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Nanoscale separations

Capillary liquid chromatography–mass spectrometry and capillary zone electrophoresis–mass spectrometry for the determination of peptides and proteins

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ABSTRACT

Nanoscale separation techniques, such as packed capillary liquid chromatography and capillary zone electrophoresis in which the column effluent flow-rates are in the nanoliter per minute range rather than microliter per minute range, have been successfully interfaced with continuous-flow fast atom bombardment and electrospray ionization. Applications of these techniques to the separation and determination of peptide mixtures, protein digests and protein mixtures are presented.

INTRODUCTION

The trend towards miniaturization has had a major, beneficial impact on separation science methodologies. The best known example is the impact that the development of open tubular capillary columns has had on gas chromatographic separations. The significantly better separation efficiencies, shorter analysis time and ease of coupling with mass spectrometry (MS) has made the use of capillary gas chromatography (GC) columns the standard GC technique.

Similar advantages have been found in liquid chromatography (LC). Smaller inner diameter columns provide greater separation efficiencies, reduced solvent con-

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sumption and increased ease in interfacing with mass spectrometers and other detector systems, such as laser-based detectors [1]. At the forefront of these developments are the nanoscale separation techniques, including packed nanoscale capillary columns and capillary zone electrophoresis (CZE).

Packed nanoscale capillary LC columns are columns with flow-rates in the nl/min range. Typically, these columns have inner diameters of 50 to 75 μm and are packed with 5–10-μm particles. Karlsson and Novotny [2] reported in 1988 on the evaluation of packed capillary columns with inner diameters as low as 44 μm and observed improved separation efficiency. This was attributed to a homogenous packing bed structure, in which the influence of the wall is felt by the entire packing bed. Kennedy and Jorgenson [3] then reported a comprehensive study on the effect of column diameter and particle size and observed that a column inner diameter to particle size ratio of $<10:1$ offered increased column performance due to decreased flow dispersion and a decrease in resistance to mass transfer in the mobile phase. This was also attributed to a more uniform packing bed density due to the wall effect being operative across the entire packing bed.

CZE separations are based on the differential migration of ionic species in an electric field [4]. Separation efficiencies of greater than $1 \cdot 10^6$ theoretical plates are possible in under 20 min using the technique [5]. Typical flow-rates associated with CZE are in the range of 500 nl/min and lower. Thus, CZE flow-rates are compatible with MS.

Two MS techniques have generally been considered to be most compatible with the flow-rates identified with nanoscale separations, continuous-flow fast atom bombardment (CF-FAB) [6] and electrospray ionization (ESI) [7]. In CF-FAB, the FAB matrix and carrier solvent flow directly onto the FAB probe tip. The analyte is introduced either by flow injection or as the effluent from an LC column. The low flow-rates required to maintain the high separation efficiencies of nanoscale LC are imcompatible with the typical CF-FAB flow-rates (5 μl/min). To circumvent this problem, we have designed a coaxial approach to CF-FAB in which the analyte and matrix streams do not meet until they reach the probe tip [8]. This coaxial design has been used successfully to interface both nanoscale capillary LC and CZE with CF-FAB with retention of the high separation efficiencies associated with the nanoscale techniques and has demonstrated high sensitivities [9–14]. CZE has also been interfaced wth CF-FAB by Caprioli *et al.* [15] and Reinhoud *et al.* [16] using a liquid junction interface.

In ESI-MS, a spray of fine droplets is formed at atmosperic pressure in the presence of a high voltage potential. The ions formed in this technique are often multiply charged which permits the molecular mass determination of relatively large molecules, such as proteins, on a low mass range quadrupole instrument. Flow-rates of 5–10 μl/min are usually optimal with ESI sources. As with CF-FAB, additional fluid must be delivered to the source for stable operation. CZE has been interfaced with ESI (and the related technique, ionspray) using either coaxial delivery [17] or liquid junction [18], and has been applied to the determination of peptides and proteins [19–21].

In this paper, we report on the application of nanoscale capillary LC and CZE combined with both coaxial CF-FAB and ESI for the determination of compounds of biological interest.

EXPERIMENTAL

MS

The CF-FAB experiments were performed on a VG ZAB 4F mass spectrometer (VG Analytical, Altrincham, UK) equipped with an Ion Tech FAB gun. Xenon was used as the FAB gas (8 kV at 1 mA).

The electrospray experiments were performed on a VG 12-250 quadropole (VG Masslabs, Altrincham, UK) equipped with a Vestec electrospray source Model 611B (Vestec, Houston, TX, USA). This source differs from other commercially available ESI sources in that the block is heated. Declustering and, possibly, ionization occurs in the electrospray chamber which is heated conductively to *ca.* 50°C. The electrospray needle voltage is 2–3 kV and the spray current is between 0.08 to 0.18 μA.

Data analysis was performed on a VG 11-250 data system with supplementary data analysis performed on a Sun 3/60 workstation (Mountain View, CA, USA) running Kratos (Ramsey, NJ, USA) Mach 3 software.

Coaxial CF-FAB

The coaxial FAB interface and probe used for the nanoscale capillary LC and CZE were fabricated in-house and have been described previously [8–14]. The CZE apparatus was built in-house and is described in detail elsewhere [8,11,13]. The packed nanoscale capillary LC columns were slurry packed using AQ-C18 (YMC, Morris Plains, NJ, USA) particles. Experimental conditions for the nanoscale capillary LC and CZE experiments are given in Table I. The linear gradients of acetonitrile–water (0.1% trifluoroacetic acid) were formed and delivered using a pair of Waters 6000A pumps and a Waters 660 gradient controller (Milford, MA, USA). The mobile phase flow was split prior to the column to produce the desired rate of flow through the column. Sample injections were performed using a pressurized injection vessel. A microvial of solution is placed in the vessel and the nanoscale capillary LC column was inserted through the lid into the vial. The vessel was then pressurized with helium to inject the sample onto the column. After the sample was injected, the column was removed from the injection vessel and reinserted into the LC system.

ESI-MS

The nanoscale capillary LC and CZE systems described for use with coaxial CF-FAB [8–14] are also used for ESI except for the probe design. The ESI probe is a standard Vestec electrospray probe that, using the main design features of our coaxial CF-FAB probe, has been modified to provide a coaxial delivery of the sheath fluid. The operating parameters are given in Table I.

RESULTS AND DISCUSSION

Nanoscale capillary LC–CF-FAB-MS

Our initial experiments investigating the performance and utility of packed nanoscale capillary columns were done with columns the entire lengths of which were filled with packing material. This prevents loss of resolution that might occur due to the large dead volume that will be present if the columns are only partially filled. (The total column length of *ca.* 1.2–2 m is dictated by the length of the CF-FAB probe and

TABLE I

NANOSCALE SEPARATION–MS PARAMETERS

Parameter	Nanoscale capillary CF-FAB	CZE–CF-FAB	Nanoscale capillary ESI	CZE–ESI
Column	50–75 μm I.D./ 150 μm O.D.	13 μm I.D. untreated or coated with aminopropylsilane	75 μm I.D. × 150 μm O.D.	75 μm I.D. × 150 μm O.D., untreated or coated with aminopropylsilane
Column packing	10 μ C_{18}	–	10 μm C_{18}	–
Column flow-rates (nl/min)	50–350	± 30	50–500	500
Sample injection volumes (nl)	0.5 to 10	0.25 to 5	0.5 to 10	0.1 to 6
Sheath column	160 μm I.D. × 350 μm O.D.	160 μm I.D. × 365 μm O.D.	600 μm I.D. stainless steel	600 μm I.D. stainless steel
Sheath composition	(Glycerol–water 25–75)	Glycerol–0.5 m*M* aqueous heptafluorobutyric acid (25:75)	Methanol–3% aqueous acetic acid (50:50)	Methanol–3% aqueous acetic acid (50:50)
Sheath flow-rate (μl/min)	0.5	0.5	5–10	5–10
Voltage drop (kV)	–	± 30	–	± 30
Buffer composition	–	5 m*M* Ammonium acetate adjusted to pH 8.5 with ammonium hydroxide or 0.01 *M* acetic acid adjusted to pH 3.4–3.5 with ammonium hydroxide	–	10 m*M* Ammonium acetate adjusted to pH 8.5 with ammonium hydroxide or 0.01 *M* acetic acid adjusted to pH 3.4 with ammonium hydroxide

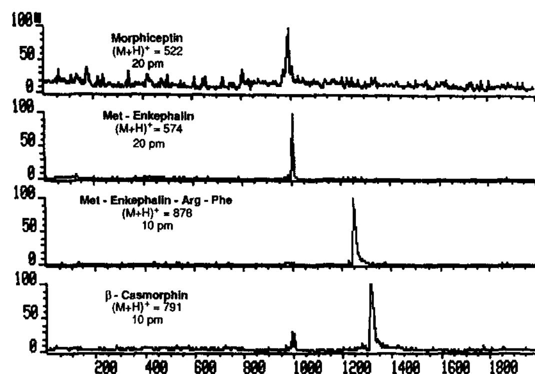

Fig. 1. Packed nanoscale capillary LC–MS separation and analysis of morphiceptin, met-enkephalin, met-enkepahlin-Arg-Phe and β-casmorphin. Selected ion chromatograms of $(M + H)^+$ from full scan data. The capillary column was 75 μm I.D. 2 m long capillary packed with 10 μm C_{18} particles. Gradient conditions were: 0% (10 min) stepped to 25%, followed by a linear gradient to 50% acetonitrile in water (0.1% TFA) over 120 min. pm = pmol.

sufficient length for maneuvering the column). For most purposes, however, optimum resolution is not necessary.

The separation of four opioid peptides is shown in Fig. 1. The flow-rate was 350 nl/min and 10–20 pmol of each component was injected. From the signal-to-noise ratio, it is apparent that the limits of detection under full scan conditions for the two enkephalins is less than 1 pmol injected. Under isocratic conditions this column exhibited a separation efficiency of 59 000 theoretical plates which compares favorably with a theoretical prediction of 66 000 plates.

A more complex example (which has previously been analyzed by microbore LC–ESI-MS [22]), a partial (*ca.* 80% of the protein remained undigested) tryptic digest corresponding to 96 pmol of undigested horse heart cytochrome *c*, was also investigated using a 2.2-m long column packed to a depth of 25 cm with 5 μm C_{18} particles. Selected ion chromatograms of major components eluting over the entire chromatograms as well as a selection of ions across the mass range scanned are shown in Fig. 2. All ions show very good signal-to-noise ratios and peak shape except for the fragment corresponding to residues 14–22 plus the iron containing heme group. The broad peak shape may be at least partially attributable to the presence of the heme moiety.

CZE–coaxial CF-FAB-MS

The CZE–coaxial CF-FAB-MS separation of a mixture of neuropeptides is shown in Fig. 3. The sensitivity of this technique can be seen from the levels of neuropeptides analyzed, ranging from 114 fmol to 172 fmol. Good resolution is observed with the number of theoretical plates ranging from 18 000 to 58 000. The separation was complete within 7 min. It should be noted that leu-enkephalin and met-enkephalin are barely separated. Given their structural similarity and that separations are based on charge, this is not surprising.

Three of these components in Fig. 3 are identical to three of the components separated by nanoscale capillary LC as shown in Fig. 1, morphiceptin, met-enkepha-

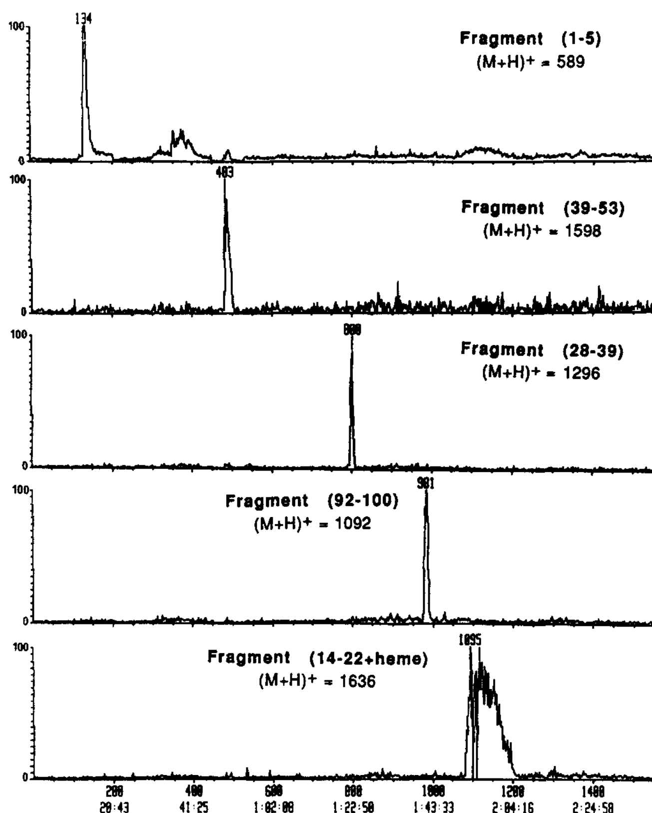

Fig. 2. Packed nanoscale capillary LC–MS separation and analysis of horse heart cytochrome *c* tryptic digest. Selected ion chromatograms of $(M + H)^+$ of selected tryptic peptides from full scan data. The capillary column was a 2 m column packed with 25 cm of 5 μm C_{18} particles. Gradient conditions were: 0% (10 min) stepped to 15% followed by a linear gradient to 35% acetonitrile in water (0.1% TFA) over 150 min. Time in h:min:s.

lin and β-casmorphin. Several observations can be made from a comparison of these two separations. The first is that the analysis time is significantly shorter for CZE than it is for nanoscale capillary LC. The second is that sensitivity by CZE is significantly better than for nanoscale capillary LC. For example, the signal-to-noise ratio for all three components is approximately the same, while the amount analyzed by CZE is approximately two orders of magnitude less than that analysed by nanoscale capillary LC. This is due to the significantly narrower peak widths observed in CZE, approximately 7 s. wide at half-height, than in nanoscale capillary LC, approximately 1 min, wide at half height, which results in a significantly greater flux of analyte into the ion source. The third observation can be made from a comparison of the elution order of the three identical components. Met-enkephalin is the second component to elute in the nanoscale capillary LC analysis while it is the last peak in the CZE analysis. As CZE separations are based on different molecular parameters than are nanoscale capillary LC separations, the separations are orthogonal and represent a valid confirmation technique.

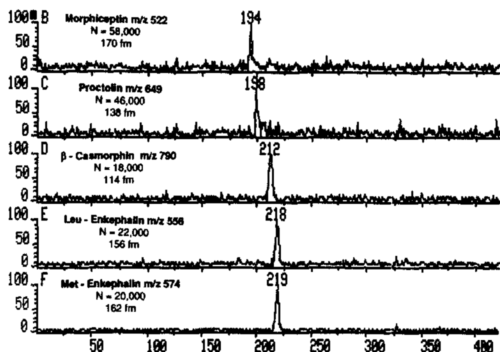

Fig. 3. CZE–CF-FAB-MS separation and analysis of neuropeptides. Selected ion chromatograms of $(M + H)^+$ from full scan data. The CZE capillary was 1 m long, 13 μm I.D. The buffer was 5 m*M* ammonium acetate (pH 8.5) containing 1% 2-propanol. The voltage drop across the column was 38 kV. *N* = Plate number.

A fourth feature that differentiates the two techniques is the loading factor. Although it is not obvious in Fig. 3, overloading of CZE–coaxial CF-FAB-MS occurs at significantly lower levels than for nanoscale capillary LC. The analysis in Fig. 3 is, in fact, close to the upper concentration limit.

Nanoscale capillary LC–ESI-MS

The analysis of the tryptic digest adrenocorticotropic hormone (ACTH) 1–24 (corresponding to 200 pmol of undigested protein) is presented as an example of the application of a packed nanoscale capillary LC–MS analysis using ESI (Fig. 4). The

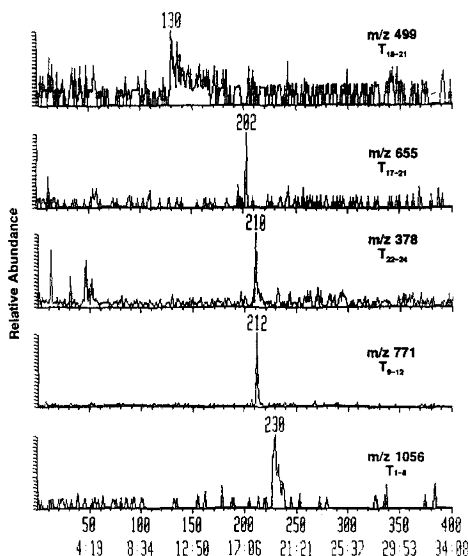

Fig. 4. Nanoscale capillary LC–ESI-MS separation and analysis of the tryptic digest of ACTH 1–24. Selected ion chromatograms of the most abundant molecular ion species from full scan data. The capillary column was 2 m × 75 μm I.D. packed to a depth of 25 cm with 10-μm AQ-C18 particles. Gradient conditions were: 0% (10 min) stepped to 15% followed by a linear gradient to 35% acetonitrile in water (0.1% TFA) over 120 min.

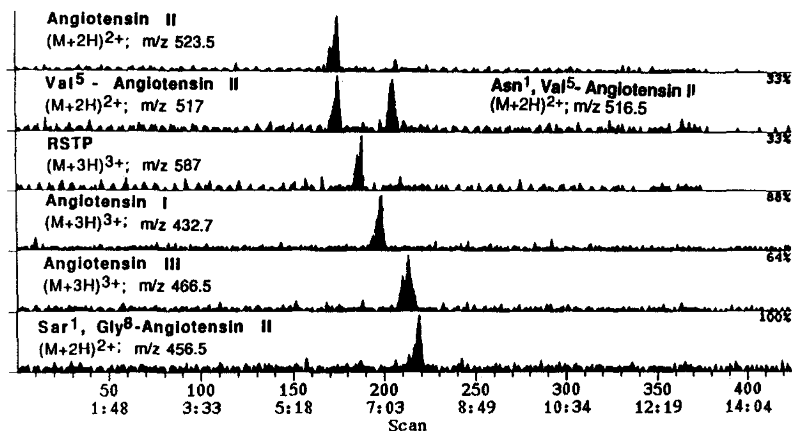

Fig. 5. CZE–ESI-MS separation and analysis of angiotensin-related peptides. Selected ion chromatograms of most abundant ion from full scan data. The CZE column was 1.1 m × 75 μm modified with aminopropylsilane. The buffer was 0.01 M acetic acid at pH 3.4. The voltage drop across the column was 30 kV.

column used in this separation is 2 m × 75 μm I.D. packed to a depth of 25 cm with 10-μm AQ-C18 particles. The significantly shorter column results in a greatly reduced analysis time compared to the 2.2 m long column. The analysis is complete within 21 min in comparison to the dead time of approximately 30 min observed with the 2.2 m columns. Peak shape, except for m/z 499 tryptic peptide T_{18-29} is still quite reasonable. The sensitivity observed here is similar to that observed with FAB detection [23].

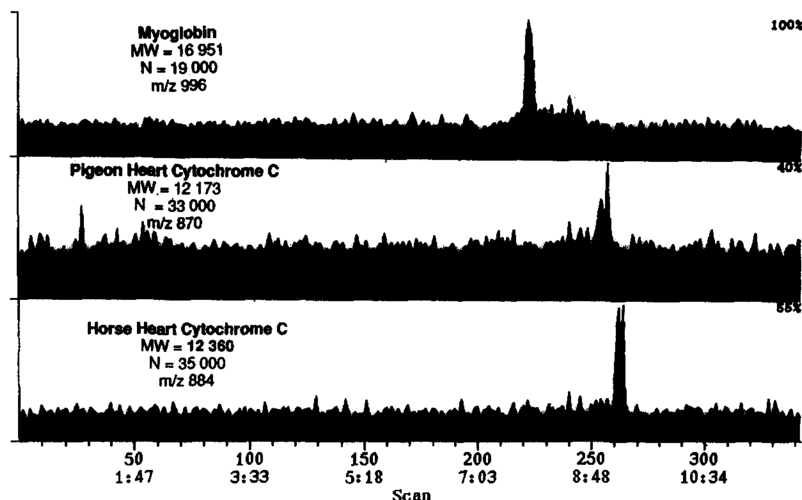

Fig. 6. CZE–ESI-MS separation and analysis of three proteins. Selected ion chromatograms of the most abundant molecular ion species from full scan data. The CZE column was 1.1 m × 75 μm I.D. modified with aminopropylsilane. The buffer was 0.01 M acetic acid at pH 3.4. The voltage drop across the column was 30 kV. MW = Molecular weight.

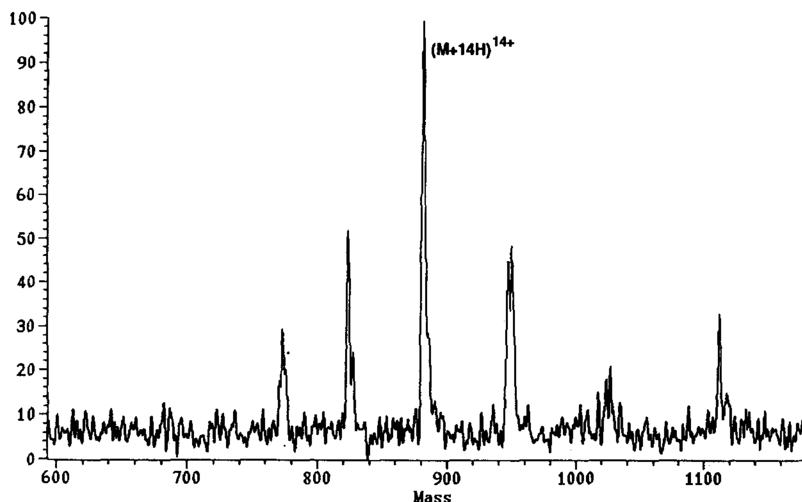

Fig. 7. CZE–ESI-MS spectrum of horse heart cytochrome *c* obtained from the separation shown in Fig. 6.

CZE–ESI-MS

There are several features of the combination of CZE with ESI-MS that make it attractive. As can be noted by comparing the column size used in the CF-FAB approach, 13 μm I.D., with that used in ESI, 75 μm I.D., the loading capacity of CZE–ESI analyses should be significantly greater. Thus, overloading the column does not present such a severe problem. The second factor is the observation of multiply charged species permits the separation and analysis of proteins. These two features are demonstrated below.

The separation and analysis of seven angiotensins are presented in Fig. 5. Each component was injected at the 2.5-pmol level. This level would cause severe overloading on a 13 μm I.D. column. Although the peaks are broader than that observed for low fmol analyses, the components are still separated.

A separation of three proteins, myoglobin, pigeon heart cytochrome *c* and horse heart cytochrome *c* is presented in Fig. 6. The amount of each protein injected was 400 fmol. Good separation (19 000 to 35 000 theoretical plates) and sensitivity is demonstrated in this analysis. As indicated above, ESI-MS spectra are dominated by multiply charged species and the spectrum of the horse heart cytochrome *c* obtained from the separation in Fig. 6 is shown in Fig. 7. The major peak is due to the $(M + 14H)^{14+}$

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