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High-performance displacement chromatography–mass spectrometry of tryptic peptides of recombinant human growth hormone

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ABSTRACT

The combination of high-performance displacement chromatography with continuous flow fast atom bombardment (FAB)-mass spectrometry (MS) offers a means of overcoming the sample capacity limitations imposed by the low flow-rates tolerated in microbore systems employed for directly coupled liquid chromatography–MS. Displacement chromatography is performed at high concentrations with the same equipment and columns as typically used in chromatography at low concentrations. By using this mode of chromatography with a solution of cetyltrimethylammonium bromide as the displacer, the capacity of a reversed-phase column can be increased 50- to 100-fold for separation of a tryptic digest of biosynthetic human growth hormone. Despite the high load, the use of displacement chromatography allowed high-resolution separation of the complex mixture of eighteen major components. On-line analysis by continuous flow FAB-MS yielded high-quality spectra of these peptides and demonstrated that sharp, single-component bands can be obtained in this separation. Along with the major fragments, the chromatogram showed other peptides originating from protein variants in the sample, from non-specific cleavage in the enzymatic digest or from autolysis of trypsin. On-line analysis also allowed selective ion monitoring of the column effluent for individual peptides and confirmed the high efficiency and resolution obtained by preparative displacement separations on HPLC columns and equipment.

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INTRODUCTION

Continuous growth over the last decade of the segment of the pharmaceutical industry based on the commercialization of therapeutic proteins produced by recombinant DNA technology has spawned other important scientific disciplines, including that of analytical biotechnology. That field has been developed to characterize the purity and identity of recombinant proteins, both as a guide to optimization of manufacturing and recovery processes and to satisfy the requirements for marketing approval by regulatory agencies. Recombinant proteins pose new and particular analytical challenges not encountered in the production of small-molecule drugs or proteins isolated from animal or human tissues. Over the last ten years, the demands of protein characterization have led to continual refinement of the tools of protein chemistry, as well as to the introduction of powerful new techniques.

One of the most useful approaches to characterization is the analysis by high-performance liquid chromatography (HPLC) of the peptide mixture produced by enzymatic digestion of a protein[1]. The specificity conferred by selection of the appropriate enzyme together with the high efficiency and selectivity of modern HPLC united in this approach to yield a sensitive, reproducible method of determining the identity and, to a certain extent, the purity of a protein. Reversed-phase HPLC is the separation system of choice in this application, since it delivers high resolution of such moderately sized analytes, and, with suitable mobile phases, offers the possibility of isolating purified individual peptides for subsequent characterization by amino acid composition analysis or sequencing, or by mass spectrometry (MS). By this strategy nearly the entire sequence of a protein can be confirmed, as has been demonstrated by many recent examples[2–4], including that of recombinant human growth hormone[5].

Fast atom bombardment (FAB) ionization[6] has become the method of choice for MS of peptides. Peptides isolated by reversed-phase HPLC with volatile mobile phases are eminently suited for transfer to the FAB matrix and deposition onto the probe tip for analysis. By this procedure highly accurate, sensitive molecular weight determinations and sequence information can be obtained for peptides up to 10 000 dalton in size. The analysis and characterization of complex mixtures can be made more efficient by coupling HPLC directly with MS[7,8]. Such an on-line analysis minimizes sample handling losses and reduces the time required for analysis of the entire mixture to little more than the time for the chromatographic separation. One constraint imposed on the HPLC in continuous FAB applications is the limitation on flow-rates due to the solvent removal capacity of the vacuum pumps in the mass spectrometer. In practice, the eluent flow is split either before[9] or after[10] the column in order to attain flow-rates in the range of 10 μl/min or less. Pre-column splitting requires the use of capillary columns and has the advantage that the entire sample passing through the column is introduced into the mass spectrometer.

The chief limitation of current LC–MS interfaces is due to the relatively low flow-rates tolerated by conventional vacuum systems. The restriction on flow-rate also effectively limits the mass load of the mass spectrometer, since the concentration of individual peptides in the traditional elution mode is governed by the sample concentration and by the dilution characteristics of the chromatographic system employed. One way to enhance the capacity of the system is to select an operating mode that results in increased concentration of the peptides in the column effluent and

more efficient utilization of the column capacity. High-performance displacement chromatography (HPDC)[11] meets certain of these requirements. Displacement chromatography (DC) was developed by Tiselius[12] as a means of efficiently resolving complex mixtures at high concentrations in order to facilitate detection by optical detectors. Although subsequently rendered obsolete for most analytical applications by the development of more sensitive detectors and the dominance of linear elution chromatography, DC carried out in columns and equipment developed for HPLC has enjoyed a renaissance recently as a preparative technique[13–22]. The displacement mode allows high-resolution separations to be carried out at concentrations and column loads that are one or two orders of magnitude higher than in conventional gradient or isocratic elution. The increased capacity afforded by the displacement mode is achieved by employing a displacer solution to mobilize the feed components after they are loaded into the column. Unlike the eluents employed in conventional elution chromatography the displacer solution contains a component that is more strongly retained than the feed components. The displacer thus saturates the stationary phase surface, displacing the feed mixture and causing the individual components to move down the column at a constant velocity and to separate into adjacent single-component bands. The mathematical description of the separation process in displacement chromatography is more involved than for the elution mode[23], but operationally the process is no more complex once the separation conditions have been established.

HPDC may offer significant advantages for micro-analytical techniques, such as capillary LC–MS, since it increases the amount of a mixture that can be separated on a given column, and thus, that can be analyzed by the mass spectrometer. In an analogous fashion, the advantages of isotachophoresis as the operating mode for capillary electrophoresis–MS have been described[24]. By increasing the sample load of the instrument, the detectability of minor components of the sample can be enhanced. The displacement of tryptic peptides of recombinant human growth hormone has been demonstrated[25] on columns and equipment identical to that employed for the gradient elution tryptic mapping procedure[5]. In this example, the displacement mode allowed an approximately 50-fold increase in the capacity of the reversed-phase column for preparative isolation of tryptic peptides. DC thus allowed the purification and recovery of relatively large amounts of minor components of the enzymatic digest. This paper describes the extension of those studies to DC–MS in a capillary HPLC system.

EXPERIMENTAL

Materials

Trifluororacetic acid (TFA) was from Applied Biosystems (Foster City, CA, U.S.A.). Recombinant methionyl human growth hormone (met-hGH) was produced as described previously[26]. L-1-Tosylamide-2-phenylethyl chloromethyl 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.). Spectrometric-grade glycerol, hydrochloric acid and cetyltrimethylammonium bromide (cetrimide) were from Aldrich (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile (ACN) was from Burdick & Jackson (Muskegon, MI, U.S.A.). Fused-

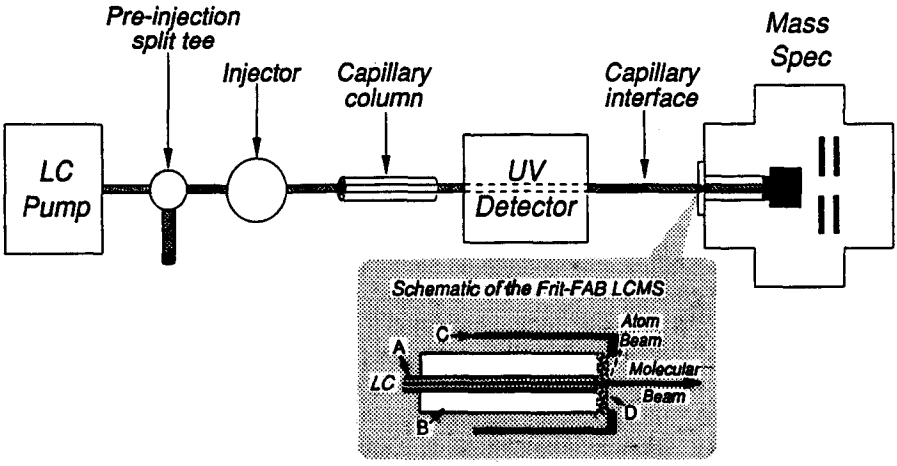

Fig. 1. Schematic diagram of the continuous flow FAB-LC–MS. The effluent from the dual syringe pump is split ahead of the injector so that 5% of the mobile phase delivered by the pump flows through the column. The UV absorbance detector and mass spectrometer are arranged in series to monitor the composition of the column effluent. Inset: Detail of the Frit-FAB target: (A) fused-silica capillary, (B) support guide, (C) cap and (D) stainless-steel frit. As the column effluent emerges from the frit, the volatile solvent components evaporate and the glycerol matrix spreads annularly towards the edge of the frit, exposing peptides to the atom beam. Reprinted with permission from ref. 9.

silica capillary tubing was obtained from Polymicro Technologies (Tucson, AZ, U.S.A.).

Instrumentation

Conventional HPLC. The conventional analytical-scale tryptic map of met-hGH was produced with a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1050 HPLC system with data analysis by a Nelson Analytical (Cupertino, CA, U.S.A.) Model 6000 software package. The reversed-phase tryptic map employed a 150 × 4.6 mm I.D. Nucleosil C_{18} column obtained from Alltech Assoc. (Deerfield, IL, U.S.A.), heated in a Flatron Laboratory Systems (Oconomowoc, WI, U.S.A.) Model CH-30 column heater.

Micro-bore HPLC. The capillary-column HPLC system is shown schematically in Fig. 1. As described previously[9], the instrument was assembled from a Brownlee (Sunnyvale, CA, U.S.A.) Model 120 dual-syringe pump and Model 783 UV absorbance detector and Rheodyne (Berkeley, CA, U.S.A.) Model 8125 injector, directly coupled to the 150 × 0.32 mm I.D. capillary column (LC Packings, Zürich, Switzerland) packed with 3-μm RP-18 silica. The pump was operated at 100 μl/min and the eluent was split before the injector to deliver approximately 5 μl/min to the column. The outlet of the column was connected to a length of 50-μm I.D. fused-silica capillary. For detection, a downstream segment of the capillary was stripped of its polyimide coating and secured in a focusing support within the detector.

LC–MS interface. Capillary tubing exiting the detector was connected to a JEOL USA (Peabody, MA, U.S.A.) Frit-FAB probe, shown schematically in the insert to Fig. 1, and consisting of a 50-μm I.D. fused-silica tubing (A) passing through an insulated support (B) and pressed against a 0.25-mm-thick sintered stainless-steel frit

(D). The column effluent passed through the frit and was bombarded with a xenon atom beam at 6 keV in the mass spectrometer source at a temperature of *ca.* 50°C.

Mass spectrometer. Mass spectra were acquired with a JEOL HX110/110 tandem mass spectrometer at a resolution of 3000 and an accelerating voltage of 10 kV. Spectra were acquired at a rate of 12 s per scan, with a mass range of 500 to 4000 mass units (m.u.).

Methods

Tryptic digest. Tryptic digestion was carried out by reconstituting 10 mg of met-hGH in 10 ml of 100 m*M* tris–acetate buffer (pH 8.3), warming the sample to 37°C, and adding 100 μg of trypsin. After 2 h, a second aliquot of 100 μg of trypsin was added. Digestion was stopped at the end of 4 h by lowering the pH of the solution below 3 with 1 *M* hydrochloric acid. The digest mixture was stored at 5°C until analyzed.

Conventional tryptic map. The tryptic map with the conventional analytical system was produced by injecting 200 μl of the digest mixture into the Nucleosil C_{18} column, equilibrated at 35°C with 0.12% aqueous TFA at a flow-rate of 1 ml/min. After a 5-min hold, a linear gradient to 38% ACN over 60 min was started. At the end of the gradient, the ACN content was increased to 57% over 10 min. The column effluent was monitored at 214 nm. Peaks were identified by comparison of retention times with previously characterized maps[5].

LC–MS tryptic map. The elution-mode tryptic digest was chromatographed by

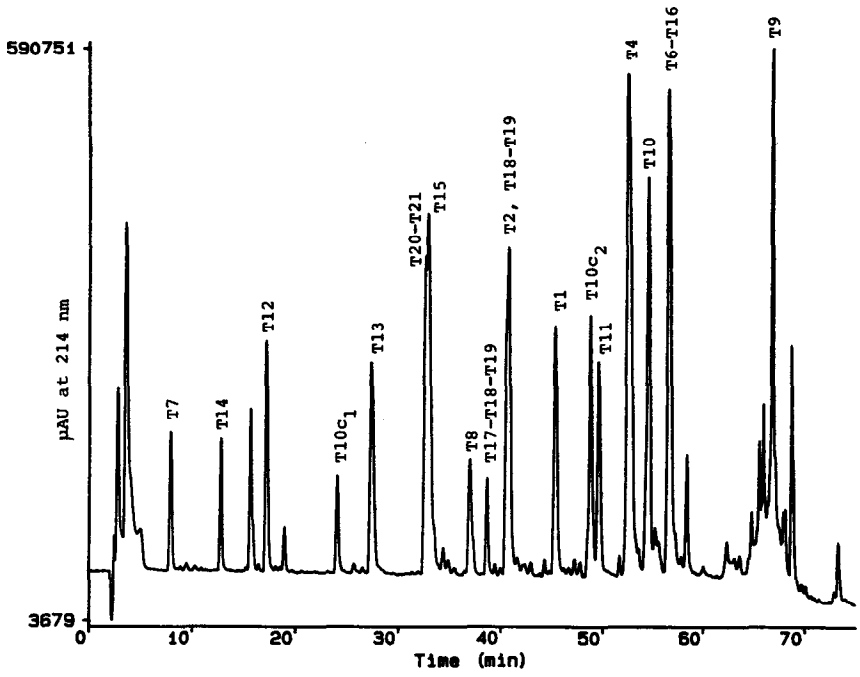

Fig. 2. Tryptic map of 200 μg of the biosynthetic human growth hormone digest mixture. Chromatographic conditions are given in the text. Peaks are labeled according to the identifiers given in Table I.

injecting 25 pmol of digested protein into the capillary column, equilibrated with a mobile phase consisting of 2% ACN, 1% glycerol and 0.1% TFA in water. After an initial 10-min hold, the gradient was started to linearly reach 60% ACN in 65 min. Detection was by absorbance at 195 nm.

DC–MS tryptic map. The displacement separation was carried out by injecting 10 nmol of the digest mixture into the capillary column, equilibrated with a mobile phase consisting of 1% glycerol and 0.1% TFA in water. After a 5-min hold, the displacer solution consisting of 2 mg/ml cetrimide, 1% glycerol and 0.1% TFA in water was pumped into the column. The switch from starting eluent to displacer solution occurred within a 1-min period. The absorbance detector monitored the column effluent at 195 nm. Data collection by the mass spectrometer commenced 150 min after flow of the displacer solution was started.

RESULTS AND DISCUSSION

The chromatogram shown in Fig. 2 represents a typical analysis of the tryptic

TABLE I

PEPTIDES PRODUCED BY DIGESTION OF METHIONYL HUMAN GROWTH HORMONE WITH TRYPSIN

$[M + H]^+$ is the calculated molecular weight for the most abundant monoisotopic species. Single-letter code for amino acids used.

Identifier	*Residues*	*$[M + H]^+$*	*Sequence*
T1	1–9	1061.58	MFPTIPLSR
T2	10–17	979.50	LFDNAMLR
T3	18–20	383.21	AHR
T4	21–39	2342.14	LHQLAFDTYQEFEEAYIPK
T5	40–42	404.22	EQK
T6[a]	43–65	2616.24	YSFLQNPQTSLCFSESIPTPSNR
T7	66–71	762.36	EETQQK
T8	72–78	844.49	SNLELLR
T9	79–95	2055.20	ISLLLIQSWLEPVQFLR
T10	96–116	2262.13	SVFANSLVYGASDSNVYDLLK
T11	117–128	1361.67	DLEEGIQTLMGR
T12	129–135	773.38	LEDGSPR
T13	136–141	693.39	TGQIFK
T14	142–146	626.32	QTYSK
T15	147–159	1489.69	FDTNSHNDDALLK
T16[a]	160–168	1148.55	NYGLLYCFR
T17	169	147.11	K
T18–T19	170–179	1253.62	DMDKVETFLR
T20[b]	180–184	618.34	IVQCR
T21[b]	185–192	785.31	SVEGSCGF
Non-tryptic cleavages			
$T10c_1$	96–100	537.27	SVFAN
$T10c_2$	100–116	1743.90	LWGASDSNVYDLLK
T17–T18–T19	169–179	1381.71	KDMDKVETFLR

[a] T6 and T16 are disulfide-linked, with a total $[M + H]^+$ of 3762.8.

[b] T20 and T21 are disulfide-linked, with a total $[M + H]^+$ of 1401.4.

digest of met-hGH obtained using the conventional-scale HPLC mapping procedure. The identity, amino acid sequence, position in the intact protein and $[M + H]^+$ of the predominant peptides in the map are given in Table I. The high resolution afforded by reversed-phase HPLC allows collection of individual peaks so that the identity of the major peaks can be determined by subsequent analysis. The identification of minor peaks is more problematic, however, since the low abundance of these peptides often demands scaling up the separation in order to obtain a sufficient quantity for further analysis. Under the chromatographic conditions shown, resolution of individual peptides is largely independent of the column load up to about 10 nmol of digested protein. Higher loads than this seriously impair resolution of certain peptides in this chromatographic system.

In order to establish the efficacy of the displacement mode for scale-up of the separation of the tryptic digest mixture, the HPLC system employed for the chromatogram shown in Fig. 2 was run in the displacement mode with cetrimide as the displacer[25]. The displacement purification was performed without modification of the analytical instrument, using 0.1% aqueous TFA or 10 m*M* aqueous phosphoric acid as the carriers. By this procedure, up to 500 nmol of digested protein were separated on the analytical column. These results demonstrated the applicability of this approach to

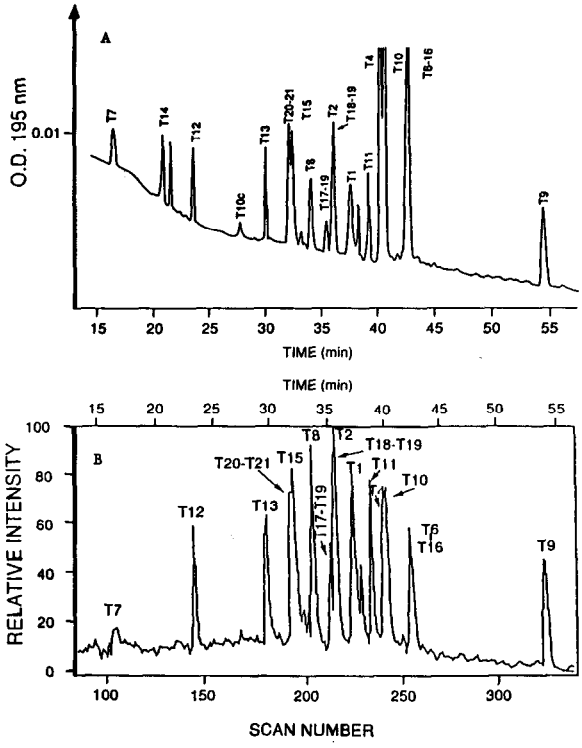

Fig. 3. Tryptic maps of 25 pmol monitored by (A) absorbance of light at 195 nm and (B) the total-ion current in the mass spectrometer. Peaks are labeled as in Fig. 2 and Table I. Experimental conditions are given in the text. Reprinted with permission from ref. 9.

the scale-up of the peptide separation, although certain of the most strongly retained hGH tryptic peptides, including T4, T10, T6–T16 and T9 were not recovered in the displacement train. Nevertheless, the remaining components of the mixture were isolated in high yield, indicating the potential of the displacement mode as an alternative in the scale-up of chromatographic purification.

As discussed above, LC–MS has an enormous potential for rapidly separating and characterizing the components of a tryptic map. The capillary LC–MS system described earlier[9] exhibits high sensitivity and resolution and, by diverting the entire column effluent into the mass spectrometer, it avoids the losses that typically accompany the handling steps associated with fraction collection and off-line FAB-MS analysis. Fig. 3 shows the total ion current (TIC) and UV absorbance chromatograms of a tryptic map of met-hGH run on the capillary system in the gradient elution mode. The small physical dimensions of the capillary LC system prevent untoward dilution of the sample components during analysis, and thereby allow high-sensitivity separations of complex mixtures, as evidenced by the analysis in Fig. 3 that was carried out on 25 pmol of digested protein. The similarity of the chromatograms in Figs. 2 and 3 despite the different provenances of the stationary phases supports the validity of the "down-scaling" of the separation to the capillary LC.

The displacement separation was also scaled down to the dimensions of the capillary LC. Fig. 4 shows the displacement chromatograms for separation of 10 nmol of digested met-hGH. The chromatogram in Fig. 4A was made by UV-absorbance detection, while that in Fig. 4B is the TIC chromatogram obtained by MS detection. Both chromatograms illustrate the "stair-case" pattern expected in displacement-mode separations. The plateau heights measured by UV absorbance of the tryptic peptides generally increase across the displacement train, as expected, since the

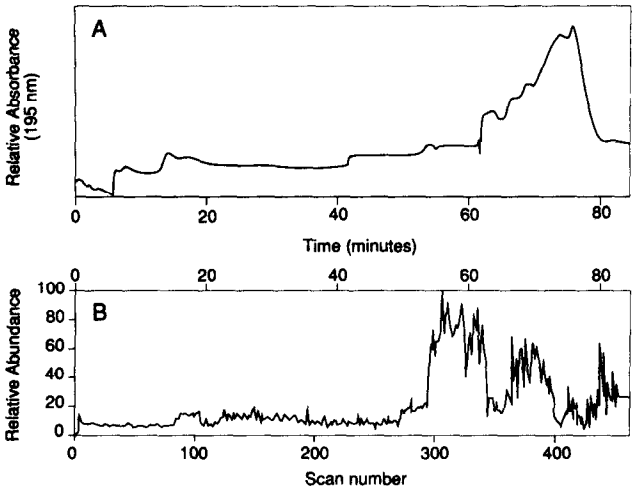

Fig. 4. Column effluent profile during displacement chromatography of 10 nmol of digested biosynthetic human growth hormone monitored by (A) absorbance of light at 195 nm and (B) the total-ion current in the mass spectrometer. Spectra were acquired in 12-s scans, and data collection commenced 150 min after the displacer solution started flowing into the column.

extinction coefficients of the peptides typically increase with increasing size. The TIC chromatogram in Fig. 4B exhibits a converse behavior, with the plateau heights decreasing in size across the displacement train. This behavior reflects the relatively poorer ionization properties of the larger, more hydrophobic peptides that is a feature of the FAB process. In addition, little ion signal was observed at the end of the displacement train, just ahead of the displacer front, so this portion of the column effluent was not analyzed in the mass spectrometer. The cause of this disruption in the

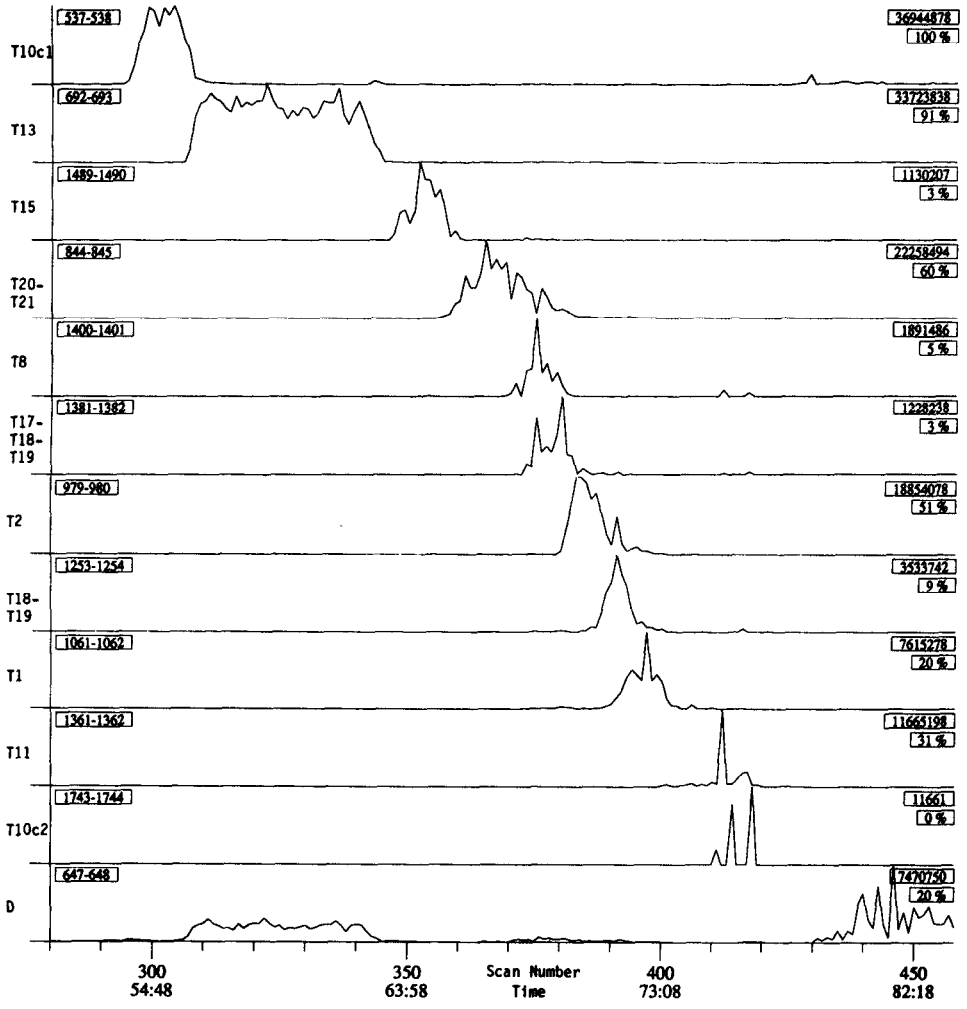

Fig. 5. Reconstructed ion-current chromatograms of the ions corresponding to the predominant peptides comprising the tryptic digest of biosynthetic hGH. The RIC chromatograms were produced by monitoring the abundance of ions within a narrow mass window measured by the mass spectrometer during the run. Each RIC thus selectively monitors a single species in the effluent. The mass range employed in the RIC and peptide identity are indicated on the left side of each chromatogram. The abundance of ions relative to that of the $T10c_1$ RIC maximum is given on the right. "D" indicates the RIC of the brominated dimer of cetyltrimethylammonium, used to monitor the displacer front.

ion current is under investigation. It may be related to the high concentrations of peptides in the glyerol at the probe tip. The TIC chromatogram also shows instability in the ion current under these conditions. Both of these untoward effects may be meliorated by optimization of the choice of ionic modifier and glycerol content in the carrier. The relatively sharp rear boundary of the UV chromatogram indicates that cetrimide, which exhibits little UV absorbance, efficiently displaces peptides under these conditions.

As has been demonstrated[13,23], interpretation of the course of the displacement separation of complex mixtures requires selective analysis of the composition along the displacement train. Such a requirement has been met previously by fraction collection and subsequent analysis of the fractions by HPLC[13,15] or thin-layer chromatography[14], or by a tandem HPLC–HPLC system[17], in which the first chromatograph performed the displacement separation and the second acted as an on-line analyzer for fast HPLC analysis of the column effluent. The results of these analyses in either case were then employed to reconstruct displacement chromatograms that defined the boundaries between components of the mixture in the displacement train. The mass spectrometer provides the opportunity to monitor the masses of peptides in the displacement train and thereby reconstruct individual species displacement chromatograms that yield the information required for interpretation of the displacement separation. Fig. 5 shows the reconstructed ion current (RIC) profiles for the predominant peptides in the tryptic digest. The RIC chromatograms demonstrate that each peptide occupies a distinct band in the displacement train that in most cases is well separated from adjacent bands. Fig. 5 also illustrates the narrowing of bands that occurs near the displacer front, where peptide concentrations are highest. The earliest-eluted peptides are at the lowest concentrations and therefore form wider bands. The more strongly retained peptides form extremely narrow, concentrated bands and, in the cases of T11 and $T10c_2$, the band is little more than one scan wide in the RIC chromatograms. The most hydrophobic peptides, as noted above, were not identified in the displacement train, owing to the interruption in ion current in the latter part of the displacement train. The displacer was monitored by the mass at 647.5 m.u., which corresponds to a brominated cetrimide dimer, since the cetrimide monomer ($[M + H]^+ = 285$) was below the mass range scanned in this experiment. The displacer ion appeared upon resumption of the ion current, evidently after the disturbance at the probe tip had been removed. All of the peptides eluted before T4 in the tryptic map shown in Fig. 2 were observed in the displacement train, although certain pairs of peptides reversed their elution order at the high concentrations employed in this mode of chromatography. Thus, T20–T21 is eluted before T15 in the chromatogram of Fig. 2, but these peptides exit the column in the reversed order in the displacement train of Fig. 5. This reversal of affinity for the stationary phase at high concentrations has previously been observed in the measurement of adsorption isotherms of proteins[27,28], but has not been reported for molecules of the size employed here.

The mass range from 500 to 4000 m.u. was scanned every 12 s, providing a "snapshot" of the composition of the column effluent at 12-s intervals. Subsequent scans can be compared to provide insight into the dynamics of the displacement process, in a manner analogous to the tandem HPLC–HPLC arrangement described previously[17]. Fig. 6 shows sequential mass spectra, starting with scan 305, which was

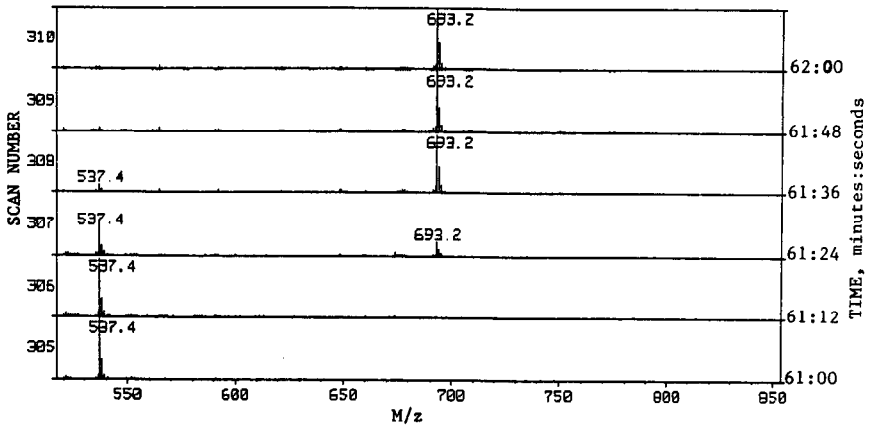

Fig. 6. Mass spectra acquired as scans 305–310 during the displacement separation. The time corresponding to the start of each scan is indicated on the right side of the figure and the scan number on the left.

started at 61 min. The dominant ion in scan 305 has a molecular mass of 537.4 m.u. and corresponds to residues 96–99 in met-hGH. This peptide arises from a chymotryptic-like clip in the T10 peptide, has been observed previously[2], and is dubbed the "T10c_1" peptide. Scan 306 is also dominated by the T10c_1 peptide, while scans 307 and 308 represent the boundary between the zone of this peptide and the next in the displacement train, an ion with a mass of 693.2 m.u. This peptide corresponds to the T13 fragment of the tryptic digest of met-hGH, as seen in Table I. Fig. 6 thus shows the small extent of overlap between bands in displacement chromatography, *i.e.*, less than two scans or 24 s wide. Few on-line or off-line analytical techniques can monitor the effluent compositon at higher scan rates than mass spectrometry, demonstrating the power of this approach in studies of the displacement process.

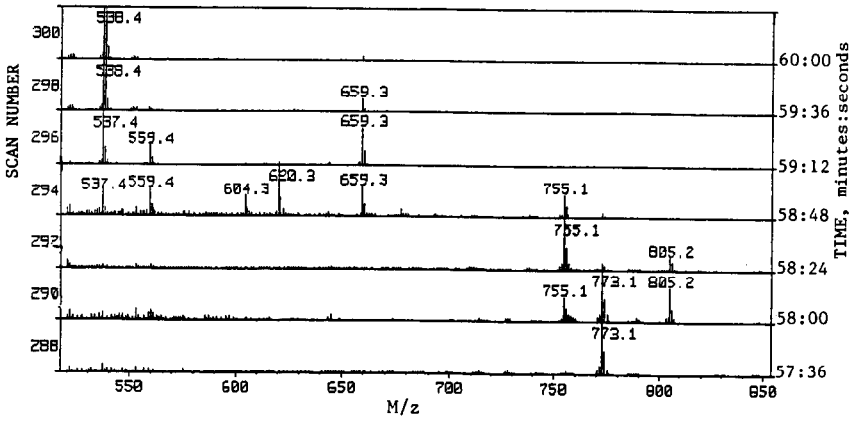

Fig. 7. Mass spectra acquired during the displacement separation by summation of pairs of scans. The spectrum labeled 288 is thus the sum of scans 288 and 289. The time corresponding to the start of the scan is indicated on the right side of the figure and the beginning scan number on the left.

In contrast to the clean transition from T10c$_1$ to T13 observed in Fig. 6, the boundary at the front of the T10c$_1$ band, which marks the transition from T12 to T10c$_1$, contained several small fragment ions, as shown in Fig. 7. The serial spectra shown in Fig. 7 illustrate that minor components of the digest, such as the 559.4 ion, which can be assigned to a non-specific cleavage of the T15 peptide, concentrate between the bands of the predominant peptides and yield intense ions in individual scans that unambiguously indicate their presence and allow precise determination of their mass. This concentrating effect illustrates the power of the displacement mode for accumulation and characterization of minor components of a complex mixture. The other ions identified in Fig. 7 are included, with their assignments, in Table II. The relative amounts of individual species in the digest mixture could be estimated roughly from the width of the zones occupied in the displacement train, as indicated by Tiselius[12]. Optimization of the FAB process may allow more accurate quantification of the peptides in the displacement train, as has been reported for other applications[8].

The dominant peptides shown in Figs. 2 and 5 include both expected tryptic cleavages, *i.e.*, peptides containing terminal arginine and lysine residues, along with the expected incomplete cleavages around T18 and the non-specific cleavage in T10. Other

TABLE II

MOLECULAR IONS OBSERVED IN DISPLACEMENT CHROMATOGRAPHY–MASS SPECTROMETRY OF METHIONYL HUMAN GROWTH HORMONE

Sequence assignments were made by comparison of the observed mass with that calculated for the most abundant isotopes of each peptide.

Observed [M + H]$^+$	*Calculated [M + H]$^+$*	*Residues*	*Sequence*
609.3	609.7	102–107	LVYGAS
773.1	773.4	129–135	LEDGSPR
805.2	804.5	98–105	trypsin fragment T7
755.1	755.3	106–112	ASDSNVY
604.3	604.3	13–17	NAMLR
620.4	620.3	13–17	NAMLR (sulfoxide)
559.4	559.4	155–159	DALLK
659.3	659.3	48–53	NPQTSL
537.4	537.3	96–100	SVFAN
693.2	693.4	136–141	TGQIFK
707.2	?	?	?
1012.1	1012.5	151–159	SHNDDALK
892.3	892.3	173–179	KVETFLR
764.2	764.4	174–179	VETFLR
1489.8	1489.7	147–159	FDTNSHNDDALLK
844.2	844.5	72–78	SNLELLR
618.3	618.3	180–184	IVQCR
785.1	785.3	185–192	SVEGSCGF
1401.4	1401.4	180–192	IVQCR-SVEGSCGF
1381.4	1381.7	169–179	KDMDKVETLR
979.3	979.5	10–17	LFDNAMLR
1253.3	1253.6	170–179	DMDKVETLR
1061.3	1061.6	1–9	MFPTIPLSR
1361.5	1361.7	117–128	DLEEGIQTLMGR
818.2	818.6	1–7	MFPTIPL

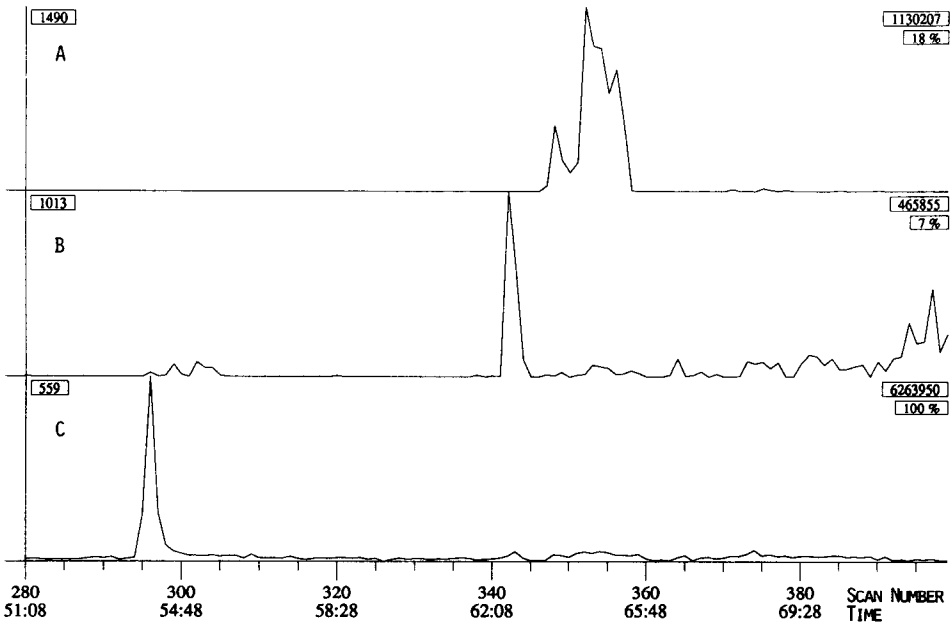

Fig. 8. (A) RIC chromatogram of T15 (residues 147–159, $[M + H]^+ = 1489.7$ m.u.), along with the chromatograms of two fragments formed by non-specific cleavage of T15 during digestion. The fragments correspond to residues (B) 151–159 ($[M + H]^+ = 1012.5$) and (C) 155–159 ($[M + H]^+ = 559.4$). The individual chromatograms are all normalized to the same scale. The relative abundance of ions is given alongside each chromatogram.

non-specific cleavages occurring during the digest were also observed as indicated above. Fig. 8 shows the RIC chromatogram of T15 (residues 147–159, predicted $[M + H]^+ = 1489.7$) in the displacement train, together with two other peptides that formed very narrow bands at earlier positions within the train. A mass spectral analysis program developed in-house at Genentech assigned the observed molecular weights of these peptides to the sequences comprising residues 151–159 (predicted $[M + H]^+ = 1012.5$) and 155–159 (predicted $[M + H]^+ = 559.4$). Thus, the two peptides arise from a non-tryptic cleavage of the T15 peptide. The relatively high abundance of these ions within their narrow bands in Fig. 8 further demonstrates the utility of displacement chromatography for concentrating minor components of a complex mixture in order to obtain a high-quality signal in on-line mass-spectrometry. Many ions were observed in the displacement separation that were assigned to other non-specific cleavages in the digest, and are summarized in Table II. Certain of these peptides have not been reported previously, owing to their relatively low abundance in the mixture that hampers their collection and characterization by conventional approaches.

Aside from non-specific cleavage arising during the enzymatic digestion, fragmentation of ions can also occur during bombardment in the continuous-flow FAB interface. Fig. 9A shows the RIC chromatograms of the T20–T21 peptide ($[M + H]^+ = 1401.4$), which contains the T20 and T21 peptides linked by a disulfide bond. The individual RIC chromatograms of T20 ($[M + H]^+ = 618.3$) and T21

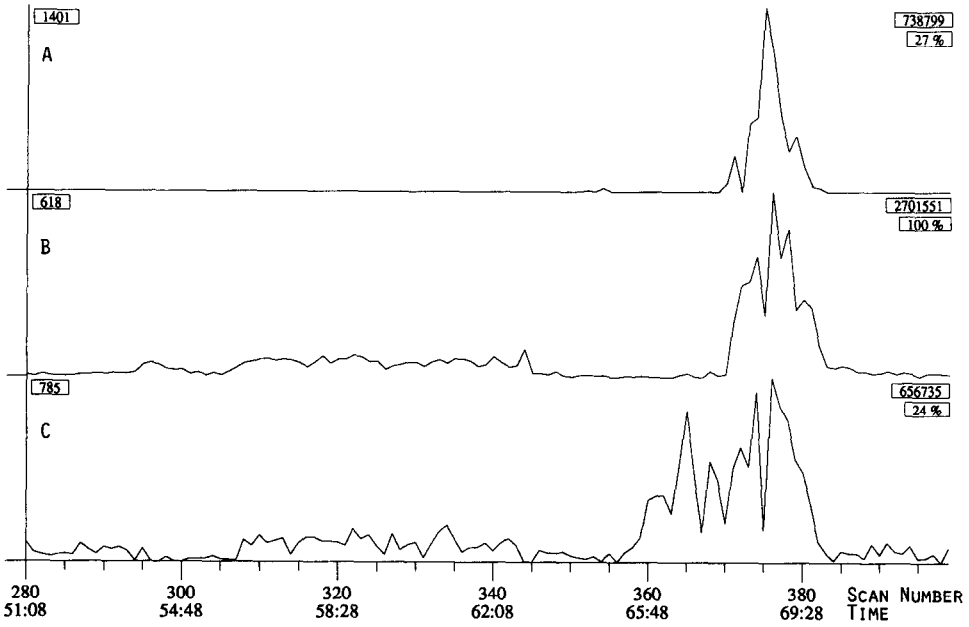

Fig. 9. (A) RIC chromatogram of T20–T21 ([M + H]$^+$ = 1401.4 m.u.), along with the chromatograms of the two fragments formed by breakage of the disulfide bond contained in this peptide. The fragments correspond individually to (B) T20 ([M + H]$^+$ = 618.3 m.u) and (C) T21 ([M + H]$^+$ = 785.1 m.u.). The individual chromatograms are all normalized to the same scale. The relative abundance of ions is given alongside each chromatogram.

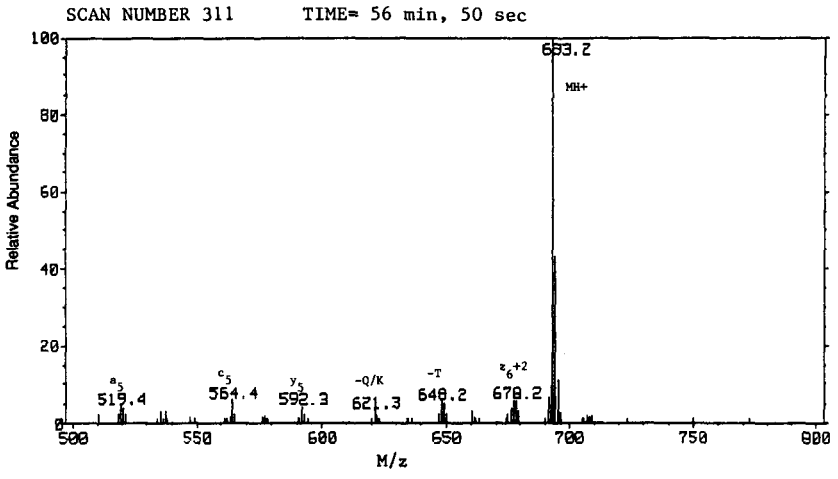

Fig. 10. Mass spectrum acquired at scan 311 during displacement chromatography. The dominant ion corresponds to T13, and smaller ions to fragments generated during xenon fast-atom bombardment on the probe. The assignment of daughter ions is indicated by the standard nomenclature[30].

($[M + H]^+ = 785.1$) are shown in Fig. 9B and C, respectively. The disulfide bond in T20–T21, like that in the other disulfide linked peptide, T6–T16, is relatively labile, so ions corresponding to the two fragments are observed together with the ion of the intact peptide. Fragmentation of other bonds within a peptide is also known to occur in the FAB ionization process[29]. Fig. 10 shows the spectrum collected at scan 311, which is dominated by the ion corresponding to the T13 peptide, with an $[M + H]^+$ of 693.2 m.u. As in the case for T20–T21 and its fragments, the fragment ions occur in the same position within the displacement train as the "parent" ion, suggesting that they arise as artifacts of the ionization process, in contrast to the peptides arising from non-specific enzymatic cleavages discussed above that appear in a different position within the displacement train. The daughter ions in Fig. 10, however, arise from breakage of bonds along the peptide backbone, or from loss of a residue side chain. The resulting ions can be assigned to fragments that are known to occur during FAB ionization[31].

CONCLUSIONS

Columns and equipment developed for reversed-phase HPLC are well suited to displacement separation of the mixture resulting from tryptic digestion of met-hGH with cetyltrimethylammonium bromide as the displacer. By this means, the capacity of the column can be increased 50-fold. Preparative purification of relatively large amounts of tryptic peptides can thus be carried out on the high efficiency equipment. Useful amounts of minor components of the digest mixture can be isolated for subsequent characterization.

Displacement chromatography offers a means of increasing the sample load in microbore LC–MS, thereby obviating some of the limitations imposed by the low flow-rates tolerated in on-line mass spectrometry, and extending the dynamic range of the analysis. In addition, the mass spectrometer acts as a highly selective detector that permits the reconstruction of the band profiles of individual species in the displacement train. It can thus aid in rapid description of the performance of the chromatographic system, to optimize the conditions for preparative and process applications.

Analysis by DC–MS of 10 nmol of recombinant human growth hormone digested with trypsin revealed the presence of most of the major expected peptides along with many smaller peptides associated with non-specific proteolysis and autolysis of trypsin. High-quality spectra were obtained for peptides present in extremely low amounts in the sample. Fragmentation of peptides in the FAB process was also observed, but could be distinguished from proteolysis arising during enzymatic digestion.

Instability in the ion signal late in the displacement train may be associated with the high concentrations characteristic of DC, and may be meliorated by optimization of chromatographic and FAB experimental conditions.

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REFERENCES

1 W. S. Hancock, C. A. Bishop, R. L. Partridge and M. T. W. Hearn, *Anal. Biochem.*, 89 (1978) 203.
2 W. G. Bennett, R. Chloupek, R. Harris, E. Canova-Davis, R. Keck, J. Chakel, W. S. Hancock, P. Gellefors and B. Pavlu, in E. E. Miller, D. Cocchi and V. Locatelli (Editors), *Advances in Growth Hormone and Growth Factor Research*, Pythagora Press, Rome-Milan, and Springer-Verlag, Berlin-Heidelberg, 1989, p. 28.
3 W. P. Blackstock, R. J. Dennis, S. J. Lane, J. I. Spards and M. P. Weir, *Anal. Biochem.*, 175 (1988) 319.
4 P. Gellerfors, K. Axelsson, A. Helander, S. Johansson, L. Kenne, S. Lindqvist, B. Pavlu, A. Skottner and L. Fryklund, *J. Biol. Chem.*, 264 (1989) 11444.
5 W. J. Kohr, R. Keck and R. N. Harkins, *Anal. Biochem.*, 122 (1982) 348.
6 M. Barber, R. S. Bordoli, R. D. Sedgwick and A. N. Tyler, *J. Chem. Soc., Chem. Commun.*, (1981) 325.
7 T. Mizuno, T. Kobayashi, Y. Ito and D. Ishii, *Mass Spectrom.*, 35 (1987) 9.
8 R. M. Caprioli, W. T. Moore and T. Fan, *Rapid Comm. Mass Spectrom.*, 1 (1987) 15.
9 W. J. Henzel, J. H. Bourell and J. T. Stults, *Anal. Biochem.*, submitted for publication.
10 R. M. Caprioli, W. T. Moore, B. DaGue and M. Martin, *J. Chromatogr.*, 443 (1988) 355.
11 J. Frenz and Cs. Horváth, in Cs. Horváth (Editor), *HPLC —Advances and Perspectives*, Vol. 5, Academic Press, New York, 1989, p. 212.
12 A. Tiselius, *Ark. Kemi Mineral Geol.*, 16A (1943) 1.
13 Cs. Horváth, A. Nahum and J. H. Frenz, *J. Chromatogr.*, 218 (1981) 365.
14 H. Kalász and Cs. Horváth, *J. Chromatogr.*, 215 (1981) 295.
15 Cs. Horváth, J. Frenz and Z. El Rassi, *J. Chromatogr.*, 255 (1983) 273.
16 G. E. Veress, Cs. Horváth and E. Pungor, in H. Kalász (Editor), *New Approaches in Liquid Chromatography*, Akadémiai Kiadó, Budapest, 1984, pp. 45–56.
17 J. Frenz, Ph. van der Schrieck and Cs. Horváth, *J. Chromatogr.*, 330 (1985) 1.
18 Cs. Horváth, in F. Bruner (Editor), *The Science of Chromatography*, Elsevier, Amsterdam, 1985, pp. 179–203.
19 S. M. Cramer and Cs. Horváth, *Prep. Chromatogr.*, 1 (1988) 29.
20 S. M. Cramer, Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 394 (1987) 305.
21 A. W. Liao, Z. El Rassi, D. M. LeMaster and Cs. Horváth, *Chromatographia*, 24 (1987) 881.
22 G. Viscomi, S. Lande and Cs. Horváth, *J. Chromatogr.*, 440 (1988) 157.
23 J. Frenz and Cs. Horváth *Am. Inst. Chem. Eng. J.*, 31 (1985) 400.
24 R. D. Smith, J. A. Loo, C. J. Barinaga, C. G. Edmonds and H. R. Udseth, *J. Chromatogr.*, 480 (1989) 211.
25 J. Frenz, unpublished results.
26 K. C. Olson, J. Fenno, N. Lin, R. N. Harkins, C. Snider, W. J. Kohr, M. J. Ross, D. Fodge, G. Prender and N. Stebbing, *Nature (London)*, 293 (1981) 408.
27 J.-X. Huang and Cs. Horváth, *J. Chromatogr.*, 406 (1987) 275.
28 J.-X. Huang and Cs. Horváth, *J. Chromatogr.*, 406 (1987) 285.
29 D. H. Williams, C. V. Bradley, S. Santikarn and G. Bojeses, *Biochem. J.*, 201 (1982) 835.
30 P. Roepstorff and J. Fohlman, *Biomed. Mass Spectrom.*, 11 (1984) 601.
31 S. Naylor and G. Moneti, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 405.