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CAPILLARY ZONE ELECTROPHORESIS OF PEPTIDE FRAGMENTS FROM TRYPSIN DIGESTION OF BIOSYNTHETIC HUMAN GROWTH HORMONE

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SUMMARY

Capillary zone electrophoresis (CZE) was applied to the separation of the 19 peptide fragments produced by enzymatic digestion of human growth hormone (hGH). The fragments of hGH produced by trypsin digestion under non-reducing conditions were identified in the electropherogram. Almost all of the fragments were resolved by CZE in less than 15 min. There is a marked difference in selectivity between reversed-phase high-performance liquid chromatography (RP-HPLC) and CZE. CZE is demonstrated to be a powerful complement to RP-HPLC for routine identification of hGH using trypsin digests.

INTRODUCTION

Human growth hormone (hGH) is an important pharmaceutical product produced by recombinant-DNA technology. Like other pharmaceuticals, hGH must be characterized for identity, potency, and purity. The identity test provides a confirmation of the correct chemical structure; potency is a measure of the activity or quantity of drug; and purity refers to the impurity content.

For biomolecules used as drugs, the initial elucidation or proof of structure is necessarily accomplished by utilizing a battery of techniques. Then, a more limited set of tests is utilized on each batch of material to verify that its structure is identical to that of previous material. Chromatography, electrophoresis and other separation techniques are frequently used for comparisons of the sample to a reference standard to confirm identity. In general, the smaller the size of a protein, the more sensitive a separation technique will be to slight differences in structure. For this reason, the chromatographic "fingerprint" of polypeptide fragments produced from proteolytic enzyme digestion of biomolecules is a powerful test of their identity. Reversed-phase high-performance liquid chromatography (RP-HPLC) is commonly used to monitor the digest fingerprint. Although the analysis time for enzymatic protein digests by RP-HPLC can be shortened to as little as 10 min^1, the most commonly used procedures for hGH require between 1 and 2 h and do not completely separate all of the fragments[2,3]. Thus, it would be desirable to have a separation technique that could

provide a fast and complete separation of all the components for routine evaluation of enzymatic digests. Alternatively, unambiguous identification of digest components could be accomplished using coupled techniques or by combination of RP-HPLC with a second, complementary technique.

Coupled techniques have been evaluated by a number of groups. O'Farrell[4] demonstrated the power of combining isoelectric focusing and sodium dodecyl sulfate (SDS) gel electrophoresis by resolving more than 1000 different components from *Escherichia coli*. RP-HPLC coupled to SDS polyacrylamide gel electrophoresis (PAGE) has been automated and applied to the analysis of protein mixtures[5]. Other examples that have been applied to the analysis of proteolytic digests are HPLC–SDS-PAGE–electroblotting[6] and HPLC–fast-atom bombardment mass spectrometry[7], the latter technique being a directly coupled, continuous flow system. However, most of these coupled techniques are difficult to quantitate, require long analysis times, and are labor intensive and/or difficult to automate. However, capillary zone electrophoresis (CZE) seemed a viable alternative as a complement to existing RP-HPLC techniques.

Extensive characterization and development of CZE for analytical separations was carried out by Jorgenson and co-workers[8–10]. CZE was applied recently to intact, highly purified biosynthetic human proteins and polypeptides[11,12]. We evaluated the applicability of CZE to generate the "fingerprint" for a trypsin digest of hGH.

EXPERIMENTAL

Reagents and materials

Biosynthetic hGH was obtained from Eli Lilly & Co. (Indianapolis, IN, U.S.A.). Morpholine was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.) and tricine from Sigma (St. Louis, MO, U.S.A.). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Tris–acetate buffer was prepared by adjusting the pH of a 0.05 *M* Tris solution to pH 7.5 with acetic acid. Trypsin (TPCK, 267 units/mg protein, 98% protein) was purchased from Cooper Biomedical (Malvern, PA, U.S.A.). Reagent-grade water obtained from a Milli-Q purification system from Millipore (Bedford, MA, U.S.A.) was used to prepare all solutions. All other reagents were analytical grade and were used without further purification. Polyimide-coated, fused-silica capillaries, 50 μm internal diameter and 360 μm outside diameter, were purchased from Polymicro Technologies (Phoenix, AZ, U.S.A.).

Methods

The trypsin digestion and subsequent RP-HPLC analysis of hGH fragments were carried out according to reported methods[2]. Aliquots of the digest mixture were frozen (−20°C) for use at a later time. Fractions were manually collected from the RP-HPLC column following injection of a large sample (0.5 ml of 8 mg/ml hGH digest mixture) onto the analytical column; the solvent elution profile was the same as used for the analytical method. Cuts were made so that fractions contained only single hGH fragments whenever possible. However, several fractions contained more than one fragment. Specifically, the following groups of fragments were co-eluted

and/or co-collected: fragments 3, 5, 7 and 17, fragments 1, 2 and 19, and fragments 8 and 18. The collected fractions were evaporated to dryness under a gentle stream of nitrogen or lyophilized and stored at 5°C. Fractions that contained more than one hGH fragment were further separated by anion-exchange chromatography.

Anion-exchange chromatography was performed on a Model 4000i Dionex (Sunnyvale, CA, U.S.A.) chromatograph using a Mono-Q HR 5/5 column (50 × 5 mm, Pharmacia LKB, Piscataway, NJ, U.S.A.). The mixed fragment fractions from RP-HPLC were dissolved in mobile phase A prior to separation by anion-exchange chromatography. The RP-HPLC fractions containing mixtures of fragments were loaded (0.1 ml) onto the anion-exchange column and eluted (flow-rate 0.5 ml/min) using the following conditions: mobile phase A, 0.01 *M* ammonium bicarbonate, pH 8.0; mobile phase B, 0.50 *M* ammonium bicarbonate, pH 8.0; gradient profile, 0% to 100% B in 40 min, hold 100% B for 10 min, and return to initial conditions over 10 min. The separation was monitored at 214 nm. In some cases, multiple injections were required to obtain sufficient amounts of material for further tests. Collected fractions were lyophilized and stored at 5°C.

The digest mixture containing all of the hGH fragments as well as the isolated fragments were examined by CZE. The thawed digest mixture was injected directly; the dried isolated fractions were dissolved in either the CZE mobile phase or Tris–acetate buffer as required. The mobile phase used in the CZE separation was 0.01 *M* tricine, 0.02 *M* NaCl, and 0.045 *M* morpholine adjusted to pH 8.0. The column was rinsed with mobile phase between injections of the isolated hGH fragments or with 0.1 *M* sodium hydroxide followed by mobile phase between injections of the digest mixture. The CZE instrumentation is the same as previously described[11] except that both CZE instruments now include vacuum injection devices and a constant temperature environment. Samples (about 10 nl of a 2 mg/ml solution) were introduced by applying vacuum (95 mmHg) to a capillary that was approximately 95 cm in length with 81 cm to the detector. Separation conditions were: 30 kV applied voltage, 25°C and approximately 40 μA current. The components were detected by UV absorbance at 200 nm. Analog data were collected directly from the absorbance detectors (10 mV outputs were amplified as necessary, typically 100-fold) on an in-house centralized chromatography computer system based on the Model 1000 Hewlett-Packard minicomputer that has storage, manipulation, and graphics capabilities.

Calculated values

The isoelectric points (p*I*) of the fragments were calculated with an in-house computer program[13]. The same program calculates the hydrophobicity parameter according to the method of Meek and Rossetti[14].

RESULTS AND DISCUSSION

The RP-HPLC analysis of the complex mixture of peptides produced by enzymatic digestion of biosynthetic hGH has been reported[2,3]. A typical chromatogram with the fragments identified is shown in Fig. 1. The structure of hGH and the 19 peptide fragments produced by the trypsin digestion are shown in Fig. 2. These fragments range in size from a single amino acid residue, lysine (fragment 17), which has a mass of 147, to a fragment of 32 residues, (fragment 6–16), which has a mass of

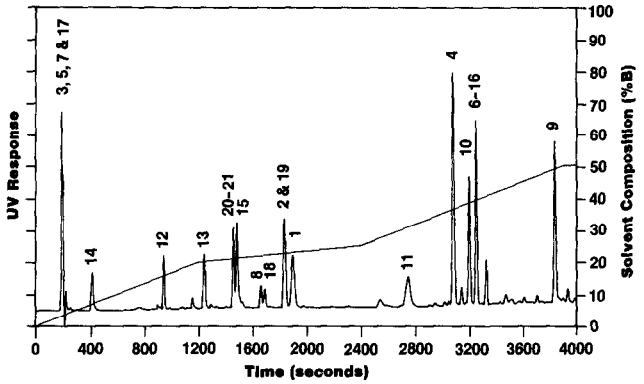

Fig. 1. Tryptic mapping of hGH by RP-HPLC. Peaks are labeled according to the fragment identification in Fig. 2.

3763. Note that two pairs of fragments (6 and 16, subsequently referred to as 6–16; and 20 and 21, referred to as 20–21) are joined by disulfide bonds. By performing the digestion under non-reducing conditions, both the correct amino acid sequence and the presence of the correct disulfide linkages can be confirmed.

Peptide fragments isolated by RP-HPLC were reinjected for verification of their integrity. The identities of the isolated fragments were assigned based on the known RP-HPLC elution profile[2]. For fragments that were further purified by anion-exchange chromatography (*i.e.*, not isolated separately by RP-HPLC alone), the composition was confirmed by comparison of data from amino acid analysis[15] to the known structural composition of the fragment.

It was found that the digest mixture could be analyzed by CZE when a well buffered mobile phase such as that described above was used. However, it was not possible to directly compare CZE migration times between electropherograms of the isolated fragments and electropherograms of the digest mixture since the complex digest matrix perturbs the electrophoretic environment. Thus, individual isolated fragments were spiked into the unseparated digest mixture for peak identification; peaks were identified by their enhancement compared to other components. Fig. 3 illustrates this process for a sample mixture containing fragments 1, 2 and 19; a similar process was followed using single-component fractions to identify all of the peaks labeled. However, the identification of two fragments deserves special comment.

Fragments of hGH were assigned peaks for the electropherogram in Fig. 4. It was found that fragment 14 gave more than one peak following isolation by RP-HPLC and evaporation of the collected fraction under reduced pressure or under a flow of nitrogen. The authentic fragment 14 elutes early in the electropherogram as labeled in Fig. 4. The other peak apparently arises from cyclization of the N-terminal glutamine (sequence, Gln-Thr-Tyr-Ser-Lys) to give pyrrolidone carboxylic acid at the N-terminal. It elutes much later in the electropherograms, labeled 14* in Fig. 4, since one positive charge has been lost. This subtle rearrangement was missed in the preliminary peak assignments for the digest[12].

Fragment 9 is practically insoluble in aqueous buffers. When introduced into

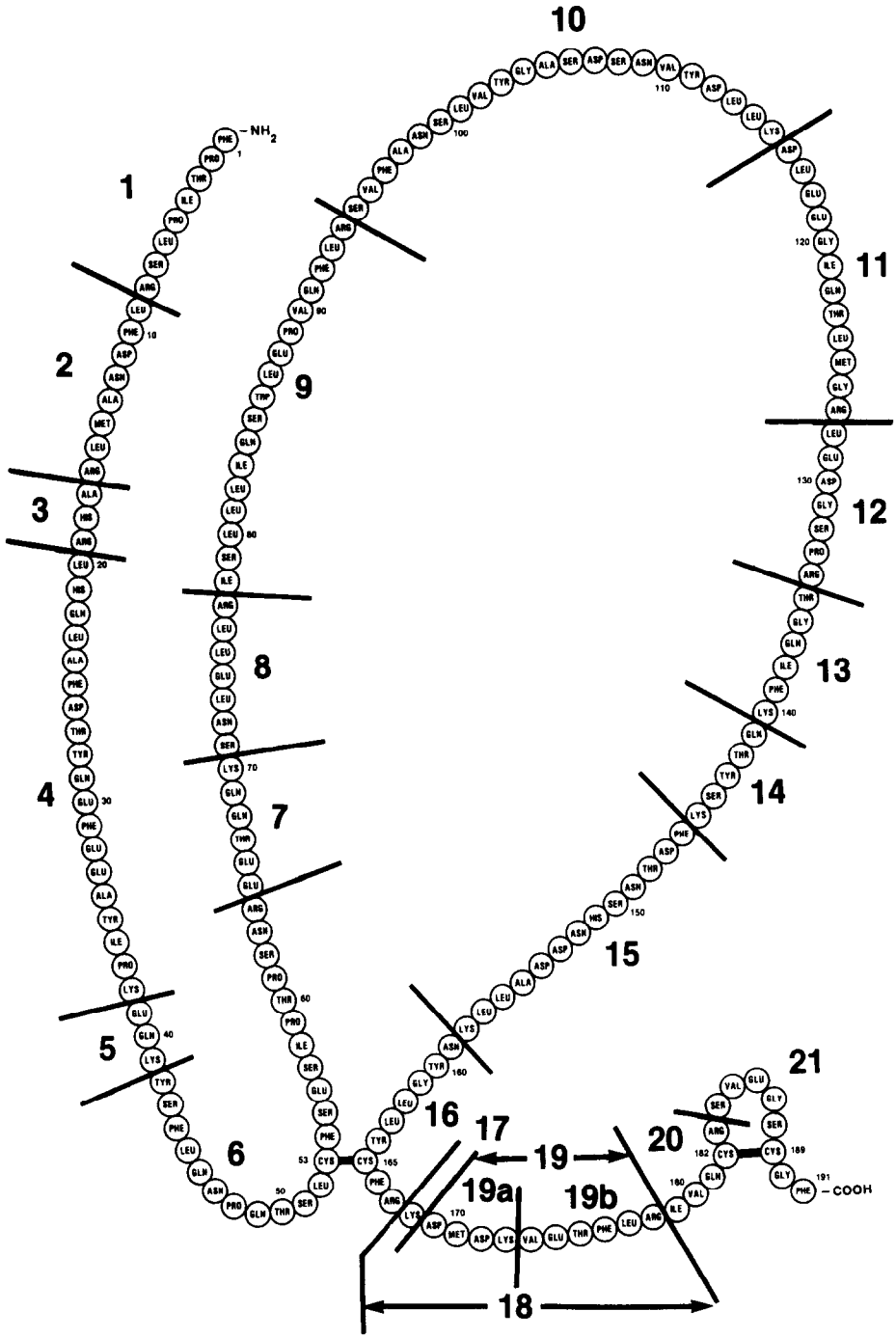

Fig. 2. Structure of hGH that shows the trypsin cleavage sites. Note that fragments 6 and 16 and fragments 20 and 21 are each joined by a disulfide bond. Fragment characteristics are given in Table I.

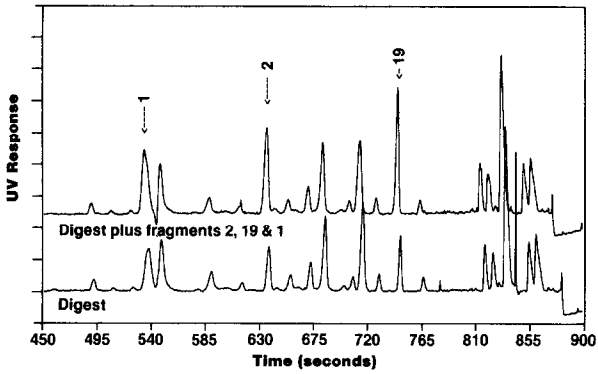

Fig. 3. Method for tryptic mapping of hGH by CZE. The digest matrix was spiked with peak fractions isolated by RP-HPLC. The peaks indicated were identified by enhancement of peak area following the addition of spikes.

the CZE capillary, it gives very sharp peaks nearly coincident with the peak for fragment 4. However, more than one peak is associated with fragment 9 and they have variable migration times. When the digest is filtered through a 0.22-μm filter, the sharp peaks are removed and no other peaks appear to be affected. Furthermore, the material washed off of the filter gives the spikes. Thus, the sharp peaks are due to insoluble particles whereas the soluble portion of fragment 9 is present at such low concentrations that it is not observed. Based on the known structure of fragment 9, it should elute early in the electropherogram near the peak for fragment 6–16.

The digest fragments are identified in Fig. 5 for the CZE and RP-HPLC separations. Differences in selectivity between CZE and RP-HPLC are readily apparent. Many peptides that co-elute or elute closely on RP-HPLC are widely separated by CZE. However, three fragments (fragments 1, 13 and 14) that separate from each other by RP-HPLC (although fragment 13 co-elutes with other fragments) elute as

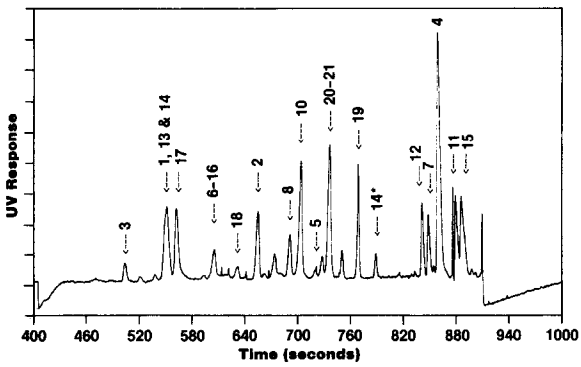

Fig. 4. Tryptic map of hGH by CZE. Fragments produced by trypsin digestion of hGH were identified as described in the text and illustrated in Fig. 3. Fragment 9 was not identified due to its insolubility in aqueous solutions.

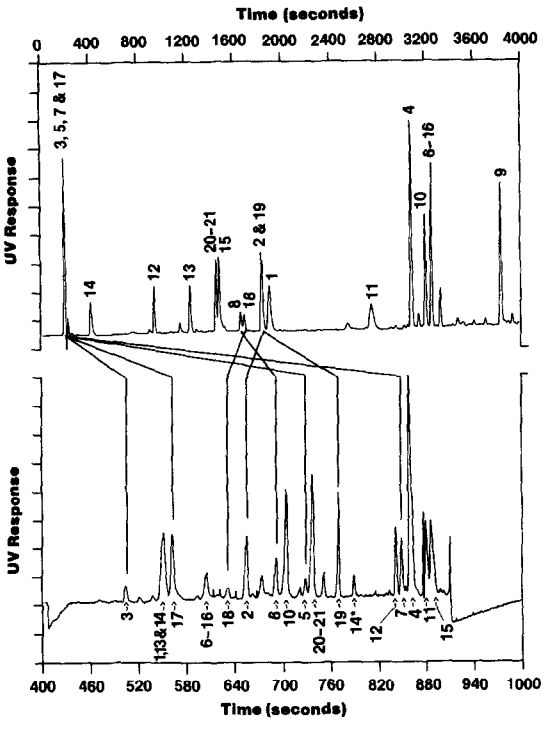

Fig. 5. Comparison of RP-HPLC and CZE separations. Differences in selectivity for these two techniques are illustrated. (Top) Tryptic map by RP-HPLC; (bottom) tryptic map by CZE.

one peak by CZE. Overall, there is no correlation between the electrophoretic migration time and the RP-HPLC elution time (correlation coefficient = 0.140). The lack of correlation or "orthogonality" of these techniques is expected since the separation mechanisms are different. That is, the peak order in RP-HPLC is determined mainly by the relative hydrophobicity of the fragments while the peak order in CZE is predominantly determined by their charge with some dependence on their size and hydrophobicity. For example, the early eluting "crash" peaks in RP-HPLC (fragments 3, 5, 7 and 17) are all highly hydrophilic (Table I). Likewise, fragments 1, 2 and 19 and fragments 8 and 18 have relatively similar hydrophobic character and are not separated by RP-HPLC. However, all of these peaks are separated by CZE. On the other hand, fragments 1, 13 and 14 have similar p*I* values (10.1, 9.2 and 9.0, respectively), similar sizes (8, 6 and 5 residues, respectively) and the same nominal charge at pH 8 (+1) and are not separated by CZE. A more extensive correlation of electrophoretic mobilities with charge and size will be reported later.

There are other differences in the separation between CZE and conventional techniques. First, the run time in CZE is much shorter than that of conventional RP-HPLC, about four-fold less in this specific case. Second, CZE can be used for small peptide fragments (relative molecular mass often less than 5000) produced by enzymatic digestion of proteins, whereas these small molecules would be difficult, if not impossible, to evaluate by conventional gel electrophoresis. Furthermore, it is

TABLE I

FRAGMENTS FROM ENZYMATIC DIGEST OF HUMAN GROWTH HORMONE

Fragment number	*Isoelectric point*[a]	*Hydrophobicity*[b]	*Molecular weight*	*Amino acid residues*
1	10.1	18.1	930	8
2	5.8	17.5	978	8
3	10.4	−12.3	382	3
4	4.2	45.5	2343	19
5	6.4	−15.7	404	3
6–16	7.3	74.8	3763	32
7	4.5	−19.0	762	6
8	5.9	9.4	844	7
9	6.4	72.1	2056	17
10	3.5	33.3	2263	21
11	4.0	13.0	1362	12
12	4.0	−3.4	773	7
13	9.2	0.4	693	6
14	9.0	−14.2	626	5
15	3.8	0.9	1490	13
17	9.0	−13.9	146	1
18	6.1	10.5	1382	11
19	4.0	12.1	1253	10
20–21	5.9	19.9	1401	13

[a] Calculated from a computer program based on Shields[13].
[b] Calculated according to Meek and Rossetti[14].

very difficult to accurately quantitate zones from gel electrophoresis, and gel techniques are much more labor intensive than CZE. Finally, CZE requires only very small sample volumes although moderate sample concentrations are needed.

On the other hand, the overall peak capacity of CZE does not match that of RP-HPLC because of the wide range of conditions that can be achieved by gradient RP-HPLC elution. Also, although CZE is noted for its high separation efficiency (large number of theoretical plates), it cannot match the separative power of coupled techniques such as two-dimensional gel electrophoresis. Overall, CZE appears to be a powerful complement to conventional techniques for the separation of complex tryptic digests of hGH. The combination of RP-HPLC and CZE produces an unambiguous verification of each of the tryptic digest fragments of hGH.

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