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CHARACTERIZATION OF HUMAN GROWTH HORMONE BY CAPILLARY ELECTROPHORESIS

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SUMMARY

Production of proteins by recombinant DNA technology for use as pharmaceuticals requires the use of the most powerful tools of analytical protein chemistry in order to confirm purity and identity of the product and reliability of the process. Capillary electrophoresis is an emerging technology that shows high sensivity and selectivity and may have promise in this application. The technique combines the instrumental control and quantification features of high-performance liquid chromatography with the separating power of electrophoresis, and thereby has attracted broad interest. In this report, human growth hormone expressed in bacteria has been analyzed by both free zone electrophoresis and isoelectric focusing in a coated capillary to demonstrate the separation of the native molecule from its deamidated variant. A capillary zone electrophoretic tryptic map has also been developed and characterized. This map complements the widely employed reversed-phase high-performance liquid chromatography tryptic mapping systems that are important in protein characterization. Certain drawbacks to capillary zone electrophoresis compared to other analytical methods are noted, including relatively poor reproducibility and low sample tolerance. For applications as demonstrated here, however, the speed, separating power and sensitivity of the technique compensate for these shortcomings.

INTRODUCTION

Human growth hormone (hGH) is one of several pharmaceutical proteins that are currently available from recombinant DNA technology and expression in either mammalian or bacterial cells[1]. Analysis of such products to support process development, manufacturing, quality control and regulatory submissions requires the use of the most powerful tools of analytical protein chemistry to ferret out contaminant species[2]. Thus high-performance liquid chromatography (HPLC) and gel electrophoresis, in all their variations, are workhorse techniques in this field. Reversed-phase (RP) HPLC is currently one of the most important tools for analysis of protein mixtures. This technique has the attributes of high resolution, nearly universal detection of proteins and high sensitivity[3]. The physico-chemical property underlying sep-

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aration, "hydrophobicity", provides adequate selectivity for many applications, although slight differences such as between a protein and its deamidated variant may be difficult to resolve[4]. In many cases a hydrophobic interaction or ion-exchange chromatography system can be developed to improve the resolution of species that are poorly separated by RP-HPLC[5].

The recent advances in and commercialization of capillary electrophoretic instruments yield an instrumental technique that now offers the control and quantification benefits already realized in HPLC[6–8] together with the separating power of electrophoresis. Capillary electrophoresis may be another alternative that has many of the same attributes of RP-HPLC, comparable efficiency and speed but with a different selectivity. Indeed, recent work has explored the potential of capillary zone electrophoresis (CZE) in analysis of biosynthetic insulin and hGH[9].

Biosynthetic proteins for pharmaceutical use are highly purified, and a broad array of chromatographic, electrophoretic and immunochemical techniques are employed to confirm purity. Recombinant DNA-derived human growth hormone (rhGH), the third major drug produced by biotechnology, has already been well characterized[10] and so provides a good model for evaluation of the capabilities of capillary electrophoresis in analytical protein chemistry. In this report, several applications of the technique in the separation of hGH variants and tryptic peptides are described in order to assess the practical utility of CZE in analytical biotechnology.

EXPERIMENTAL

Instrumentation

Capillary electrophoresis was performed in a Bio-Rad (Richmond, CA, U.S.A.) HPE 100 unit, with data collection by a Nelson Analytical (Cupertino, CA, U.S.A.) Model 6000 software package. Capillaries were supplied by Bio-Rad mounted in cartridges with an integral flow cell for on-column optical detection. All capillaries were covalently coated at the internal wall with a hydrophilic polymer. Detection was by UV absorbance, and the capillary was unthermostatted.

RP-HPLC was performed on a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1050 instrument equiped with a Flatron Laboratory Systems (Oconomowoc, WI, U.S.A.) Model CH-30 column heater and a 150 × 4.6 mm Nucleosil C_{18} column obtained from Alltech (Deerfield, IL, U.S.A.).

Ion-exchange chromatography was performed on a Hewlett-Packard 1090 instrument with a 75 × 7 mm TSK DEAE-3SW column purchased from Novex (Encinitas, CA, U.S.A.).

Materials

Growth hormone derived from human pituitaries (Crescormon) was a gift from Kabi (Stockholm, Sweden). Biosynthetic human growth hormone (met-hGH) was produced as described previously[11]. This protein has an additional methionine present as the N-terminal residue. Met-hGH was aged by storage in a pH 7.8 solution for 2 weeks, or in a pH 9 solution for 2 months, at 5°C. These treatments induce differing degrees of deamidation at asparagine residues in the protein.

N-Tosyl-L-phenylalanylchloromethyl ketone (TPCK)-treated trypsin was obtained from Worthington (Freehold, NJ, U.S.A.). Water was purified with a Milli-Q

system from Millipore (Bedford, MA, U.S.A.). HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.) and dithiothreitol from Sigma (St. Louis, MO, U.S.A.). Phosphoric acid, hydrochloric acid, trifluoroacetic acid (TFA), mono- and dibasic potassium phosphate, sodium acetate and Tris base were supplied by Aldrich (Milwaukee, WI, U.S.A.). Hydrochloric and acetic acids were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Buffers for capillary electrophoresis —100 m*M* sodium phosphate, pH 2.56; 50 m*M* sodium phosphate, pH 8.0; 10 m*M* phosphoric acid; 20 m*M* sodium hydroxide and 80 m*M* sodium chloride in 20 m*M* sodium hydroxide— along with pH 3–10 and 4–6 BioLyte 2% ampholyte solutions for isoelectric focusing were from Bio-Rad. The electrophoresis buffers contained hydroxypropyl methylcellulose.

Methods

Capillary zone electrophoresis. Free zone electrophoresis was carried out with the polarity of the internal power supply of the instrument set such that the sample components would migrate toward the detector either as cations at pH 2.56 or anions at pH 8.0, *i.e.*, the cathode was at the detector end of the capillary for the lower pH runs, and at the inlet end for the higher pH runs. At the start of an analysis the capillary and the electrode reservoir at the detector end of the capillary were filled with the phosphate buffer, and the inlet-side electrode reservoir was flushed with distilled water. A 10-μl volume of the sample solution with an ionic strength approximately one-tenth that of the electrophoresis buffer was then placed by syringe into the reservoir just ahead of the inlet of the capillary. The power supply was turned on, and the sample electrophoresed into the capillary for 5–10 s at 8 kV. The inlet electrode reservoir was then flushed with the electrophoresis buffer, and the power supply turned back on at either a constant voltage up to 12 kV or a constant current up to 25 μA. Electropherograms were made by monitoring absorbance at 200 nm. Following a run, the capillary was flushed with buffer to remove uneluted components.

Isoelectric focusing. Isoelectric focusing (IEF) in the capillary was also a two-step procedure. In the first step, the sample solution at a concentration of 1 mg/ml was mixed in a 3:5 ratio with a 2% BioLyte pH 3–10 or 4–6 solution and flushed into a 12 cm × 25 μm I.D. coated capillary with a syringe. The buffer reservoir at the cathode was filled with 20 m*M* sodium hydroxide, and the anode reservoir with 10 m*M* phosphoric acid. The power supply was turned on with the anode at the detector end of the instrument, and the proteins focused at 8 kV until the current through the capillary fell to 0.3 μA. In the second step, the cathode buffer was replaced with 80 m*M* NaCl in 10 m*M* phosphoric acid, and the proteins mobilized at 8 kV. The IEF electropherogram was produced by monitoring the protein bands at 280 nm as they moved through the detector flow cell. Between runs the capillary was flushed with water to remove residual sample and ampholytes.

*Tryptic digest.*Digestion of hGH was carried out in a 100 m*M* Tris–acetate buffer, pH 8.3, at an initial trypsin substrate ratio of 1:100 and a temperature of 37°C. After 2 h, additional trypsin was added to double its concentration. Digestion was stopped after 2 more hours by addition of 1 *M* hydrochloric acid. Aliquots of the digestion mixture were stored frozen at −60°C, and thawed just before analysis.

HPLC tryptic mapping. The tryptic map was produced by injecting 200 μl of the digest mixture onto a 150 × 4.6 mm Nucleosil C_{18} column at 35°C equilibrated with

0.1% TFA in water. The mobile phase flow-rate was 1 ml/min. After a 5-min hold, a linear gradient to 38% acetonitrile over 120 min was started. At 125 min, the gradient slope increased to reach 57% acetonitrile over 10 minutes. The column effluent was monitored at 214 nm, and individual peaks were manually collected in volumes of 700–1200 μl. Peaks were identified by retention time[12].

CZE tryptic mapping. The free zone electrophoretic map was prepared by electrophoresis as described above with the pH 2.56 buffer. Peaks in the map were identified by electrophoresis of aliquots of fractions isolated from the HPLC tryptic map.

Ion-exchange chromotography was carried out in a 66 m*M* potassium phosphate buffer, pH 5.5, operated at 45°C. A 10-μl volume of an aged met-hGH sample was injected onto the column, and eluted with a gradient of sodium acetate to 0.15 *M* over 30 min. The chromatogram was made by monitoring absorbance at 280 nm.

RESULTS AND DISCUSSION

In order to examine the utility of capillary electrophoresis in the analysis of rDNA-derived met-hGH it was run along with its variants under a variety of conditions. Fig. 1 compares electropherograms of met-hGH and of growth hormone extracted from human pituitaries. The material produced by recombinant DNA technology yields a single peak, compared to the several unresolved peaks of the tissue-extracted material, indicating the higher degree of purity of the former. The homogeneity of met-hGH reflects its origin and relatively large-scale production. Biosynthetic protein pharmaceuticals are produced to higher purity specifications than are extracts from human tissue. Recombinant proteins are also available in much greater quantities so the recovery losses entailed by additional purification steps can be tolerated. As demonstrated by these results, capillary electrophoresis may be a rapid, supplemental method for confirmation that the desired level of purity has been achieved.

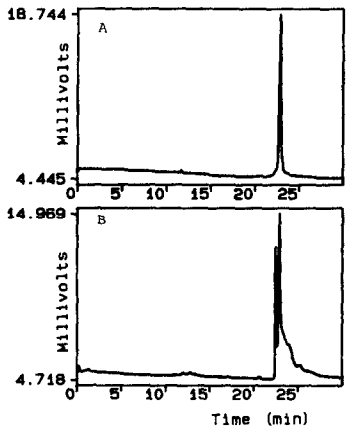

Fig. 1. Electropherograms of (A) recombinant and (B) pituitary-derived hGH. The samples were electrophoresed in a 100 m*M* pH 2.56 phosphate buffer at 8 kV in a 50 cm long × 50 μm (I.D.) coated capillary. Detection was by absorbance at 200 nm, at the cathode end of the capillary, and the analog output to the integrator was scaled 1 V per absorbance unit (V/AU).

Free zone electrophoresis as described here separates species according to both mass and charge. hGH stored in solution, particularly at elevated pH, has been shown[13] to undergo deamidation at asparagine residues 149 and 152. The conversion of asparagine to aspartic acid introduces an additional negative ionizable group on the molecule. This change allows deamidated hGH to be separated from the native molecule by anion-exchange HPLC, as shown in Fig. 2A. At pH 8.0, as shown in Fig. 2B, free zone electrophoresis yields a similar resolution of deamidated protein. Quantification is similar by the two techniques, with anion exchange measuring 2.6% deamidated met-hGH, and CZE measuring 3.1%. Part of the quantitative difference may result from the sample introduction procedure in CZE, as relatively more of the faster migrating deamidated species electrophoreses into the capillary during sample loading. Under the conditions employed here, the more acidic deamidated variant elutes ahead of the native protein.

In Fig. 2A, the identity of the deamidated species, peak D, was confirmed by tryptic digestion of the collected peak and characterizing the tryptic peptides. The deamidated peak in the electropherogram of Fig. 2B was identified by injection of a sample collected from the ion-exchange system as a standard (data not shown). The low sample tolerance of CZE makes preparative separations difficult, so injection of standards is the simplest means of identifying peaks. It is doubtful that sufficient materal could be collected from the capillary to allow characterization by tryptic mapping. Direct interfacing to a mass spectrometer might permit identification of

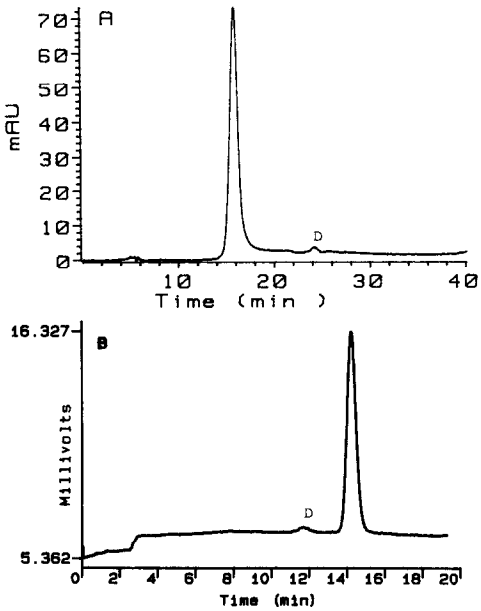

Fig. 2. (A) Ion-exchange chromatography of hGH stored in solution for two weeks. The separation was performed on a silica-based DEAE column with an acetate gradient. The more acidic peak, labelled "D", corresponds to deamidated growth hormone, as determined by sequencing and mass spectrometry of tryptic fragments. (B) Capillary electrophoresis at high pH of the sample shown in (A). The sample was loaded and run at 8 kV in a 20-cm coated capillary, with a pH 8.0 phosphate buffer. Detection was by absorbance at 200 nm at the anode end of the capillary, at 1 V/AU.

some protein variants, although a single unit of relative molecular mass change, such as resulting from deamidation, might be difficult to resolve.[14]. When an external standard is available, however, the speed and sensitivity of CZE in this application may make it a method of choice[9].

Another means of determining charge-variant species is by isoelectric focusing in the capillary. The focusing step in this technique is most conveniently carried out in coated capillaries in which electroendoosmotic flow is minimized. In uncoated capillaries the convection caused by electroendoosmotic flow interferes with the focusing process[15]. Fig. 3A shows an IEF electropherogram of the aged protein sample, after focusing in pH 4–6 ampholytes. Peaks corresponding to monodeamidated and dideamidated met-hGH are labelled "D" and "DD", respectively. Resolution of deamidated species is superior when the narrower range is used, compared to a run with pH 3–10 ampholytes (data not shown). Fig. 3B shows a free zone electropherogram at pH 8 of this sample, and more closely approximates the relative amounts of monodeamidated (D) and dideamidated (DD) species. The relative heights of the deamidated and native peaks in Fig. 3A do not accurately reflect their amounts in the sample because precipitation occurs during focusing when the concentration within bands reaches a high level. Focusing thus may allow analysis of trace amounts of species, but quantification may be hampered by precipitation of proteins present in higher amounts. This problem may be alleviated by addition of urea, detergents or ethylene glycol to the buffers used for IEF[16].

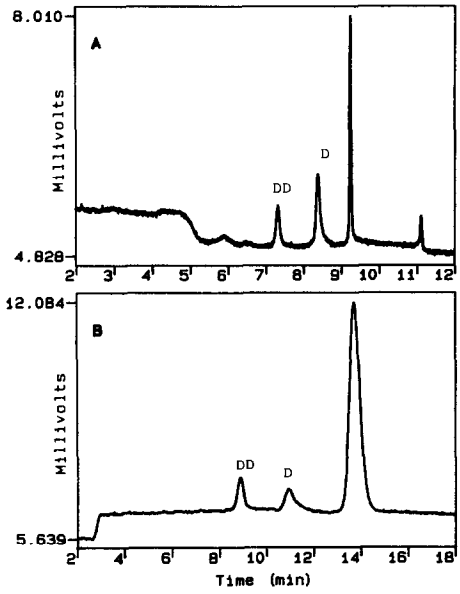

Fig. 3. (A) Isoelectric focusing in a 12-cm coated capillary of an hGH sample stored two months at 5°C in a pH 9 buffer. Detection was at 280 nm. (B) Capillary electrophoresis at high pH of the aged hGH sample shown in (A). The sample was loaded and run at 8 kV in a 20-cm coated capillary, with a pH 8.0 phosphate buffer. Detection was by absorbance at 200 nm, 1 V/AU. The identities of the peaks corresponding to deamidated hGH variants were confirmed by electrophoresis of fractions obtained by anion exchange chromatography, and are indicated by "D" for monodeamidated hGH and "DD" for the dideamidated species.

A useful tool for identifying variants is the peptide map obtained by RP-HPLC of an enzymatic digest of the protein sample[17]. By careful standardization of digest and chromatography conditions, the RP-HPLC tryptic map can be used to monitor degradation products in a sample and to isolate variant proteins for the characterization of contaminants. Fig. 4 shows the RP-HPLC map of met-hGH, with identification of the major peptides resulting from digestion. The peptides are labelled numerically according to their position in the intact protein such that T1 is the N-terminal tryptic peptide and T20–T21 are the two C-terminal peptides that are joined by a disulfide bond. The characterization of the deamidation appearing in the T15 peptide of hGH has been described previously[4]. This map is a relatively sensitive analytical method that can be used to determine, in most cases, the presence of variants constituting as little as 5% of a sample.

Capillary electrophoresis, with selectivity greatly different to that of RP-HPLC, offers a convenient alternative means of producing a tryptic map of a protein. Fig. 5 shows the CZE map of hGH, with peptides labelled according to the convention described above. The peptides were identified by running aliquots of the fractions collected from the RP-HPLC map shown in Fig. 4. The separation in Fig. 5 was performed on a 20-cm capillary, and yielded a map with a run time of less than 12 min with resolution of most peptides. As in the RP-HPLC map, small unidentified peaks may represent non-tryptic cleavages or contaminants from the trypsin preparation used for the digestion. Better resolution can be achieved by using a 50-cm long capil-

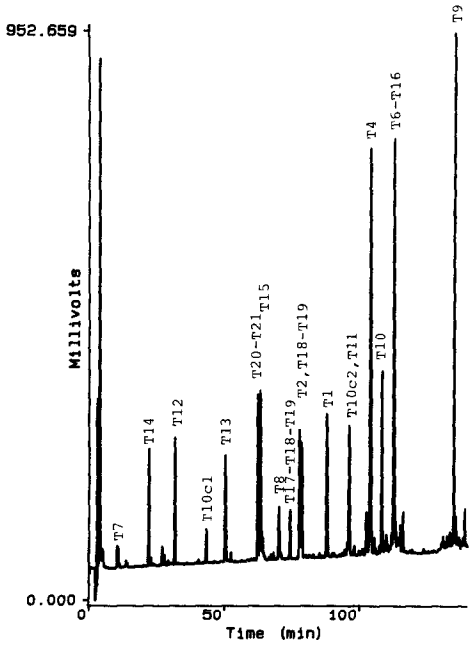

Fig. 4. RP-HPLC of peptide fragments from tryptic digestion of recombinant human growth hormone. Tryptic fragments are numbered sequentially from the N-terminus of the protein, and the suffix "c" indicates a peptide fragment resulting from a chymotrypsin-like cleavage. Detection was at 214 nm, 1 V/AU.

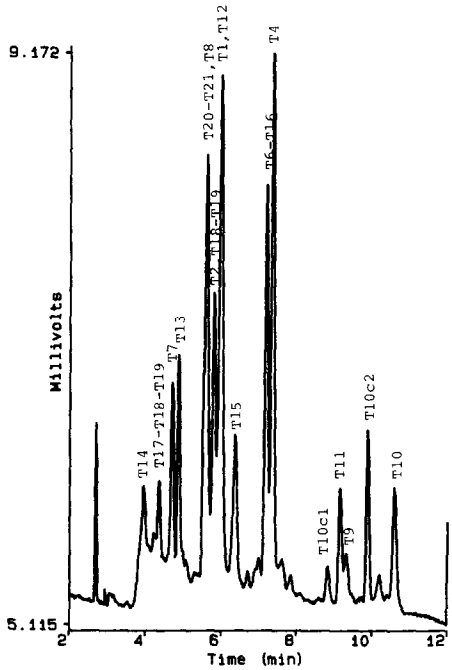

Fig. 5. Capillary electrophoresis of peptide fragments from tryptic digestion of recombinant hGH. The cathode chamber and 20 cm × 25 μm (I.D.) capillary contained a pH 2.5 phosphate buffer. A 10-μl aliquot of the tryptic digestion mixture, stopped by addition of HCl, was loaded into the sample chamber at the anode of the instrument. Peptides were introduced into the capillary by electromigration for 5 s at 8 kV. After flushing the anode chamber with the pH 2.5 buffer, the peptides were electrophoresed at 8 kV and detected by absorbance at 200 nm, 1 V/AU. Peaks were identified by capillary electrophoresis of 10-μl aliquots from individual fractions collected from the reversed-phase tryptic map.

lary, as demonstrated in Fig. 6. The pattern obtained is similar to that in Fig. 5. The separation time is much longer with the 50-cm capillary, but remains less than the time for the RP-HPLC map. Thus this may be a promising tool for confirmation of identity, although as noted above it has the disadvantage compared to HPLC of not being able to collect a sufficient amount of a peak for subsequent sequence analysis.

A second problem observed in tryptic map analysis of met-hGH by capillary electrophoresis is the poor reproducibility of retention or transit times[a]. Fig. 7 shows sequential injection of the same sample. While the pattern is reproducible from run to run, transit times shift appreciably. This drift impedes automatic peak identification by software programs such as those developed for HPLC. These programs establish a relatively narrow retention time window for each identified peak in the map, thus

[a] From a phenomenological aspect "retention" adequately describes the differential migration characteristic of the movement of solutes in electrophoresis. However, since this term is closely associated with chromatography, and evokes the adsorptive "binding" features of chromatography, the phrase "transit time" is advanced here to describe the amount of time from the commencement of electrophoresis to the passage of a solute past the detector portion of the capillary.

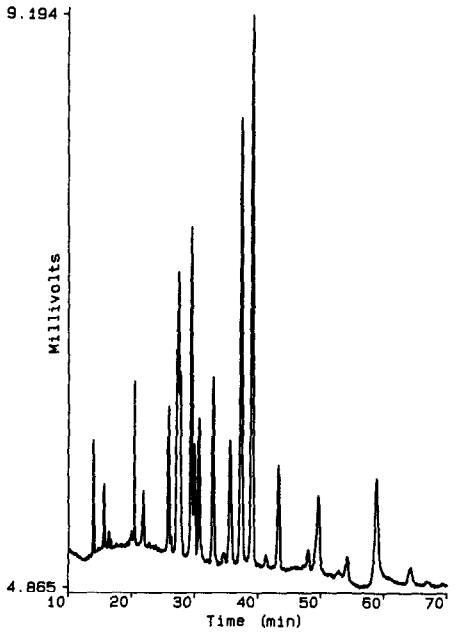

Fig. 6. Capillary electrophoresis of the tryptic digest of hGH in a 50 cm × 50 μm I.D. capillary. Conditions as in Fig. 5, except that the sample was loaded for 10 s.

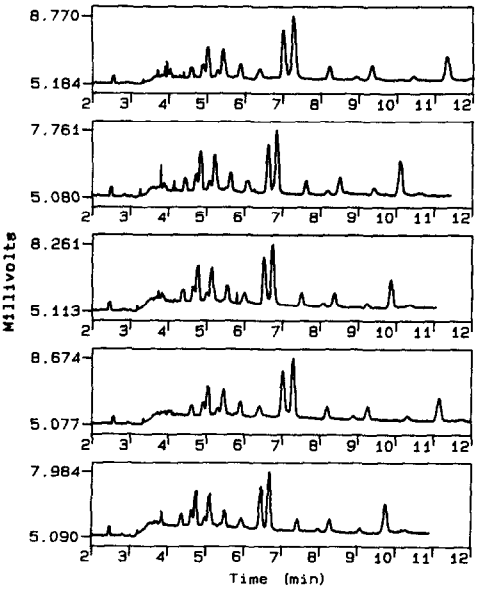

Fig. 7. Sequential runs of the hGH tryptic digest in a 20-cm long capillary. Conditions as in Fig. 5.

reproducibility is essential for unattended operation. The cause of the observed drift in transit times has not been determined, but may involve adsorption of charged protein fragments onto the internal wall thus inducing electroendosmotic flow, or temperature changes in the capillary and its environment. Automation of capillary washing and sample introduction steps may alleviate some of the irreproducibility observed here.

In spite of these problems, CZE shows promise in analysis of the peptides generated by tryptic digestion, especially as it separates according to different solute properties than RP-HPLC and thereby complements the more established technique. A useful application of these complementary techniques is shown in Fig. 8, where one of the fractions collected from the RP-HPLC tryptic map has been reanalyzed by HPLC and CZE. As seen in the expansion of the chromatogram in Fig. 8A, the fraction appears to contain two components that are unresolved by HPLC. The electropherogram of the same fraction in Fig. 8B shows two well-resolved peaks in under 12 min. The high sensitivity of CZE is also reflected in Fig. 8B, which was made by taking only 10 μl of the 800-μl fraction. The speed and sensitivity of this approach make it promising as a second dimension for analysis of complex mixtures. The complementarity of these two analytical techniques is illustrated by the plot in Fig. 9 of the transit times in CZE *versus* retention times in RP-HPLC of the tryptic peptides. The broad scatter in this plot indicates that these two techniques complement one another well.

An empirical relation between electrophoretic transit time and the molecular weight and net charge of a peptide[18], can be used to aid optimization of the sep-

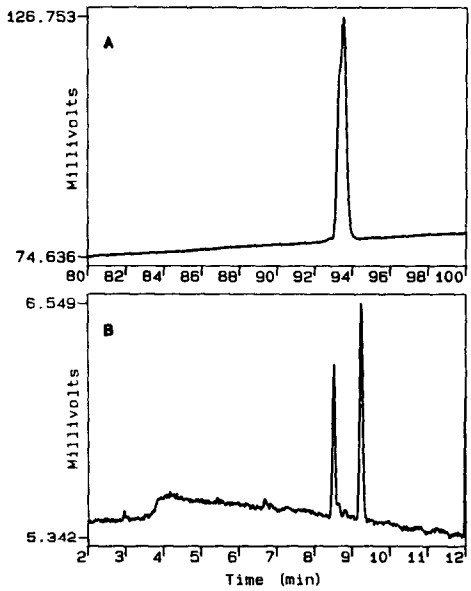

Fig. 8. Analysis of the "T11, T10c2" fraction from the RP-HPLC tryptic map by (A) HPLC and (B) capillary electrophoresis. A 10-μl volume of the 0.8-ml fraction collected on re-chromatography of the two peptides was used to produce the electropherogram. The conditions were as in (A) Fig. 4 and (B) Fig. 5.

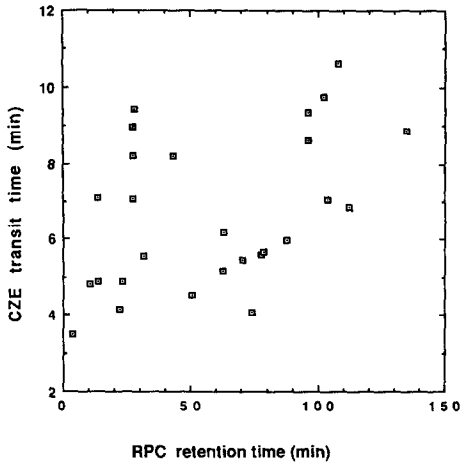

Fig. 9. Correlation of retention times of peptides produced by tryptic digestion of recombinant hGH in RP-HPLC (RPC) with their transit times in CZE.

aration, as shown in Fig. 10. The abscissa in Fig. 10 is $MW^{2/3}/z$ where MW is the molecular weight of the peptide and z is its net charge. At the low pH employed here, the charge on a peptide is expected to be positive and due only to protonation of its amino-terminus and any lysine, arginine or histidine ($pK_a \approx 6$, ref. 19) residues it contains. The net charge on a peptide (and so $MW^{2/3}/z$) is also most accurately determined at low pH. Fig. 10 shows a good correlation between this parameter and transit time. Many of the peptides arising from tryptic digestion of hGH have similar transit times, as already seen in Fig. 4, and Fig. 10 provides an explanation. Since

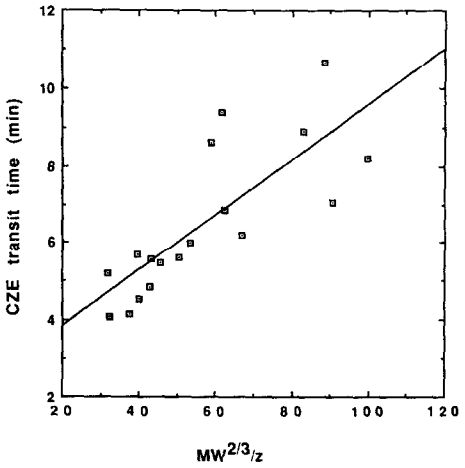

Fig. 10. Correlation of capillary electrophoretic transit times of tryptic peptides with $MW^{2/3}/z$, where MW is the molecular weight of the peptide and z is its net charge in the buffer used. The peptides were assumed to carry charges only at the N-terminus and on lysine, arginine and histidine side chains in order to calculate z.

trypsin cleaves a protein after lysine and arginine residues, and hGH contains only two histidine residues, most of the peptides contain two positive charges. Exceptions to this, aside from histidine-containing peptides, arise from chymotrypsin-like cleavages ($z=1$), peptides joined by disulfide bonds ($z=3,4$) and incompletely digested peptides ($z=3,4$). As the tryptic peptides have similar molecular weights and net charges, many elute in a window from 4–6 min from the 20-cm capillary. Better resolution is achieved with the 50-cm capillary, but an even better separation could be expected by digesting the protein either chemically or enzymatically with an agent that cleaves at residues other than lysine, arginine or histidine. Such digestion would result in peptides carrying variable numbers of charges.

CONCLUSIONS

Free zone electrophoresis in coated capillaries is a fast, sensitive method for detection of deamidation in protein pharmaceuticals such as hGH. The resolution by this method is comparable to anion-exchange HPLC and requires less sample, although the maximum sample tolerance is much lower.

Isoelectric focusing in coated capillaries minimizes electroendoosmotic flow and yields sharp bands upon subsequent mobilization past the detector. The IEF mode allows rapid detection of low-level contaminants or variants in a sample, based on p*I* differences. Care must be taken to regenerate the capillary between runs, particularly for relatively insoluble samples.

Electrophoresis in coated capillaries with low pH buffers yields good separation of tryptic peptides, but closely related protein variants may not be resolved.

Capillary electrophoresis is complementary to HPLC and provides a rapid, convenient method to check the identity and purity of collected fractions.

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