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Original Article

Separation of Somatropin and Its Degradation Products by High-Performance Liquid Chromatography Using a Reversed-Phase Polymeric Column

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Abstract

The accurate prediction of protein stability is one of the most challenging goals in protein formulation and delivery. In this study, a gradient RP-HPLC method is described for the separation of human growth hormone (hGH) variants as deamidated and oxidized forms. The methodology employed a polymeric poly (styrene-co-divinylbenzene) column and a 1mL/min flow rate of a linear gradient of 0.1% v/v TFA/acetonitrile and TFA/Water (pH=2.0) mixture as the mobile phase. The overall run time of this method was 12 min and the average retention times were about 8.7 min for the native somatropin, 7.2 min for the deamidated form and 1.6 and 5.3 min for oxidized variants. The method was also validated in terms of selectivity, linearity, intra- and inter-day variations.

In conclusion the method was found to have the potential for being applied as an initial and rapid evaluation method for assessing the quality and quantity of hGH during downstream processing, formulation and storage.

Keywords: Somatropin; Degradation products; HPLC; Gradient; Polymeric column.

Introduction

Human growth hormone (hGH) is a responsible protein for a wide range of growth-promoting effects in the body. Somatropin, the major component of growth hormone produced by the human pituitary gland, is a single-chain peptide composed of 191 amino acids and two disulfide bridges with a molecular mass of approximately 22000 daltons (1, 2). Since 1980's, the availability of recombinant DNA (rDNA)-derived hGH in sufficient quantities and high purity allowed scientists to prepare various pharmaceutical formulations for the fast increasing clinical applications (1).

Like most large proteins, growth hormone readily undergoes various chemical and physical instability reactions (3). The predominant degradative reactions of hGH are deamidation, oxidation, amino-terminal degradation and physical instability, which produce dimers or more aggregated forms. A number of publications have referred to the various routs through which hGH is degradated and described the mechanisms and kinetics of its degradation (1-7).

The development of stability-indicating analytical methods is critical for manufacturing, formulation and storage of protein drugs. Complex structures of proteins make their characterization difficult. Meanwhile, small structural changes in a protein can influence the physicochemical properties as well as its activity and potency (8). Therefore, rapid, accurate and reliable methods are necessary for the characterization and purity determination of proteins (8, 9).

Reversed-phase high performance liquid

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chromatography (RP-HPLC) is the most frequently used chromatographic technique for the analysis of peptides and intact proteins, especially for the determination of their purity (8) as well as for the analysis of peptide fragments (peptide mapping) obtained after enzymatic digestion or chemical cleavage (8, 10, 11). It is notable that dimers and more aggregated forms of proteins are studied by other methods such as size exclusion chromatography, which is also applied for the protein assay (1, 3).

Several different RP-HPLC methods for the analysis of hGH and its variants and degradation products have been reported in the literature (1, 6, 12-14) and also in the European Pharmacopoeia (EP) (2) and US Pharmacopeial forum (7). According to the EP monograph, the suggested run time for each analysis is around 55 min. The retention time of the native somatropin is about 33 min and desamido-somatropin appears after 28 min (2). Other reported methods are able to separate different variants within at least a 25 min-run time (6, 12-14).

The polymeric reversed-phase packing materials, specifically the poly (styrene-co-divinylbenzene) sorbents, have been introduced as excellent alternatives to the alkyl-bonded silicas for RP-HPLC of some drugs and proteins. Extended column life, reduced analysis time and inherent stability to a wide range of pH are some of the claimed advantages of these columns (15).

The aim of this study was to develop a rapid and sensitive RP-HPLC method for conducting stability studies on the recombinant hGH (rhGH) degradation products, using a polymeric column.

Experimental

Materials

Somatropin coded NIBSC-98/574 (Hertfordshire, UK), accepted by the Expert Committee on Biological Standardization of WHO in 2001 as the 2nd International standard of hGH, was obtained in vials containing 1.95 mg of Somatropin and 26.5 mg of excipients (glycine, mannitol, lactose and sodium bicarbonate). Norditropin® vials (NovoNordisk, Denmark), a pharmaceutical dosage form of Somatropin (1.3 mg) formulated with glycine, mannitol and sodium bicarbonate, was also used. Water was purified with a Millipore system (Bedford, USA). HPLC-grade acetonitrile and trifluoroacetic acid (TFA), and all other analytical grade reagents were obtained from Merck Chemical Co. (Darmstadt, Germany).

Methods

Sample preparation

Stock solution with a concentration of 1 mg/ml was prepared by adding an adequate amount of purified water to each vial. Solutions with lower concentrations were prepared by serial dilution of the stock solution. All protein solutions were stored at 4°C and used within 24 h.

Deamidation and Oxidation of hGH

Somatropin/desamido-somatropin mixture (1 mg/ml) was prepared according to the EP monograph (2) by adding 0.1 mg sodium azide to each ml of a freshly-prepared hGH solution, and allowing the solution to stand at room temperature for 24 h. This sample was then maintained at 2-8°C and used within 24 h.

The oxidation was performed by adding 2 or 20 µL hydrogen peroxide (30%, v/v) to each ml of a hGH solution (1mg/ml) and incubating at 2-8°C, overnight. The obtained solution was used within 24 h (6).

Instrumental and chromatographic conditions

A computer-controlled Shimadzu HPLC system (Japan) consisting of two pumps (LC-10 ADvp), a system controller (SCL-10Avp), a degasser (DGU-14A), a diode array detector (SPD-M10 vp) and a column oven (CTO-10A vp) was used. The separation was performed on a PRP-3 polymeric reversed-phase column (10 µm, 300°A, 150*4.1 mm I.D.) (Hamilton, Switzerland). A proper guard column (12-20 µm, 20*2.3 mm I.D.) (Hamilton, Switzerland) was also applied. Mobile phase was passed through the column with a flow rate of 1 ml/min as a linear gradient of 0-60% of solution A (0.1%

v/v TFA/acetonitrile) in solution B (TFA/Water, pH=2.00) within 5 min followed by holding for 5 min. Mobile phase solutions (A and B) were prepared freshly for each day. During separation, the column temperature was set at 35°C. The injection volume of the sample was 20 µL. Detection was carried out by measuring UV absorbance at 215 nm.

Method validation

Validation of the method was performed in terms of selectivity, linearity, intra- and inter-day variations and percentage of recovery.

Selectivity of the method was assessed by separate injection of each excipient within the sample (as described above) and observing any interference with somatropin peak. Selectivity assessment was also extended to the evaluation of any interference from degradation (deamidation and oxidation) products of the protein. Precision of the method was evaluated by repeated analysis of solutions containing known concentrations of somatropin. Linearity was studied over a range of 0.05-1 mg/ml at 6 different concentration levels of somatropin, each prepared freshly before injection.

Inter- and intra-day variations were determined at three protein concentration levels, namely 0.1, 0.5 and 1 mg/ml.

Results And Discussion

Mobile phase constituents and pH have been shown to influence the reversed-phase HPLC separation of proteins (5). Modifiers are substances that are added to the mobile phase, usually in a relatively low concentration, and interact with both the stationary phase and sample constituents in order to alter retention. Trifluoroacetic acid (TFA) is the modifier most frequently used for peptide separation in RP-HPLC, usually at a concentration of 0.1%. It has the most important effect of improving peak shapes and exerts its effects by pairing with positively charged and polar groups on peptides and proteins to mask these sites from polar interactions and bringing them to the reversed-phase surface. The UV absorbance spectrum of TFA occurs below 200 nm and thus creates minimal interference with the detection of peptides at low wavelengths (16). The inherent stability of the applied polymeric column in low pH extreme allowed taking benefit of TFA as a modifier in the mobile phase. On the other hand, the polymeric support is pressure stable and cross-linked to prevent shrinkage or swelling

Table 1. Reproducibility of the analysis of freshly prepared somatropin solutions (n=3)

Expected concentration (mg /ml)	Measured concentration (mean±S.D.)	
	Intra-day	Inter-day
0.1	0.087 ± 0.005(5.9)	0.083 ± 0.02(2.3)
0.5	0.402 ± 0.008(2)	0.405 ± 0.005(1.2)
1	1.019 ± 0.03(2.9)	1.068 ± 0.045(4.3)

Values in parentheses are coefficients of variation (%).

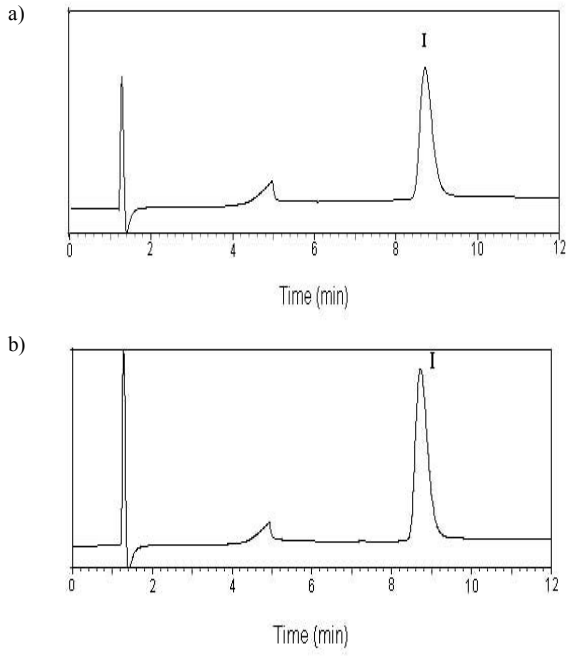

Figure 1. Chromatograms of freshly prepared 1mg/ml aqueous solutions of (a) standard hGH and (b) Norditropin® separated on a Hamilton PRP-3 polymeric column. Peak I is related to the native somatropin.

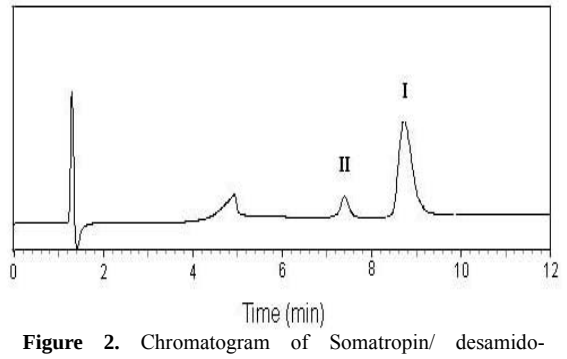

Figure 2. Chromatogram of Somatropin/ desamido-somatropin mixture) 0.5 mg/ml). Peaks I and II are related to native somatropin and deamidated form of protein, respectively.

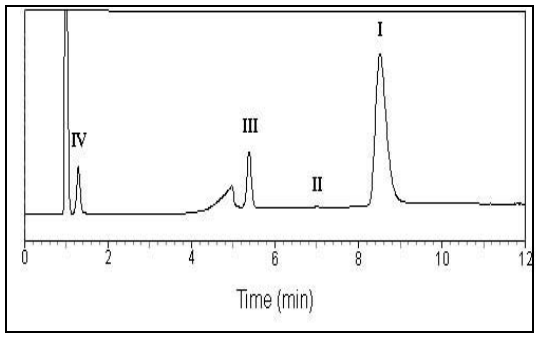

Figure 3. Chromatograms of a hydrogen peroxide-treated 0.5 mg/ml hGH solution. Peak I corresponds to native somatropin and peaks III and IV are attributed to the oxidized forms of hGH. The small peak numbered as II is related to desamido-somatropin.

when the mobile phase is changed (15). This helped to run a rapid linear gradient from 0 to 60% of acetonitrile in water within 5 min. Thus, compared to the EP method, the proposed method allowed a shorter analysis time for hGH by reducing the run time from 55 min to 12 min. The average retention time of the native somatropin peak (I) was 8.7 min (Figure 1). Meanwhile, a shorter column length and larger particle size of the packing material, along with higher flow rate of the mobile phase contributed to shorter run time. It is also evident that the solution-driven conformational changes resulted from the addition of organic modifiers like 1-propanol (in EP method) or acetonitrile (in the present method), significantly influence the retention behavior of the protein. For example, 1-propanol at a concentration of >10% induces the formation of a molten globule state, which is an intermediate in the unfolding pathways of a globular protein and provides additional hydrophobic surfaces for interaction in RP-HPLC (17).

Under the chromatographic conditions described, no interferences due to the presence of excipients used within the samples were observed. As shown in Table 1, the coefficients of variation for repeated analysis of freshly prepared samples containing known concentrations of protein were less than 6%, which is acceptable for the routine measurement of somatropin.

The calibration curve for the determination of somatropin in freshly prepared samples was linear over the studied range and the equation intercept was not statistically different from zero. The correlation coefficient of the standard curve was 0.9980 and the corresponding equation was: Y=2X-0.014, where Y is the peak area of somatropin and X is the somatropin concentration. Moreover, baseline separation of deamidated and oxidized forms of hGH demonstrates selectivity of the method used.

Figure 2 shows separation of native somatropin and the deamidated form, in which retention of the latter was 7.2 min. The earlier elution of this form is suggested to occur as a result of its higher polarity.

The chromatograms of hydrogen peroxide-incubated hGH samples presented two peaks (peaks III & IV in Figure 3) at 1.6 and 5.3 min. Oxidation of methionine residues of proteins generally results in the formation of more polar compounds. Therefore, the species that contains the oxidized methionine group is eluted prior to the non-oxidized species (8). Identity of the oxidized forms of the protein (peaks III & IV) was revealed by rendering the oxidation reaction under higher concentration of H_2O_2 (as described in the Experimental section). Larger amounts of H_2O_2 led to the acceleration of oxidation reaction, which resulted in an increase in the peak heights of corresponding oxidized products.

Conclusion

In this work, a selective, rapid and reproducible HPLC method, based on reversed-phase polymeric column, was presented for the identification of somatropin and its degradation products. The method is potentially applicable for the quality control of this protein during purification, formulation or stability studies.

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