Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 291-299

MICROBORE HPLC / MASS SPECTROMETRY FOR THE ANALYSIS OF PEPTIDE MIXTURES USING A CONTINUOUS FLOW INTERFACE

Richard M. Caprioli, Beverly DaGue, Terry Fan, and William T. Moore

Department of Biochemistry and Molecular Biology,
and the Analytical Chemistry Center,
The University of Texas Medical School at Houston,
Houston, Texas 77030

Received June 4, 1987

SUMMARY. Microbore HPLC techniques have been combined with fast atom bombardment mass spectrometry to provide HPLC/MS capabilities for the analysis of mixtures of peptides and small proteins. The interface between the liquid chromatograph and mass spectrometer is a continuous flow direct insertion probe which contains a fused silica capillary that delivers the eluting solvent to the FAB source of the mass spectrometer at a rate of 5-10 μL/min. Data are presented for the analysis of several mixtures of peptides ranging in molecular weights from about 900 to 6000 daltons. In addition, the analysis of 100 pmol of a tryptic digest of whale myoglobin is shown where 16 of the possible 19 peptides were identified in the mass range m/z 2200-250. The advantages of this approach to HPLC/MS are a relatively high sensitivity because of the low flow rates and low background, and the ability to detect high molecular weight compounds.

High performance liquid chromatography has become the method of choice for the separation and isolation of a wide variety of biological compounds, particularly for peptides and proteins. Sample detection of peptides is usually accomplished by spectrophotometric means at a wavelength of approximately 210 nm where absorption of the peptide bond is monitored. Although this can be done simply and with high sensitivity, it is far from the ideal detector for these compounds because of its lack of specificity. Mass spectrometry offers some unique advantages as a detector for HPLC because it is able to perform a mass specific analysis of the compounds eluting from the column.

There have been a number of approaches designed to interface a high performance liquid chromatograph to a mass spectrometer. These include thermospray (1), direct liquid injection (2), supercritical fluid (3) and

0006-291X/87 $1.50

moving belt systems (4), among others. Although these approaches have been successful for certain types of analyses, they have not proved useful for the analysis of peptides in the 2000-5000 molecular weight range where many peptide fragments are produced from proteolytic digestions.

Fast atom bombardment mass spectrometry (FABMS), as well as a number of other desorption ionization techniques, has provided a means for the direct analysis of peptides and other biological compounds without the need for chemical derivatization. Although FABMS analyses are usually done in solutions containing 95% or more glycerol, it has recently been shown (5,6) that, using a continuous flow sample probe, analysis of aqueous solutions containing small amounts of glycerol can be accomplished effectively and offers particular advantages. These include significantly higher sensitivity due to the decrease in the chemical background characteristic of high glycerol containing solutions and increased ion yields due to the lower viscosity of the aqueous sample solution being bombarded. In addition, the ion suppression effect (7), caused by the tendency for hydrophobic ions to populate the surface of the sample and suppress ions from more hydrophilic compounds is significantly reduced.

The HPLC/MS interface used in this work utilizes a fused silica capillary column to transport the eluate from the column to the tip of the sample probe in the ionization chamber of the mass spectrometer. The probe tip is continuously bombarded with energetic Xe atoms, causing sputtering of the sample solution as it emerges from the tip of the capillary. Molecular ions from the sample produced in this process are then mass analyzed by the instrument. Ito et al.(8) described a capillary device for interfacing an HPLC to a FAB mass spectrometer where the capillary was terminated with a fine stainless steel mesh to disperse the mobile phase and concentrate the solute and glycerol.

We report on the use of a continuous flow interface for use with FABMS and microbore HPLC. The technique was evaluated by analyzing a number of mixtures of peptides in the molecular weight range of 1000-6000 daltons, including the

separation and molecular identification of several species of intact insulins. In addition, data are presented which shows the HPLC/MS identification of sixteen specific molecular fragments from the tryptic digestion of sperm whale myoglobin.

MATERIALS AND METHODS

Apparatus: An Applied Biosystems model 130A microbore HPLC system fitted with a 0.1 x 25 cm Brownlee RP-300 column was used for the chromatographic separations. The unit was modified by installing a Rheodyne 7410 injector with a 0.5 μL injection loop. The end of the column was connected directly to the continuous flow FAB probe (5) by a one meter length of 0.075 mm (i.d.) fused silica capillary. The end of the capillary terminated at the probe tip where it was bombarded by energetic xenon atoms inside the ionization chamber of the mass spectrometer. This arrangement is shown schematically in Figure 1.

The microbore HPLC was operated at a flow of 5 μL/min. Gradient elutions were accomplished using a mixture of 5% glycerol in water with 0.1% TFA as solvent A, and %5 glycerol, 60% acetonitrile, and 0.1 % TFA as solvent B.

A Kratos MS50RF high resolution mass spectrometer equipped with a DS90 (S/280) data system was used. The instrument was operated at 4 keV accelerating voltage and a resolution of about 1200. The probe tip was maintained at 40°C. The Ion Tech B11NF saddle field gun was operated at 8 keV and 40 μa current using xenon gas. Data acquisition was done using the raw data multi-channel analyzer program available with the data system. Cesium iodide was used for mass calibration.

Chemicals: All chemicals and reagents were obtained from the Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Angiotensin II and III, substance P, and bombesin were obtained from the Vega Chemical Co. (Phoenix, AZ). The sample of trypsin hydrolyzed sperm whale myoglobin was obtained from Applied Biosystems Inc. (Foster City, CA).

RESULTS AND DISCUSSION

LOW MOLECULAR WEIGHT PEPTIDE MIXTURES:

A mixture of 125 pmol each of angiotensin II (MW 1045), angiotensin III (MW 930), substance P (MW 1347), and bombesin (MW 1618) was injected onto the

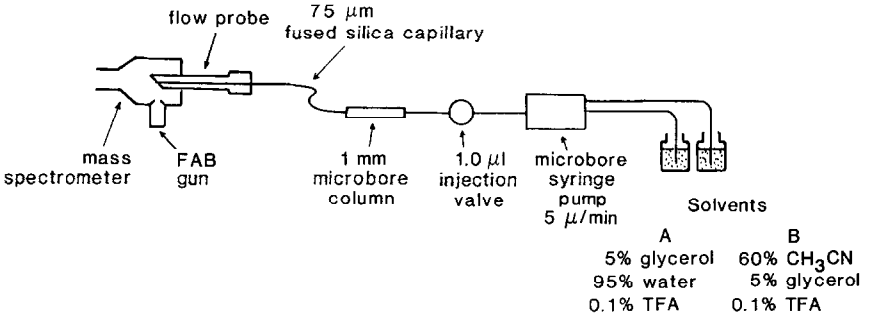

FIGURE 1. Microbore HPLC/MS arrangement using the continuous flow FAB probe interface.

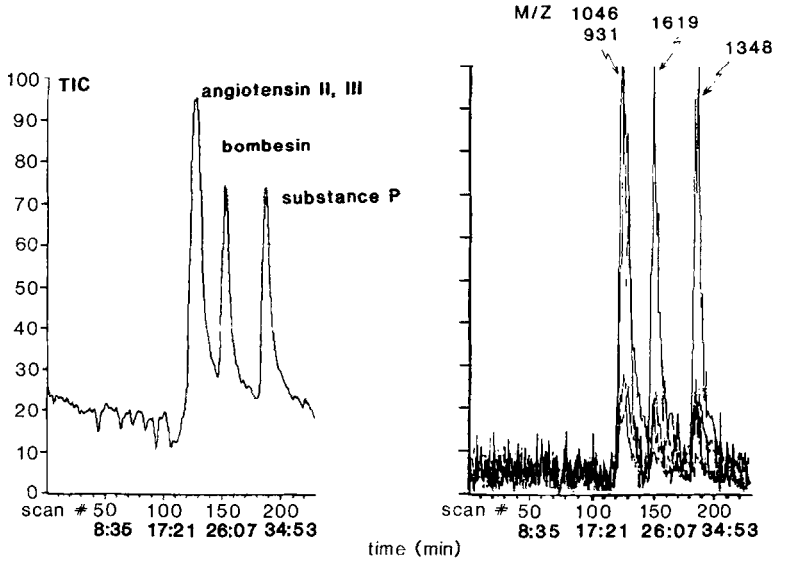

FIGURE 2. Total ion chromatogram (top) and several selected ion chromatograms (bottom) for the microbore HPLC/MS analysis of 125 pmol each of angiotensin III, angiotensin II, substance P, and bombesin. The protonated molecular ions for these compounds appear at m/z 931, 1046, 1348, and 1619, respectively.

microbore RP300 column. The mass spectrometer was continuously scanned from m/z 1700 - 900 at a rate of 10 sec/scan. Figure 2 shows the total ion chromatogram and the selected ion chromatograms for the individual (M+H)+ ions of the peptides. A reasonable separation of the peptides was achieved except for Angiotensins II and III which were not separated from each other. The eluted peaks, at 10% peak height, were approximately of two minutes duration.

ANALYSIS OF TRYPTIC DIGEST OF MYOGLOBIN:

Figure 3 shows the total ion chromatogram produced from the analysis of 100 pmol of tryptic digest of sperm whale myoglobin. The mass spectrometer was continuously scanned form m/z 2300-250 at a rate of about 20 sec/scan. Figure 3 also shows the selected ion chromatograms for the higher molecular weight peptide fragments. The $(M+H)^+$ region of the FAB mass spectrum for one of these, m/z 1393, is shown in Figure 4. The complete digestion of this protein would be expected to give 19 peptides ranging from 2 to 17 residues in length. A total of 16 peptides were identified in the HPLC/MS analysis, as summarized in TABLE I. Only two dipeptides and one tripeptide were missed in

Electronics, Hialeah, FL) was used for data acquisition and analysis. Each cell suspension contained 10^6 cells/ml in complete medium. The $DiOC_6(3)$ dye was added in DMSO (1% v/v, maximum) to a concentration of 25 nM. This was not toxic during experiments: fluorescence intensity maintained a steady-state level indicating no change in membrane potential (no decreases in cell viability). The correlation of fluorescence intensity with the membrane potential was tested by changing extracellular potassium concentrations ($[K^+]$): Increasing $[K^+]$ from 6 mM up to 130 mM proportionately decreased the fluorescence intensity, reaching a minimum 130 mM (At 130 mM, the extracellular and intracellular $[K^+]$ equalizes in lymphocytes). It was also tested by ionophore molecules with known effects (37): gramicidin, 10 ug/ml, considerably decreased while valinomycin, 1 ug/ml, increased the membrane potential. That $DiOC_6(3)$ measured only cytoplasmic and not mitochondrial membrane potential changes was tested by using 30 mM sodium azide (43): after 5 min incubation, fluorescence intensity changes resembled those without mitochondrial enzyme decoupling.

Flow cytometric determination of membrane potential using $DiBaC_4(3)$ dye: The relevance of the fluorescence intensities as measured with $DiOC_6(3)$ dye was doubly checked by applying $DiBaC_4(3)$ (38). Flow cytometric analysis was performed as above and by Wilson et. al. (43). Cells were washed with PBS to remove extraneous protein, treated with biologic test reagents then stained with the dye (150 nM). In contrast to $DiOC_6(3)$ which is a positively charged dye, $DiBaC_4(3)$ is a negatively charged dye. Thus, a fluorescence intensity decrease signifies hyperpolarization. Fluorescence intensity of the $DiBaC_4(3)$-stained cells is stable for 10 min only, so that histograms were taken 2 min after staining. The sample tubing was equilibrated with a 150 nM solution of $DiBaC_4(3)$ before a histogram was taken.

RESULTS

Effect of rIFa on the fluorescence intensity of $DiOC_6(3)$ stained Daudi cells: Cells in complete medium were equilibrated with $DiOC_6(3)$, then treated with varied concentrations of rIFaA. The rIFaA, from 10 U/ml to 1000 U/ml, decreased the fluorescence intensity $DiOC_6(3)$-stained Daudi-IF^S cells (i.e. depolarized) in a dose dependent manner (Fig 1A). Untreated controls showed no decrease. Gramicidin (10 ug/ml) decreased the fluorescence intensity of the $DiOC_6(3)$-stained Daudi-IF^S cells, even beyond that measured with 1000 U/ml of rIFaA (Fig 1C). Typical bit maps, ungated and gated, are shown in Fig 1B and 1C. No change was observed in the light scatter with the 1000 U/ml rIFaA treatment, indicating stable cell geometry. Neither of the Daudi-IF^r subclones exhibited decreased fluorescence intensity when treated with up to 1000 U/ml of rIFaA (not shown). These had been grown without IFa for several doublings (39).

Comparisons of fluorescence intensity changes of $DiOC_6(3)$-and $DiBaC_4(3)$-stained Daudi-IF^S cells treated with rIFa, IFaA, IFg or rIL-2: Aliquots of Daudi-IF^S cells pretreated with 100 U/ml rIFaA for 16 hr and untreated control cells were stained with 25 nM $DiOC_6(3)$. Fluorescence histograms are compared in Fig 2A: rIFaA pretreated cells exhibited lower fluorescence intensities than controls and results with IFa were similar. As expected, gramicidin pretreatment (5 ug/ml) lowered and valinomycin pretreatment (1 ug/ml) increased the fluorescence intensities of control cells indicating depolarization and hyperpolarization respectively (Fig. 2A). Other aliquots

TABLE I

Sperm Whale Myoglobin Sequences	$(M+H)^+$ Calculated	$(M+H)^+$ Found HPLC/MS
VLSEGEWQLVLHVWAK	1894	1894
VEADVAGHGQDILIR	1593	1593
LFK	407	407
SHPETLEK	940	940
FDR	437	437
FK	294	?
HLK	397	?
TEAEMK	708	708
ASEDLK	662	662
K	147	NA
HGVTVLTALGAILK	1392	1393
K	147	NA
K	147	NA
GHHEAELK-PLAQSHATK	1855	1856
HK	284	284
IPIK	470	470
YLEFISEAIIHVLHSR	1927	1928
HPGNFGADAQGAMNK	1518	1519
ALELFR	748	748
K	147	NA
DIAAK	517	517
YK	310	?
ELGYQG	665	665
TOTAL: 19 peptides (2-17)		
FOUND: 16		

about 11 sec/scan. Elution times were; S peptide, 9.4 min; B-chain, 37.8 min; glucagon, 41.8 min. The mass spectra of the molecular ion region for the two higher molecular weight compounds are shown in Figure 5 with the measured value of $(M+H)^+$ indicated.

ANALYSIS OF INSULINS:

In order to evaluate the high mass capability of the microbore HPLC/FABMS system, a mixture of 400 pmol each of bovine, ovine, equine, and porcine insulins were analyzed using the gradient system described above. The mass spectrometer was scanned from m/z 6100 - 5500 at a rate of 3.2 sec/scan. The four proteins were reasonably well separated and eluted between 22 and 50 min. Figure 6 compares the elution profile obtained using both a mass spectrometer and a UV detector. Since the resolution of the mass spectrometer was set to about 1200, the isotopic molecular ion species were not resolved from each other, and were recorded as a single peak approximately 6 masses wide.

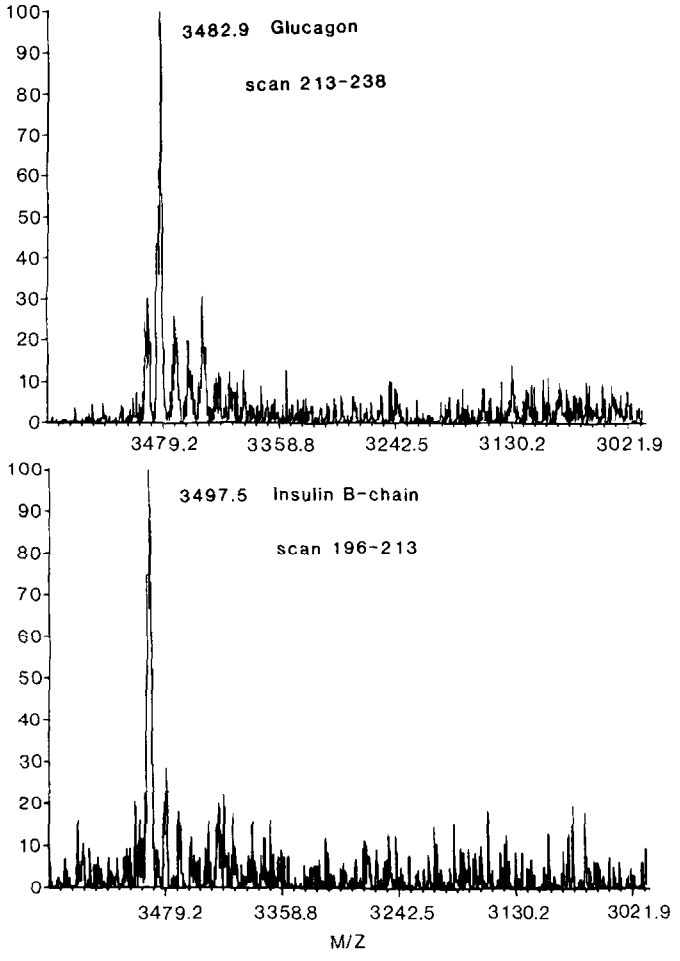

FIGURE 5. FAB mass spectra of glucagon (top) and oxidized bovine insulin B chain (bottom) from the microbore HPLC/MS analysis of a mixture of 1 nmol each of several polypeptides. The spectra shown are the sum of individual mass spectra taken over the eluted peak.

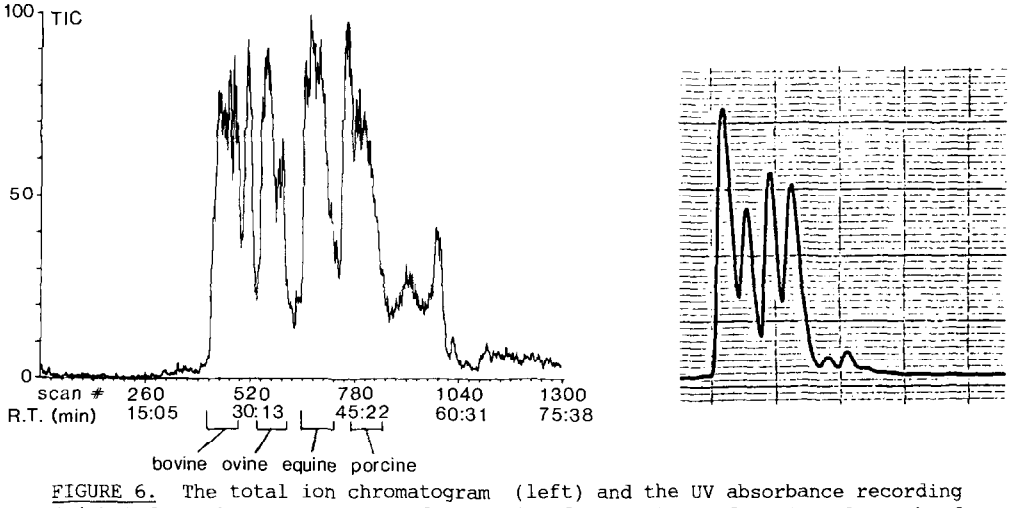

FIGURE 6. The total ion chromatogram (left) and the UV absorbance recording (right) for the separation and analysis of a mixture of 400 pmol each of bovine, ovine, equine, and porcine insulins using microbore HPLC techniques.

TABLE II. HPLC/MS ANALYSIS OF INSULINS

	$(M+H)^+$ CENTROID		
Scan #	measured	calc.	species
415-532	5735	5734.6	bovine
535-624	5748	5748.2	ovine
650-728	5705	5704.7	equine
750-832	5779	5778.2	porcine

Average mass values were calculated from these peaks using centroiding programs available with the software and the results given in Table II. Mass assignments were made to an accuracy of about +/- 0.4 mass units.

CONCLUSION

Data presented here have shown that microbore HPLC and fast atom bombardment mass spectrometry can be effectively coupled using a continuous flow interface. The mass detection system operates with high sensitivity up to about m/z 6000, and appears to have no high mass limitations other than that normally imposed by the FAB process itself. Microbore HPLC is an ideal separation technique for this application because it operates at relatively low flow rates and is compatible with the normal pumping capacities of most mass spectrometers. Peaks are eluted in relatively small volumes, typically about 10 μL, and therefore can be detected at high sensitivity. The chromatography presented here was done using columns designed to operate at flow rates of 100 μL/min or more; thus, these examples illustrate the type of separations which are minimally possible with current microbore columns. Future work will be focused on the production of microbore packed columns and also fused silica capillary columns for application to the continuous flow FAB interface HPLC/MS instrument.

ACKNOWLEDGEMENTS

The authors wish to thank the National Science Foundation, Grant number PCM-8404230, and the National Institutes of Health, Grant number RR-01720, for their support of this work.

REFERENCES

1. Blakely, C. R., Carmody, J. J., and Vestal, M. L. (1984) Anal. Chem. 56, 1236-1239.
2. Arpino, P. J., Bounine, V. P., Dedieu, M., and Guiochon, G. (1983) J. Chromatogr. 271, 43-48.
3. Smith, R. D., Fjeldsted, J. C. and Lee, M. L. (1982) J. Chromatogr. 247, 231-243.
4. Stroh, J. G., Cook, C. C., Milberg, R. M., Brayton, L., Kihara, T., Huang, Z. and Rhinehart, K. L. (1985) Anal. Chem. 57, 985-991.
5. Caprioli, R. M., Fan, T. and Cottrell, J. S. (1986) Anal. Chem. 58, 2949-2954.
6. Caprioli, R. M., and Fan, T. (1986) Biochem. Biophys. Res. Comm. 141, 1058-1065.
7. Caprioli, R. M., Fan, T. and Moore, W. T. (1987) Rapid Comm. Mass Spectrom. 1, 1-8.
8. Ito, Y., Takeuchi, T., Ishi, D. and Goto, M. (1985) J. Chromatogr. 346, 161-166.