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High-sensitivity peptide mapping using packed-capillary liquid chromatography and electrospray ionization mass spectrometry

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Abstract

The use of packed-capillary LC with electrospray (ES) ionization mass spectrometry (MS) has been explored for the separation and detection of a tryptic digest of myoglobin. Useful m/z information and a complete tryptic map was obtained from as little as 2 pmol of digested protein. Using methionine enkephalin as a model peptide, as little as 190 fmol could be detected in the scan mode and 18 fmol could be detected in the selected-ion monitoring (SIM) mode of MS operation. Finally, the ion signals obtained from identical injections on column diameters of 0.25, 1.0, 2.1 and 4.6 mm were compared. The 0.25 mm I.D. column was found to give a signal that was 163 times greater than that obtained using the 4.6 mm I.D. column.

Keywords: Peptide mapping; Peptides; Myoglobin; Methionine enkephalin

1. Introduction

Electrospray ionization (ES) mass spectrometry (MS) has rapidly matured into a powerful detection method for liquid separation systems such as liquid chromatography (LC) and capillary electrophoresis (CE). Especially useful in the area of biological analysis, ES-MS has proven successful for the characterization of unknown species such as proteins, peptides, glycoproteins, carbohydrates, nucleic acids and others. ES has been successfully interfaced to most types of mass spectrometers including quadrupole [1–6], magnetic [7–12], Fourier-transform [13–15], ion-trap [16,17] and time-of-flight [18–20].

A unique feature of ES-MS is its ability to be used with a wide variety of liquid flow-rates (well below 1 μl/min to over 1000 μl/min), and thus a wide variety of LC or CE column geometries, while maintaining optimum performance. This expanded operating range is largely the result of the development of assisted ES techniques which overcome the natural limitation of ES restricting its usable flow-rate to a few μl/min. Examples of these techniques include pneumatically assisted ES [21–24] and ultrasonically assisted ES [25,26]. They allow higher flow-rates to be used by mechanically creating the fine spray of liquid droplets that are required for the ionization and desolvation processes that occur in the ES ion source. Similarly, these techniques also allow for the use of aqueous and conductive liquids which are difficult, if not impossible, to use with conventional ES.

While the improvements in ES droplet formation techniques have allowed for the use of increased flow-rates that are amenable to conventional bore (4.6 mm I.D.) LC, these improvements have not increased the sensitivity available from ES-MS. This result is due to the fact that electrospray generally acts as a concentration-sensitive detector, much like UV absorbance, fluorescence, conductivity, etc., and not as a mass-sensitive detector. The speculation for

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this behavior is the occurrence of droplet space–charge repulsion in the ES-MS source. As the liquid droplets carrying the sample analyte are formed and charged, they repel each other in space as they travel in the ES source towards the orifice which will transport the emerging ions into vacuum. Because there is a limit or a maximum density of droplets due to like-charge repulsion, only a small portion of the droplets created will be sampled. As the liquid flow-rate increases, the droplet spacing near the vacuum orifice is conserved due to the repulsion, so the number of droplets contributing to the ion signal does not increase. In practice then, at high flow-rates, the liquid sample is split internally after it enters the source [27].

Because of this effect, a reasonable direction to pursue in order to increase mass sensitivity, or actual quantity of sample consumed, is to use separation methods that operate optimally at the lowest possible sample flow-rates. Packed-capillary LC and CE are two such methods. Both of these advanced separation techniques exhibit high chromatographic efficiencies and increased mass sensitivity when concentration-sensitive detectors are used. In the work presented here, the performance of ES-MS with packed-capillary LC is evaluated in comparison to conventional bore LC and the advantages available from using reduced diameter separation methods with ES-MS will be demonstrated. This technique will then be used to analyze a tryptic digest of myoglobin.

2. Experimental

2.1. Apparatus

The pump used for mobile phase delivery was a dual syringe type, model ABI 140A from Applied Biosystems (Foster City, CA, USA). In order to deliver linear gradients at flow-rates suitable for microcolumn LC, a flow-splitting device consisting of a simple tee with a fused-silica restrictor was constructed. With this system, the pump was operated at 75 μl/min, and the resulting flow to the column was measured at 1.8 μl/min. This system was rugged and provided reproducible results, so it was used for all of these studies. The analytical column was fabricated from 250 μm I.D. fused-silica from Polymicro Technologies (Phoenix, AZ, USA) and was 30 cm in length. The reversed-phase packing material used was Capcell Pak C_{18}, 5 μm particle size from Shiseido (Tokyo, Japan). Sample injection was accomplished with a Valco Instruments (Houston, TX, USA) CI4W valve. The microbore or 15 cm×1 mm I.D. column as well as the 25 cm×2.1 mm I.D. column were purchased from Vydak (Hesperia, CA, USA) and were packed with 5 mm, C_{18} particles. A 4.6-mm I.D. column packed with 5 mm, C_{18} Microsorb particles was obtained from Rainin (Woburn, MA, USA) and was 25 cm in length.

The electrospray ionization source was from Analytica of Branford (Branford, CT, USA). This particular source design allowed independent control of the source potentials on the needle (V_{need}), cylindrical electrode (V_{cyl}), nosepiece (V_{nose}) and capillary entrance (V_{ent}). With this configuration, the system was always operated with V_{need}=ground, in order to protect the user from electrical shock and isolate the LC system from high voltage. The needle itself was an Analytica of Branford microspray probe. Drying-gas temperature was always set to 300°C. Needle gas was set at 60 p.s.i. (1 p.s.i.=6894.76 Pa). The mass spectrometer (HP89A) was from Hewlett-Packard (Palo Alto, CA, USA) and was scanned from 100–1000 m/z units.

2.2. Materials

Methionine enkephalin was obtained from Sigma (St. Louis, MO, USA). In order to provide the best possible quantitation, the trypsin digest of horse heart myoglobin was purchased in a prepared form from Michrom BioResources (Auburn, CA, USA). At the time of injection, these samples were dissolved at the appropriate concentration in a mobile phase composition which was identical to the starting material of the LC gradient used. All water was obtained from a Barnstead (Boston, MA, USA) NANOpure II system. Acetonitrile (ACN) was purchased from Mallinckrodt (Paris, KY, USA). All solvents were filtered through nylon 66 membranes from Anspec (Ann Arbor, MI, USA). Trifluoroacetic acid (TFA) was also obtained from Mallinckrodt.

3. Results and discussion

3.1. Effect of sample flow-rate on ES-MS signal

This figure of merit was evaluated by directly infusing into the ES-MS system a 10-pmol/μl solution of methionine enkephalin (molecular mass= 574) dissolved in methanol–water (1:1, v/v) containing 0.1% acetic acid. The mass spectrometer was scanned from 100–1000 m/z units in about 1 s. Fig. 1 shows the ion signal obtained across a wide range of sample flow-rates. It is important to note that the ion signal does not increase with sample flow-rate, even though at 1000 μl/min, 1000 times more sample is being consumed per unit time than at 1 μl/min. The most reasonable explanation for this result is the droplet repulsion effect discussed earlier, coupled with a finite droplet drying potential available from the heated gas flow in the ES ion source. Thus, the number of microscopic droplets and ions available for sampling by the MS system is limited. Finally, at the highest flow-rate, 1 ml/min, the absolute ion signal has in fact decreased by approximately 30%. This observation is also most likely the result of some incomplete droplet drying.

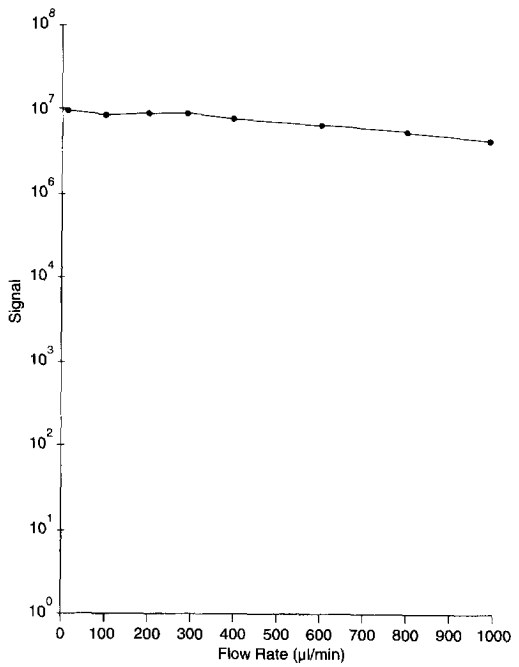

Fig. 1. Ion signal from the direct infusion of a 10 pmol/μl solution of methionine enkephalin as a function of sample flow-rate.

These facts have strong repercussions on the ultimate sensitivity available from ES-MS and the choice of chromatographic methods used with ES-MS. It is clear that employing separation techniques that operate optimally at lower flow-rates will greatly reduce the amount of sample required for analysis. Alternatively, conventional column sizes and flow-rates may be employed but with a post column split prior to the ES-MS system inlet followed by fraction collection. In this way, most of the sample can be conserved.

3.2. Effect of column diameter

In order to evaluate the effect of LC column diameter on LC–ES-MS signal, identical quantities of methionine enkephalin (50 pmol) were injected onto four columns with I.D. sizes of 0.25 mm, 1.0 mm, 2.1 mm and 4.6 mm, respectively. Each column was operated with its particular optimum flow-rate, this being 1.8, 50, 200 and 1000 μl/min, respectively. A linear gradient of 5–75% ACN in water containing 0.1% TFA over 30 min was used in each case. These experiments were performed in triplicate to ensure reproducibility. The resulting peaks from the total ion current (TIC) of each run were then combined into the single TIC trace shown in Fig. 2. The results show that the signal-to-noise ratio in the TIC from the mass analyzer scans can be greatly increased through the use of smaller bore LC columns. This is in agreement with theory, which states that the concentration sensitivity of LC columns of varying diameters is equal to the inverse of the square of the two radii of the columns or:

$$\frac{\text{Sensitivity Column 2}}{\text{Sensitivity Column 1}} = \frac{(\text{Column 1 radius})^2}{(\text{Column 2 radius})^2}$$

assuming that the length, efficiency, porosity and particle size are identical in each column. By measuring the signal-to-noise (S/N) ratio of the peaks in Fig. 2, an increase of 163 times in sensitivity is calculated between the 4.6 mm I.D. column and the 0.25 mm I.D. column. From the column diameters and the relation above, a theoretical value of 339

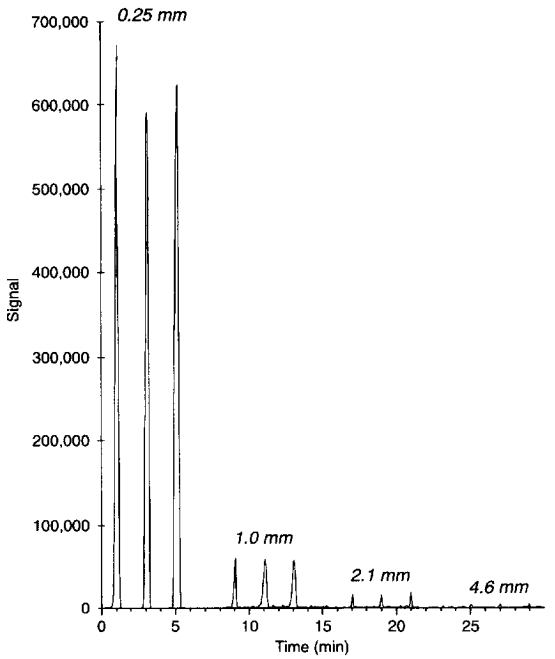

Fig. 2. TICs from methionine enkephalin injections (50 pmol each) on columns with different diameters.

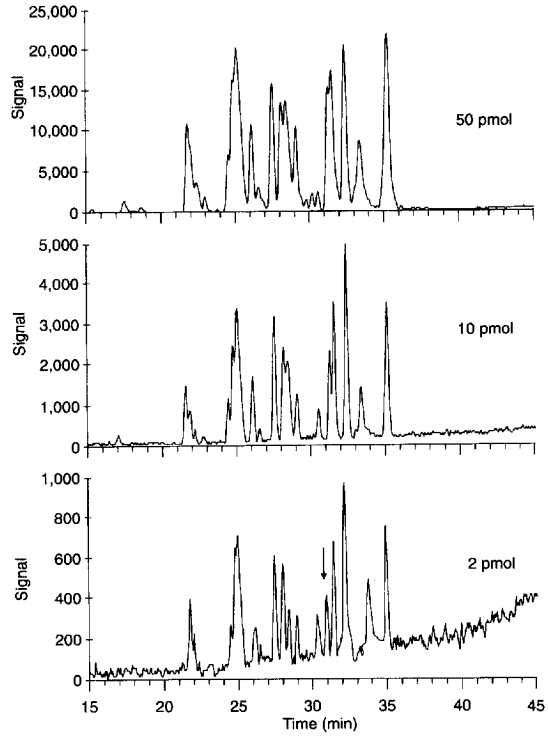

Fig. 3. TICs from the LC–ES-MS analysis of 50, 10 and 2 pmol of a myoglobin tryptic digest using a 0.25-mm I.D. packed-capillary column.

is expected. This is a reasonable result since in the work described here, the assumption that the remaining physical properties of the columns and packing material are identical is only partially valid. Also, the assumption that the signal response of the ES-MS system is equal across the flow-rate range used is again only partially valid.

3.3. Separations using packed-capillary columns

As a demonstration of the capability of analyzing tryptic digests by capillary LC–ES-MS, Fig. 3 shows the TICs from the separation of 50, 10 and 2 pmol of a trypsin digest of myoglobin. The LC and MS conditions used were the same as described above. Even at the 2 pmol level, nearly all of the components are visible in the TIC. The separation quality is also considerably improved at the 2 pmol level since this smaller amount of material is not overloading the column. This level of sensitivity is comparable to that shown with UV detection of tryptic digests using nearly identical packed-capillary LC methods [28]. This is encouraging, as MS is sometimes criticized for not offering the same sensitivity as spectroscopic methods of detection, especially with capillary LC separations. The sensitivity also compares well to that of Kassel et al. [29] who used 180 μm I.D. columns with 10 pmol of digested protein. The averaged mass spectrum for the peak marked in Fig. 3 is shown in Fig. 4 and gives significant peaks at $m/z = 340$ and 679. These correspond to the doubly and singly charged species derived from a tryptic fragment having a molecular mass of 678. The peak at $m/z = 391$ is a commonly observed phthalate contaminant. Even with 2 pmol of total digest, sufficient information is present to identify protein fragment masses generated by the trypsin digestion. Assuming the existence of approximately 20 fragments, an estimation of the quantity present of just this particular fragment would be 100 fmol.

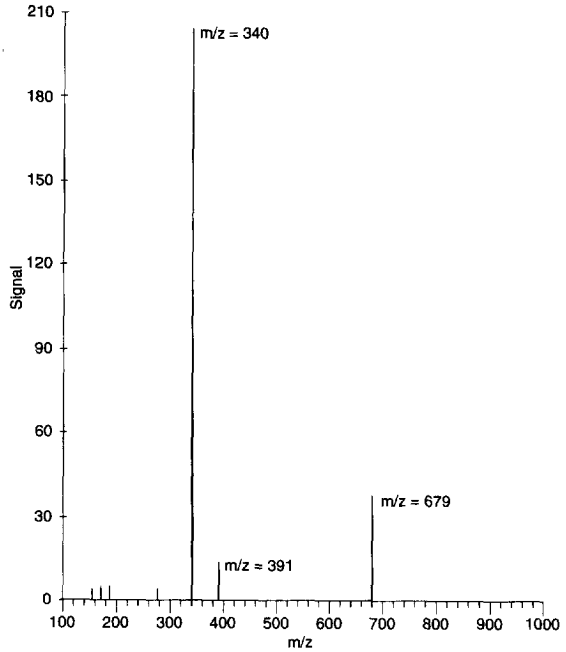

Fig. 4. Mass spectrum of peak marked in 2 pmol digest.

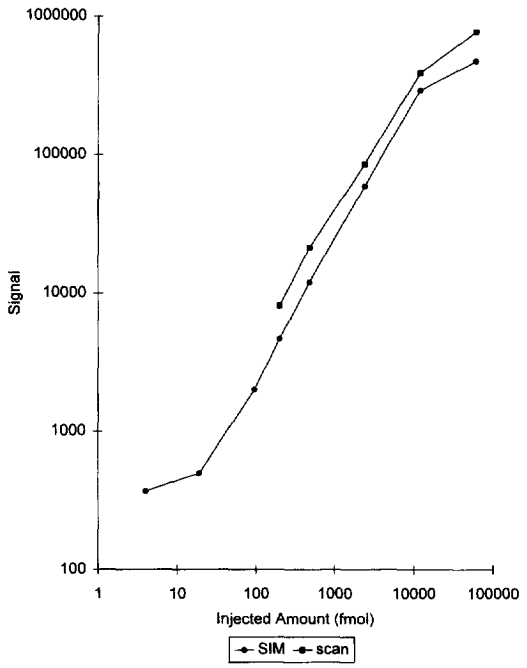

Fig. 5. Ion signal from methionine injections on a 0.25-mm I.D. column in scan and SIM modes of operation.

3.4. Sensitivity

In order to investigate the absolute sensitivity of this system, methionine enkephalin was injected onto the 250 μm I.D. column in increasing amounts. All LC and MS conditions were the same as those used above, except that a second series of experiments was performed using the MS in single-ion-monitoring (SIM) mode only. Fig. 5 shows a graph of the ion signal in scan and SIM modes vs. the amount injected. In the scan mode, the minimum amount that could be detected on the linear portion of the graph was 190 fmol, while in the SIM mode, the limit of detection was 18 fmol. This sensitivity compares well with that of Hunt et al. [30] who showed MS–MS spectra from as little as 300 fmol of a single peptide.

4. Conclusions

The use of capillary separations, specifically, packed-capillary LC, with electrospray ionization MS provides for greatly increased sensitivity compared to that obtained using conventional 4.6 mm I.D. columns. Using a 0.25-mm I.D. column, as little as 190 fmol of methionine enkephalin can be detected in scan mode. In SIM mode however, a detection limit of 18 fmol is possible. When applied to a tryptic digest of myoglobin, this method can give useful information from as little as 2 pmol of digested material.

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