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Separation of oxidized and deamidated human growth hormone variants by isocratic reversed-phase high-performance liquid chromatography

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Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) was utilized for the separation of recombinant human growth hormone (hGH) variants on a C_{18} silica column at 55°C using an isocratic mobile phase which contained 27% 1-propanol in a 25 m*M* potassium phosphate buffer, pH 6.5. Three of the obtained peaks were characterized by tryptic mapping and mass spectrometry; two of the peaks were found to contain oxidized hGH (dioxy Met14/Met125 and Met125 sulfoxide) while the third contained a deamidated form (Asn149→Asp149 or Asn152→Asp152). Compared to the European Pharmacopoeia RP-HPLC method of hGH analysis, this new method gives two additional peaks and a 50% reduction in the analysis time.

Keywords: Growth hormone; Proteins

1. Introduction

Human growth hormone (hGH) consists of a single polypeptide chain containing 191 amino acid residues and two disulfide bridges [1]. Modifications of hGH may arise during the fermentation and purification processes, as well as during storage of the protein. These products may or may not influence the biological effect of the hormone. Methionine residues at positions 14 and 125 have been reported to be prone to oxidation [2–4], while the only remaining methionine, Met170, which is located in the interior of the protein [5] is not oxidized in the native peptide [6]. Becker et al. [2] reported a greater susceptibility for oxidation of Met14 relative to Met125 in an oxidized fraction that was purified by means of neutral pH reversed-phase high-performance liquid chromatography (RP-HPLC). Hydrogen peroxide generated oxidation [7] and light exposure [8] of hGH has been reported to give a higher degree of oxidation of Met125 than Met14. However, a more mild hydrogen peroxide treatment gives mainly oxidation of Met14 [3]. Other forms of hGH such as dimers [9], deamidated variants of hGH (Asn149→Asp149 and Asn152→Asp152) [4], N-terminal truncated des-Phe [4], cleaved variants (Thr142–Tyr143) [4], and more recently a trisulfide (Cys182–SSS–Cys189) variant [10] have been described. The European Pharmacopoeia [11] as well as Riggin et al. [12] describe isocratic C_4 RP-HPLC

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methods using a pH of 7.5 at 45°C. Capillary RP-HPLC methods have also been used to identify various forms of hGH [13]. The present study describes a neutral pH RP-HPLC method that allows for the separation of several hGH variants, two of which were characterized as oxidized hGH and one which was characterized as a deamidated form, all three were characterized by means of tryptic mapping and mass spectrometry (MS). Compared to the European Pharmacopoeia RP-HPLC method the proposed method gives two additional peaks and a 50% reduction in the analysis time.

2. Experimental

2.1. Materials

A laboratory sample of recombinant hGH was obtained from Pharmacia and Upjohn. Analytical grade 1-propanol and far UV acetonitrile were from LabScan, suprapur hydrogen peroxide was purchased from Kebo, catalase (catalogue no. C30) was obtained from Sigma, and affinity purified trypsin (catalogue. no. 8444) was from Merck. Phosphoric acid (85%), potassium phosphate, sodium phosphate and ammonium bicarbonate (all p.a.) were obtained from Merck.

2.2. Oxidation and deamidation of hGH

The sulfoxide derivatives were produced in a similar manner to that described by Gellerfors et al. [4]. Briefly, hGH was incubated with 300 m*M* hydrogen peroxide in 100 m*M* ammonium bicarbonate for 5 h at 0°C, followed by addition of catalase [ratio of catalase:hGH was 1:100 (w/w)] and incubation for 5 min at room-temperature, to quench the reaction. The deamidation was performed by incubating hGH, 1 mg/ml, in 30 m*M* ammonium hydrogencarbonate for 24 h at 37°C.

2.3. RP-HPLC

The hGH samples were chromatographed using RP-HPLC on a Hewlett-Packard HP 1090 equipped with a Vydac 218 TP C_{18} (5 μm, 300 Å, 250×4.6 mm I.D.), analytical column that was equilibrated with 27% (v/v) 1-propanol in 25 m*M* potassium phosphate, pH 6.5. The injection volume of hGH (1.3 mg/ml) was 25 μl, and the flow-rate was 1.0 ml/min at 55°C. Detection was carried out by measuring UV absorbance at 220 nm. The method was also run at 45 and 50°C, and at pH 7.0 and 7.5 to determine changes in resolution. The main peaks were characterized using electrospray mass spectrometry (ES-MS).

2.4. Tryptic peptide map analysis

The peaks of hGH that were collected from RP-HPLC (Section 2.3) were concentrated and desalted by using Centricon microconcentrators from Amicon (Danvers, MA, USA) with an M_r 10 000 cut-off, in 1% (w/v) ammonium hydrogencarbonate buffer, followed by incubation of 40 μg of the collected hGH with affinity purified trypsin for 4 h at 37°C in a total volume of 70 μl; the enzyme to substrate ratio was 1:70. The digestion was stopped by the addition of 12 μl of 9% (v/v) phosphoric acid. The trypsin digested hGH fragments were then chromatographed using RP-HPLC with a Hewlett-Packard HP 1090 that was equipped with a Vydac 218 TP C_{18} column. The injection volume was 25 μl of 0.7 mg/ml hGH, and elution was performed by using a gradient of acetonitrile (5–60%) in 100 m*M* sodium phosphate buffer, pH 2, at a flow-rate of 1.0 ml/min [14]. Detection was carried out by measuring by UV absorbance at 210 nm. Tryptic peptides were characterized by using matrix-assisted desorption ionization MS with time-of-flight detection (MALDI-TOF).

2.5. Electrospray MS of hGH

The main peak fractions from the isocratic RP-HPLC of hGH were diluted 1.4 times with water and 1.2 ml of this solution was then injected at 0.2 ml/min onto a protein trap cartridge (10×1 mm) from Michrom Bioresources (Auburn, CA, USA) that was mounted in the loop position of a Valco six-port injector. The column was washed with 0.15 ml of an aqueous solution containing 5% acetonitrile and 0.2% (v/v) formic acid. The valve was switched to the inject position and the analyte was eluted with 60% acetonitrile, 0.2% formic acid, in water,

pumped at 0.02 ml/min by an LC-10AD pump from Shimadzu (Kyoto, Japan). The column effluent was diverted to the electrospray ion-source of a Q-TOF instrument from Micromass (Manchester, UK). The quadrupole was set to the transmission mode and the electrospray mass spectrum was recorded by the time-of-flight mass analyser. The electrospray spectrum was transformed to the mass scale by the MASSLYNX software from Micromass.

2.6. MALDI-TOF MS of tryptic peptides

The peptides that were isolated from the tryptic maps of the different hGH fractions were diluted four times with 0.1% (v/v) trifluoroacetic acid (TFA) in water and extracted on 80 mg Oasis columns from Waters (Milford, MA, USA). The peptides were eluted with 1 ml of an aqueous solution containing 80% acetonitrile, 0.1% formic acid, and evaporated under vacuum. The samples were reconstituted in 10 μl of 0.1% TFA in water and 1 μl of the solution was spotted onto a MALDI target along with 1 μl of the matrix solution (50% acetonitrile, 0.1% TFA saturated with α-cyano-4-hydroxycinnamic acid, in water) plus 1 μl of the internal standard solution (bradykinin and adrenocorticotropic hormone (ACTH) fragment 18–39). MALDI-TOF mass spectra were recorded on a TofSpec-SE instrument from Micromass operated in the reflectron mode.

2.7. RP-HPLC of hGH according to the European Pharmacopoeia

A Hewlett-Packard HP 1090 equipped with a Vydac 214ATP54 Protein Pak C_4 column (5 μm, 300 Å, 250×4.6 mm I.D.), analytical column which was equilibrated with about 29% (v/v) 1-propanol in 35.5 m*M* tris(hydroxymethyl)aminomethane–HCl, pH 7.5 was utilized in this work. The injection volume was 20 μl of 2.0 mg/ml hGH, and the flow-rate was 0.5 ml/min at 45°C. Detection was carried out by measuring UV absorbance at 214 nm [11].

3. Results and discussion

The reversed-phase chromatographic method that was utilized allowed for a high degree of selectivity in the analysis of several hGH variants, as shown in Fig. 1. The oxidized sample gave seven distinct peaks, of which the main peak gave 5400 theoretical plates (N) per meter with a retention factor (k') of about 6. The resolution between the deamidated peak and the main peak was 1.1 at pH 6.5 and 0.9 at pH 7.5. Similarly, N was higher at pH 6.5 than at 7.5 (Table 1). The European Pharmacopoeia [11] as well as Riggin et al. [12] have described isocratic C_4 RP-HPLC methods which utilize a Tris buffer, pH 7.5, at 45°C. In the analysis of hGH we chose pH 6.5, at 55°C because of the improved resolution, peak shape, and better stability of the silica column. According to the Vydac manufacturer, a maximum pH of 6.5 is recommended for maximum column lifetime. The method that is described in this paper gives two additional peaks (peaks A and C) when compared with the European Pharmacopoeia RP-HPLC method (Fig. 3). In addition, the analysis time was reduced by approximately 50%, primarily due to the increased flow-rate at elevated temperature. When the hGH laboratory sample was analyzed six different times, the new method gave a mean of 94.0% (0.7% RSD) of the total integrated area for the main peak, while the corresponding value for the European Pharmacopoeia method was 93.6% (1.2% RSD). This indicates an acceptable intermediate precision and accuracy for the new method. The resolution of 1.1 between the deamidated peak and the main peak in this study (Table 1) may be compared with the value of 0.9 obtained in a study using the European Pharmacopoeia method [15]. This improved method may be seen as an alternative to the method described in the European Pharmacopoeia. The ES-MS of the peaks A and B (Fig. 1, II) and peak D (Fig. 1, III) exhibited mass differences relative to reference hGH of +32, +16 and +1 u, respectively (Table 2). The increases of 32 and 16 u are consistent with the oxidation of two and one methionine residues, respectively, while the increase of 1 u indicates a deamidation.

Tryptic mapping was carried out in order to localize the sites of oxidations and deamidations. Tryptic cleavage of peak A, followed by RP-HPLC, gave decreased retention times for both the T2 and T11 fragments (Fig. 2, II). These fragment peaks were then identified as being the oxidized (+16 u in

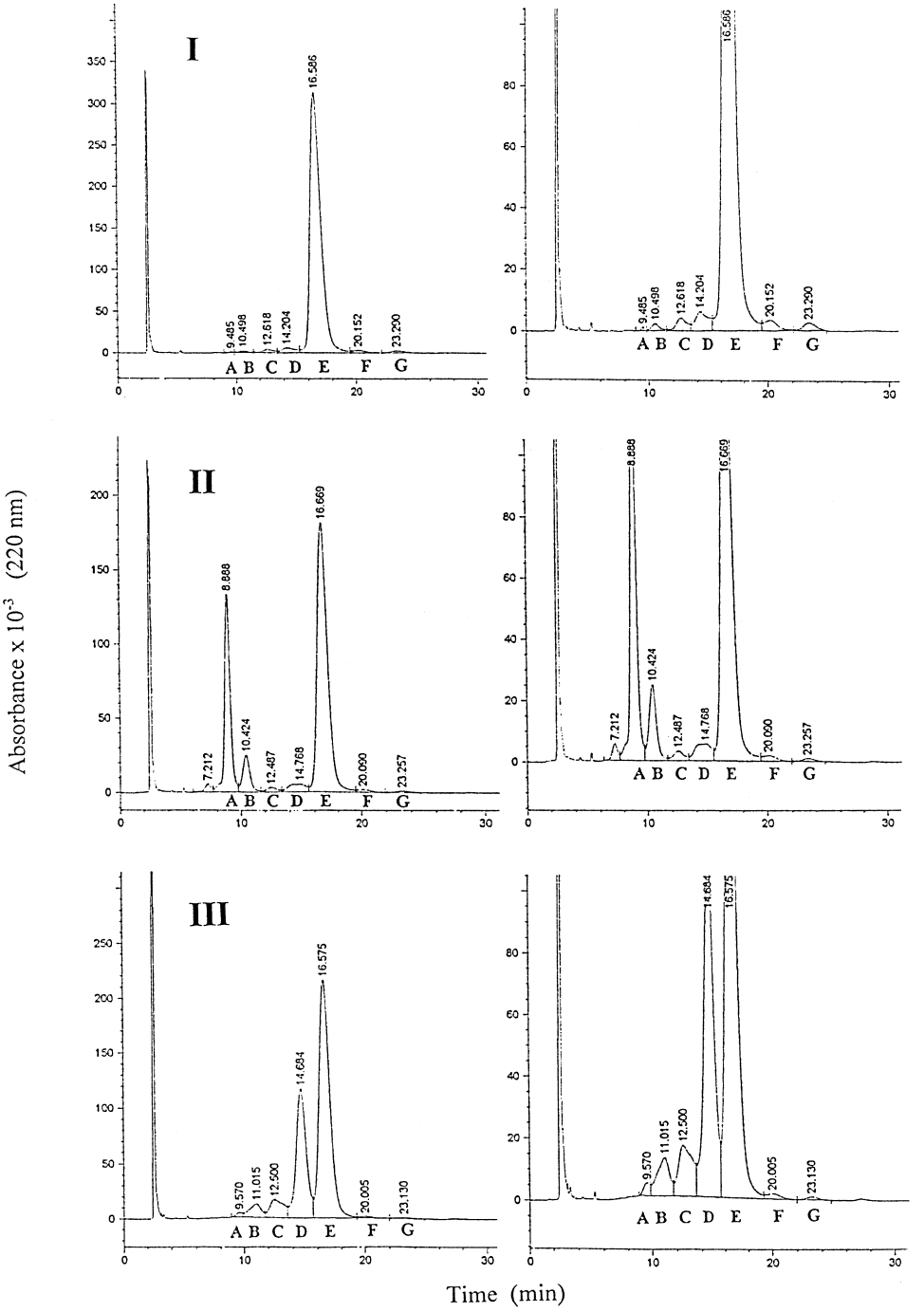

Fig. 1. Reversed-phase isocratic HPLC of hGH using a Vydac C_{18} column equilibrated with 27% (v/v) 1-propanol in 25 m*M* potassium phosphate, pH 6.5, at a flow-rate of 1.0 ml/min and temperature 55°C. A 25-μl volume of hGH, 1.3 mg/ml, was injected. Detection of absorbance at 220 nm. (I) Untreated hGH (II) oxidized hGH mixed with untreated hGH in a ratio of 1:2. (III) deamidated hGH. The chromatograms to the right are enlargements of the full-scale chromatograms. Oxidation and deamidation of hGH were performed as described in Section 2.

Table 1
Chromatographic parameters for hGH peaks at different pH and temperature[a]

Peak		pH 6.5		pH 7.0	pH 7.5	
		45°C	55°C	50°C	45°C	55°C
A B	(R_s)	1.5	1.3	1.2	1.3	1.3
D E	(R_s)	1.1	1.1	0.8	0.9	0.9
A	(N/m)	3480	5240	3220	2870	4630
B	(N/m)	3150	3920	3340	2960	4110
D	(N/m)	3780	4950	2390	2480	3330
E	(N/m)	4480	5390	3470	3190	4650
A	(t_R)	12.1	8.7	8.6	10.9	7.8
B	(t_R)	14.8	10.2	10.1	13.3	9.2
D	(t_R)	22.5	14.5	14.1	19.3	12.4
E	(t_R)	25.9	16.4	15.9	22.1	13.9

[a] Resolution $(R_s)=1.175(t_{R2}-t_{R1})/(W_{h1}+W_{h2})$, where t_{R2}/W_{h2} and t_{R1}/W_{h1} are the retention times (min)/width at half the peak height, of the more retained and less retained components, respectively. Number of theoretical plates $(N/\text{m})=(t_R/W_h)^2\times5.54\times4$. Peaks A and B were from the oxidized hGH sample, while peaks D and E (the major peak of hGH) were from the deamidated hGH sample. Experimental conditions are as described in Section 2.

mass) T2 and T11 by MALDI-MS (Table 3), thus indicating that peak A (Fig. 1, II) is the dioxy Met14/Met125 form of hGH. In a similar manner, peak B (Fig. 1, II) was characterized as Met125 sulfoxide (Table 3). Although the oxidized form of T11 coeluted with T1, this did not cause any problems in the MALDI-MS analysis. The T11 fragment does not contain other amino acids prone to oxidation than Met125. Fragment T17 (residue 169–178) was found to be identical to the hGH reference in the oxidized peak samples (Fig. 2, II), indicating that no oxidation of Met170 occurred. This is in accordance with earlier studies [4,6] and is most likely a result of Met170 being located within the interior of the native protein, as shown by the hGH crystal structure [5] and confirmed by a crystal study of hGH Gly120→Arg mutant [16]. Oxidation using high concentrations of hydrogen peroxide [7], which was also was used in this study, and light exposure [8] of hGH have both been reported to give a higher degree of oxidation of Met125 than Met14, while in the native hGH Met14 is the primary site of oxidation [2,3].

We also have experimental findings which support the fact that peak D contains the oxidized Met14 form of hGH. This was deduced by comparing the obtained retention time with that which was obtained after injecting a collected fraction of oxidized Met14 hGH; this oxidation was done according to Teshima and Canova-Davis [3]. In a similar manner, the elution position of des-Phe1 hGH was attributed to peak C, the clipped form (Thr142–Tyr143) to the rear part of peak D and the trisulfide (Cys182–SSS–Cys189) hGH form coeluted with the main component (data not shown). These analyzed forms were obtained by chromatographical methods which were

Table 2
Electrospray ionization mass spectral analyses of the collected hGH peaks; peaks A and B were from the oxidized hGH sample, while peak D was from the deamidated hGH sample

Sample	t_R (min)	Observed mass (u)	Mass difference to hGH reference (u)
hGH, reference		22 126.3[a]	0.0
hGH, peak A	8.9	22 157.8	31.5
hGH, peak B	10.4	22 142.4	16.1
hGH, peak D	14.7	22 127.2	0.9[b]

[a] Average theoretical mass for hGH is 22 125.
[b] Not statistically significant ($P>0.05$).

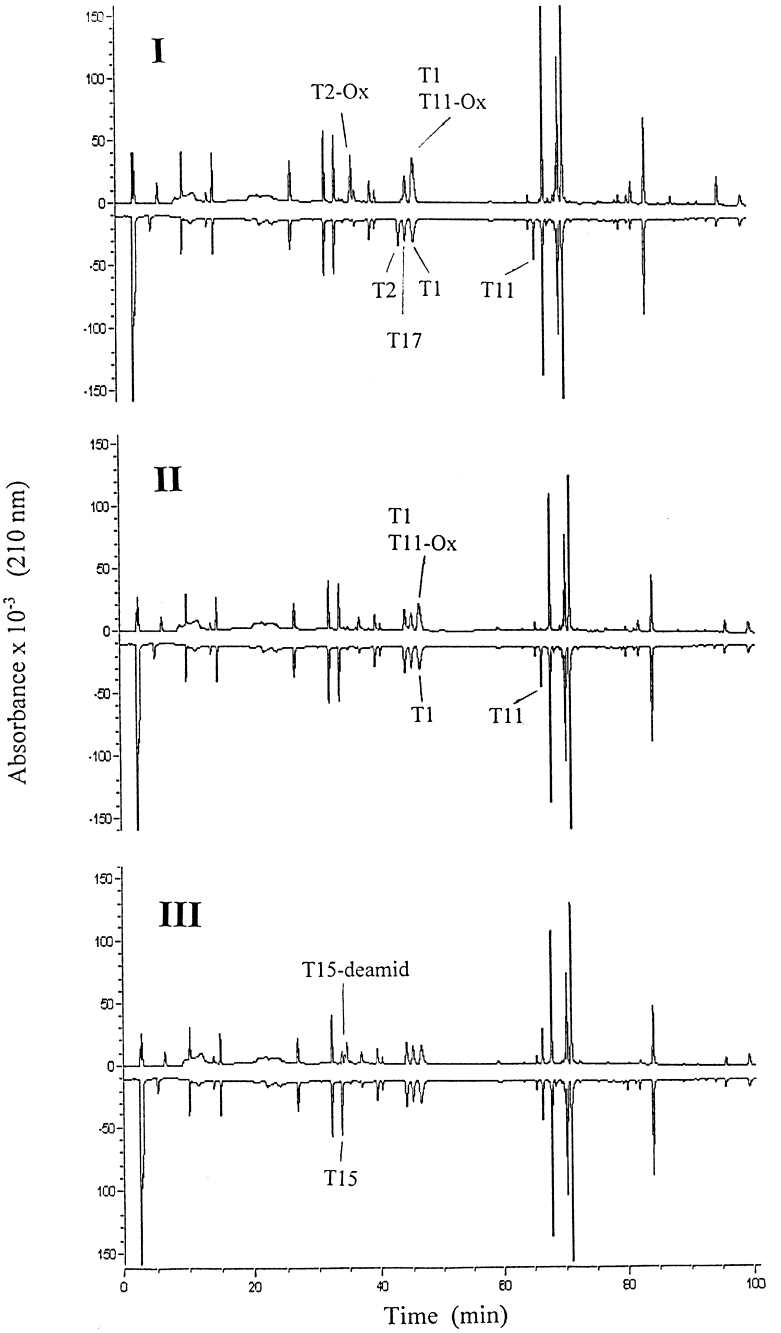

Fig. 2. RP-HPLC tryptic maps of oxidized and deamidated hGH fractions compared with untreated hGH (inverted chromatograms). Chromatography was performed on a Vydac C_{18} column equilibrated with 100 m*M* sodium phosphate, pH 2, and eluted with an acetonitrile gradient, as described in Section 2. (I) Peak A (Fig. 1, II) (II) Peak B (Fig. 1, II). (III) Peak D (Fig. 1, III).

Table 3
Mass spectral analyses of tryptic peptides of hGH by MALDI-TOF[a]

Sample	Peptide	Sequence	t_R[b] (min)	Expected monoisotopic mass (u)	Observed mass (u)	Identification
Reference	(146–158)	FDTNSHNDDALLK	34.1	1488.7	1488.7	T15
Reference	(9–16)	LFDNAMLR	44.4	978.5	978.4	T2
Reference	(1–8)	FPTIPLSR	46.8	929.5	929.6	T1
Reference	(116–127)	DLEEGIQTLMGR	66.7	1360.7	1360.7	T11
Peak A			36.8		994.5	T2+16 u
Peak A			46.7		929.6	T1
Peak A			46.7		1376.8	T11+16 u
Peak B			46.7		929.8	T1
Peak B			46.7		1376.7	T11+16 u
Peak D			34.6		1489.6	T15+1 u

[a] New identified tryptic fragments from the collected peaks: Peak A, T2+16 u and T11+16 u; Peak B, T11+16 u; Peak D, T15+1 u. Peaks A and B were both from the oxidized hGH sample and peak D was from the deamidated hGH sample.
[b] See Fig. 2.

based upon those by Gellerfors et al. [4,10,14]. These forms were also analyzed by means of the European Pharmacopoeia method (data not shown). The elution positions for the mentioned variants are presented in Fig. 3. One main difference between the methods is that the trisulfide form (Cys182–SSS–Cys189) elutes in the main peak in this new method, while in the European Pharmacopoeia method this form elutes before the clipped hGH form and is separated from the main peak. Aside from this difference, the elution patterns of the two methods are very similar. One difference between the methods, when analyzing the same hGH laboratory sample, is that the new method gives an area of about 1.0%, of the total integrated area, for peak C (the elution position for des-Phe1 hGH), while the peak in the corresponding elution position is almost absent in the European Pharmacopoeia method (Fig. 3). However, this may be explained by the fact that the des-Phe1 hGH elutes close to or as a part of the Met14 sulfoxide hGH impurity peak in the European Pharmacopoeia method. This additional peak (peak C), together with the two peaks which elute after the main peak should be further characterized in the new method.

A tryptic map of peak D (Fig. 2, III) exhibited a splitting of the T15 fragment into two smaller fragments which eluted about 1 min after the T15 reference. The first of these fragments was identified as deamidated (+1 u) T15. Since Asn149 and Asn152 are the only possible candidates for deamidation in T15 one of these amino acids must have been deamidated to aspartic acid or to isoaspartic acid [17]. It has been shown by Becker et al. that Asn149 is the amino acid most susceptible to deamidation [2]. The amino acids aspargine and glutamine are the most sensitive to degradation, becoming deamidated to aspartic and glutamic acid, respectively. Deamidation reactions have been shown to be influenced by pH, temperature, and ionic strength, as well as by the adjacent amino acid sequence [18]. In this study increased temperature and alkaline pH were used to induce deamidation. These identified variants, i.e. the oxidized and deamidated forms, are as biologically active as the native form of hGH [2], which is not the case for some other hGH variants, e.g. dimeric hGH [9].

4. Conclusion

A neutral pH RP-HPLC method has been described which allows for the rapid separation of several hGH variants, two of which were characterized as oxidized hGH (dioxy Met14/Met125 and Met125 sulfoxide) and one as a deamidated form (Asn149→Asp149 or Asn152→Asp152) by means of tryptic mapping and mass spectrometry. Compared to the European Pharmacopoeia RP-HPLC method of hGH analysis this new method gives two

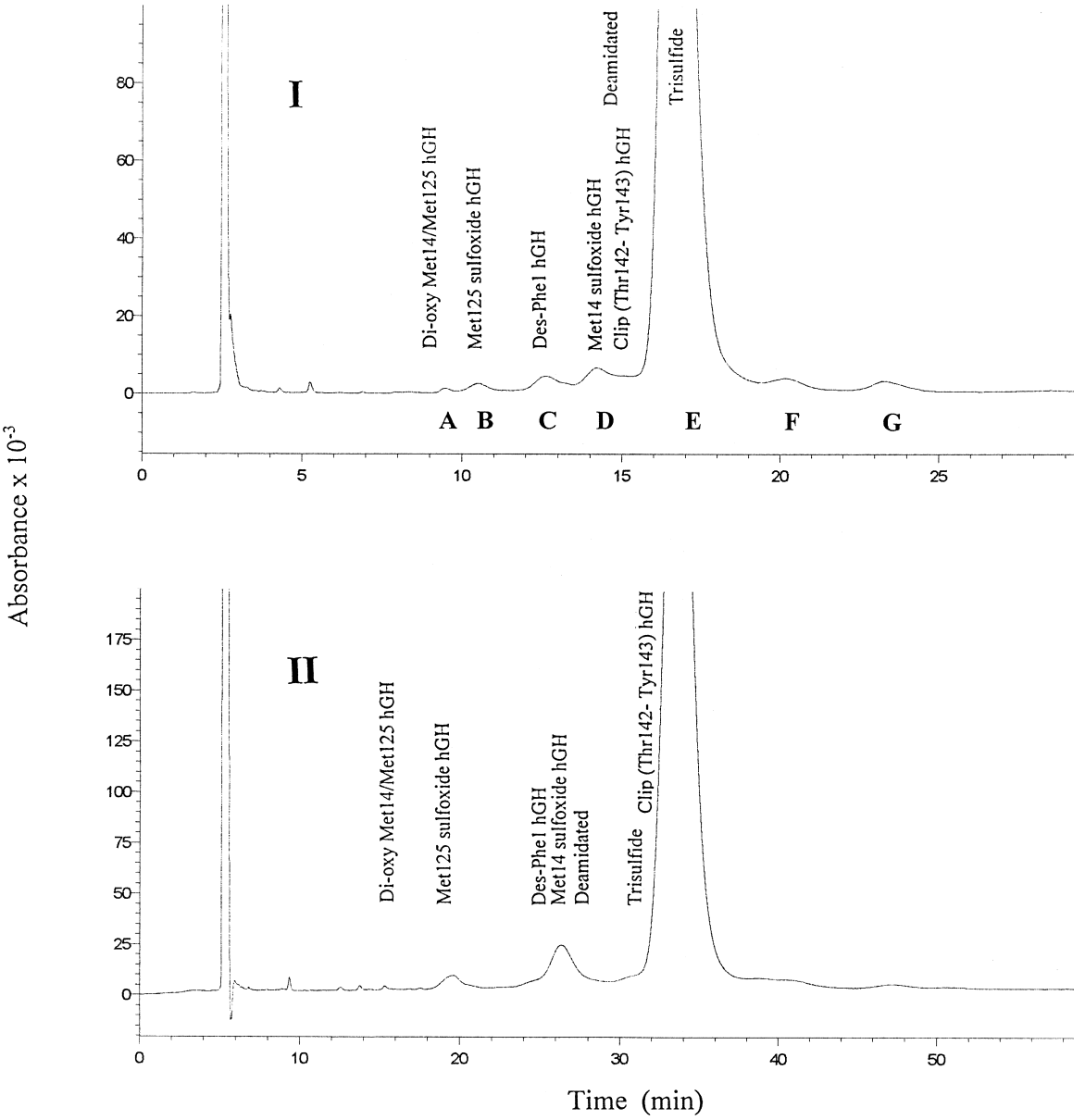

Fig. 3. Comparison between the new RP-HPLC method (I) and the method as described in the European Pharmacopoeia (II) when analyzing the same untreated hGH sample. In the new method (I) 25 μl hGH, 1.3 mg/ml, was injected, the flow-rate was 1.0 ml/min and absorbance at 220 nm was detected. The corresponding parameters for the European Pharmacopoeia method (II) were injection of 20 μl hGH, 2.0 mg/ml, flow-rate 0.5 ml/min and detection at 214 nm. See Section 2 for further details. The elution positions of the hGH variants are indicated in the figure. Deamidated indicates the (Asn149→Asp149 and/or Asn152→Asp152) form, while trisulfide indicates the trisulfide (Cys182–SSS–Cys189) form of hGH.

additional peaks and a 50% reduction in the analysis time.

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References

[1] T.A. Bewley, C.H. Li, Adv. Enzymol. 42 (1975) 73–166.
[2] G.W. Becker, P.M. Tackitt, W.W. Bromer, D.S. Lefeber, R.M. Riggin, Biotech. Appl. Biochem. 10 (1988) 326–337.
[3] G. Teshima, E. Canova-Davis, J. Chromatogr. 625 (1992) 207–215.
[4] P. Gellerfors, B. Pavlu, K. Axelsson, C. Nyhlen, S. Johansson, Acta Paediatr. Scand. [Suppl.] 370 (1990) 93–100.
[5] A.M. De Vos, M. Ultsch, A.A. Kossiakoff, Science 255 (1992) 306–312.
[6] R.A. Houghten, C.B. Glaser, C.H. Li, Arch. Biochem. Biophys. 178 (1977) 350–355.
[7] L.C. Teh, L.J. Murphy, N.L. Huq, A.S. Surus, H.G. Friesen, L. Lazarus, G.E. Chapman, J. Biol. Chem. 262 (1987) 6472–6477.
[8] E. Canova-Davis, R.C. Chloupek, I.P. Baldonado, J.E. Battersby, M.W. Spellman, L.J. Basa, B. O'Connor, R. Pearlman, C. Quan, J.A. Chakel, J.T. Stults, W.S. Hancock, Am. Biotechnol. Lab. 6 (1988) 10–17.
[9] G.W. Becker, R.R. Bowsher, W.C. Mackellar, M.L. Poor, P.M. Tackitt, R.M. Riggin, Biotech. Appl. Biochem. 9 (1987) 478–487.
[10] C. Andersson, P.O. Edlund, P. Gellerfors, Y. Hansson, E. Holmberg, C. Hult, S. Johansson, J. Kördel, R. Lundin, I. Mendel-Hartvig, B. Norén, T. Wehler, G. Widmalm, J. Öhman, Int. J. Peptide Protein Res. 47 (1996) 311–321.
[11] European Pharmacopoeia, 3rd ed., Supplement 1999, Convention on the Elaboration of a European Pharmacopoeia, Strasbourg, Monography 0951.
[12] R.M. Riggin, G.K. Dorulla, D.J. Miner, Anal. Biochem. 167 (1987) 199–209.
[13] J.E. Battersby, A.W. Guzzetta, W.S. Hancock, J. Chromatogr. B. 662 (1994) 335–342.
[14] B. Pavlu, P. Gellerfors, Bioseparation 3 (1993) 257–265.
[15] T. Tojo, C. Yomota, S. Okada, J. Kawamura, Iyakuhin Kenkyu 27 (1996) 815–820.
[16] M. Sundström, T. Lundqvist, J. Rödin, L.B. Giebel, D. Milligan, G. Norstedt, J. Biol. Chem. 271 (1996) 32 197–32 203.
[17] G. Teshima, W.S. Hancock, E. Canova-Davis, in: D.W. Aswad (Ed.), Deamidation and Isoaspartate Formation in Peptides and Proteins, CRC Press, Boca Raton, FL, 1995, pp. 167–189.
[18] R.C. Stephenson, S. Clarke, J. Biol. Chem. 264 (1989) 6164–6170.