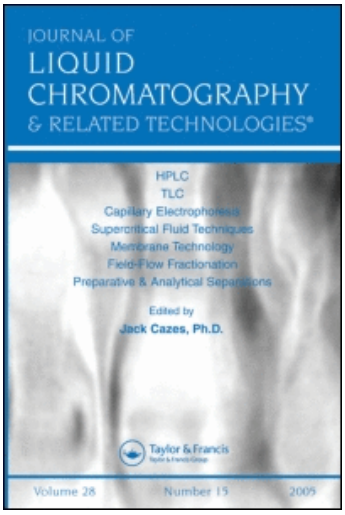

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ANALYSIS OF HUMAN GROWTH HORMONE BY HYDROPHOBIC INTERACTION HPLC. METHOD DEVELOPMENT, VALIDATION, AND COMPARATIVE SELECTIVITY TO REVERSED PHASE HPLC

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ABSTRACT

A hydrophobic interaction HPLC method was developed and validated for the analysis of human growth hormone (hGH) preparations. Separations were carried out on a TSK-Phenyl 5PW column using a descending linear gradient of 100% 1M ammonium phosphate dibasic/n-propanol (99.5:0.5), pH 8.0 to 100% 0.1M sodium phosphate/n-propanol (97.5:2.5), pH 8.0 over 30 minutes. The method proved highly reproducible for retention times, peak areas and peak heights, and the detector response for the main peak was linear between 1 and 1000 µg hGH/mL. Under these conditions, several hGH variants were resolved from the main hGH peak. A number of hGH preparations including the first WHO International Standard for hGH of pituitary origin and the new International Standard for hGH of recombinant origin were analysed.

Of particular interest was the detection of a major impurity in the latter preparation. This impurity could not be detected by other conventional chromatographic or electrophoretic techniques. The same preparations were also analysed using a previously reported, neutral pH reversed phase HPLC method. A comparison of the impurity profiles detected by the two methods demonstrated that they have complementary selectivities.

INTRODUCTION

An increasing number of therapeutic proteins are being produced on large scale through recombinant DNA technology. While health concerns related to vector-derived contaminants (e.g. host cell proteins, endotoxins, or residual DNA) were raised early in the development of this new technology, it has become well accepted that active protein variants are potential risk factors as they may lead to undesirable immunogenic responses or to greatly diminished biological activity.[1] Variants resulting from covalent (e.g. oxidation) or non-covalent (e.g. aggregation) modifications have been identified and shown to occur throughout the manufacturing process. Modifications may occur at more than one site of the protein, leading to complex mixtures and rendering separation, and ultimately quantitation difficult. While modern high performance chromatographic and electrophoretic techniques have been increasingly used to monitor protein heterogeneity,[1] no single technique has been found to provide sufficient information with respect to drug purity. Typically, it is necessary to resort to an array of analytical techniques.

Hydrophobic interaction HPLC (HI-HPLC) has been used successfully for the separation of proteins and other biomolecules.[2] This chromatographic mode is generally recognized for its mild separation conditions, a situation that allows most proteins to retain their native structure and, consequently, their biological activity. When combined with the high performance nature of modern stationary phases, these properties have made HI-HPLC an attractive alternative for the detection of variants. In particular, the absence of high concentrations of organic modifiers such as those used in reversed-phase HPLC (RP-HPLC) often prevents the breaking of weak intermolecular or intramolecular bonds, or those arising from alterations of the protein three-dimensional structure, and reveals the presence of otherwise undetected variants.

Commercial preparations of recombinant human growth hormone (hGH) are available from several manufacturers. hGH variants resulting from covalent modifications of the polypeptide chain have been identified and characterized and include deamidated, oxidized, and clipped forms. The protein has also been shown to self-associate under specific conditions to a non-covalent dimeric species (ncd-hGH) with greatly diminished biological activity.[3] Because it is

readily dissociated in the presence of high concentrations of organic modifiers or chaotropic agents (e.g., SDS), ncd-hGH has only been detected by size exclusion chromatography.[3]

HI-HPLC has been used previously to study human growth hormone. Wu *et al.*[6] studied the effect of column support and temperature on the separation of two recombinant hGH products, the N-terminus methionine derivative, met-hGH, and the native 22-kD protein. In our hands, this method was found to lack repeatability. Widely variable retention times, with RSD ≥ 50% were observed when the method was applied on different days. The same group[7] later reported on the optimization of their method and showed that it could separate closely related hGH clipped forms. However, the method was not validated with respect to precision, linearity, and repeatability. More recently Jespersen et al.[8] used HI-HPLC to separate and characterize a trisulfide hGH variant from *E.coli* fermentation broth. Herein we report on the development of a fully validated HI-HPLC method capable of separating a wide range of hGH variants. Analysis of various preparations and comparison of the selectivity of the method with that of a neutral pH RP-HPLC method that has been widely used for purity determination of hGH by numerous groups is also reported. Results were published in part elsewhere.[4]

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a Waters 600E Multisolvent Delivery System, a Waters 700 Satellite WISP Autoinjector, and a Waters 990+ Photodiode Array Detector. Data were accumulated between 200 and 300 nm and processed using Powerline and Waters 990 software.

Chemicals

Ammonium phosphate dibasic (J. T. Baker Inc.) was Baker Analysed grade and sodium phosphate dibasic (J. T. Baker Inc.) was Ultrapure Bioreagent. HPLC grade n-propanol (Aldrich Co.) was used. Deionized water was obtained from a Millipore Milli-Q water purification system. Human growth hormone and methionyl human growth hormone (met-hGH) preparations were obtained from the Bureau of Biologics and Radiopharmaceuticals, Health Canada. Non-covalent dimeric hGH (ncd-hGH) and a resolution mixture containing approximately 12% monodesamido hGH (des-hGH) were generously donated by Dr. R.M. Riggin, Eli Lilly and Company, Indianapolis (USA).

Chromatography

HI-HPLC separations were effected on Tosohaas Progel TSK-Phenyl 5PW, 75 x 7.5 mm i.d. columns (Supelco Canada). Mobile phases were as described in text. Optimized separation conditions consisted of Mobile phase A: 1M ammonium phosphate dibasic, pH 8.0 / n-propanol (99.5:0.5), and Mobile phase B: 0.1M sodium phosphate dibasic, pH 8.0 / n-propanol (97.5:2.5) at 30°C. Mobile phases were filtered through a 0.45 μm filter and degassed with helium. Prior to analysis, the column was equilibrated with mobile phase A until a stable baseline was obtained.

Separations were carried out with a 30 minutes linear gradient from 100% mobile phase A to 100% mobile phase B and maintaining the latter for 20 minutes; the flow rate was constant at 1.3 mL/min. Return to initial conditions and re-equilibration were achieved over 15 minutes. The elution profile is summarized as follows:

Time (min)	**%A**	**%B**	**Gradient**	**Temp. (°C)**
0	100	0	linear	30
30	0	100	isocratic	30
50	0	100	linear	30
51	100	0	isocratic	30
65	100	0	----	30

While the gradient was initiated at injection time, a combined 6.1 min gradient delay caused by the 2.7 min post-injector void volume delay and the 3.4 min gradient system volume delay inherent to the solvent delivery system used was observed. Effective salt concentrations were evaluated on the basis of that delay.

The RP-HPLC method was that described by Riggin et al.[5] and was used under isocratic elution conditions on a Vydac C4 214TP54 columns (Separations Group), 250 X 4.6 mm i.d. In normal conditions, a saturating silica column was placed between the pump and the injector. In specific experiments (see Results and Discussion), this column was removed.

In a typical run, aliquots (20 μL) of reconstituted hGH preparations (approx. 1 mg/mL) were injected and chromatograms were monitored at 220 nm. Blank injections were made at regular intervals in order to assess protein carry-over. Duplicate injections were made for all samples analysed.

RESULTS AND DISCUSSION

Development of HI-HPLC Separation Conditions

Recombinant met-hGH and hGH were used to set up the HI-HPLC separation conditions on a TSK-Phenyl 5-PW column. The appropriate conditions were developed upon examination of chromatographic criteria: (a) peak shape evaluated from the tailing factor, Tf, (b) peak efficiency measured from the number of theoretical plates, N, and (c) resolution, R. The choice of anti-chaotropic salt in mobile phase A was investigated and data are shown in Table 1. Under identical gradient conditions and irrespective of salt used or eluent pH (entries 2, 4, 5, 6, 7, and 8) substrates consistently eluted in a narrow range, between 22-27 minutes and 23-30 minutes for hGH and met-hGH respectively. The order of elution was identical for all conditions. Eluent pH appeared to have a significant effect on peak shape. In three cases where pH < 6 (entries 1, 2, and 7) significant peak tailing (Tf > 1.8) was observed. Good peak shape (Tf < 1.2) was obtained at pH >6 (entries 3, 4, 5, 6, 8, and 9). Increasing the length of the descending gradient (entries 8 and 9) slightly improved resolution while efficiency was not greatly affected. Separation conditions using 1.0M ammonium phosphate dibasic with a 30 minute linear gradient (entry 9) provided best results for the pair of substrates. These conditions were retained for further optimization.

Using the same selection criteria the effect of buffer salts, gradient profiles, temperature, and organic modifiers in mobile phase B were measured (Table 2). Various buffer salts were investigated of which ammonium sulfate and sodium phosphate dibasic gave the best results (entries 1 and 2). Peaks were generally symmetrical with Tf varying between 1.0 and 1.2 (data not indicated). The largest increment on resolution came from increasing the temperature of the column (entries 4, 5, and 6, and entries 10 and 11). These results are similar to that reported by Wu et al.[6]

Although separations at 45°C provided the greatest number of theoretical plates and good resolution, these conditions led to longer elution times and an apparent decrease in peak areas (~10%) indicating a possible loss of compound through irreversible adsorption onto the silica support or degradation (data not shown). The addition of acetonitrile and propanol as organic modifiers significantly decreased elution times while maintaining resolution and efficiency (entries 2, 7, 8, and entries 2, 3, 9, respectively). Addition of 0.5% propanol to mobile phase A was found to improve baseline stability. Thus, optimized conditions were found to be 1.0 M ammonium phosphate dibasic , pH 8.0 / propanol (99.5:0.5) and 0.1 M sodium phosphate dibasic, pH 8.0 / propanol (97.5:2.5) for mobile phase A and B respectively, with a descending gradient

Table 1

Effect of Mobile Phase A Antichaotropic Salt Composition[a] on hGH and Met-hGH Retention Time (t), Tailing Factor (Tf), Plates Number (N) and Resolution (R)[c]

					hHG			Met-hGH			
Salt R[b]	Entry	[M]	pH	Gradient (Min)	t (Min)	Tf[b]	N[b]	(Min)	t	Tf	N
$(NH_4)_2SO_4$	1	1.8	5.2	3.0	35.6	1.85	4300	36.6	1.84	5100	0.49
	2	0.8	5.5	20	22.6	2.43	1430	23.7	2.45	1730	0.45
Na_2SO_4/ 0.03M TRIS[3]	3	1.0	7.5	45	19.2	1.13	2520	20.2	1.12	2800	0.67
NaH_2PO_4	4	1.0	7.0	20	23.9	1.17	4600	24.9	1.17	4750	0.71
$(NH_4)H_2PO_4$	5	1.0	8.0	20	25.3	1.10	5830	26.3	1.12	6300	0.71
	6	1.0	7.0	20	23.1	1.08	3500	24.5	1.05	3275	0.85
	7	1.0	3.9	20	27.0	1.85	1870	29.5	1.90	1720	0.91
$(NH_4)_2HPO_4$	8	1.0	8.3	20	25.8	1.25	6200	26.8	1.20	6700	0.75
	9	1.0	8.3	30	32.6	1.10	6150	34.1	1.12	6710	0.85

[a] Unless otherwise indicated mobile phase B consisted of Milli-Q-water.

[b] Plate number (N expressed as plates per column), tailing factor (Tf) and resolution (R) were calculated according to USP 23.

[c] Conditions adapted from B. Pavlu et al., J. Chromatogr., **359**, 449-460 (1986).

Table 2

Effect of Mobile Phase B Composition[a] on hGH and Met-hGH Retention Time (t), Plates Number (N) and Resolution (R)

Mobile						hHG			Met-hGH	
				Gradient	T	t		t		
Phase B	Entry	[M]	pH	(Min)	°C	(Min)	N[b]	(Min)	N	R[b]
$(NH_4)_2SO_4$	1	0.1	8.0	20	20	27.4	5800	28.6	6400	0.85
Na_2HPO_4	2	0.1	8.0	20	20	27.8	6060	28.9	6600	0.85
Na_2HPO_4/	3	0.1	8.0	20	20	21.1	2520	22.3	2800	0.66
5%PrOH	4			30	20	26.4	2250	28.1	2400	0.73
	5			30	30	27.8	4450	29.5	4630	0.97
	6			30	45	32.7	9500	34.1	9580	1.17
Na_2HPO_4/ 5%ACN	7	0.1	8.00	20	20	22.0	2680	23.2	2975	0.71
Na_2HPO_4/ 2.5%ACN	8	0.1	8.0	20	20	22.7	2850	23.9	3165	0.75
Na_2HPO_4/	9	0.1	8.0	20	20	21.8	2625	26.3	2920	0.80
2.5%PrOH	10			30	20	27.6	5210	29.4	5910	0.96
	11			30	30	29.1	6900	31.2	7600	1.24

[a] Unless otherwise indicated mobile phase A consisted of 1.0 M ammonium phosphate dibasic, pH 8.0.
[b] Plate numbers (N expressed as plates per column) and resolution (R) were calculated according to USP 23.

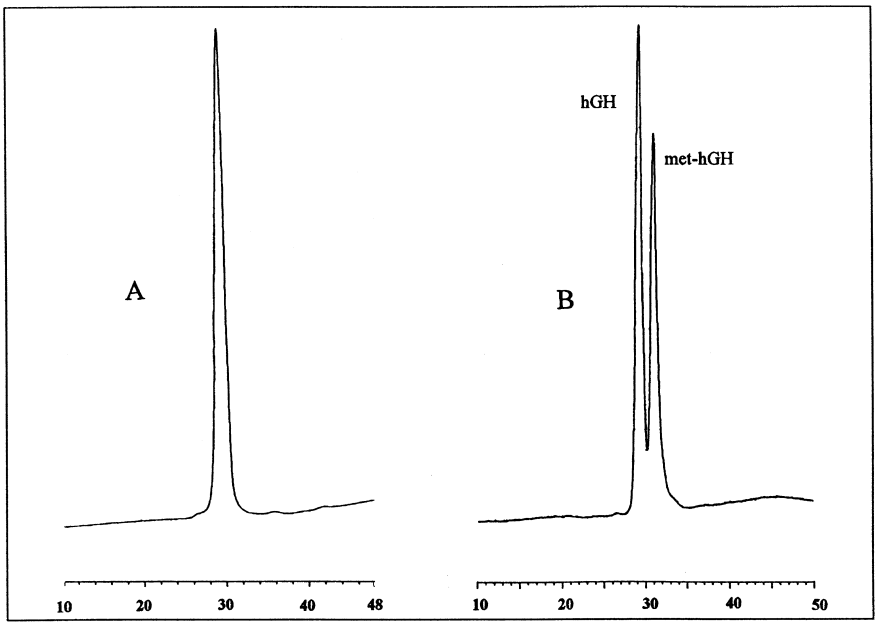

Figure 1. Chromatograms of (A) in-house hGH standard and (B) a mixture of hGH and met-hGH, obtained under optimized HI-HPLC conditions.

from 100% A to 100% B over 30 minutes and at a column temperature of 30°C. Representative chromatograms of hGH and a mixture of hGH and met-hGH obtained under optimized conditions are presented in Figure 1 A and B respectively.

The system suitability was monitored by injecting a mixture of hGH and met-hGH. It was found adequate when $R \geq 1.10$ and $N \geq 6000$ plates per column were obtained for each peak.

Method Validation

The linearity of the method was assessed from standard curves of the detector response against the concentration of hGH main peak from serial dilutions of a stock solution at 1.0 mg / mL. Five dilutions ranging from 0.1 to 1.0 mg/mL were prepared and chromatographed. Plots indicated that the responses for both peak area and peak height were linear for the entire range studied (Figure 2). Coefficient of determinations, R^2, were greater than 0.999 and the RSD of the slopes was less than 3% (n=3). The precision of the method

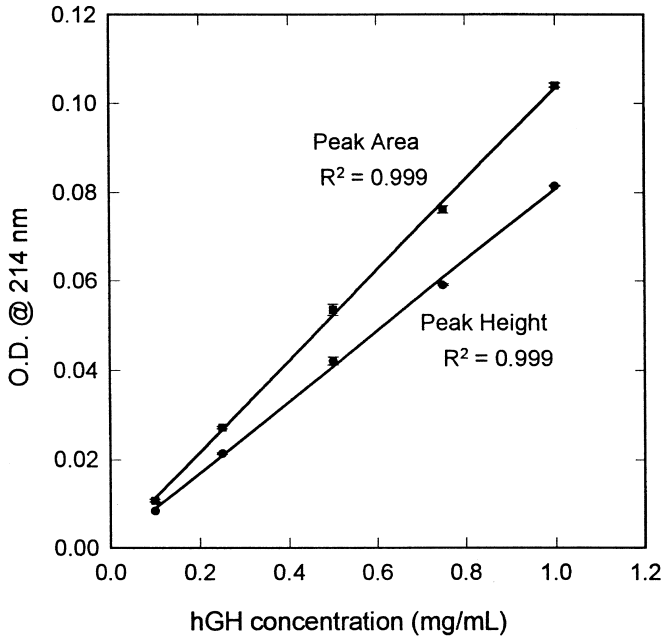

Figure 2. Linear regression plots of peak area and peak height vs hGH concentration from optimized HI-HPLC.

was assessed by measuring intra- and inter-day variations of migration time, peak height and peak area for replicate injections of an hGH solution at 1 mg/mL (Table 3). Components of within-batch variance, reported as RSDs, were consistently below 2.5% while those of between-batch variance were less than 8%.

The variation of migration time was low (RSD < 0.2% for between-day assays), a good indication of the stability of the column under these elution conditions. These data also indicated that the method was highly reproducible. The limit of quantitation (LOQ) was determined at 0.15 mg/mL, the concentration at which the signal-to-noise ratio was approximately 10:1 as determined by replicate injections (n=4).

When assayed on other columns from the same manufacturer, similar overall results were obtained indicating to the repeatability and robustness of the methodology.

Table 3

Precision Data for Migration Time, Peak Area and Peak Height for hGH Main Peak Measured by HI-HPLC

	Migration Time (min)	Peak Area (AU·min)	Peak Height (AU)
Day 1			
Mean (n=10)	30.44	0.1216	0.0924
SD	± 0.0057	± 0.0014	± 0.0008
RSD (%)	± 0.02	± 1.18	± 0.87
Day 2			
Mean (n=10)	30.42	0.1042	0.0810
SD	± 0.0375	± 0.0005	± 0.0005
RSD (%)	± 0.12	± 0.48	± 0.68
Day 3			
Mean (n=10)	30.38	0.1107	0.0855
SD	± 0.0053	± 0.0022	± 0.0019
RSD (%)	± 0.02	± 2.03	± 2.23
Between-day			
Mean (n=3)	30.41	0.1122	0.0863
SD	± 0.0306	± 0.0088	± 0.0057
RSD (%)	± 0.10	± 7.84	± 6.65

Analysis of Standard hGH Preparations and Comparative Selectivity to RP-HPLC

In addition to hGH and met-hGH, four other standard hGH preparations were analysed using the optimized HI-HPLC conditions. Figures 3a-d present typical chromatograms of: (a) a partially deamidated hGH sample, des-hGH, containing approximately 12% monodesamido-hGH, (b) the non-covalent hGH dimer, ncd-hGH, (c) the first WHO International Standard (IS) for hGH of pituitary origin, pit-hGH, and (d) the first WHO IS for somatropin (hGH of recombinant origin), who-hGH. Each preparation was also analysed using a neutral-pH RP-HPLC method[5] widely used for hGH purity determination (Figure 3e-h). This allowed a comparison of their respective selectivities for the separation of variants.

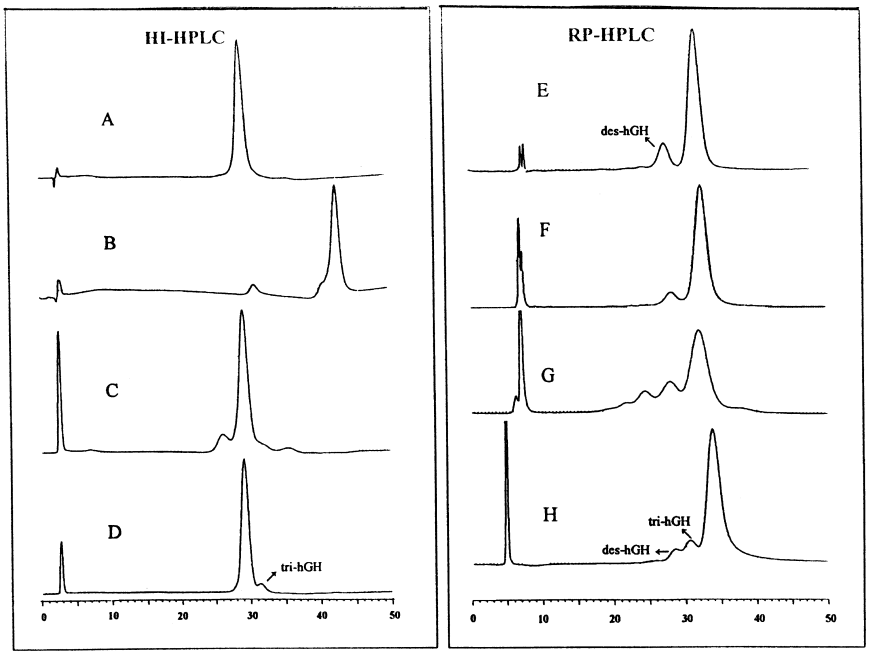

Figure 3. Chromatograms of standard hGH preparations obtained by HI-HPLC (A-D) and by RP-HPLC (E-H): des-hGH (A and E), ncd-hGH (B and F), pit-hGH (C and G) and who-hGH (D and H).

Analysis of des-hGH by HI-HPLC showed a single peak (Fig. 3a), with the same retention time as that of the main peak of the hGH sample shown in Fig. 1. This indicated that des-hGH and hGH had very similar hydrophobicities under these separation conditions and were not resolved. However, under RP-HPLC des-hGH was completely resolved from hGH (Fig.3e) as previously demonstrated by Riggin et al.,[5] eluting prior to hGH at RRt = 0.86. Quantitatively, the des-hGH peak ranged between 11.3-13.6% of the total peak area, in good agreement with the expected amount of 12%.

On the other hand, analysis of ncd-hGH showed a reversal of the previous situation. While under RP-HPLC ncd-hGH was not separated from the monomeric species (Fig. 3f), the HI-HPLC profile showed the major peak eluting at RRt = 1.42 (relative to average retention time of monomeric hGH as shown in Fig.1) and some minor components. Assuming the major peak to be the non-covalent dimer it was noteworthy that it eluted at significantly less salt concentration than the monomeric species indicating an increased hydrophobic

character. Furthermore these results suggested that under RP-HPLC conditions the labile ncd-hGH likely reverted back to the monomeric species, a situation that likely resulted from the presence of large amounts of organic modifier which break up the weak hydrophobic bonds between the monomeric species. The milder conditions used in HI-HPLC allowed the compound to retain its structural integrity.

Analysis of the two WHO International Standards showed peak profiles unlike that observed in the other preparations. Analysis of pit-hGH by the two chromatographic modes showed the major peak in each chromatogram corresponded to native hGH (Figures 3c and 3g for HI- and RP-HPLC respectively). However, large amounts of impurities were detected by both methods. Some of these impurities could be ascribed to deamidated and oxidized variants on the basis of their relative retention times.[5]

The pituitary preparation has recently been replaced by the first WHO IS for somatropin, a recombinant DNA version of the main 22 kD hGH product. The preparation, coded 88/624, was introduced for its increased purity making it more amenable to standardization.[9] Analysis by HI-HPLC (Figure 3d) revealed, apart from the main hGH peak, the presence of a major impurity which eluted at RRt =1.10 and corresponded to approximately 4.5% of the total peak area. We first observed this impurity during the International Collaborative study of the candidate hGH preparation, 88/624 (4b). Other chromatographic or electrophoretic test methods (e.g., IEF, native and SDS-Page, SE-HPLC) failed to provide evidence of the presence of this impurity, later identified as a trisulfide variant of hGH (tri-hGH) (see below). RP-HPLC analysis showed that the separation of tri-hGH was possible and was dependent on specific chromatographic conditions. Thus, in the presence of a saturating silica column positioned between the pump and the injector, chromatograms showed the presence of two major impurities at RRt = 0.85 and RRt = 0.92, corresponding to des-hGH and tri-hGH respectively (Fig. 3h). However, removal of the silica column led to loss of resolution and co-elution of the two impurities at RRt = 0.87 (data not shown).

Identification of Unknown Impurity in WHO International Standard for Somatropin

Upon isolation by semi-preparative HI-HPLC, the collected impurity was re-injected under analytical HI-HPLC conditions and eluted at the expected retention time demonstrating that it was not an artefact of the chromatographic conditions. Partial structural information was obtained on the purified product. Amino acid sequence analysis revealed intact N-terminus sequence for the first 20 amino acids. Mass information from mass spectrometry showed the product as having an average mass of 22,162.58 ± 1.20 D, approximately 36 mass units

heavier than that measured from amino acid analysis (22,126 D). RP-HPLC analysis[10] of the tryptic digest of the unreduced material suggested that the structural modification occurred near the C-terminus, with the disappearance of the peak corresponding to peptide T20-T21 and the appearance of a peak at longer retention time (data not shown). These data were in close agreement with those reported recently for a hGH variant featuring a trisulfide bridge linking Cys182-Cys189[8] (tri-hGH).

CONCLUSION

The detection and quantitative assessment of impurities in protein pharmaceuticals has become a corner stone of the concept of well-characterized biotechnology pharmaceuticals. While it is widely accepted that these products may not be characterized by a single technique, this study further demonstrated the utility of using a wide range of methods for their analysis. The HI-HPLC method described herein showed improved selectivity for the detection of the trisulfide and the non-covalent dimer hGH variants over the conventional neutral pH RP-HPLC method. This methodology was found to provide complementary information to other chromatographic methods.

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REFERENCES

1. G. S. Srivatsa,"Biotechnology Products," in **Development and Validation of Analytical Methods**, C. M. Riley, T. W. Rosanke, eds., Pergamon, London, 1996, pp. 209-248.

2. R. E. Shansky, S.-L. Wu, A. Figueroa, B. L. Karger, "Hydrophobic Interaction Chromatography of Biopolymers," in **HPLC of Biological Macromolecules,** K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 95-144.

3. G. W. Becker, R. R. Boesher, W. C. Mackellar, M. L. Poor, P. M. Tackitt, R. M. Riggin, Biotech. Appl. Biochem., **9**, 478-487 (1987).

4. (a) M. Girard, N. Mousseau, H. P. Bietlot, L. W. Whitehouse, Pharmaceutical Sciences, **3**, 9-14, (1997); (b) H. Rode, M. Girard, Report on the WHO International Collaborative Study on Somatropin, July 1991.

5. R. M. Riggin, G. K. Dorulla, D. J. Miner, Anal. Biochem., **167**, 199-209 (1987).

6. S.-L. Wu, W. S. Hancock, B. Pavlu, P. Gellerfors, J. Chromatogr., **500**, 595-606 (1990).

7. B. Pavlu, P. Gellerfors, Bioseparation, **3**, 257-265 (1993).

8. A. M. Jespersen, T. Christensen, N. K. Klausen, P. F. Nielsen, H. H. Sorensen, Eur. J. Biochem., **219**, 365-373 (1994).

9. (a) A. F. Bristow, D. Schulster, S. L. Jeffcoate, Pharmeuropa, **6(1)**, 60-67 (1994); (b) A. F. Bristow, S. L. Jeffcoate, Biologicals, **20**, 221-231 (1992); A. F. Bristow, S. L. Jeffcoate, Pharmeuropa, **3(Special Issue, March)**, 3-20 (1991).

10. J. Frenz, S.-L. Wu, W. S. Hancock, J. Chromatogr., **480**, 379-391 (1989).

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