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Application of capillary high-performance liquid chromatography to biotechnology, with reference to the analysis of recombinant DNA-derived human growth hormone

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Abstract

Using capillary HPLC, femtomole amounts of recombinant DNA-derived human growth hormone (rhGH) have been successfully detected from solutions at nanomolar concentrations. The separation used capillaries of 15 cm × 320 μm I.D. and detection was with a UV absorbance detector containing a capillary Z-shaped flow-cell. A sample of rhGH that was recovered from rat serum was analyzed by capillary reversed-phase HPLC, using both acidic- and neutral-pH mobile phases, as well as by capillary ion-exchange chromatography. When compared to HPLC separations performed at flow-rates of 1 ml/min, the sensitivity of the detection was increased 200 times, without any loss in resolution. Sub-microgram amounts of rhGH were also analyzed by tryptic mapping using capillary HPLC and peptides were identified by capillary LC–MS.

1. Introduction

A recent advance in the field of high-performance liquid chromatography (HPLC) has been the popularization of capillary columns [1–8]. It has been shown that the packing of efficient capillary columns is relatively straightforward and such columns are now commercially available [9,10]. One significant advantage of using capillary columns is their reduced solvent requirement. Environmental concerns are addressed by the decreased solvent consumption and disposal of a minimal amount of solvent waste. Additionally, the reduced sample requirement of capillary HPLC eliminates the need to prepare large amounts of sample by repetitive, preparative techniques and thus this technique is particularly useful in studies where only limited amounts of sample are available.

The growth of hyphenated technologies, e.g. liquid chromatography–mass spectrometry (LC–MS), has also promoted the application of capillary separations. The possibility to use low flow-rates make capillary HPLC the system of choice. Previously, these hyphenated technologies reduced solvent delivery rates by the use of a post-column stream splitter, however, this approach not only splits the flow but also the sample and therefore is problematic for samples that are available in limited amounts. A barrier to the widespread use of capillary chromatography has been the lack of suitable instrumentation.

Capillary HPLC systems must be configured

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with low dead volumes, be able to deliver reproducible gradients at low flow-rates and require a low-volume sensitive detector. Recently such instrumentation has become available [8].

To date, most studies on capillary HPLC have used protein standards or, following enzymatic digestion, peptide mixtures derived from these standards, to generate information on the applicability of this technique [1–8]. Although these studies have been useful, the application of this technology to small amounts of proteins isolated from biological samples, has been limited. For example, issues such as handling of small sample amounts, specific detection problems, and the selection of separation methods and appropriate mobile phases, have not been explored for the study of protein metabolism. A biotechnological application for capillary HPLC is the analysis of host-cell contaminants in a final product, which typically requires detection below the 0.1% level. While such analyses may first require a concentration step, such as affinity chromatography, the use of subsequent capillary analysis will reduce the amount of sample required.

This study will examine the application of capillary HPLC in analytical biotechnology by studying variants of rhGH which are analyzed using reversed-phase HPLC, ion-exchange HPLC and peptide mapping.

2. Experimental

2.1. Materials

rhGH is a product of Genentech (South San Francisco, CA, USA). Two-chain rhGH was isolated and purified from the manufacturing process. DesPhe^{1}Pro^{2}-rhGH was purified from an aged sample of rhGH using hydrophobic interaction chromatography [11].

A rhGH mutant that contained an aspartic acid substitution for an asparagine (residue 149) was produced by point mutation. This mutant was representative for deamidated rhGH. Trypsin was purchased from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA), HPLC/spectro grade, was obtained from Pierce (Rockford, IL, USA). Acetonitrile (HPLC grade) was purchased from Burdick and Jackson (Muskegon, MI, USA). Water was purified by a Millipore, Milli-Q system (Bedford, MA, USA).

2.2. Instrumentation

The capillary liquid chromatographic system was configured as shown in Fig. 1. The solvent delivery system was a Hewlett-Packard Model HP1090 liquid chromatograph (Palo Alto, CA, USA), operating at a flow-rate of 200 to 400 μl/min. Isocratic and gradient elutions were performed. The solvent delivery system was connected to an Acurate Model AC-70 micro-flow processor purchased from LC Packings (San Francisco, CA, USA). A 0.5-μm filter was placed between the solvent delivery system and the micro-flow processor. Sample loading was performed with a manual Rheodyne Model 8125 injector (Cotati, CA, USA) fitted with a 5-μl sample loop. Either commercially available capillary columns (LC Packings) or columns packed in our own laboratory were connected directly to the injector. Detection was performed with an Applied Biosystems Model ABI 785A UV absorbance detector (Foster City, CA, USA), fitted with a capillary Z-shaped flow-cell purchased from LC Packings. The absorbance was monitored at 214 nm. Data acquisition was performed with a Hewlett-Packard Model HP 1000 computer.

2.3. Neutral-pH RP-HPLC

Neutral-pH reversed-phase HPLC separations were performed on a capillary column (10 cm ×

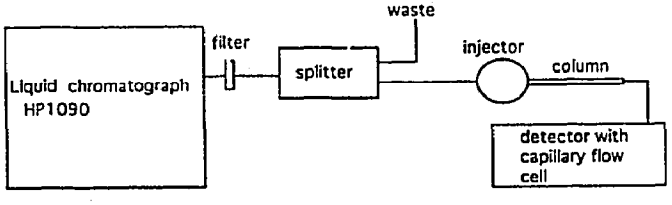

Fig. 1. Arrangement of a capillary HPLC system. Details are given in the Experimental section.

320 μm I.D.) that was packed in our laboratory with packing material removed from a Polymer Laboratories PLRPS-4000 column (Shropshire, UK). Solvent A was 50 m*M* potassium phosphate, pH 7.5. Solvent B was acetonitrile. The sample (0.1 μg of each rhGH variant, in a total of 5 μl) was loaded onto the column equilibrated with 25% solvent B, and run isocratically for 5 min. A rapid gradient was run to 34% solvent B in 1 min. After a 10-min hold, a 1%/min gradient was run to 35% solvent B, followed by a 0.2%/min gradient to 37% solvent B to complete elution. The flow-rate was 3.5 μl/min. Separations were also performed using a Hewlett-Packard Model HP1090 liquid chromatograph, using the same support and mobile phases, but with column dimensions of 15 cm × 4.6 mm I.D. and a flow-rate of 0.5 ml/min. These separations were performed isocratically at 45% solvent B and at a column temperature of 50°C.

2.4. Acidic-pH RP-HPLC

Acidic-pH reversed-phase HPLC separations were performed on a capillary column (10 cm × 320 μm I.D.) packed in our laboratory with Vydac RPC4, 300 Å pore-size, 5-μm particle diameter (The Separations Group, Hesperia, CA, USA). Solvent A was 0.1% TFA and solvent B was 0.09% TFA in acetonitrile. The column was equilibrated in 40% solvent B and a 5-μl sample was loaded onto the column. After allowing the sample to concentrate at the head of the column for 10 min, a gradient was run to 60% solvent B in 10 min, and held for 10 min to complete elution. The flow-rate was 3.5 μl/min.

2.5. Ion-exchange HPLC

Ion-exchange separations were performed using a 10 cm × 320 μm I.D., glass-lined stainless steel capillary, packed in our laboratory with TSK-gel, DEAE 5-PW (Tosoh Corporation, Tokyo, Japan). Elution was performed isocratically using 33 m*M* potassium phosphate pH 6.5, with the addition of 20% acetonitrile. The flow-rate was 3.5 μl/min.

2.6. Trypsin digestion and peptide mapping

rhGH (0.5 μg/1 μl) was diluted with digest buffer (5 μl), pH 8.3. Trypsin (0.025 μg) (1:20, w/w, protein:enzyme (P:E)) dissolved in digest buffer (1 μl) was added. The digest mixture was incubated at 37°C for 2 h followed by a second enzyme addition (1:10, w/w P:E). After a further 2 h at 37°C digestion was stopped by the addition of 2% TFA (3 μl). The released tryptic fragments were resolved on a custom packed Nucleosil C_{18}, 5 μm capillary column, 15 cm × 320 μm I.D. (LC Packings). Solvent A was 0.1% TFA and solvent B was 0.08% TFA in acetonitrile. The digest mixture (2 μl containing 0.09 μg of digested rhGH) was loaded onto the capillary column equilibrated in 100% solvent A. Separation of the tryptic fragments was achieved by running a 0.3%/min gradient to 40% solvent B, followed by a 2%/min gradient to 60% solvent B. The flow-rate was 3.5 μl/min.

2.7. LC–MS

The eluate from the reversed-phase column (see section 2.6 above) was analyzed with a SCIEX API III triple quadrupole mass spectrometer (Thornhill, Ont., Canada). Quadrupole one was scanned from 300–2000 Da with a scan time of 3.59 s, using a step size of 0.5 Da and a 1-ms dwell time per step.

3. Results and discussion

A schematic of the capillary HPLC system used in this study is shown in Fig. 1. This capillary system was assembled from commercially available components and demonstrated reproducible separations and a reasonable degree of ruggedness. However the inability to control the column temperature prevented the examination of the effect of temperature on resolution while the absence of an automatic sample injector limited sample throughput. The liquid chromatograph used, i.e. the Model HP1090 can deliver reproducible gradient flow-rates of 100 μl/min, and when coupled with a

stream splitter this flow-rate can be reduced to 2–5 μl/min. The injector and detector are configured so that the total delay volume of the system is approximately 15 μl. Typical sample volumes that can be injected without loss of separation efficiency are 0.5–5.0 μl, although larger volumes can be loaded by repeated injection if the sample can be concentrated at the head of the column, e.g. a hydrophobic sample on a reversed-phase column. Little band broadening is observed with the capillary detector and excellent sensitivities down to the femtomole range are obtained. Typical peak volumes are in the range of 1–5 μl, which is convenient for subsequent micro manipulations such as enzyme digestion or infusion into an electrospray mass spectroscopy.

A capillary reversed-phase HPLC gradient analysis of a rhGH standard is shown in Fig. 2. The separation conditions used for this analysis can resolve closely related variants such as aggregated material, norleucine variants, proteolytically clipped and oxidized methionine variants [12–14]. The analysis showed the absence of rhGH variants, which is consistent with previous studies using this purified material [12]. However the capillary analysis was performed using a 5-ng (230 fmol) sample, whereas the previous separations used 10–100 μg. The use of reversed-phase HPLC is particularly valuable in the capillary HPLC configuration since components can be readily concentrated at the top of the reversed-phase column. In this example the sample was 5 ng of rhGH in a volume of 5 μl which corresponds to a concentration of $4.6 \cdot 10^{-8}$ *M*. Since the sample is concentrated at the head of the column before elution, the sample volume can be increased to 50 μl, and at this concentration, nanomolar sensitivity can be achieved. Sample loading at these low flow-rates can become a lengthy process (up to 20 min), however, by using porous packing materials, e.g. PLRPS 4000 reversed-phase, 4000 Å pore-size (Polymer Laboratories), that allow higher flow-rates, this process may be sped up significantly. A sample may be loaded at a high flow-rate and then, in order to regain the high sensitivity of the capillary system, the flow-rate can be reduced for elution. A nanomolar detection limit achieved with a commercially available UV absorbance detector combined with a capillary Z flow-cell presents an excellent result, especially when one considers that this is equal to the detection limit of most capillary electrophoresis systems and is also at the limit of detection for conventional electrospray mass spectrometry [15,16].

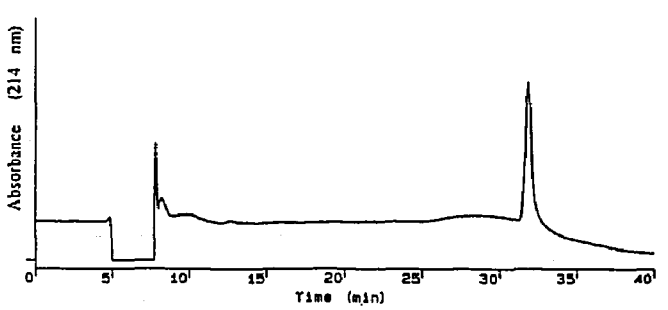

Fig. 2. Capillary RP-HPLC analysis of rhGH (5 ng) on a Vydac RPC4 column (10 cm × 320 μm I.D.). Solvent A was 0.1% TFA and solvent B was 0.09% TFA in acetonitrile. The sample was loaded at 40% solvent B. After a 10-min hold a linear gradient was run to 60% solvent B over 10 min. The flow-rate was 3.5 μl/min.

Two clipped variants of growth hormone are two-chain rhGH and desPhe^{1}Pro^2-rhGH. The two-chain variant is formed during the manufacturing process by a single proteolytic clip between tyrosine 142 and threonine 143, while a chemical degradation process, resulting in the loss of two N-terminal residues, phenylalanine and proline, forms the desPhe^{1}Pro^2-rhGH variant. Fig. 3A is a chromatogram showing the resolution of two-chain rhGH, desPhe^{1}Pro^2-rhGH and intact rhGH performed on a 4.6 mm I.D. column with a flow-rate of 0.5 ml/min [11]. Using the same packing material and mobile phase system in a capillary format, similar resolution was obtained, however with a 100-fold increase in sensitivity (Fig. 3B, upper profile). The lower profile shows the analysis of rhGH (0.1 μg) isolated from a rat blood sample [17]. This result demonstrates that capillary HPLC is useful for the analysis of protein metabolites which can normally only be obtained in small amounts after purification from in vivo systems. Small variations in retention times (0–30 s) were observed between analyses, which in part could

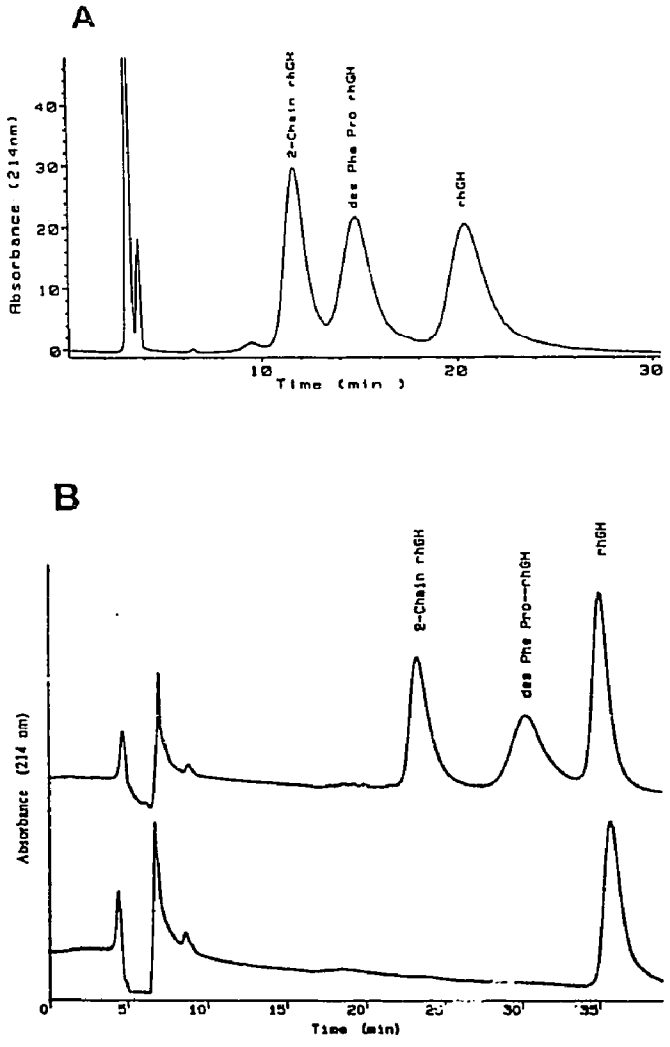

Fig. 3. (A) Reversed-phase HPLC analysis of rhGH and variants (10 μg) performed on a PLRP-S 4000 Å pore size, 15 cm × 4.6 mm I.D. column with a flow-rate of 0.5 ml/min. Solvent A was 50 m*M* potassium phosphate, pH 7.5 and solvent B was acetonitrile. The separation was performed isocratically at 45% solvent B and at a column temperature of 50°C. (B) Neutral-pH capillary RP-HPLC analysis of rhGH and variants (0.1 μg) performed on PLRP-S 4000 Å pore size, 10 cm × 320 μm I.D. column, using 50 m*M* potassium phosphate, pH 7.5 as solvent A and acetonitrile as solvent B (upper profile). The lower profile is a similar analysis of rhGH recovered from rat serum. The flow-rate was 3.5 μl/min and the gradient is described in the Experimental section.

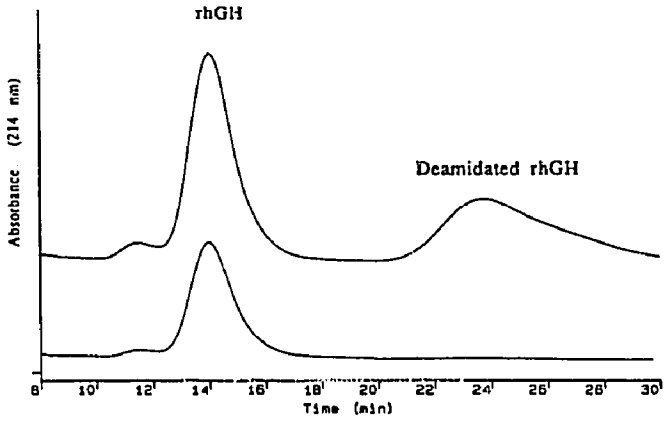

Fig. 4. Capillary ion-exchange separation of rhGH and deamidated rhGH performed on a TSK-gel DEAE 5-PW, 10 cm × 320 μm I.D. column, using 33 m*M* potassium phosphate, pH 6.5 with the addition of 20% acetonitrile as the mobile phase. The separation was performed isocratically with a flow-rate of 3.5 μl/min. The lower profile is a similar analysis of rhGH recovered from rat serum.

be attributed to the absence of column temperature control.

The ion-exchange analysis of a rhGH mutant (containing an aspartic acid substitution for asparagine, residue 149, and thus simulating deamidated rhGH), is shown in Fig. 4. The resolution is comparable to that obtained using a 4.6 mm I.D. column operating at a flow-rate of 1 ml/min (data not shown). The upper profile shows a baseline separation of the rhGH deamidated variant (more acidic peak eluting at 24 min) from rhGH (elution time, 14 min). The lower profile shows a similar analysis of rhGH isolated from rat serum [17]. The small peak eluting prior to the main peak in both profiles was also present in a blank run (data not shown).

A preliminary identification of a particular variant may be made by observing its chromatographic behavior in different systems, for example, deamidated rhGH has been observed to be unresolved in a low-pH RP-HPLC system, but emerges as a pre-peak in a basic-pH RP-HPLC system and as a post-peak in anion-exchange chromatography [18,19]. Positive identification of such a variant, however, requires that a secondary method such as peptide mapping and/or mass spectrometry be performed.

Fig. 5 shows the capillary separation (flow-rate, 3.5 μl/min) of peptides generated by

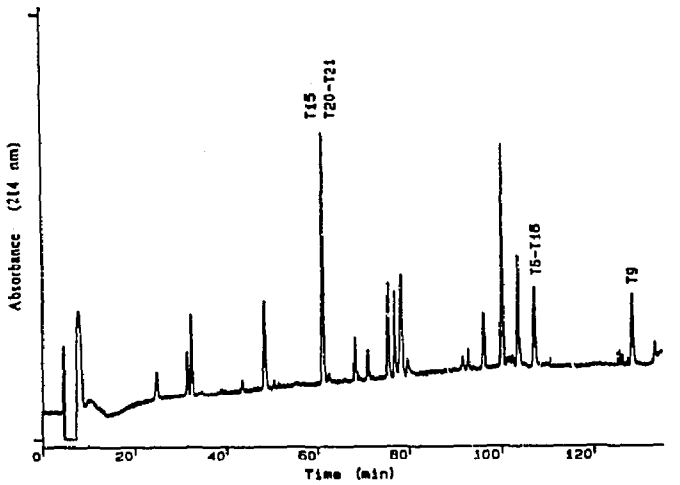

Fig. 5. Capillary peptide mapping of rhGH (90 ng) resolved on a Nucleosil C_{18} column (15 cm × 320 μm I.D.). Solvent A was 0.1% TFA and solvent B was 0.08% TFA in acetonitrile. The peptide mixture was loaded at 100% solvent A. The gradient was 0.3%/min to 40% solvent B, followed by a 2%/min gradient to 60% solvent B. The flow-rate was 3.5 μl/min.

trypsin digestion of rhGH. A similar RP-HPLC result using a 4.6 mm I.D. column and a flow-rate of 1 ml/min has been published elsewhere [18].

The primary structure of rhGH and the numbering of the tryptic peptides have been published previously [18]. This study was carried out with 90 ng (4.1 pmol) of material and achieved baseline resolution of all but one pair of tryptic peptides (T15 and T20–T21, retention time 61 min). With further optimization of the separation parameters, e.g. flow-rate and temperature, resolution of all tryptic fragments could be expected. The elution order of the peptides (identified using mass spectrometry) was the same as previously observed and the signal-to-noise ratio was acceptable, despite using only one thousandth the amount of sample usually used [18].

A problem typically observed in the analysis of sub-microgram amounts of sample is the low

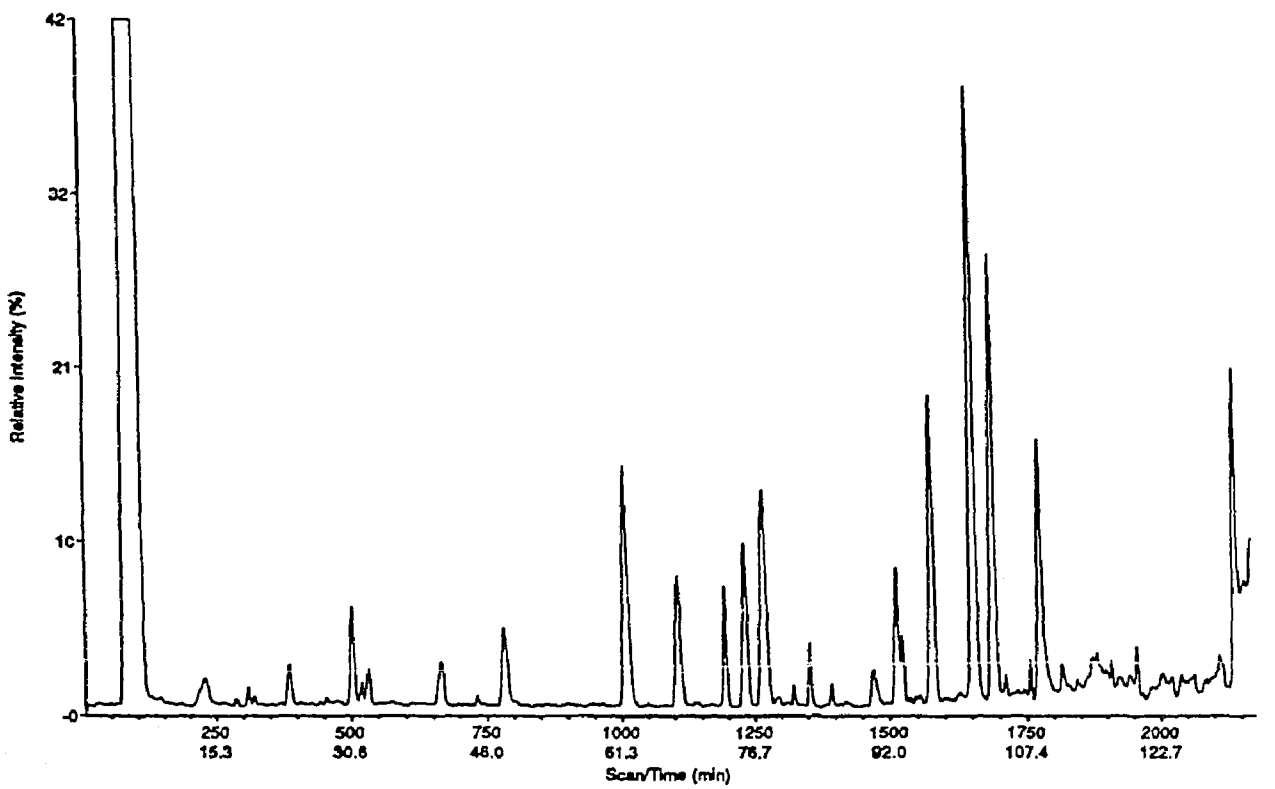

Fig. 6. Total-ion current profile of trypsin digested rhGH produced by capillary LC–MS. The tryptic peptides were first resolved using conditions as given in Fig. 5 before mass analysis.

recovery of hydrophobic peptides. When the capillary tryptic map is compared with a conventional map (data not shown), low recoveries of the peptides eluting at approximately 108 and 128 min are observed in the capillary separation. These peptides were identified as T6–T16 and T9 which are known to give recovery problems even under some standard analytical conditions [20]. Modifications of the chromatographic conditions which may reduce the severity of this problem, e.g. the use of detergents, are currently under study.

The capillary tryptic map shown in Fig. 5 shows a significant improvement in the state-of-the-art for analytical biotechnology. In a typical analytical format, using 4.6 mm I.D. columns, 50 μg or 500-fold more material is usually the minimum amount used to perform a mapping experiment.

The total-ion current produced as a result of coupling capillary peptide mapping with electrospray mass spectrometry (in stead of UV detection) is shown in Fig. 6. It can be seen that the profile of the total-ion current is similar to the UV absorbance profile shown in Fig. 5. Mass data were obtained for all expected tryptic fragments and excellent agreement between observed and theoretical masses were observed

Table 1
Capillary LC–MS data obtained from the trypsin digest of rhGH

Amino acid residue		Tryptic peptide	Expected mass (Da)	Observed mass (Da)	Delta from expected (Da)	Retention time (min)	Peptide amino acid sequence
from	to						
1	8	T1	930.1	929.5	0.6	76.4	FPTIPLSR
9	16	T2	979.2	978.5	0.7	74.2	LFDNAMLR
17	19	T3	382.4	382.7	−0.3	8.5	AHR
20	38	T4	2342.6	2342.9	−0.3	99.4	LHQLAFDTYQEFEEAYIPK
39	41	T5	403.4	403.7	−0.3	6.0	EQK
42	64	T6–T16	3763.2	3763.2	0.0	106.7	YSFLQNPQTSLCFSESIPTTPSNR
159	167	disulfide					NYGLLYCFR
65	70	T7	761.8	761.4	0.4	13.5	EETQQK
71	77	T8	844.0	843.5	0.5	66.7	SNLELLR
78	94	T9	2055.5	2055.2	0.3	131.6	ISLLLIQSSWLEPVQFLR
95	115	T10	2262.5	2262.6	−0.1	101.7	SVFANSLVYGASDSNVYDLLK
116	127	T11	1361.5	1360.7	0.8	94.9	DLEEGIQTLMGR
128	134	T12	772.8	772.4	0.4	30.2	LEDGSPR
135	140	T13	692.8	692.4	0.4	47.1	TGQIFK
141	145	T14	625.7	625.3	0.4	23.1	QTYSK
146	158	T15	1489.6	1488.8	0.8	60.8	FDTNSHNDDALLK
168	178	T17–T18–T19	1381.7	1380.8	0.9	72.0	KDMDKVETFLR
168	172	T18–T19	1253.6	1253.2	0.4	76.4	DMDKVETFLR
179	183	T20–T21	1400.6	1399.8	0.8	60.8	IVQCR
184	191	disulfide					SVEGSCGF
141	145	T14a	607.7	607.3	0.4	27.2	pQTYSK
141	143	T14c	410.6	410.7	−0.1	28.6	QTY
95	99	T10c1	536.3	536.3	0.0	40.0	SVFAN
95	103	T10c2	1742.9	1743.0	−0.1	91.1	SVFANSLVY
1	6	T1c	686.4	686.4	0.0	88.6	FPTIPL

(Table 1). The advantage of on-line mass spectrometry is that the mass data greatly aids in the identification of peptides as well as in indicating any coeluting peptides. The peptides expected to coelute in the capillary tryptic map (T20–T21 and T15, as indicated by UV absorption) were confirmed by mass spectrometry. Using ion reconstruction to plot the elution positions of peptides with masses corresponding to tryptic fragments T20–T21 and T15 we observed the same scan numbers for both fragments, confirming the coelution of this pair (mass data not shown) [21].

4. Conclusions

This study has shown that capillary HPLC can separate intact rhGH variants as well as tryptic fragments at the femtomole level, resulting in a 100-fold increase in sensitivity. Such separations can be readily achieved with commercially available instrumentation and columns. We have demonstrated capillary HPLC separations using both reversed-phase and ion-exchange modes. We have also shown that capillary HPLC coupled on-line with electrospray mass spectrometry can give femtomole sensitivity. The application of capillary HPLC will have a significant impact on analytical biotechnology.

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