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

Effect of mobile phase pH and organic modifier

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ABSTRACT

Human growth hormone (hGH), a polypeptide of 191 amino acids, contains three methionine residues, two of which are susceptible to oxidation by both chemical and photochemical means (Met14 and Met125). To date, no method has existed for resolving the various mono- and di-oxidation products. We report on the resolution of these oxidized variants and native hGH at weakly (pH 3.5) acidic pH. Although all of the oxidized species can be resolved at pH 3.5, use of low pH and neutral pH mobile phases confer some advantages. For example, the Met14 oxidized variant (MetSO-14) and native hGH are best resolved at neutral pH and the mono-oxidized variants (MetSO-125 and MetSO-14) are best resolved at low pH. The effect of organic modifier on the separation of the oxidized variants was also evaluated. 1-Propanol was more effective than acetonitrile in the separation of MetSO-14 and native hGH while acetonitrile was more effective than 1-propanol in the separation of MetSO-125 and MetSO-14. In summary, mobile phase pH and organic modifier were shown to be important parameters in the separation of oxidized hGH variants.

INTRODUCTION

Oxidation of methionine to methionine sulphoxide is a common chemical reaction known to occur in proteins [1–5]. Human growth hormone (hGH), produced by recombinant DNA technology, contains three potential sites for methionine oxidation; Met14, Met125, and Met170. Oxidation has been detected at both Met14 and Met125 in hGH [6–9], although there has been some discrepancy concerning the relative rates of oxidation at the two sites. Met170 is not oxidized in the native state [10] consistent with its location in the interior of the molecule according to the recently published hGH-receptor crystal structure [11].

Teh *et al.* [6] have reported a greater susceptibility of Met125 to chemical oxidation whereas Becker *et al.* [7] detected oxidation primarily at Met14. In the study by Teh *et al.* [6], relative rates were deduced from the peak heights of tryptic peptides generated from digestion of hydrogen peroxide-treated hGH. In the study by Becker *et al.* [7], relative rates were deduced from the peak heights of tryptic peptides generated from digestion of an oxidized fraction isolated by neutral pH reversed-phase high-performance liquid chromatography (HPLC). Canova-Davis *et al.* [8] isolated and characterized a Met125 oxidized hGH variant produced from exposure to light. The oxidized variant was isolated by reversed-phase HPLC at low pH. The results from the studies

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of Becker *et al.* [7] and Canova-Davis *et al.* [8] suggest that the Met14 and Met125 oxidized hGH variants have different mobile phase pH selectivities; that is the Met14 oxidized variant is better resolved from native hGH at neutral pH whereas the Met125 oxidized variant is better resolved from native hGH at low pH. To better understand the separation mechanism and chromatographic behavior of human growth hormone and its oxidized variants, the effect of mobile phase pH and type of organic modifier was evaluated.

EXPERIMENTAL

Preparation of oxidized hGH

An amount of 15 mg of recombinant hGH (lyophilized) was dissolved in 1.5 ml water (final pH 7.4). An aliquot of 30 μl of 3% hydrogen peroxide (J. T. Baker) was added. The reaction was carried out at ambient temperature for 4 h.

Preparative reversed-phase chromatography at neutral pH

The oxidized hGH solution (15 mg) was loaded onto a Polymer Labs PLRP-S 10 μm, 300 Å (300 × 7.5 mm) column. Buffer A consisted of 25 m*M* ammonium acetate, pH 7.5. Solvent B was 1-propanol. Protein was eluted with a linear gradient from 34% B to 39% B over 100 min. Column temperature was 40°C and the flow-rate was 1 ml/min.

Rechromatography and characterization of oxidized fractions

Oxidized and main peak fractions isolated by preparative reversed-phase chromatography at neutral pH were analyzed on a Vydac C_{18} column (5 μm, 300 Å, 250 × 4.6 mm I.D.). Buffer A was aqueous 0.1% trifluoroacetic acid (TFA). Solvent B was 0.1% TFA in acetonitrile. Protein was eluted with a gradient from 57% B to 77% B in 40 min. Column temperature was 40°C and the flow-rate was 0.5 ml/min. The oxidized fraction from neutral pH reversed-phase chromatography was separated into two peaks. The less retained, minor fraction (12% by peak area) and major fraction were dried using a Savant Speed-vac. The dried fractions were reconstituted in a 10 m*M* Tris buffer, pH 8.2, containing 100 m*M* sodium acetate and 0.1 m*M* calcium chloride, and digested with L-1-tosylamido 2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) at a 1:50 enzyme to substrate weight ratio for 4 h at 37°C. The trypsin-digested protein was loaded onto a Nucleosil C_{18} column (150 × 4.6 mm I.D.) equilibrated with 0.1% aqueous TFA and eluted with a linear gradient to 40% acetonitrile over 120 min at a flow-rate of 1 ml/min. UV absorbance was monitored at 214 nm. The native and oxidized tryptic peptides were collected, dried, and reconstituted in methanol–water (50:50) in preparation for analysis by electrospray ionization mass spectrometry (MS). This solution was infused at 10 μl/min into the ionspray probe of a Sciex API 111 triple quadrupole mass spectrometer. The mass axis was previously calibrated with a mixture of polypropylene glycols. The mass spectrum was analyzed using the Sciex Hypermass program to calculate the molecular mass from multiple-charged ions.

Analytical chromatography

A mixture of the oxidized and main peak fraction from the preparative reversed-phase HPLC separation were analyzed isocratically by reversed-phase HPLC on a Polymer Labs PLRP-S 8 μm, 300 Å (250 × 4.6 mm I.D.) column. Column temperature was 40°C and the flow-rate was 0.5 ml/min. The following mobile phase systems were evaluated: (1) Buffer A: 0.1 *M* sodium phosphate, pH 2.0, 1% sodium chloride. Solvent B: 1-propanol or acetonitrile. (2) Buffer A: 0.1 *M* sodium phosphate, pH 3.5, 1% sodium chloride. Solvent B: 1-propanol or acetonitrile. (3) Buffer A: 0.1 *M* sodium phosphate, pH 7.0, 1% sodium chloride. Solvent B: 1-propanol or acetonitrile.

RESULTS

Hydrogen peroxide-treated hGH (15 mg) was separated by reversed-phase HPLC at neutral pH on a PLRP-S (Polymer Labs) column. Lesser retained minor (peak a, Fig. 1) and major (peak b, Fig. 1) peaks were resolved from native hGH (peak c, Fig. 1). The two side fractions (peak a and b) and the main peak (native hGH) were then rechromatographed by reversed-phase HPLC on a Vydac C_{18} column using an 0.1% aqueous TFA–acetonitrile solvent system (Fig. 2). HPLC analysis of the minor

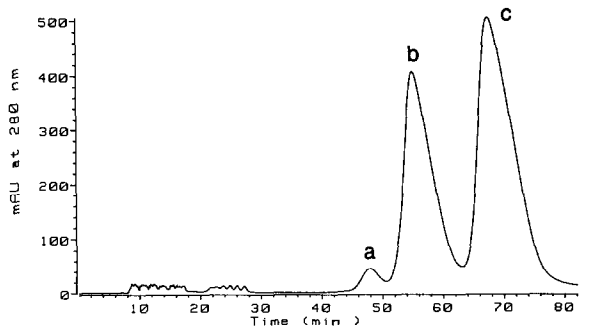

Fig. 1. Preparative reversed-phase HPLC at neutral pH of hydrogen peroxide-treated hGH (15 mg). Chromatography was performed on a Polymer Labs PLRP-S column equilibrated with ammonium acetate and eluted with a 1-propanol linear gradient as described in Experimental section.

TABLE I

ELECTROSPRAY IONIZATION MASS SPECTRAL ANALYSES

	Mass (dalton)	Theoretical mass
T2 (residues 9 to 16)	979.1	979.5[a]
T2 (oxidized at Met14)	995.1	995.5[a]
T11 (residues 116 to 127)	1361.3	1361.7[a]
T11 (oxidized at Met125)	1377.3	1377.67[a]
hGH (native)	22 126.5	22 125[b]
hGH (oxidized at Met125)	22 143.1	22 141[b]

[a] Mono-isotopic.
[b] Average.

side fraction (peak a, Fig. 1) resulted in the separation of a lesser retained peak (peak a-1, Fig. 2A). The corresponding fraction was collected, dried, and analyzed by tryptic mapping (Fig. 3A). The native T2 (residues 9 to 16, Leu-Phe-Asp-Asn-Ala-Met-Leu-Arg) and T11 (residues 116 to 127, Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg) peptides are absent in the map and two new peptides represented by their oxidized counterparts appeared indicating that the minor peak from the neutral pH separation (Fig. 1) was a di-oxidized hGH variant.

HPLC analysis of the major side fraction (peak b, Fig. 1) resulted in the separation of an earlier eluting peak (peak b-1, Fig. 2B) in addition to a peak with the same retention time as native hGH (peak b-2, Fig. 2B). The fraction representing the earlier eluting fraction (peak b-1, Fig. 2B) was collected, dried, and analyzed by electrospray ionization MS. The mass of the earlier eluting component was 22 143.1 u as compared to 22 126.5 u for native hGH (Table I). The increase of 16.5 u is consistent with the oxidation of a single methionine residue. Tryptic mapping (Fig. 3) was carried out in order to localize the site of oxidation, since hGH contains three potential sites for methionine oxidation (residues 14, 125, and 170). The T11 peptide (residues 116 to 127) was absent from the tryptic map and a new peptide appeared on the ascending shoulder of T2 (residues 9 to 16) (Fig. 3B, inset). The new peptide had a mass of 1377.3 u as compared with 1361.3 u for the native T11 peptide (Table I). The increase in mass of 16 u is consistent with the oxidation of Met125 to methionine sulphoxide. Other amino acids which can react with hydrogen peroxide at neutral pH (cysteine, tryptophan, histidine, or tyrosine residues) are not present in the T11 peptide. A fraction representing the major peak (peak b-2, Fig. 2B), which co-eluted with native hGH (peak c-1, Fig. 2C), was also analyzed by tryptic mapping and electrospray ionization MS. The T2 peptide (residues 9 to 16) was absent from the tryptic map and a new peptide eluting at 56 min appeared (Fig. 3C). The new peptide had a mass of 995.1 u as compared with 979.1 u for the native T2 peptide. This increase in mass of 16 u is consistent with the oxidation of Met14 to methionine sulphoxide. The tryptic map of native hGH (peak c-1, Fig. 2C) is shown in Fig. 3D. The T2 and T11 peptides elute at 66 min and 81 min, respectively, as compared to 56 min and 63 min for their oxidized counterparts. In summary, the tryptic mapping and mass spectrometry results demonstrate that the major side fraction isolated from neutral pH reversed-phase HPLC consists of two mono-oxidized hGH variants; a minor variant (12%) oxidized at Met125 and a major variant oxidized at Met14.

To better understand the effect of pH and organic modifier on the separation of the oxidized variants; acidic, weakly acidic, and neutral pH mobile phases were evaluated with either acetonitrile or 1-propanol as the organic modifier (Fig. 4). The sample analyzed was a mixture of the di-oxidized, mono-

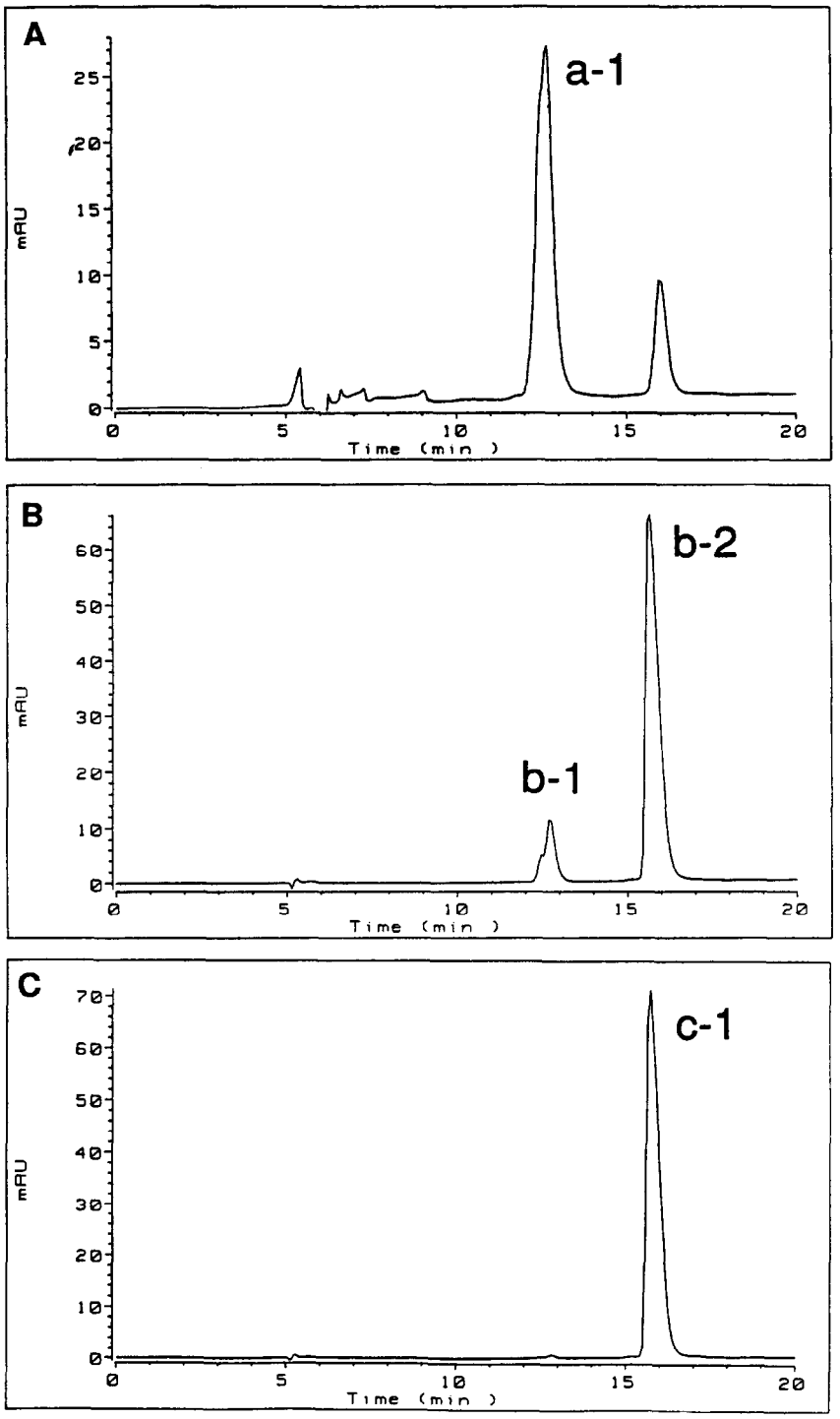

Fig. 2. Rechromatography at acidic pH of fractions isolated from reversed-phase HPLC at neutral pH. Chromatography was performed on a Vydac C_{18} column equilibrated with 57% acetonitrile containing 0.1% TFA and eluted with a linear acetonitrile gradient as described in Experimental section. (A) Reversed-phase HPLC of peak a in Fig. 1; (B) reversed-phase HPLC of peak b in Fig. 1; (C) reversed-phase HPLC of peak c in Fig. 1.

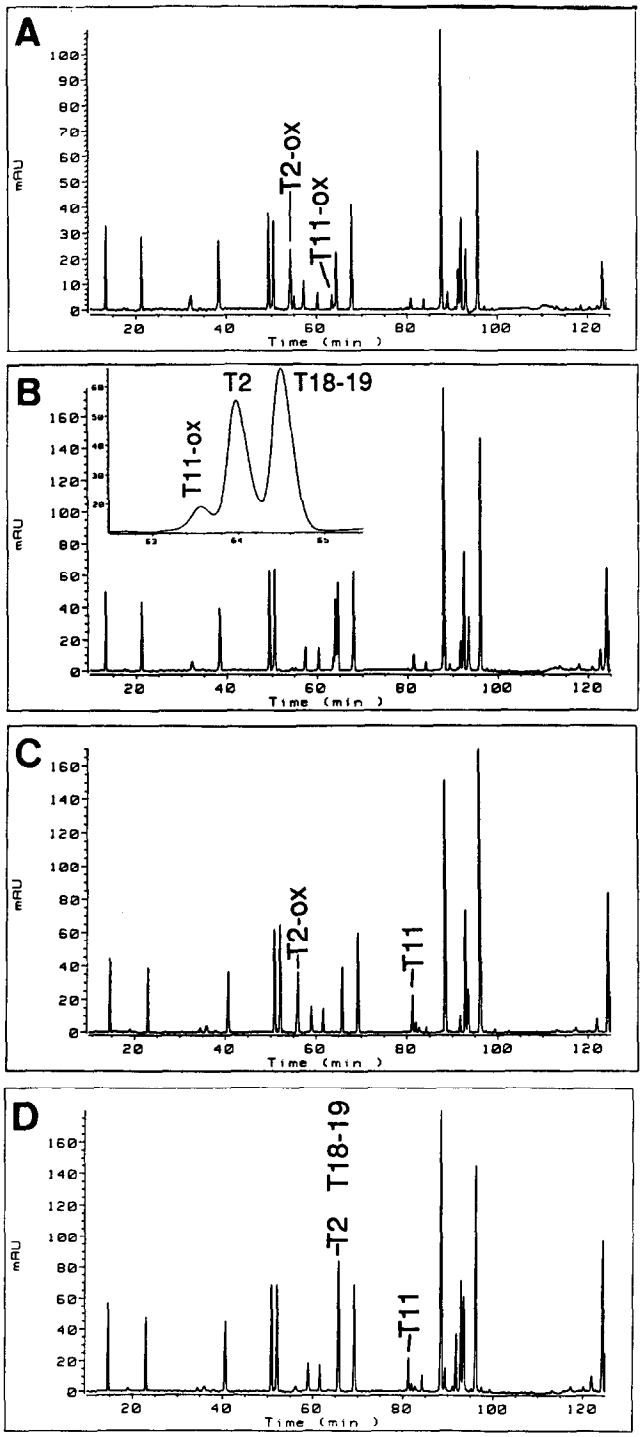

Fig. 3. Reversed-phase HPLC tryptic maps of oxidized and native hGH fractions. Insert: blow-up of the chromatogram between $t = 62.5$ min and $t = 65.5$ min. Chromatography was performed on a Nucleosil C_{18} column equilibrated with 0.1% TFA and eluted with a linear acetonitrile gradient as described in Experimental section. (A) Tryptic map of di-oxidized hGH (peak a-1, Fig. 2A); (B) tryptic map of mono-oxidized (methionine sulphoxide-125) hGH (peak b-1, Fig. 2B); (C) tryptic map of mono-oxidized (methionine sulphoxide-14) hGH (peak b-2, Fig. 2B); (D) tryptic map of native hGH (peak c-1, Fig. 2C).

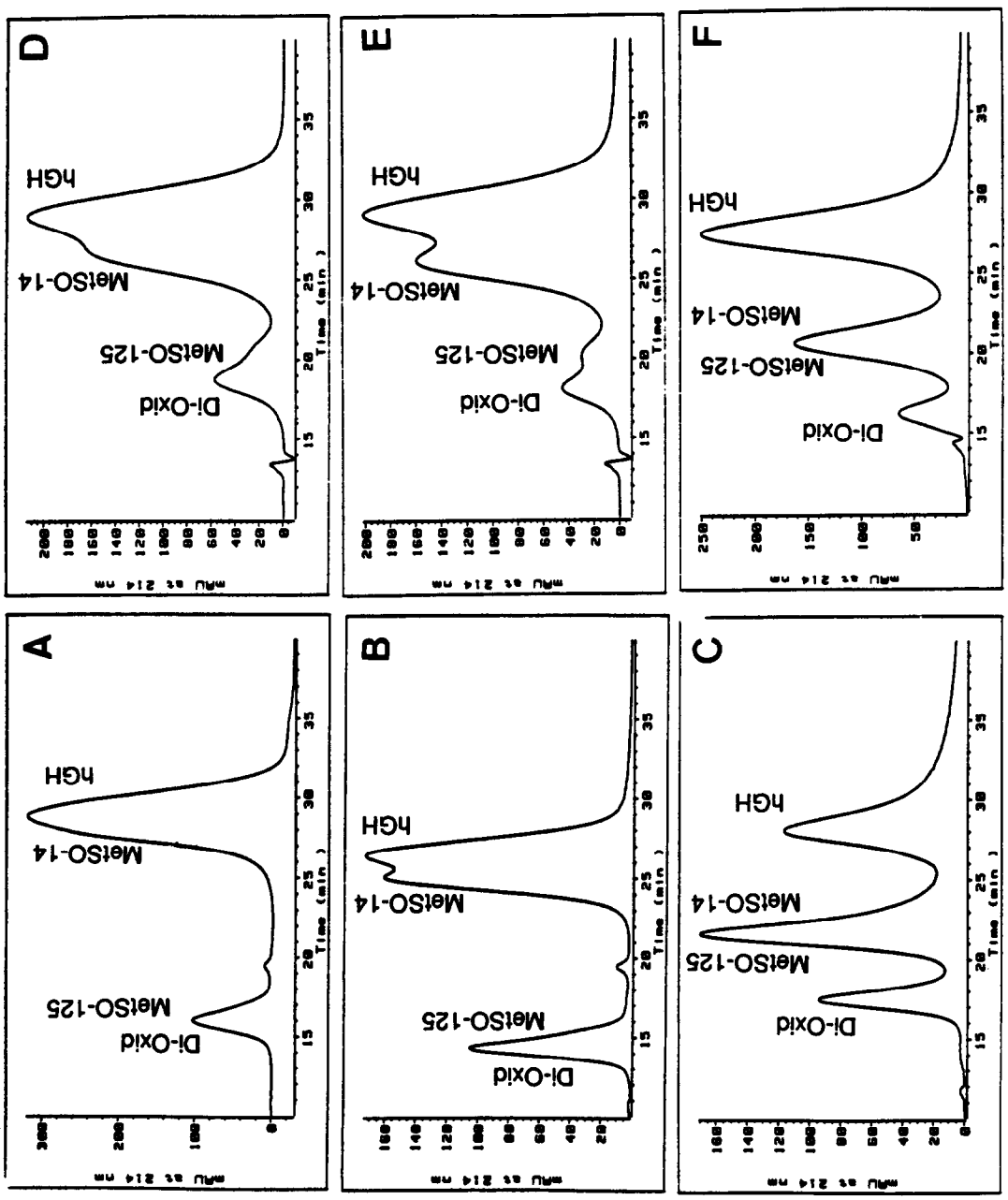

Fig. 4. The effect of pH and organic modifier on the separation of hGH and its oxidized variants by reversed-phase HPLC. The sample analyzed was a mixture of the di-oxidized, mono-oxidized and main peak fractions isolated from the preparative reversed-phase HPLC separation at neutral pH. The sample (100 μg) was eluted isocratically from a Polymer Labs PLRP-S 8 μm, 300 Å (250 × 4.6 mm I.D.). The column temperature was 40°C and the flow-rate was 0.5 ml/min. The concentration of organic modifier in the mobile phase was adjusted to maintain a constant k' (4.2 ± 0.3). The following mobile phase systems were evaluated: (A) 0.1 *M* sodium phosphate pH 2.0–acetonitrile; (B) 0.1 *M* sodium phosphate pH 3.5–acetonitrile; (C) 0.1 *M* sodium phosphate pH 7.0–acetonitrile; (D) 0.1 *M* sodium phosphate pH 2.0–1-propanol; (E) 0.1 *M* sodium phosphate pH 3.5–1-propanol; (F) 0.1 *M* sodium phosphate pH 7.0–1-propanol.

TABLE II

SELECTIVITY FACTORS (α) FOR hGH VARIANTS

Selectivity factor $(\alpha) = (t_{R2} - t_0)/(t_{R1} - t_0) = k'_2/k'_1$, where t_{R2} is the retention time of the more retained component and t_{R1} is the retention time of the lesser retained component.

	Acetonitrile			1-Propanol		
	pH 2.0	pH 3.5	pH 7.0	pH 2.0	pH 3.5	pH 7.0
B/A[a]	1	1	1.34	1	1.14	1.42
C/B[a]	2.20	2.18	1	1.6	1.39	1
D/C[a]	1	1.06	1.40	1	1.13	1.44
k' (native)[b]	4.44	3.99	4.31	4.46	4.38	4.20
% Organic	47.5	47.5	40.8	36	36	32.1

[a] A = Di-oxidized hGH; B = hGH methionine sulphoxide at 125; C = hGH methionine sulphoxide at 14; D = native hGH.
[b] Capacity factor $(k') = t_R - t_0$, where t_R is the retention time and t_0 is the void time.

oxidized, and main peak fractions collected from the preparative neutral pH separation. Elution was done in the isocratic mode and retention times held constant from run to run. The selectivity factors for the separation of (MetSO-125-containing and di-oxidized hGH), (MetSO-14 hGH and MetSO-125 hGH), and (native hGH and MetSO-14 hGH) are shown in Table II.

The optimal pH for the separation of MetSO-125-containing and di-oxidized hGH was 7.0 ($\alpha = 1.34$ with acetonitrile, $\alpha = 1.42$ with 1-propanol). Partial resolution was attained at pH 3.5 with 1-propanol as the organic modifier ($\alpha = 1.14$). MetSO-125 and di-oxidized hGH co-eluted at pH 2.0 and at pH 3.5 with acetonitrile as the organic modifier. A substantially lower organic concentration eluted hGH at pH 7 (40.8% acetonitrile, 32% 1-propanol) than at pH 2 and 3.5 (47.5% acetonitrile, 36% 1-propanol) indicating that hGH is less denatured at neutral pH than at low pH.

MetSO-14 and MetSO-125 hGH were not separated at pH 7.0 ($\alpha = 1$) but were well resolved at pH 2 and 3.5 using either acetonitrile or 1-propanol. Resolution was considerably better using acetonitrile ($\alpha = 2.18$ at pH 3.5) as compared to 1-propanol ($\alpha = 1.39$ at pH 3.5).

MetSO-14 and native hGH were best resolved at pH 7.0 using either acetonitrile or 1-propanol as the organic modifier ($\alpha = 1.40$ with acetonitrile, $\alpha = 1.44$ with 1-propanol). Partial resolution was achieved at pH 3.5, with 1-propanol giving a slightly better separation than acetonitrile ($\alpha = 1.13$ with 1-propanol, $\alpha = 1.06$ with acetonitrile). MetSO-14 and native hGH co-eluted at pH 2.0.

Sodium phosphate, pH 3.5, with 1-propanol was the only mobile phase system capable of resolving the mono- and di-oxidized variants and native hGH. In order to further improve the separation, the column residence time was increased (Fig. 5). The oxidized hGH variants are sufficiently well resolved to allow quantitation of the various oxidized species. Using this method, it was estimated that there was 2% di-oxidized hGH, 5% mono-oxidized at Met125, and 35% mono-oxidized at Met14 in the hydrogen peroxide-treated hGH sample.

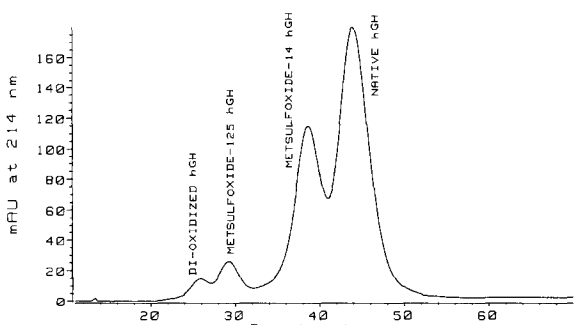

Fig. 5. Optimized reversed-phase HPLC analysis of hydrogen peroxide-treated hGH. Hydrogen peroxide-treated hGH (100 μg = 10 μl of 10 mg/ml solution) was eluted isocratically from a Polymer Labs PLRP-S 8 μm, 300 Å (250 × 4.6 mm I.D.) column. Column temperature was 40°C. The mobile phase consisted of 32.5% 1-propanol in 100 m*M* sodium phosphate, pH 3.5, with 1% sodium chloride. The flow-rate was 0.5 ml/min.

DISCUSSION

Mobile phase parameters such as pH, type of organic modifier, and column temperature have been shown to influence the reversed-phase HPLC separation of proteins [12,13]. Although reversed-phase chromatography is denaturing due to interaction of the protein with the hydrophobic matrix, the extent of denaturation can be modified by the mobile phase selection [14]. Use of less denaturing conditions (neutral pH) has been employed in the separation of native hGH and hGH with an additional methionine at the N-terminus [12,13]. More denaturing conditions (low pH and acetonitrile) failed to resolve the pair. Partial resolution was attained at low pH with 1-propanol as the organic modifier and at intermediate pH (3.5–4.5).

In our study, less denaturing conditions (neutral pH) favored the resolution of the Met14 oxidized hGH variant and native hGH while more denaturing conditions (low pH) favored the separation of the Met125 oxidized hGH variant and native hGH. These results suggest that Met125 is less solvent accessible than Met14; that is more denaturing conditions are required to expose it for interaction with the matrix.

Since Met14 is most likely solvent accessible under all of the mobile phase conditions evaluated, more strongly denaturing conditions which expose additional residues for interaction with the matrix results in a poorer separation. A possible explanation is that exposure of these additional residues dilutes out the effect of the modification at Met14. A similar explanation was described by Canova-Davis *et al.* [12] and Frenz *et al.* [13] for the separation of native hGH and hGH with an additional N-terminal methionine. It is interesting to note that the selectivity factors for the separation of the Met14 oxidized hGH variant and native hGH and the N-terminal hGH variant and native hGH are similar. For example, both are well resolved at neutral pH, partially resolved at intermediate pH and at low pH with 1-propanol as the organic modifier and not resolved at low pH with acetonitrile as the organic modifier. This suggests that they have similar solvent accessibilities in the column matrix-mobile phase environment. In contrast, Met14 and Met125 appear to have different solvent accessibilities in the column matrix-mobile phase environment.

Changing the conformation (by varying the mobile phase composition as discussed above) and therefore the contact region such that a lesser or greater number of residues are involved in binding to the matrix is one mechanism through which selectivity could be altered. Other possible mechanisms involve a change in local conformation due to oxidation at Met14 and/or Met125, local pH changes affecting the ionization of nearby residues (*i.e.*, Asp11 and His18), and a disruption of secondary interactions, in particular, the salt bridge between His18 (one turn remove from Met14 in α-helix 1) and Glu174. It cannot be determined with any degree of certainty what mechanism is involved in the separation of oxidized and native hGH, however, the fact that a substantially lower organic concentration eluted hGH at neutral pH as compared to low pH suggests that conformational changes induced by the mobile phase play a major role.

Use of a weakly acidic pH (3.5) mobile phase provided a compromise between the contrasting selectivities obtained at low *versus* neutral pH and resulted in the separation of the Met125 and Met14 mono-oxidized hGH variants, the di-oxidized hGH variant and native hGH. Development of a single method for separating the mono- and di-oxidized variants and native hGH at weakly acidic pH (3.5) with 1-propanol was useful for directly determining the relative reactivity of Met14 and Met125 in hGH rather than indirectly by measurement of the peak heights of tryptic peptides as was the case in the study by Teh *et al.* [6]. Our results are in agreement with Becker *et al.* [7] that Met14 is the primary site of oxidation in hGH. This was not unexpected since our reaction conditions (pH, temperature, concentration of hydrogen peroxide) were the same as used by Becker *et al.* [7]. Perhaps the different reaction conditions (*i.e.*, higher concentration of hydrogen peroxide) used by Teh *et al.* [6] explains the greater reactivity of Met125 reported in their study. Our tryptic map of the oxidized hGH fraction separated by reversed-phase HPLC at neutral pH appeared similar to the one shown by Becker *et al.* [7]. A modified T2 peptide containing methionine sulphoxide at residue 14 was identified; however, the methionine sulphoxide-125 containing T11 peptide was not detected nor was a significant decrease in the native T11 peptide observed. Rechromatography of the oxidized fraction at low pH resulted in the

separation of the Met14 and Met125 oxidized hGH variants. The Met125 variant represented 12% of the mono-oxidized hGH fraction by peak area calculations. The fact that its corresponding oxidized peptide was not detected in the tryptic map emphasizes the importance of a direct chromatographic assay for monitoring oxidation in hGH.

CONCLUSIONS

We have shown that the selectivity factors for the separation of native hGH and its oxidized variants can be varied by manipulating the mobile phase conditions. Both pH and type of organic modifier were shown to be important parameters in the separation of the oxidized hGH variants and native hGH.

Met14 and Met125 appeared to have very different solvent accessibilities under reversed-phase chromatographic conditions since more denaturing mobile phase conditions favored the separation of MetSO-125 and less denaturing conditions favored the separation of MetSO-14.

This suggests a general strategy for separating protein variants assuming that the residues involved have dissimilar solvents accessibilities; that is one could vary the strength of protein interaction by adjusting the pH and type of organic modifier.

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