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Characterization of recombinant human growth hormone variants from sodium hyaluronate-based sustained release formulation of rhGH under heat stress

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ABSTRACT

This study provides the findings of investigations of potential product-related variants on recombinant human growth hormone (rhGH) in a once-weekly sustained release formulation (SR–rhGH) of sodium hyaluronate microparticles and on the rhGH bulk solution used as the active ingredient for SR–rhGH under extreme stress conditions of 24 h at 60 °C. The extent of rhGH degradation was much higher in solution (33%) than in SR–rhGH (10%). The degradation products, especially Met14 sulfoxide and deamidated rhGH variants, were separated and quantified by a modified reversed-phase high-performance liquid chromatography (RP–HPLC) method at reduced flow rate. The primary degradation product of rhGH was found to be deamidated rhGH, although an unknown peak was also detected. In contrast, the primary degradation product of SR–rhGH was Met14 sulfoxide rhGH, with no unknown peaks. Using a cell proliferation assay, the biological activities of the isolated products of SR–rhGH degradation were found to be equivalent to those of native hGH, as determined by comparison with a National Institute for Biological Standards and Control standard. In conclusion, SR–rhGH is structurally and functionally stable and maintains the intactness of rhGH.

Recombinant DNA technology has resulted in the production of proteins for use as therapeutic agents [1]. One promising protein drug is recombinant human growth hormone (rhGH)[1], which is used to treat growth failure in children and metabolic dysfunction in adults with growth hormone deficiency [2,3]. However, conventional growth hormone therapy requires daily subcutaneous injections, resulting in poor patient compliance [4], and various attempts have been made to develop a long-acting formulation of rhGH [5,6]. LG Life Sciences in Korea has developed a sodium hyaluronate (HA) microparticle-based sustained release formulation of rhGH (SR–rhGH) [7,8] to be injected once a week and to provide enhanced therapeutic efficacy and patient convenience. To date, this formulation is the only commercially available type of SR–rhGH. In the development of protein pharmaceuticals, a challenge is their susceptibility to physicochemical degradation [9]. Protein drugs are sensitive to various environmental factors, including temperature, light, oxidizing agents, pH, freezing, shaking, and shear stress [10]. These factors can influence proteins during the production process of the active ingredients as well as during the manufacture and storage of the drug product. Residual moisture and excipients in a formulation may also affect the chemical stability of proteins in the solid state [11]. In addition, analytic methods are required to identify, separate, and quantify impurities in these drugs both for their thorough physicochemical characterization and for quality control [12,13].

rhGH is a single-chain protein consisting of 191 amino acid residues with two disulfide bridges and a mass of approximately 22 kDa [14]. Product-related rhGH variants isolated by reversed-phase high-performance liquid chromatography (RP–HPLC) include primarily sulfoxide rhGH and deamidated rhGH, as well as Des-Phe-Pro rhGH, cleaved rhGH and trisulfide rhGH. The deamidated and oxidized forms of rhGH are the primary degradation products. Oxidation of methionine to methionine sulfoxide is a common chemical reaction in proteins [15–17]. rhGH contains three methionine residues susceptible to oxidation: Met14,

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[1] *Abbreviations used:* rhGH, recombinant human growth hormone; HA, sodium hyaluronate; SR–rhGH, sustained release formulation of recombinant human growth hormone; RP–HPLC, reversed-phase high-performance liquid chromatography; Ph. Eur., European Pharmacopoeia; USP, United States Pharmacopoeia; hGH, native human growth hormone; RWG, rat weight gain; hGHR, hGH receptor; CD, circular dichroism; UV, ultraviolet; NIBSC, National Institute for Biological Standards and Control.

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Met125, and Met170. Oxidation has been detected at methionine residues 14 and 125, with the former showing greater susceptibility. In contrast, Met170, located in the interior of the molecule, is not oxidized when the protein is in its native state [18]. Deamidation of asparagine residues, via a cyclic imide intermediate and resulting in the formation of isoaspartic acid on hydrolysis, also occurs frequently in proteins. The formation of the cyclic imide depends on the flexibility of the neighboring C-terminal amino acid. Potential sites of asparagine deamidation are Asn149 and Asn152, with the former being more susceptible [15,17]. N-terminal truncation is a minor chemical modification occurring in proteins, with Des-Phe-Pro rhGH produced by the removal of the N-terminal phenylalanine and proline residues from rhGH [19].

Current European Pharmacopoeia (Ph. Eur.) and United States Pharmacopoeia (USP) guidelines recommend using RP–HPLC to quantify the related proteins derived from rhGH [20,21]. The RP–HPLC method is well-suited for the analysis of rhGH, and especially sulfoxide rhGH can be quantified only by RP–HPLC [22,23]. However, the RP–HPLC method has a limitation of not being able to clearly separate Met14 sulfoxide rhGH and the neighboring deamidated rhGH form when using the elution conditions provided by the Ph. Eur. and USP monographs. Degradation of a liquid formulation of rhGH results primarily in deamidated rhGH, overshadowing Met14 sulfoxide rhGH. To overcome this drawback, a modified RP–HPLC method was applied to SR–rhGH, with the flow rate changed from 0.5 to 0.15 ml/min to better resolve the two overlapping peaks (Met14 sulfoxide and deamidated rhGH). The effects of heat stress (60 °C for 24 h) on rhGH bulk solution and SR–rhGH were compared, with the degradation products being analyzed by the modified RP–HPLC method. Three batches of rhGH bulk solution and SR–rhGH were analyzed to confirm batch-to-batch consistency.

Although the biological activity of protein degradation products may be lower than that of the native forms of most proteins, the biological activities of the oxidized and deamidated forms of rhGH are equal to that of the native protein. The deamidated and oxidized forms of rhGH are structurally similar to native human growth hormone (hGH), as determined using anti-hGH antibodies, and have biological activities in hypophysectomized rats comparable to those of native hGH [15]. The potency of rhGH is usually measured using a rat weight gain (RWG) bioassay [24], which assesses rhGH-mediated growth induction in hypophysectomized rats [25]. However, dose responses in this assay may be influenced by various parameters, including rat strain, age, sex, time elapsed hypophysectomy, and breeding environment (e.g., diet, temperature, stress), which can result in poor reproducibility. An alternative bioassay for rhGH [26,27] is based on its ability to induce the proliferation of Ba/F3 cells, a murine interleukin-3-dependent pro-B cell line, transfected with a plasmid encoding full-length hGH receptor (hGHR) [28] and expressing this receptor on its cell surface. Cell proliferation is quantified using the eluted stain assay (ESTA), permitting rapid assays of numerous samples using 96-well plates and a microtiter plate reader [29]. This in vitro cell proliferation bioassay was recently described in the USP General Chapter [30]. In this study, the biological activities of SR–rhGH degradation products were measured using the cell proliferation assay and compared with international standards.

Materials and methods

Modified RP–HPLC method

rhGH samples were assayed by RP–HPLC using a Hewlett–Packard HP 1100 (Agilent Technologies, USA) and a Vydac214ATP54 C4 column 300 Å (250 × 4.6 mm i.d., 5 μm, Grace Vydac, Hesperia, CA, USA), with the column temperature maintained at 45 °C. The operating conditions were identical to those described in the Ph. Eur. and USP monographs except for the flow rate. The concentration of rhGH sample was 1 mg/ml, the injection volume was 40 μl, and eluents were detected at 220 nm. The mobile phase was a 71:29 (v/v) mixture of 0.05 M Tris (hydroxymethyl) amino-methane at pH 7.5 (Merck, USA) and *n*-propanol (Sigma, USA), and the flow rate was 0.15 ml/min.

Preparation of rhGH bulk solutions for stress test

Three batches of rhGH (LG Life Sciences, Korea) were each diluted to 4.0 mg/ml in a buffer consisting of 0.5% glycine (Aldrich, USA), 2.25% mannitol (Sigma), and 10 mM sodium phosphate (Sigma) at pH 7.5. Six 1.0-ml aliquots of each test solution were added to six test tubes. Three of the tubes, one from each batch, were incubated in a dry oven at 60 °C for 24 h, whereas the other three were considered the zero-time point. Each of the six test solutions was diluted with 3.0 ml of the above buffer, yielding solutions of 1.0 mg/ml rhGH. Aliquots of each were injected into the RP–HPLC system to quantify rhGH degradation products.

Preparation of SR–rhGH for stress test

Two test vials were prepared from each of three batches of SR–rhGH dry powder (LG Life Sciences, Korea). Three vials, one from each batch, were incubated in a dry oven at 60 °C for 24 h, whereas the other three were considered the zero-time point. A 30-mg sample of powder, containing approximately 6 mg of rhGH, was removed from each vial and placed in a test tube, where it was dissolved in 3 ml of extraction buffer consisting of 1 M NaCl (Sigma) and 10 mM sodium phosphate (pH 7.5) by stirring for 1 h at room temperature. To each tube was added 3 ml of ethanol (J.T. Baker, USA), followed by stirring at room temperature for 10 min to precipitate HA. The tubes were centrifuged at 13,000 rpm for 10 min to remove HA precipitate, and the supernatants, containing approximately 1 mg/ml rhGH, were injected into the RP–HPLC system to quantify rhGH degradation products.

CD analysis

Circular dichroism (CD) is a specialized form of absorption spectroscopy that measures differences in the absorption of left- and right-handed polarized light due to structural asymmetry. The presence of ordered secondary structures can be evaluated from far-UV (ultraviolet) CD. CD was analyzed in three of the above-described samples: rhGH bulk solution at zero-time (positive control) and SR–rhGH at zero-time and after incubation for 24 h at 60 °C. Prior to testing, the protein concentrations of the samples were adjusted to 0.2 mg/ml with purified water. CD spectra were determined at room temperature on a spectropolarimeter (J-815 CD instrument, Jasco, USA) in quartz cuvettes. Far-UV CD spectra were recorded in 0.1-mm-path cells at 0.2 nm step resolution. Each data point was recorded at a scan speed of 100 nm/min. The baseline-corrected spectra were converted to mean residue ellipticity using a residue mean weight of rhGH of 115.7. Secondary structure was calculated by the CONTINLL method, which applied a locally linearized model to selecting proteins from the reference database [31–33].

Preparation of rhGH variants for cell proliferation assay

Four rhGH products separated by the modified RP–HPLC method—Met125 sulfoxide rhGH, Des-Phe-Pro rhGH, Met14 sulfoxide rhGH, and deamidated rhGH—were individually isolated from the samples stressed at 60 °C for 24 h. Each sample was

concentrated to 0.5 mg/ml rhGH using Centricon (Ultracel YM-10, Millipore, USA), and their identities and quantities were confirmed by modified RP–HPLC.

Cell proliferation assay

Ba/F3-hGHR cells (LG Life Sciences) were grown in selection medium (10 nM or 20 ng/ml hGH), harvested, and washed with 30 ml of Dulbecco's phosphate-buffered saline (DPBS, Gibco-BRL, USA). The cells were resuspended in assay medium (RPMI-1640 supplemented with 5% fetal bovine serum [FBS] and 0.5% antibiotics, Gibco-BRL) and counted with a hemacytometer (Hausser Scientific, USA). Aliquots of 50 µl containing cells at a concentration of 8×10^5/ml in assay medium were added to each well of a 96-well plate containing hGH diluent. The plates were incubated for 42 h at 37 °C in a humidified atmosphere containing 5% CO_2. To each well was added 20 µl of a solution containing 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, USA) and 0.92 mg/ml phenazine methosulfate (PMS, Sigma), and the plates were incubated for 4 h at 37 °C in 5% CO_2. The absorbances of each solution at 490 and 650 nm were measured using a microplate reader. The potency of rhGH samples was compared with that of the somatropin international reference standard (National Institute for Biological Standards and Control [NIBSC] 98/574, UK).

Results

Stress tests of rhGH

SR–rhGH is a microparticular powder prepared by spray drying technology using HA polymer. Analysis of the active ingredient in SR–rhGH requires extraction of rhGH from the microparticular powder. Samples of rhGH bulk solution at zero-time (T_0) and after storage in a drying oven at 60 °C for 24 h (T_{24}) were analyzed by modified RP–HPLC. At T_0, the rhGH bulk solution had a purity of approximately 98%, with very small amounts of impurities (Table 1 and Fig. 1A). At T_{24}, however, the total impurity level was approximately 33% (net 30% increase compared with T_0) and approximately 25% of the starting rhGH was in the deamidated form (Table 2 and Fig. 1B). A slight amount of Met125 sulfoxidized rhGH was present, but Met14 sulfoxide rhGH could not be quantified because its peak overlapped with that of the deamidated rhGH. Furthermore, an unknown peak was observed between the Met125 sulfoxide rhGH and Des-Phe-Pro rhGH forms. The three batches of rhGH showed good agreement in the types and percentages of individual degradation products.

Stress tests of SR–rhGH

Similar to rhGH solutions, vials of SR–rhGH powder were (T_{24}) or were not (T_0) incubated in a drying oven at 60 °C for 24 h. These samples were subsequently dissolved in solution, HA was removed, and the amounts of rhGH in SR–rhGH were measured by RP–HPLC. At T_0, the extracted rhGH from SR–rhGH contained four rhGH products: Met125 sulfoxide rhGH, Des-Phe-Pro rhGH, Met14 sulfoxide rhGH, and deamidated rhGH (Table 3 and Fig. 2A). Although the overall level of impurity was higher for SR–rhGH than for rhGH bulk solution at T_0, the purity was approximately 95%. The primary degradation product was Met14 sulfoxide rhGH, whereas the primary degradation product in rhGH bulk solution was deamidated rhGH.

Stressed samples showed completely different patterns from stressed rhGH bulk solutions (Table 4). The total impurity level was approximately 10%, and the net increase was just 5% compared with T_0. Although the levels of the two sulfoxide rhGH variants were relatively high, the levels of deamidated rhGH and Des-Phe-Pro rhGH were unchanged from baseline (T_0). These findings indicated that the major chemical degradation product of rhGH in SR–rhGH powder was the Met14 sulfoxide form. In addition, the chromatograms showed no evidence for the induction of unknown new peaks, even under stress conditions (Fig. 2B). Similar to rhGH bulk solution, the percentages and peak patterns of individual impurities in the three batches of SR–rhGH at both T_0 and T_{24} were highly reproducible and consistent.

CD analysis

The structural integrity of stressed samples of SR–rhGH was investigated using CD absorption spectroscopy, a method that is extremely sensitive to protein conformation. The presence of ordered secondary structures can be evaluated from far-UV CD. Fig. 3 shows the CD spectra of rhGH extracted from SR–rhGH at T_0 and T_{24}. These spectra exhibited two intense negative maxima near 208 and 222 nm, bands characteristic of α-helices. The CD spectrum of rhGH extracted from SR–rhGH overlapped that of rhGH bulk solution. Table 5 shows analytical results of the secondary structures of test samples. The right-handed α-helix [H(r) + H(d)] contents and the β-structure contents [sum of S(r), S(d), and Turn] of SR–rhGH at T_0 and T_{24} were similar to that of rhGH bulk solution, with the three samples having similar profiles. These findings indicated that the secondary and tertiary structures of SR–rhGH were identical to those of the original rhGH solution, with no structural change even under extremely stressful conditions.

Table 1
Results of stress test for rhGH drug substance at zero-time (T_0).

Batch	Number	Individual peak% of rhGH drug substance at T_0					
		Principal	Met125	Des-Phe-Pro	Met14	Deamidated	Others
Batch 1	1	97.63	0.07	0.63	0.69	0.97	0.0
	2	97.69	0.07	0.61	0.68	0.96	0.0
	3	97.71	0.06	0.63	0.60	0.99	0.0
	Mean	97.68	0.07	0.62	0.66	0.97	0.0
Batch 2	1	97.64	0.08	0.60	0.66	1.02	0.0
	2	97.63	0.07	0.60	0.73	0.97	0.0
	3	97.62	0.07	0.61	0.73	0.97	0.0
	Mean	97.63	0.07	0.61	0.70	0.99	0.0
Batch 3	1	97.72	0.07	0.61	0.67	0.93	0.0
	2	97.70	0.07	0.64	0.67	0.92	0.0
	3	97.68	0.07	0.61	0.71	0.93	0.0
	Mean	97.70	0.07	0.62	0.68	0.92	0.0

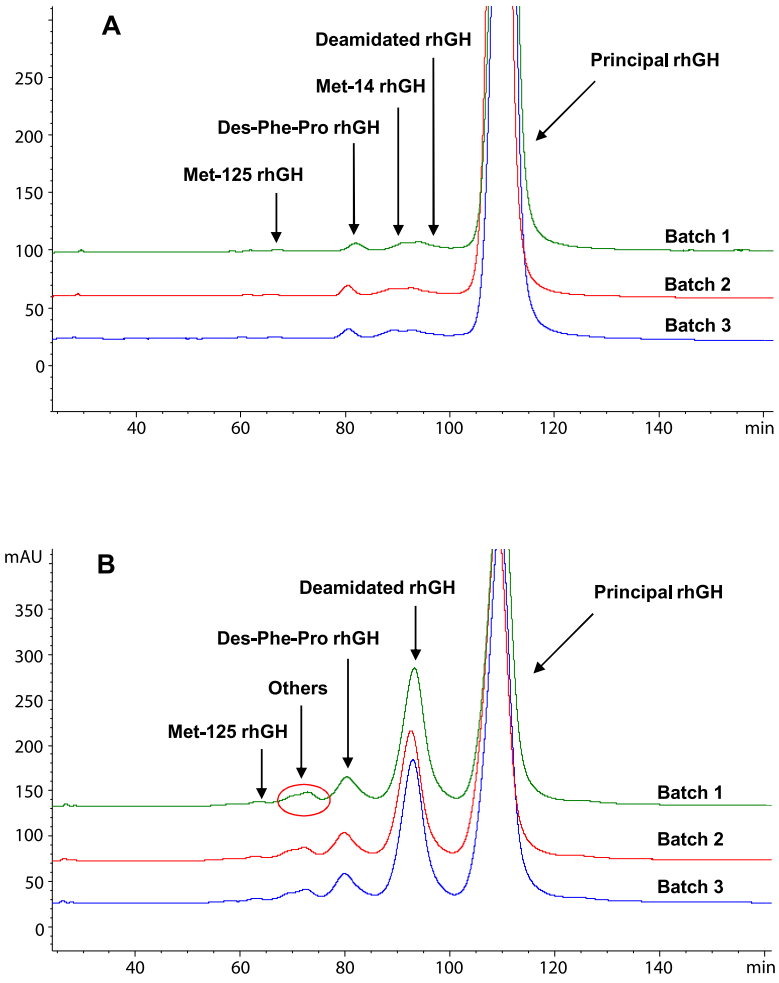

Fig.1. Modified RP–HPLC chromatograms of stress test for rhGH bulk solution three batches at T_0 (A) and T_{24} (B).

Table 2
Results of stress test for rhGH drug substance after storage for 24 h (T_{24}).

Batch	Number	Individual peak% of rhGH drug substance at T_{24}					
		Principal	Met125	Des-Phe-Pro	Met14	Deamidated	Others
Batch 1	1	67.57	0.36	4.64	–	25.08	2.35
	2	67.51	0.36	4.67	–	25.08	2.38
	3	66.92	0.42	4.96	–	25.11	2.58
	Mean	67.34	0.38	4.76	–	25.09	2.44
Batch 2	1	66.78	0.39	4.85	–	25.50	2.47
	2	66.76	0.39	4.86	–	25.51	2.48
	3	66.77	0.40	4.87	–	25.49	2.48
	Mean	66.77	0.39	4.86	–	25.50	2.48
Batch 3	1	67.08	0.37	4.87	–	25.19	2.49
	2	66.98	0.37	4.92	–	25.22	2.51
	3	66.92	0.37	4.94	–	25.23	2.54
	Mean	66.99	0.37	4.91	–	25.21	2.51

Biological activity of rhGH variants in SR–rhGH

Four rhGH degradation products—Met125 sulfoxide rhGH, Des-Phe-Pro rhGH, Met14 sulfoxide rhGH, and deamidated rhGH—separated by the modified RP–HPLC were individually isolated from the samples stressed at 60 °C for 24 h. The biological activity of each compound was measured using a Ba/F3-hGHR cell proliferation assay. Each degradation product was added to microtiter plate wells containing Ba/F3-hGHR cells, and the potency of each was compared with the NIBSC international reference standard (3 IU/mg). The activity of each individual rhGH product of SR–rhGH was comparable to that of native hGH (Table 6). The potency of each of the four products of SR–rhGH was sufficiently high to satisfy the acceptance criteria of the USP ($\geqslant$2.0 IU/mg).

Discussion

In general, protein drugs, including rhGH, are susceptible to chemical degradation by a variety of environmental factors. Temperature is a major factor contributing to chemical

Table 3
Results of stress test for the extracted rhGH from SR–rhGH at zero-time (T_0).

Batch	Number	Individual peak% of extracted rhGH from SR–rhGH at T_0					
		Principal	Met125	Des-Phe-Pro	Met14	Deamidated	Others
Batch 1	1	94.70	1.01	0.62	2.01	1.66	0.0
	2	94.66	1.01	0.62	2.06	1.66	0.0
	3	94.73	0.98	0.64	2.04	1.61	0.0
	Mean	94.69	1.00	0.63	2.04	1.64	0.0
Batch 2	1	94.35	1.04	0.70	2.58	1.32	0.0
	2	94.35	0.98	0.72	2.66	1.30	0.0
	3	94.25	1.09	0.70	2.53	1.43	0.0
	Mean	94.32	1.04	0.71	2.59	1.35	0.0
Batch 3	1	94.30	1.15	0.78	2.43	1.34	0.0
	2	94.24	1.12	0.73	2.48	1.43	0.0
	3	94.12	1.10	0.73	2.54	1.50	0.0
	Mean	94.22	1.12	0.75	2.48	1.42	0.0

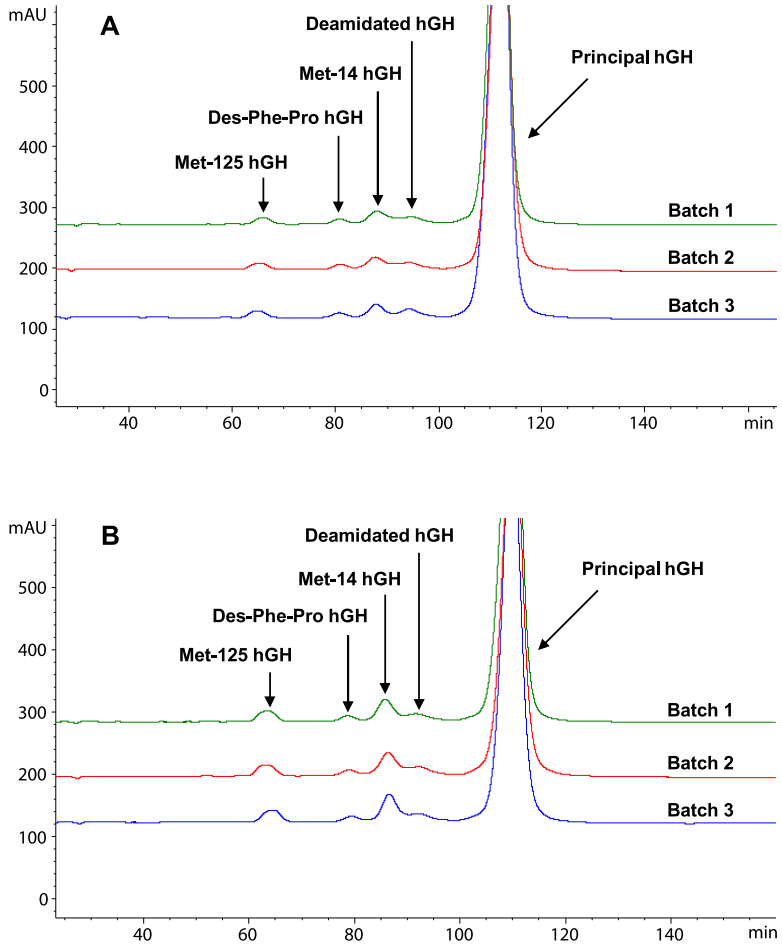

Fig.2. Modified RP–HPLC chromatograms of stress test for SR–rhGH drug product three batches at T_0 (A) and T_{24} (B).

degradation. The current study assessed the effects of long-term high temperature on rhGH bulk solution and SR–rhGH (60 °C for 24 h). This study required a modification of the RP–HPLC method cited by the Ph. Eur. and USP. Reducing the flow rate was found to detect potential impurities as well as to consistently quantify known impurities. Several methods can improve the resolution of the RP–HPLC method, including modifications in temperature, mobile phase ingredients, gradient (or isocratic) flow, and flow rate. Modifications in all conditions other than flow rate resulted in poor resolution or different peak patterns. Because an RP–HPLC method has been developed for hGH and peak identity has been established, this study was not designed to develop a tool for the identification of major impurities derived from SR–rhGH. Rather, it was designed to characterize the levels of these impurities and to compare their amounts quantitatively with those of the products derived from rhGH bulk solution. Nevertheless, RP–HPLC methods have the limitation that they are unable to clearly separate the Met14 sulfoxide rhGH and deamidated rhGH peaks, the

Table 4
Results of stress test for the extracted rhGH from SR–rhGH after storage for 24 h (T_{24}).

Batch	Number	Individual peak% of extracted rhGH from SR–rhGH at T_{24}					
		Principal	Met125	Des-Phe-Pro	Met14	Deamidated	Others
Batch 1	1	89.93	2.39	0.85	5.16	1.66	0.0
	2	89.75	2.53	0.89	5.15	1.67	0.0
	3	90.18	2.38	0.86	5.07	1.52	0.0
	Mean	89.96	2.44	0.86	5.10	1.62	0.0
Batch 2	1	90.82	1.92	1.10	4.06	2.10	0.0
	2	90.61	1.89	1.11	4.09	2.31	0.0
	3	90.50	1.90	1.16	4.07	2.37	0.0
	Mean	90.64	1.90	1.12	4.07	2.26	0.0
Batch 3	1	90.65	2.23	0.85	4.56	1.72	0.0
	2	90.03	2.28	0.90	4.88	1.92	0.0
	3	89.99	2.18	0.91	4.89	2.03	0.0
	Mean	90.22	2.23	0.89	4.77	1.89	0.0

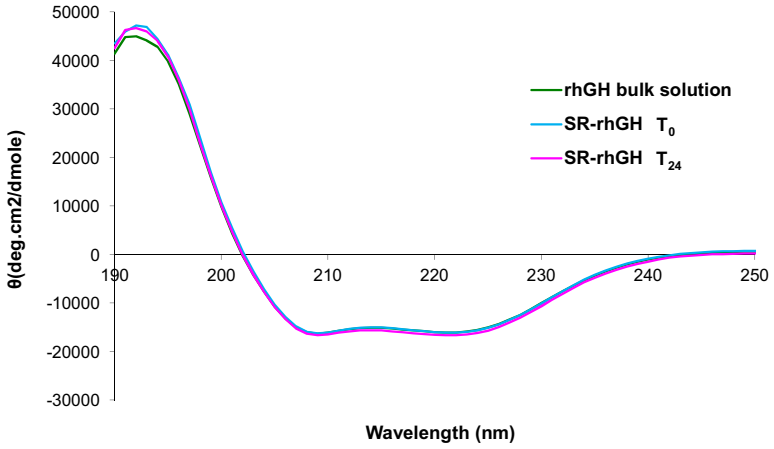

Fig.3. Comparison of CD spectra of rhGH bulk solution and SR–rhGH drug product at T_0 and T_{24}.

Table 5
Comparison of secondary structure profiles of rhGH bulk solution and SR–rhGH drug product at T_0 and T_{24}.

Test sample	α-Helix		β-Structure			Unordered
	H(r)	H(d)	S(r)	S(d)	Turn	
rhGH bulk solution	45.1	21.9	0.6	2.8	10.2	19.5
SR–rhGH at T_0	45.6	20.9	0.8	2.7	9.6	20.4
SR–rhGH at T_{24}	45.3	20.5	0.9	2.7	9.8	20.8

Note. Values in table are percentages (%). H(r), regular α-helix; H(d), distorted α-helix; S(r), regular β-strand; S(d), distorted β-strand.

Table 6
Biological activities of individual rhGH variants isolated from SR–rhGH stressed at 60 °C for 24 h by cell proliferation assay.

rhGH variant	95% confidence limit		Potency (IU/mg)	Relative %[a]
	Lower	Upper		
Met14 sulfoxide rhGH	94.6	122.9	3.2	108
Met125 sulfoxide rhGH	77.0	104.2	2.7	90
Des-Phe-Pro rhGH	95.1	138.5	3.4	114
Deamidated rhGH	80.5	120.9	3.0	100

[a] Relative % indicates the percentage of potency against 3 IU/mg NIBSC international reference standard.

primary degradation products of rhGH. Accordingly, the elution conditions of the RP–HPLC method were modified to overcome this limitation.

The stress test showed that the chemical degradation patterns of rhGH induced by heat stress were similar for individual compounds but different in the extent between aqueous state (rhGH bulk solution) and solid state (SR–rhGH) rhGH. Deamidation was the primary degradation pathway in the aqueous state, whereas sulfoxidation was the primary degradation pathway in SR–rhGH powder. These results showed that the modified RP–HPLC method was well-suited for rhGH, ensuring the separation and quantification of impurities. An unknown peak was observed in rhGH bulk solution, but not in SR–rhGH powder, subjected to heat stress, indicating that the latter does not contain any unknown impurities arising during processing or the somewhat harsh conditions used to extract rhGH.

In addition, the CD spectra showed that SR–rhGH was structurally identical to rhGH bulk solution, with no differences observed following heat stress of SR–rhGH, indicating that the increase in chemically modified variants in SR–rhGH did not induce any changes in secondary and tertiary structures. Structural intactness was strongly correlated with the maintenance of biological activity, as determined using the cell proliferation assay. The biological activities of the individual rhGH degradation products isolated and separated from SR–rhGH were equivalent to that of native hGH.

HA-based SR–rhGH was developed to allow weekly injections, enhancing patient convenience by providing a less frequent dosing regimen than the daily rhGH formulation. The findings of this study indicate that rhGH in SR–rhGH is similar to native hGH in terms of structural and biological integrity.

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