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APPLICATION OF HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY TO THE CHARACTERIZATION OF RECOMBINANT DNA-DERIVED HUMAN GROWTH HORMONE[a]

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SUMMARY

This publication analyzes different preparations of recombinant human growth hormone (rhGH) by hydrophobic-interaction chromatography (HIC). The effect of temperature on the separation was investigated as well as a series of commercially available HIC columns (TSK-phenyl-5PW, TSK-ether-PW, Beckman CAA-HIC and polypropyl A). The TSK-ether column gave the best results in the analysis of rhGH samples at different temperatures, as well as allowing an efficient separation of methionyl-hGH from rhGH. The TSK-phenyl column can be effectively used in the examination of different human growth hormone variants. The details of sample preparation have been demonstrated to be important in HIC analysis of hGH on the TSK-ether-5PW column. Injection volume and the solvent used to dissolve the protein sample are both crucial factors in this analysis. Also protein aggregation may play a role in these observations. The effect of temperature, protein concentration and spectroscopic data on the eluted protein suggest, however, that aggregation is not the cause of frontal peaks

INTRODUCTION

Hydrophobic-interaction chromatography (HIC) was recently introduced for rapid separation of proteins under mild adsorption and elution conditions and yielded protein fractions in a biologically active state[1–6]. This contrasts with reversed-phase high-performance liquid chromatography (RP-HPLC) where many proteins elute in a denatured state[7–10]. The mobile phase used in HIC is composed of a high

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concentration of a salting-out agent (solvent A) decreasing to a low concentration of the agent (solvent B). The pH is usually close to neutrality and subambient temperature can be used for maintaining the folded protein structure[11,12]. Retention is a function of three major variables: the stationary phase, the composition of the mobile phase and the unique characteristics of the solute. Although retention in both RP-HPLC and HIC columns is based on hydrophobic interactions, HIC columns are much less hydrophobic. In some cases, HIC columns are so weakly hydrophobic that they behave like size-exclusion chromatography columns at low ionic strengths[5]. It is only at high ionic strengths that they begin to retain proteins. The retention of proteins on HIC columns has been shown to be proportional to alkyl chain length on a series of HIC columns having the same ligand density[3]. This is the opposite of RP-HPLC columns where alkyl chain length has little influence on protein retention. The chromatography of a number of recombinant human growth hormone (rhGH) variants on different types of commercialized HIC columns are examined in this paper.

Spectroscopy is a well developed technique for characterizing protein conformational changes[13–15]. An on-line UV photodiode array detector has been employed to monitor protein conformational changes that may occur during HPLC separations[11,12]. hGH has 191 amino acid residues including eight tyrosine residues and one tryptophan residue. The optimal ratio of tyrosine to tryptophan is between one and five for accurate determination of the second-derivative γ ratio[11,16]. Although growth hormone has a high ratio of tyrosine to tryptophan (8:1), the use of large sample loads can sufficiently improve the signal-to-noise ratio of the second-derivative UV spectrum between 245–320 nm to allow monitoring of conformational changes. This publication will use these spectra to evaluate the success of a given separation and provide some practical guidelines for the use of the diode array detector for examining protein conformations.

EXPERIMENTAL

Equipment

A Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1090 M liquid chromatograph with a 1040A photodiode array detector, an HP 9000 work station, HP Color Pro graphics plotter, HP 9153 disc drives, HP 4.05 version software and HP think jet printer was used in this work. A Model VWR 1140 water bath (VWR Scientific, San Francisco, CA, U.S.A.) was also included, to control the temperature to ± 0.1°C.

The chromatographic columns were purchased from several different manufacturers, *e.g.*, TSK-phenyl-5PW (75 × 7.5 mm I.D.) and TSK-ether-PW (75 x 7.5 mm I. D.) were obtained from Tosoh (Tokyo, Japan), polypropyl A (200 × 4.6 mm I.D.) was obtained from Poly LC (Columbia, MD, U.S.A.). Beckman CAA-HIC (100 × 4.6 mm I.D.) was obtained from Beckman (Berkeley, CA, U.S.A.).

Chemicals

Milli Q water was obtained from a Milli Q water-filtering system. Grade III ammonium sulfate and ultra-pure potassium phosphate were obtained from Sigma (St. Louis, MO, U.S.A.). Protropin® (met-hGH) which has one N-terminal methionine residue more than the authentic hGH and rhGH with the same sequence as pituitary growth hormone were obtained from Genentech (South San Francisco, CA,

U.S.A.), Humatrope® (having the same sequence as rhGH) was obtained from Eli Lilly (Indianapolis, IN, U.S.A.) and Crescormon® (pituitary hGH), Genetropin® (rhGH) and Somatonorm® (met-hGH) were obtained from Kabi Vitrum (Stockholm, Sweden).

Chromatographic procedures

Mobile phases were prepared by adding the correct weights of salt and buffer to a volumetric flask containing previously vacuum-degassed Milli Q water. The pH was adjusted to the appropriate value with either ammonium hydroxide or phosphoric acid and a small amount of degassed Milli Q water was added to the mark. Solutions containing high salt concentrations were not allowed to remain in the column or pump for long periods of time. Standard mobile phase conditions consisted of 2 *M* ammonium sulfate, 0.1 *M* potassium phosphate (pH 7) as mobile phase A and 0.1 *M* potassium phosphate (pH 7) as mobile phase B. A standard 20-min linear gradient from 0 to 100% B was used in most experiments with a flow-rate of 1.0 ml/min. Exceptions are noted in the paper. Protein solutions (5–10 mg/ml) were freshly prepared. When not in use, the samples were stores at −60°C. An alternative mobile phase was used to differentiate some variants of rhGH. Mobile phase A consists of 0.03 *M* Tris–HCl (pH 8), 0.5 *M* sodium sulfate and 0.5% acetonitrile. Mobile phase B consists of 0.03 *M* Tris–HCl (pH 8), 5% acetonitrile and 0.075% Brij 35.

Spectroscopic procedures

The normal UV spectrum and its second derivative were recorded on the HP 1040A photodiode array detector. The spectra were processed by the recently developed spline software function for the HP 9000 work station which constructs a cubic (splined) curve through the existing data points by generating new data points at each 0.2-nm intervals between the original values. The splined curve still goes through all of the original data points as was confirmed by comparison with the normal plots. However the spline function did facilitate visual comparison of different spectra.

RESULTS AND DISCUSSION

Spectroscopic and chromatographic characterization of rhGH

Wu *et al.*[11] have previously studied the use of subambient temperature in HIC for the stabilization of labile proteins, such as α-lactalbumin and β-lactoglobulin A. We are therefore using 5°C as the column temperature for the comparison of different samples of hGH in an attempt to relate the analytical results as closely as possible to the protein's native state. Fig. 1 shows the elution profile of rhGH from three different manufacturers, Genentech (rhGH), Lilly Humatrope (rhGH) and Kabi Crescormon (pituitary hGH) on a TSK-phenyl-5PW column at 5°C. The three hGH samples eluted with very similar retention times (Fig. 1A). However, the on-line UV spectra (Fig. 1B) do show a slight but significant difference at the wavelength range of 282 nm and 288 nm between the pituitary hGH and the recombinant hGHs, (Genentech rhGH and Lilly Humatrope). The absorption chromophores of tryptophan and tyrosine amino acid residues (282 to 288 nm) have a relatively high extinction coefficient and therefore observed differences in this range are more likely to be significant. The reason for the spectral difference may well be due to impurities present in pituitary

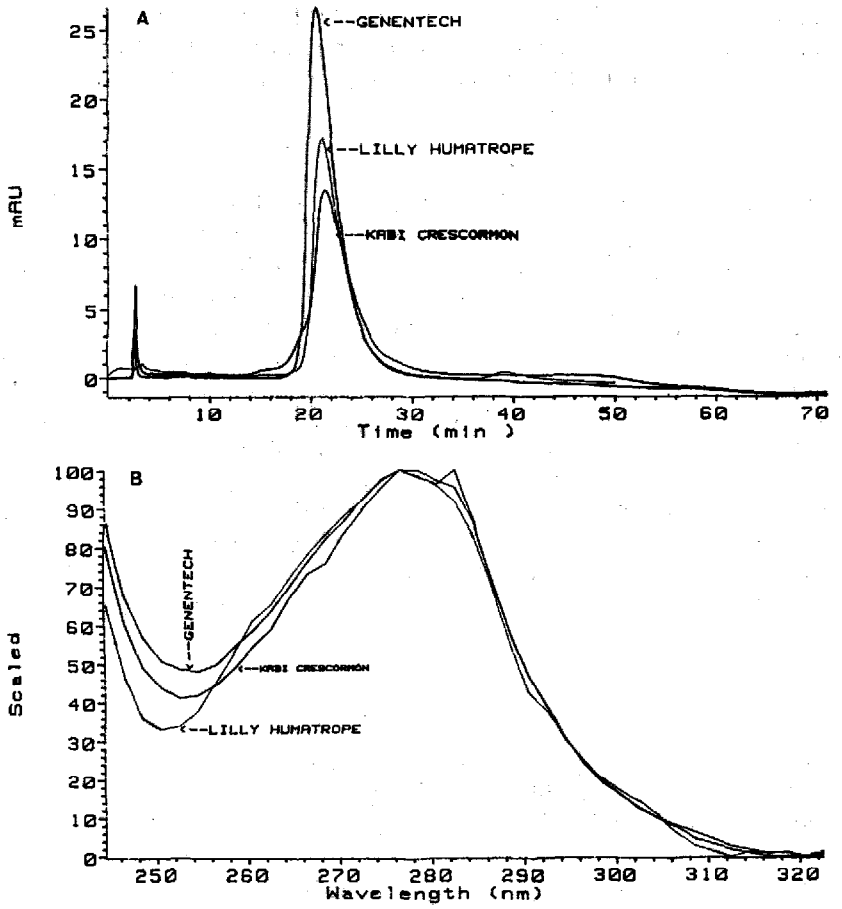

Fig. 1. (A) Comparison of the chromatography of Lilly Humatrope (rhGH), the Genentech rhGH and the Kabi Vitrum Crescormon pituitary hGH on the TSK-phenyl-5PW column at 5°C. Conditions: mobile phase A, 0.7 *M* ammonium sulfate, 0.1 *M* potassium phosphate (pH 7); mobile phase B, 0.1 *M* potassium phosphate (pH 7); gradient time, 60 min (linear); flow-rate, 1 ml/min. (B) Comparison of UV spectra of each growth hormone peak measured at the peak maximum (in A) on TSK-phenyl-5PW column at 5°C.

hGH and not to the rhGH samples. The other spectral difference is found in the wavelength range of 250–255 nm. The differences are seen in all three hGH samples. However, the extinction coefficients are relatively low in this range and the observed differences may be due to variability in the photodiode array detector.

Fig. 2 displays an increase in retention of rhGH with increasing temperature (5 to 50°C) when chromatographed on the TSK-phenyl-5PW column. It can be seen that the peak shape is similar at all temperatures with a slight increase in amounts of late eluting material at 50°C which is probably due to aggregated rhGH. An increase in retention time with increasing temperatures was generally found in HIC[5,11] and has been attributed to entropy controlled adsorption[11,12,18].

Fig. 3 displays elution profiles at three temperatures (5, 25 and 50°C) for the separation of a pair of rhGHs (met-hGH and rhGH) on the TSK-ether-5 PW column.

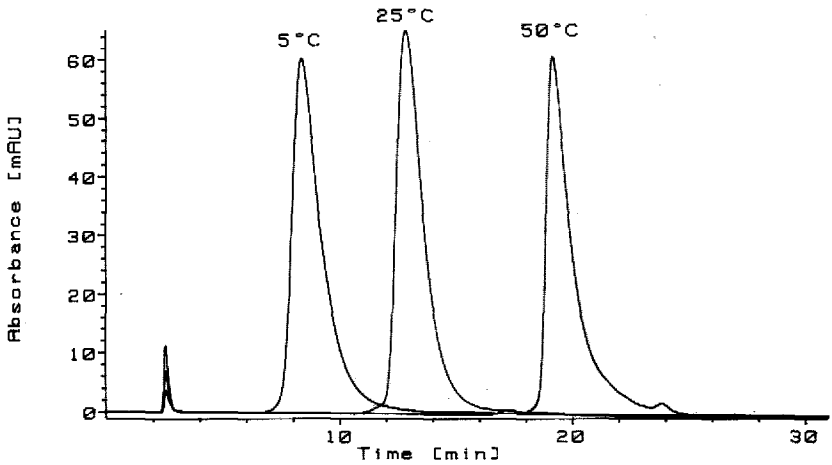

Fig. 2. Effect of temperature on chromatographic behavior of rhGH (Genentech) on the TSK-phenyl-5PW column. Conditions: mobile phase A, 0.7 *M* ammonium sulfate, 0.1 *M* potassium phosphate (pH 7); mobile phase B, 0.1 *M* potassium phosphate (pH 7); gradient time, 20 min (linear); flow-rate, 1 ml/min.

The only difference between met-hGH and rhGH is the extra methionine residue at the N-terminus of met-hGH. That the HIC method can discriminate between met-hGH and hGH with only one amino acid difference out of 191 amino acid residues is one of the best examples to date of the high resolving power of HIC. The peak shapes of met-hGH and rhGH are significantly sharper on the TSK-ether-PW than on the phenyl column (narrower band width over the range from 5 to 50°C). The difference may be related to the lower hydrophobicity of the ether column. Fig. 3B and C display the separation of met-hGH and rhGH on two other different HIC columns. In general, at 50°C, hGH shows a second peak which may be due to some structural changes such as protein unfolding or aggregation. Other analytical techniques, such as gel-permeation chromatography, however, have shown that this sample contains very low levels of aggregated material. Thus, the HIC conditions at high temperatures might have induced some structural changes in the growth hormone sample.

With the advent of the diode array detector it is possible for the spectral properties of the proteins to be analyzed immediately after the chromatographic separation. In case of met- and rhGH this information was used to examine the potential of the diode array detector to measure the effect of differences in chromatographic conditions *e.g.*, changes in temperature and hydrophobicity of the column on the protein structure. Also the correlation between differences in spectral properties of the chromatographed proteins and the degree of separation was examined.

Tables I and II summarized the on-line spectroscopic characteristics of met-hGH and rhGH as they elute from two different columns. The wavelength ratio and second-derivative γ ratio of met-hGH and rhGH were very similar on the TSK-ether-PW column at 5 and 50°C, but differ significantly on the polypropyl column. One possible explanation is that since the TSK-ether-PW column is less hydrophobic than the polypropyl A column the spectral difference can be related to differences in the degree of protein denaturation. On the TSK-ether-PW column both met-hGH and rhGH may exist in a native-like folded state with similar spectroscopic characteristics.

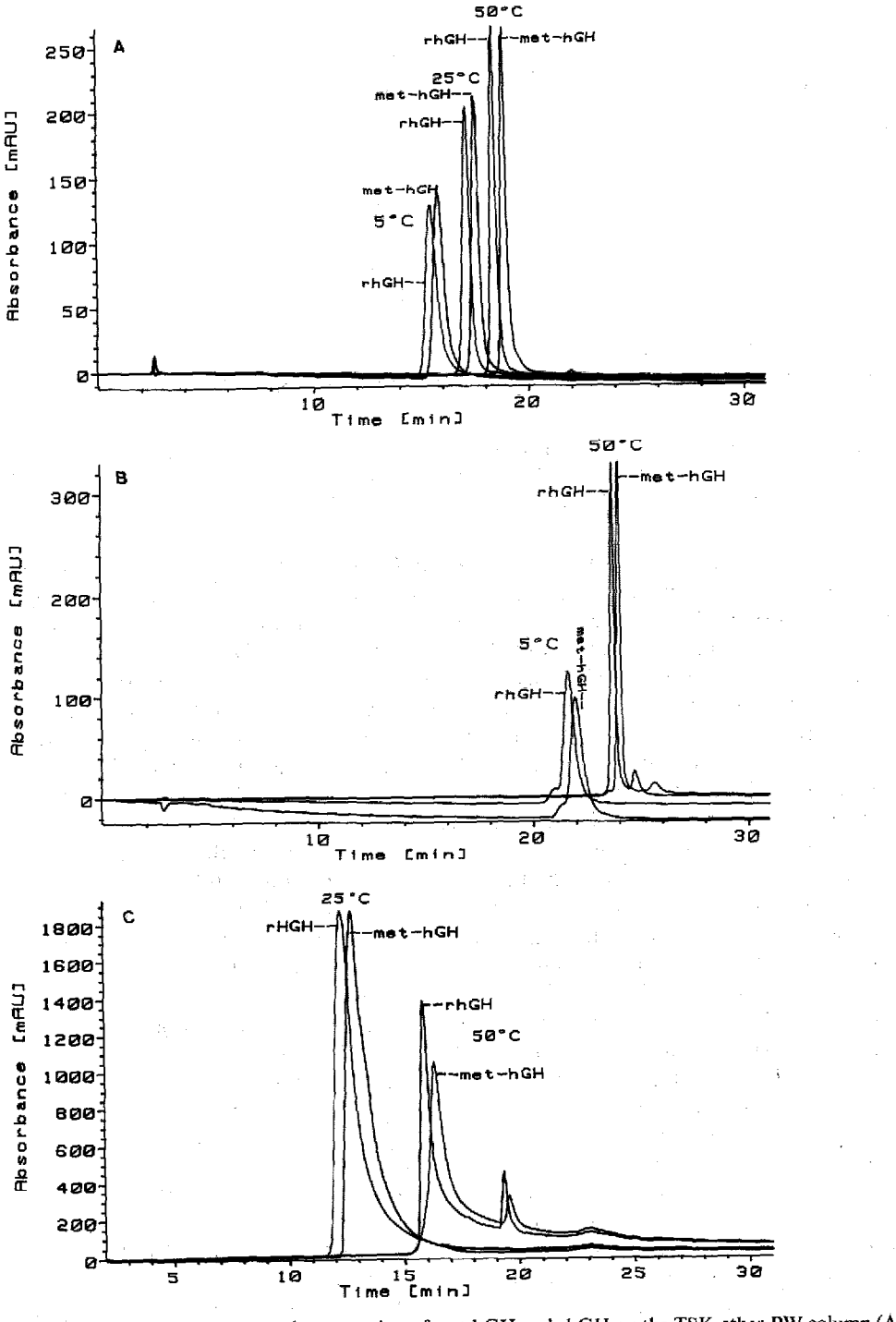

Fig. 3. Effect of temperature on the separation of met-hGH and rhGH on the TSK-ether-PW column (A), polypropyl A (Poly LC) column (B) and a Beckman CAA-HIC column (C). All conditions as in Fig. 2, except the ammonium sulfate concentration was 2.0 *M* in mobile phase A.

TABLE I

SPECTROSCOPIC CHARACTERISTICS OF met-hGH and rhGH WITH TSK-ETHER-PW COLUMN AT 5°C AND 50°C

Conditions: TSK-ether-PW column; solvent A, 2 *M* ammonium sulfate and 0.1 *M* potassium phosphate (pH 7); solvent B, 0.1 *M* potassium phosphate (pH 7). Wavelength absorbance ratios and second-derivative γ ratios are reported together with standard deviations and the number of assay in parentheses.

Temperature (°C)	*Protein*	*275.5/293*[a]	*293/255.5*[a]	γ[b]
5	met-hGH	3.31 ± 0.01(5)	0.610 ± 0.001(3)	6.9 ± 0.6(3)
5	rhGH	3.34 + 0.01(5)	0.610 + 0.002(3)	7.7 + 0.5(3)
50	met-hGH	3.32 + 0.02(5)	0.611 + 0.002(3)	7.3 + 0.2(3)
50	rhGH	3.33 + 0.02(5)	0.611 + 0.003(3)	7.27 + 0.05(3)

[a] Absorbance ratio at the wavelengths (nm) indicated.
[b] Second-derivative characteristics, defined in Fig. 2 in ref. 11.

However, on the more hydrophobic polypropyl A column the two proteins may have unfolded, with the resulting differences shown in Table II. These results also suggest that the separation of met-hGH and rhGH is not only influenced by the additional N-terminal methionine residue in met-hGH but also by differences in methionine stability between the two closely related proteins. Apparently, the slight differences in protein stability are only observed under the more denaturing conditions provided by the polypropyl column.

This study also allows the opportunity to investigate which spectroscopic parameter is most useful in monitoring changes in protein structure during the separation. The more hydrophobic polypropyl A column showed a significant difference in the γ ratio between 5 and 50°C for both met-hGH and rhGH (3.6 *vs.* 6.7 and 5.3 *vs.* 7.0, respectively). In addition the polypropyl A column showed increased wavelength ratios at 275.5/293 for met-hGH and rhGH from 5 to 50°C (3.17 *vs.* 3.28 and 3.16 and 3.27 respectively). However the ratio of 293/255.5 was insensitive to both temperature and to the nature of the sample. Previously Wu *et al.*[11] noted that the 275.5/293 ratio indicated changes in tyrosine exposure and 293/255.5 of tryptophan exposure. Also the γ ratio (peak-to-peak distance between the maximum at 288.5 nm and the mini-

TABLE II

SPECTROSCOPIC CHARACTERISTICS OF met-hGH and rhGH IN POLYPROPYL COLUMN ON 5°C AND 50°C.

All conditions are the same as in Table I.

Temperature (°C)	*Protein*	*275.5/293*	*293/255.5*	γ
5	met-hGH	3.17 + 0.03(6)	0.620 + 0.001(6)	3.6 + 0.5(3)
5	rhGH	3.28 + 0.01(6)	0.620 + 0.001(6)	5.3 + 0.9(3)
50	met-hGH	3.16 + 0.02(3)	0.620 + 0.001(3)	6.7 + 0.8(3)
50	rhGH	3.27 + 0.02(3)	0.620 + 0.001(3)	7.0 + 1.0(3)

mum at 282.5 [Tyr] and between the maximum at 296.5 nm and the minimum at 292.5 [Trp]) was seen as a sensitive indicator of tyrosine exposure. Again the γ ratio showed an increase at 50 compared to 5°C. Since growth hormone has a preponderance of tyrosine over tryptophan (ratio 8:1) it is not surprising that the wavelength ratio 293/255.5 was insensitive, whereas the γ ratio and 275.5/293 wavelength ratio were able to detect conformational changes. Also it was suggested[12] that increases in the values of these ratios for a given separation could be related to an increase in the less folded form. This is consistent with that of the increase observed in this separation in both γ value and 275.5/293 ratio with increasing temperature.

The separation of met-hGH and rhGH was correlated with the differences in the wavelength and γ ratios. The results presented in Fig. 3A and B as well as Tables I and II suggest that the γ ratio is a better predictor of a separation than the wavelength ratio. In fact sometimes there is no correlation between a separation and response of the wavelength ratio. It may be that the structural differences that are responsible for the separation, for example the presence of a N-terminal methionine residue may not effect the degree of exposure of the aromatic residues and thus the separation would be expected to be unrelated to the diode array detection results. However, the fact that the γ ratio can indeed be correlated with the observed separations suggests that the N-terminal difference does result in changes in stability of the three-dimensional structure of the protein, with consequent differences in unfolding rates under different conditions. However, in some cases the changes are relatively small and can only be detected by the more sensitive γ ratio.

An additional analytical HIC method was developed for the separation and quantitation of variants of rhGH using the TSK-phenyl-5PW column. Better separations and recoveries were obtained by (1) using sodium sulfate instead of ammonium sulfate, (2) increase in pH to 8.0 and (3) the addition of acetonitrile and Brij 35 to the mobile phase (see Fig. 4 for a typical elution profile). As well as the main rhGH peak, two new hGH peaks were observed at a retention time of 10 and 19 min, respectively. Two additional peaks at 5 and 35 min were system artifacts and attributed to salts and Brij 35 micelles, respectively. The rhGH variant eluting at 10 min was analyzed by RP-HPLC tryptic mapping and the only change observed in the map was a shift to an earlier retention time for the N-terminal tryptic peptide (T1). The modified peptide was shown to be the desPhe variant lacking the N-terminal phenylalanine residue by N-terminal sequencing and by fast atom bombardment mass spectrometry (mass of 783.4 for the molecular ion of the modified instead of 930.5 for the correct T1 peptide). The rhGH variant eluting at 19 min was shown to be a two-chain form in which the peptide bond was cleaved between residues 142 and 143 (Thr-142 and Tyr-143). This variant was characterized by a combination of N-terminal sequencing, electrophoretic analysis under reducing conditions and tryptic mapping[21]. In addition to excellent separations of rhGH variants the new chromatographic conditions could be used for the resolution of met-hGH and rhGH (see Fig. 5).

Sample preparation and injection volume

Another important factor to be considered in HIC is sample preparation —that is, the sample concentration and injection volume. A HIC method generally starts from a high concentration of a salting-out agent with a decreasing gradient of salt concentration. Thus water (low ionic strength) in HIC can be considered as a strong

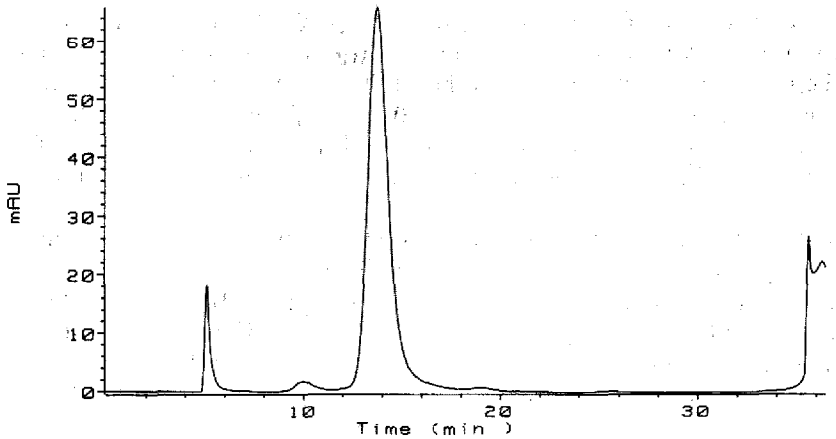

Fig. 4. The elution profile of a sample of rhGH that contains proteolytically clipped variants on a TSK-phenyl-5PW column at 25°C. Mobile phase A, 0.5 *M* sodium sulfate, 0.03 *M* Tris-HCl, 0.5% acetonitrile (pH 8); mobile phase B, 0.03 *M* Tris-HCl, 5% acetonitrile, 0.07% Brij (pH 8); gradient time, 60 min (linear); flow-rate, 0.5 ml/min.

solvent for elution analogous to the role of organic solvent in RP-HPLC. The injection of large volumes of aqueous samples may cause distortion of peak shapes or lack of retention of some of the protein samples. This effect can be related to the strong solvent effect of water and may be minimized by an increase in the hydrophobicity of the column. Fig. 6 shows an example of the effect of sample concentration and the injection volume on the resultant chromatography. In this figure, rhGH is dissolved in water at two different concentrations, either 1 or 10 mg/ml. The volume of injection is 25 μl for 10 mg/ml and 250 μl for 1 mg/ml solutions respectively, which results in the same amount of the rhGH injected in each case. A frontal peak is

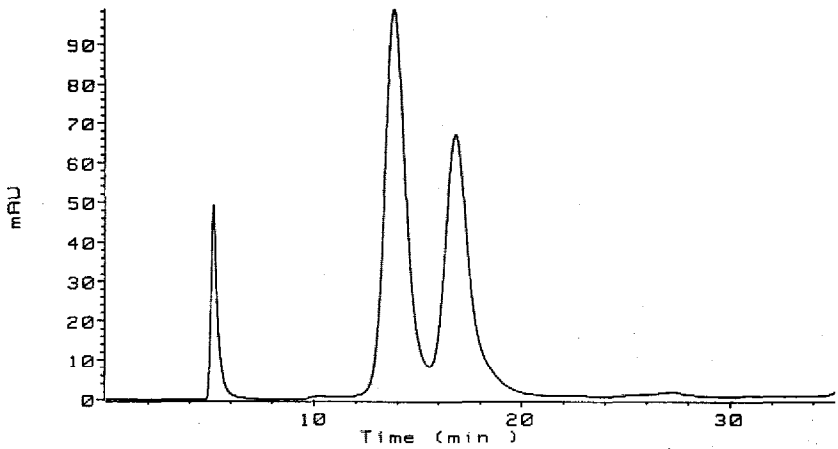

Fig. 5. The elution profile of mixed Genentropin rhGH (14 min) and Somatonorm met-hGH (17 min). All conditions as in Fig. 4.

observed for the protein sample at 1 mg/ml but not at 10 mg/ml. Also the sample concentrations were decreased to 0.5 mg/ml and 0.1 mg/ml respectively while sample volume was kept at 250 μl. Fig. 6A showed that frontal peaks were still observed at 0.5 mg/ml concentration, but disappeared at the 0.1 mg/ml concentration. All three peaks from the separation at 1 mg/ml were collected and directly reinjected. The elution time of each peak was now identical to the original main peak, as shown in Fig. 7.

Two possible reasons for this result are as follows. Either the protein exhibits association or aggregation at a particular concentration (*e.g.*, 1 mg/ml and 0.5 mg/ml), or the large sample volume may cause these frontal peaks. In order to address these possibilities, the following experiments were conducted. For the 1 mg/ml concentration the injected sample volume was decreased to 25 μl for 1 mg/ml and the frontal peaks disappeared. Also the frontal peaks disappeared if the sample was

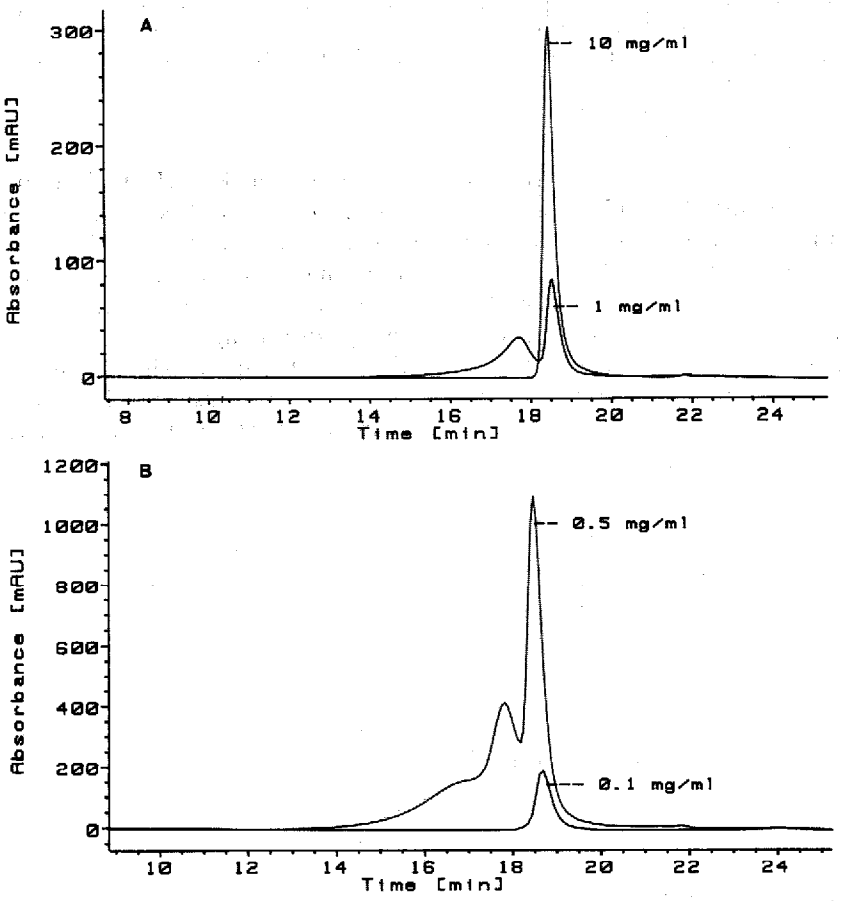

Fig. 6. Effect of the sample (rhGH) concentration and the injection volume on the TSK-ether-PW (Tosoh) column at 50°C. Mobile phase conditions were the same as in Fig. 3, except for the sample concentrations. (A) concentration 10 mg/ml, injection volume 25 μl and 1 mg/ml, injection volume 250 μl. (B) concentration 0.5 mg/ml, injection volume 250 μl and 0.1 mg/ml, injection volume 250 μl.

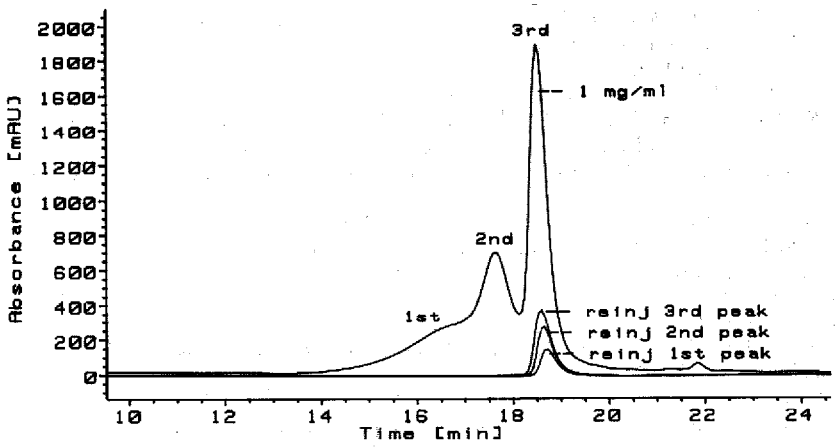

Fig. 7. Reinjection of fractions collected from a separation as shown in Fig. 6. The top profile shows the original separation of sample of rhGH, 1 mg/ml with an injection volume 250 μl. The lower traces were obtained from collection of the third fraction of top chromatogram (labeled 3rd) and the sample was reinjected under the same chromatographic conditions, then collection of the second fraction of chromatogram (labeled 2nd) and reinjected under the same chromatographic conditions and finally collection of the first fraction of chromatogram (labeled 1st) and reinjected under the same chromatographic conditions.

dissolved in 1 *M* ammonium sulfate instead of water (250 μl was still injected at a concentration of 1 mg/ml). This result correlates with the disappearance of the frontal peaks on reinjection because these samples were dissolved in the mobile phase which contains ammonium sulphate. If the formation of frontal peaks was due to aggregation the chromatographic phenomenon should be concentration and temperature dependent and may result in different spectral properties for each of the species. However, smaller volumes (25 μl instead of 250 μl) of the most concentrated solution studied (10 mg/ml) did not show the frontal peaks. Also, temperature studies from 5 to 50°C showed the same pattern of peaks as in Fig. 6 at a concentration of 0.1 mg/ml. The study was repeated on another sample of rhGH produced by an alternative rDNA process (Humatrope) and gave similar results, which demonstrated that the chromatographic effect was not specific to a particular sample of this protein.

Using the same conditions as on the ether column, the more hydrophobic columns, *e.g.*, TSK-phenyl-PW and polypropyl A do not show the phenomenon of frontal peaks. It would be expected that the more hydrophobic column binds the protein more strongly and therefore a problem in adsorption kinetics (caused by weaker binding as for example with the ether column used in this study) is less likely to occur. From these results, it was concluded that the observation of the frontal peak was an artifact caused by the loading conditions.

An additional study is currently planned to use on-line low angle laser light scattering to directly measure the molecular weight of the frontal peaks as well as the main peak[22].

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