volume ($V_t$) was 6.2 and 15 min, respectively. Figure 3 shows a typical chromatogram obtained from GEN samples immediately after reconstitution by method #1. Peaks of glycine and D-mannitol additives appeared around the elution time as $V_t$. Using a molecular weight (MW) calibration standard, peaks 1 (11.9 min), 2 (10.7 min) and 3 (7.2 min) were assigned to monomeric and dimeric r-hGH, and to higher molecular weight substances, respectively. Peak 4 might be derived from particulate matter that passed through the filter prior to injection. The elution patterns in GEN did not differ among the reconstitution methods although the results of the visual inspection differed.

Figure 4 shows typical chromatograms of three commercial samples immediately after reconstitution by method #1. The elution profile was different among preparations, especially in the ratio of peak area.

Table 2 shows the peak areas obtained from three commercial r-hGH preparations reconstituted by each method. There was no definite difference in the amounts of r-hGH (peak 1) of each preparation among the three reconstitution methods. Looking at the concentrations of r-hGH in the three preparations, the values of peak 1 were in the order of NOR > HUM > GEN, but those of peak 3 were in the opposite order. These corresponded to the visual inspection and SEM results, suggesting that opalescence and gel formation in the solution decrease the content of r-hGH.

**Electrophoresis** Figure 5 shows silver stained electrophoretic profiles of r-hGH preparations stored for 0, 1, and 7d at 4°C after reconstitution by method #1. Immediately after reconstitution (day 0), there were several bands on all lanes of GEN, HUM and NOR, suggesting that these r-hGH preparations contained compounds other than r-hGH itself.

Silver stained IEF revealed a band at pI 5.0 in all preparations beside the main peak at pI 5.2, and these
became more marked over time after reconstitution. This suggested the progressive denaturation of r-hGH. The band at pH 5.0 immediately after reconstitution was marked in the order of NOR > HUM > GEN, which was in inverse correlation with the pH of the solution. The desamidation of hGH is more rapid in alkaline medium.\(^5\,^7\). The degradation products include a fragment having an amino residue due to the cleavage of a peptide bond,\(^13\) a charge isomer due to a conformational change,\(^14\) a covalently-linked polymer due to interchain disulfide linkage\(^15\) and a sulfoxide derivative due to oxidation of methionine residues.\(^7\,\,^{16}\). We observed only the main single band (MW about 22000) in SDS-PAGE. The sulfoxide might not be separable by native-PAGE or IEF, because the net charge is the same as that of r-hGH. These results suggested that the band at pH 5.0 is desamide r-hGH and that degradation products are also produced during manufacturing, storage or supplying.

In some commercial lyophilized r-hGH we confirmed that the reconstitution procedure affects the nature of the preparation. In addition, we believe that these preparations contained particulate matter arising during manufacture or supply. The degree of particulate matter and degradation products of r-hGH differed among commercial preparations. NOR included less particulate matter than GEN or HUM when examined immediately after reconstitution, but this preparation was easily denatured after storage in solution. Further improvement of the manufacturing procedure is required to maintain the safety and efficacy of r-hGH preparations.

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**REFERENCES AND NOTES**

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