Micro-Electrospray Mass Spectrometry: Ultra-High-Sensitivity Analysis of Peptides and Proteins

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A "micro-electrospray" ionization source has been developed that markedly increases the sensitivity of the conventional electrospray source. This was achieved by optimization of the source to accommodate nanoliter flow rates from 300 to 800-nL/min spraying directly from a capillary needle that, for the analysis of peptides, contained C18 liquid chromatography packing as an integrated concentration–desalting device. Thus, a total of 1 fmol of methionine enkephalin was desorbed from the capillary column spray needle, loaded as a 10-μL injection of 100-amol/μL solution. The mass spectrum showed the $[M + H]^+$ ion at m/z 574.2 with a signal-to-noise ratio of better than 5:1 from a chromatographic peak with a width of about 12 s. A narrow range (15-u) tandem mass spectrum was obtained for methionine enkephalin from the injection of 500 amol, and a full-scan tandem-mass spectrum was obtained from 50 fmol. For proteins, the average mass measurement accuracy was approximately 100–200 ppm for the injection of 2.5 fmol of apomyoglobin and 20–40 ppm for 200 fmol. Carbonic anhydrase B and bovine serum albumin showed similar mass measurement accuracies. *(J Am Soc Mass Spectrom 1994, 5, 605–613)*

Electrospray (ES) ionization mass spectrometry is becoming widely used for the analysis of biological molecules, especially peptides and proteins [1, 2]. It is a continuous-flow ionization device that has the capability to accurately measure the masses of large proteins as well as small peptides and other organic molecules. ES can also operate under variable solvent conditions, which allows it to be used as an interface for coupling separation techniques such as liquid chromatography (LC) to the mass spectrometer. Reverse-phase LC can also be used effectively to desalt [3] and concentrate a sample prior to its introduction into the mass spectrometer—steps that are sometimes essential to the success of an ES analysis.

Some of the most challenging applications of modern analytical technology in biological research requires the analysis of compounds such as endogenous metabolites and hormones at low femtomole concentrations and in small (microliter) volumes of solutions that contain relatively high salt concentrations. Mass spectrometry can be used with such samples and can provide analytical capabilities at the molecular level with high mass measurement accuracy. The specificity of mass spectrometry is even further enhanced in the tandem mass spectrometry mode. Applications in the neurosciences for monitoring physiologically active substances such as neuropeptides is one area where mass spectrometry has proven to be essential. The majority of neuropeptide release studies have been performed by radioimmunoassay (RIA) or radioreceptorassay (RRA), which have the needed sensitivity, but lack specificity due to antibody cross-reactivity [4, 5]. Mass spectrometry previously has been used to identify neuropeptides isolated from pooled brain tissues [6]. After extensive chromatographic purification procedures, samples were analyzed by fast-atom bombardment tandem mass spectrometry with a detection sensitivity of approximately 50 pmol. Previous constant-infusion ES analysis of proteins in this and other laboratories [7] resulted in sensitivities in the range of 200 fmol/μL to 1 pmol/μL. ES ionization coupled to either LC or capillary zone electrophoresis (CZE) has greatly increased sensitivity for both peptides and proteins [8–10]. Previously reported detection limits for peptides from LC-ES procedures are typically in the 100–500-fmol range, which is in agreement with our initial measurements. CZE-ES results show attomole sensitivity [10] for the total amount of analyte loaded, although because of low load volumes, sample solutions had to be of relatively high concentration (picomoles per microliter). However, biological extracellular concentrations of neurosubstances are 4 to 6 orders of magnitude lower at the low attomole per microliter level.

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1044–0305/94/$7.00

Received March 14, 1994
Revised April 12, 1994
Accepted April 13, 1994

In this article, we describe a micro-ES source that operates with nanoliter flow rates and yields attomole per microliter sensitivity for peptides and low femtomole per microliter sensitivity for proteins in the 70,000 MW range. The sensitivity and mass measurement accuracy is demonstrated with the small peptide methionine enkephalin (M-enk) and the proteins apomyoglobin, carbonic anhydrase B, and bovine serum albumin. In addition, the coupling of micro-ES with tandem mass spectrometry is demonstrated. When necessary, desalting and concentration of dilute solutions were accomplished by on-line low flow rate LC methods.

Materials and Methods

Electrospray Mass Spectra

Mass spectra were obtained with a TSQ 70 triple-quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) with a 20-kV dynode and software updated to TSQ 700 status. The pumping system was the original configuration that consisted of two Balzers (Balzers, Hudson, NH) TPH 330 turbomolecular pumps with the addition of a 590-L/min TriVac (Leybold-Heraeus, Export, PA) D25B rotary vane rough pump. An ES source (PerSeptive Biosystems, Vestec Products, Houston, TX) was used and modified as described below and as shown in Figure 1. The ES source also had its own two-stage pumping system that consisted of two 323-L/min Balzers UNO-016B rotary vane pumps. To reduce the volume of liquid flowing into the source, a small inner diameter capillary needle similar to that previously reported [11–15] was used. Needles were constructed from fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ) with inner diameters ranging from 5 to 250 μm. The polyimide coating was burned away from the tip (approximately 0.5 cm) and the tip was then lowered into a 49% solution of hydrofluoric acid (HF) while distilled H_2O was pumped through the capillary at 500 nL/min [11]. The progress of the etching was monitored with a microscope until a tapered needlelike tip was obtained. The tip was then carefully ground by hand to a flat end by using the flat side of a ceramic cutting stone. For preconcentration and desalting of peptide samples, unetched capillary needles were packed with reverse-phase C-18 (10-μm) particles as described below. The "needle" end of the capillary was supported by a PEEK (polyether ether ketone) sleeve that was secured in the probe tip by a hard rubber support. The opposite end of the needle was attached to a stainless-steel zero dead-volume fitting with a 0.5-mm through-hole. High voltage was applied to the spray needle through the solvent at this union. Depending on experimental conditions, the needle voltage varied from 2.0 to 3.5 kV. Electrical current leakage to other components was insignificant because of the high resistance of the (~ 0.5-m-long) capillary transfer line and by additional grounding of the

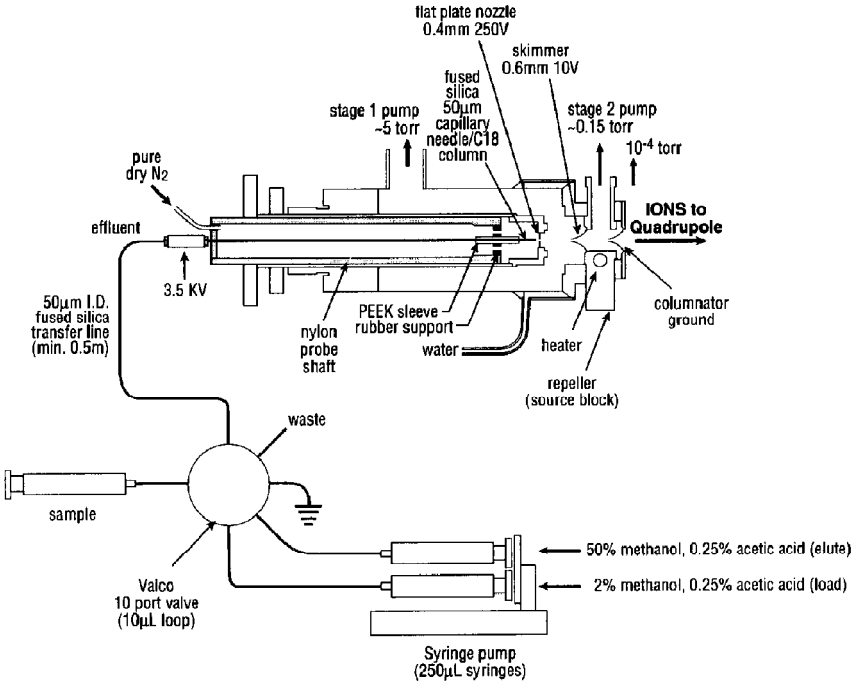

Figure 1. Schematic drawing of the micro-ES source coupled to a Valco 10-port valve with a 10-μL sample loop.

switching valve and/or syringe pump. Another modification involved replacement of the original conical nozzle with a 0.4-mm through-hole flat nozzle (see Figure 1). Voltages on the nozzle averaged between 200 and 250 V and the repeller voltage was set to 10 V. Tandem mass spectra were produced by using argon gas in the collision cell at a pressure of 1.6 mtorr.

Samples were introduced into the source by constant infusion or direct injection via a valve-loop system. Constant infusions were made by loading the sample (dissolved in 50% methanol–0.25% acetic acid) into a 250-μL gas-tight Hamilton syringe. The sample was presented to the source at a rate of 0.3–6.4 μL/min via a Harvard Apparatus (South Natick, MA) Model 2274 syringe pump. Loop injection was accomplished by using either a Rheodyne (Cotati, CA) 8125 injector valve with a 5-μL loop or, for column concentration, a Valco (Houston, TX) 10-port valve equipped with a 10-μL loop. In both cases a Harvard syringe pump equipped with Hamilton 250-μL syringes provided the solvent flow at 820 nL/min.

The original ES source did not employ counterflowing gas to help desolvate the sample, but did allow ambient air to aid in drawing the ions through the converging nozzle en route to the analyzer [16, 17]. However, we found that in our environment this arrangement produced relatively high background signals, which were often sufficient to mask the signal of interest in high-sensitivity applications. This deficiency was remedied by making the probe gas tight and supplying ultra-pure dry N_2 at 970 mL/min (~ 1 atm). When LC mass spectrometry methods were required for desalting or sample preconcentration, fused-silica capillaries with 50-μm i.d. and 220-μm o.d. were packed as previously described [18, 19] with the following modification. The capillary was cut into a 30-cm length, washed with 2-propanol, and dried with a stream of helium. Then the polyimide coating was burned away with a butane lighter (~ 0.5 cm) and the end of the capillary was tapped lightly into a vial containing a small amount of 5-μm spherical silica (SIL 120A-S5, YMC Corp., Morris Plains, NJ). The amount of silica (~ 100–200 nM in length) pressed into the capillary was monitored with a stereoscope. The silica frit was sintered in place with a butane microtorch. The tip of the capillary was then ground by hand to a perpendicular flat end with the flat side of a ceramic cutting stone. The grinding process was monitored under a stereoscope. For the preparation of capillary column needles, etching was not done because of the difficulty of maintaining the frit after etching. To prepare packed needles, a slurry of 20-mg/mL C18 (10-μm spherical C18 particles, ODS-AQ 120A S10, YMC Corp., Morris Plains, NJ) in 100% methanol was prepared. The slurry was then poured into a brass packing bomb that contained a 1.9-cm-deep 1.5-cm-diameter reservoir. A Teflon-coated stir flea kept the slurry uniform. Two high-performance liquid chromatography (HPLC) fittings that protrude from the top of the bomb allow the connection of an HPLC pump (130A, Applied Biosystems Inc., Foster City, CA) on the inlet side and the column to be packed on the outlet side. The HPLC pump was used to pump 100% methanol into the packing bomb. A microscope was used to monitor the packing of the column. The column was packed slowly until a bed volume of 40 nL was obtained (2 cm in length). The column was disconnected from the packing bomb, directly pressurized with 100% methanol to ~ 3500 lb/in.^{2}, and then flushed with and stored in 30% methanol until used.

Reagents

All reagents were reagent grade or better. All peptides and proteins were from Sigma Chemical Co. (St. Louis, MO) or Peninsula Laboratories (Belmont, CA). Proteins used were apomyoglobin from horse skeletal muscle, carbonic anhydrase B from human erythrocytes, and albumin from bovine serum.

Results

Optimization of Micro-Electrospray

For optimization of spray conditions, several capillary needles of various internal diameters were etched with HF to form tapered needles. These needles were tested with a constant infusion of 100 fmol/μL M-enk in 50% methanol that contained 0.25% acetic acid to optimize source voltages and flow rates at levels needed to maintain a steady spray. To measure the detection level of the source with each needle, a constant infusion of 10 fmol/μL M-enk solution in 50% methanol–0.25% acetic acid was used. The results of these experiments, given in Table 1, show that as the inner diameter of the capillary needle increases, the flow rate increases and the minimum high voltage needed to establish a steady spray decreases. A stable spray could not be routinely established for 5- and 10-μm-i.d. capillaries, although they produced acceptable spray conditions intermittently. The outer diameter of the capillary did not significantly affect the spray stability because each capillary had been etched to its minimal possible outer diameter (wall thicknesses were ~ 10–20 μm at the tip). The sensitivity [i.e., the amount of peptide needed to provide a spectrum with a signal-to-noise ratio (S/N) of 2.5 or better] increased as the inner diameter decreased and is a consequence of the decrease in flow rate with smaller inner diameter needles and consumption of less sample. For the experiments described below, we chose to work with needles that had a 50-μm inner diameter because of their high sensitivity, low flow rate capability, and relative ease of use (capillaries with inner diameters of less than 20 μm were too susceptible to clogging by minute particulate matter to be of routine utility).

The replacement of ambient air with ultra-pure N_2 as the flow-through gas in the ES probe had a dramatic

Table 1. Effect of Needle Size on ES Performance

Capillary dimensions (μm)		Flow rate	High voltage	Nozzle Voltage	Sensitivity with M-enk[a]
i.d.	o.d.[b]	(μL/min)	(kV)	(V)	(amol)
20	140	0.30	3.5	250	125
20	350	0.30	3.5	250	125
50	220	0.82	3.5	250	342
50	350	0.82	3.5	250	342
100	350	1.15	3.0	273	479
200	350	1.64[c]	2.4	238	683
250	350	2.29[c]	2.2	228	954

[a] Amount of M-enk consumed from a constant infusion of 10 fmol/μL to produce spectra from five scans with an average S/N of 2.5 or better.
[b] Before etching. After etching, the wall thickness at the tip was ~ 10–20 μm.
[c] Flow rates up to 6.4 μL/min were possible, but 10-fmol sensitivity was not obtained at the higher flow rates.

effect on S/N measurements at low analyte levels. Figure 2 shows spectra from a constant infusion of 100-fmol/μL M-enk in 50% methanol that contained 0.25% acetic acid at a flow rate of 820 nL/min. The spectra were obtained with room air and prepurified N_2 via a 50-μm i.d. and 220-μm o.d. capillary needle. Because background is high with room air, m/z 574, $[(M + H)^+$ of M-enk] is barely discernible in the background signal. In contrast, the spectrum obtained by using prepurified dry N_2 shows an approximately 50-fold decreased background signal and a S/N of about 8:1 for m/z 574.

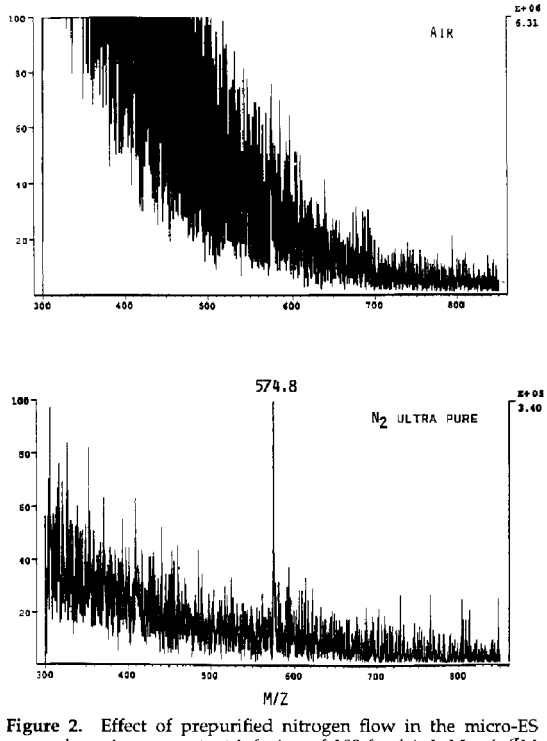

Figure 2. Effect of prepurified nitrogen flow in the micro-ES source by using a constant infusion of 100-fmol/μL M-enk ($[M + H^+] = 574.7$). The solvent was 50% methanol–0.25% acetic acid at a flow rate of 820 nL/min in (top) ambient air and (bottom) prepurified dry nitrogen.

Flow-Injection Analysis of Peptides

One of the most useful modes of operation for trace analysis of peptides and proteins is the direct injection of small volumes of analyte into a carrier solvent that continuously flows into the ES source. To provide preconcentration and desalting procedures, we have integrated LC capability into the micro-ES source. This was done by packing the needle with 10-μm C-18 particles to give an ~ 2-cm-long column at the tip of the spray needle. By spraying from the end of the column itself, all postcolumn dead volumes were eliminated.

Performance of the integrated LC-ES source was tested on several peptides, including M-enk, oxytocin, neurotensin, and substance P. Figure 3a shows the spectrum of a total of 50 fmol of M-enk (28.75 pg) in Ringer's solution (5-mM KCl, 120-mM NaCl, 1.2-mM $MgCl_2$, 1.8-mM $CaCl_2$, 0.15% phosphate buffered saline, pH 7.4) in a total volume of 10 μL. Figure 3b illustrates the current limit of detection of the technique for M-enk, that is, a total of 1 fmol of M-enk (570 fg) in 10 μL (100 amol/μL or 57 fg/μL) was loaded onto the column. M-enk was eluted with 50% methanol–0.25% acetic acid to give a peak width of approximately 12 s. The signal intensity was linear between 1 and 200 fmol of M-enk with a linear coefficient of 0.995. Similar results were obtained for the other peptides analyzed, and we have taken M-enk to be representative. For example, neurotensin was more sensitive (< 30-amol detection limit) than M-enk, and oxytocin and substance P were slightly less sensitive.

The performance of the micro-ES source was also tested in the tandem mass spectrometry mode. Figure 4 shows the tandem mass product ion spectrum pro-

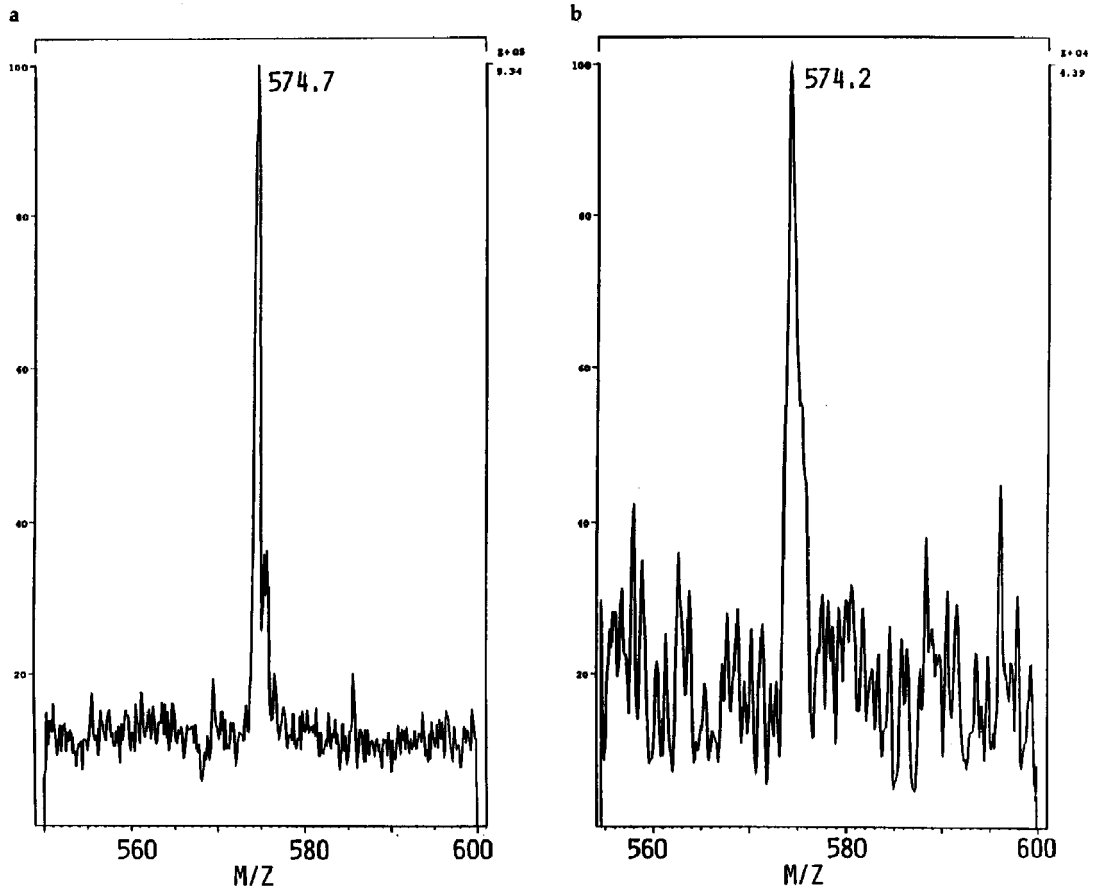

Figure 3. Micro-ES spectrum of M-enk desorbed from a C-18 packed spray needle with (a) 10 μL of 5 fmol/μL in Ringer's solution and (b) 10 μL of 100 amol/μL in H_2O.

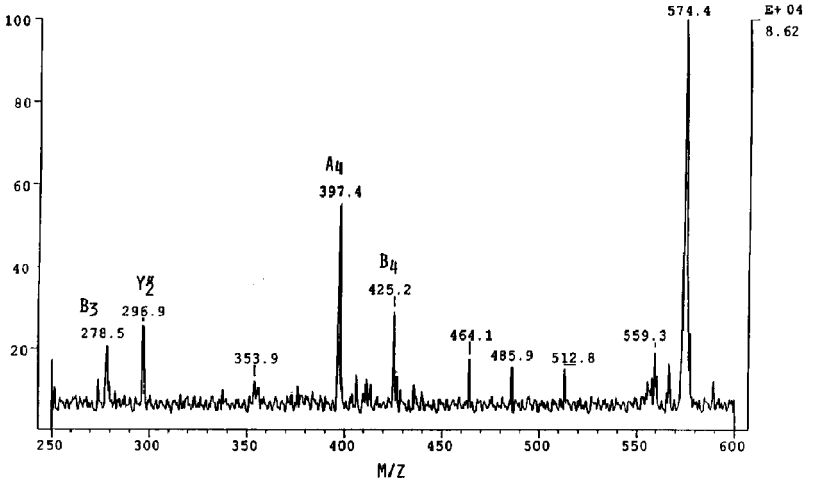

Figure 4. Tandem mass spectrum of 50 fmol of M-enk from an injection of 10 μL of 5 fmol/μL in H_2O, desorbed from a C-18 packed spray needle.

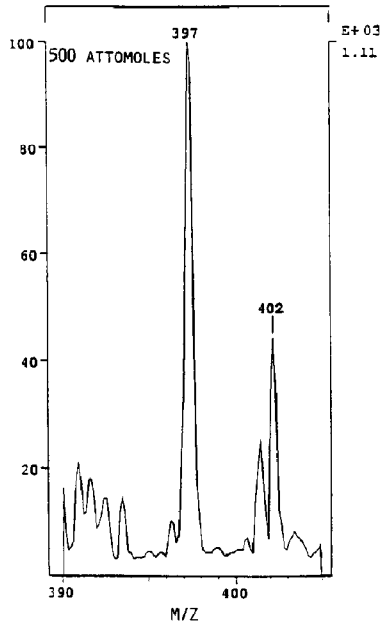

Figure 5. Tandem mass spectrum of 500 amol of M-enk from an injection of 10 μL of 50 amol/μL in H_2O, desorbed from a C-18 packed spray needle.

duced from the injection of a total of 50 fmol (28.5 pg) of M-enk followed by elution from the capillary column. The spectrum shows strong product ions of m/z 278, 297, 397, and 425 that correspond to fragments B_3, Y_2'', A_4, and B_4, respectively [6, 20]. When the m/z 397 product ion was recorded over a 15-u range in the tandem mass spectrometry experiment, detection below 500 amol could be achieved for M-enk. Figure 5 shows the product ion spectrum for m/z 397 obtained from a total of 500 amol of M-enk eluted from a capillary column (10 μL of a 50-amol/μL solution of M-enk was loaded on the column). The peak at m/z 402 is derived from the background.

Analysis of Proteins

Apomyoglobin, carbonic anhydrase, and bovine serum albumin were used to test the performance of the micro-ES source with protein samples. For apomyoglobin, both the constant-infusion and direct-injection techniques were used to determine detection limits, whereas for carbonic anhydrase and serum albumin, only direct injection data are presented. The authors prefer to use the direct-injection method, although it is not as sensitive as constant infusion, because it allows discrete amounts of sample to be used and provides a situation more closely related to routine protein analyses.

For apomyoglobin, spectra were acquired for the injection of 2.5 μL each of 10, 20, 50, 100, and 1000 fmol/μL in 1:1:0.1 (v, v, v) methanol–water–acetic acid flowing at 590 nL/min. The spectra for the lower concentration sample are shown in Figure 6a, where the molecular weight peak had about a 5:1 S/N and a calculated value of 16,952.7 $\pm$1.9 u (actual average 16,951.5 MW). The five most intense ions were chosen for this calculation because of high background signals. The spectrum for the direct injection of 2.5 μL of 100-fmol/μL apomyoglobin is shown in Figure 6b and gives a molecular weight calculation of 16,951.8. Generally, for total injections above 200 fmol, mass measurement accuracies of $\pm$20–40 ppm could be achieved with care, whereas at the 25-fmol level and below, 100–200-ppm accuracies were obtained. In comparison to direct injection, constant infusion of protein solutions allows lower concentrations to be analyzed, although the infusion technique does not necessarily use lower total amounts of protein. Figure 7 shows spectra for the analyses of 480 amol/μL and 4.8 fmol/μL of apomyoglobin in solvent consisting of 1:1:0.1 water–methanol–acetic acid flowing at 590 nL/min. The lower concentration is near the limit of detection of the technique for infused samples and gives a noisy but identifiable molecular weight peak centroided at 16,955.1 at a S/N of about 4:1. It is noteworthy in this example that a coherent spectrum of multiply charged ions can be identified in the raw data with computer algorithms although they are not obvious by eye. The higher concentration (4.8 fmol/μL) provided a molecular weight calculation of 16,952.0 at a S/N of approximately 20:1. Longer scan times of 20 s/scan (32 scans averaged) were used for the lower concentration sample, compared to 2 s/scan (32 scans averaged) for the other samples.

Direct injections of two higher molecular weight proteins were also performed at or near the detection limits of the instrumentation to further test its capabilities. For carbonic anhydrase (28,781.1 MW), the injection of 2.5 μL of a solution at 5.0 fmol/μL produced the spectra shown in Figure 8a with a measured molecular weight of 28,779.0. Injection of 2.5 μL of a solution containing 10 fmol/μL of bovine serum albumin (66,430.3 MW) produced the spectra shown in Figure 8b with a measured molecular weight of 66,433.3 for the major species. The albumin sample was not prepurified and was found to be a mixture of several protein species as seen in the deconvoluted spectrum. In both cases, reasonably accurate molecular weight measurements were obtained on injections of a total of low femtomole amounts of these proteins.

Discussion

Modifications to the ES source to allow it to operate with solvent flow rates as low as 300 nL/min also allows the spray needle to be positioned much closer to the nozzle than in the conventional source. The decreased distance and low flow rate give a spray pattern of narrow dispersion, which allows more analyte to be drawn into the analyzer with less solvent.

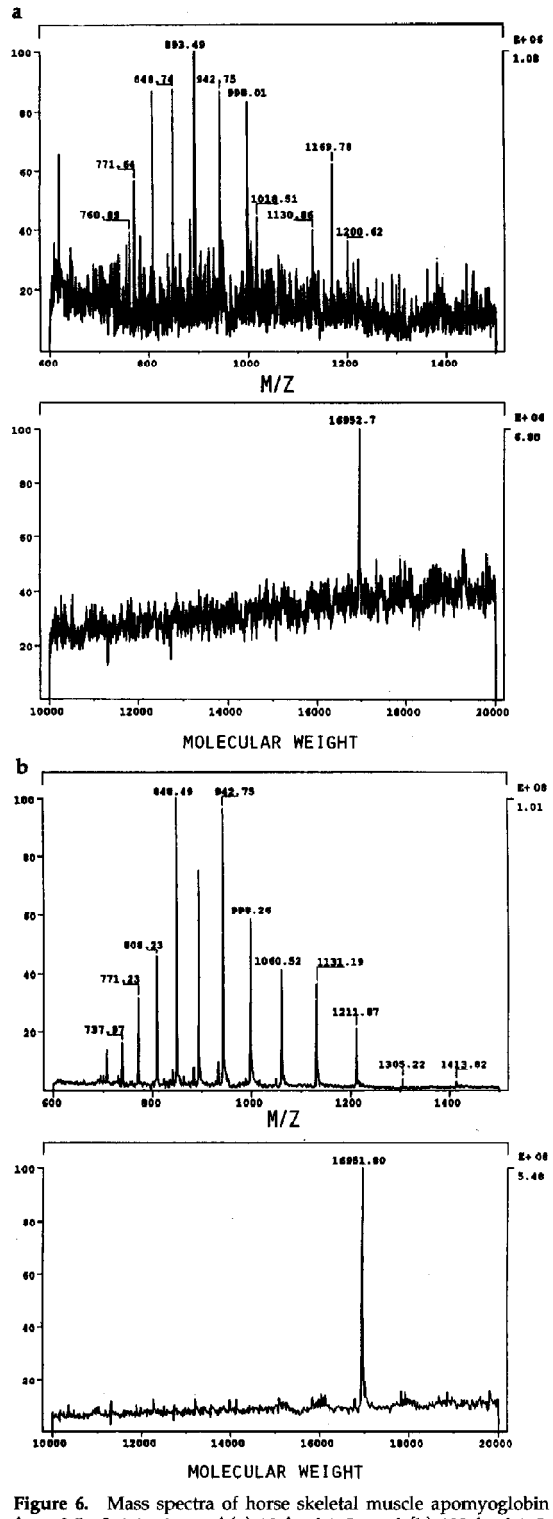

Figure 6. Mass spectra of horse skeletal muscle apomyoglobin from 2.5-μL injections of (**a**) 10-fmol/μL, and (**b**) 100-fmol/μL solutions. Upper spectra show raw data obtained at 5 s/scan with 16 scans averaged, and the lower spectra show the deconvoluted data.

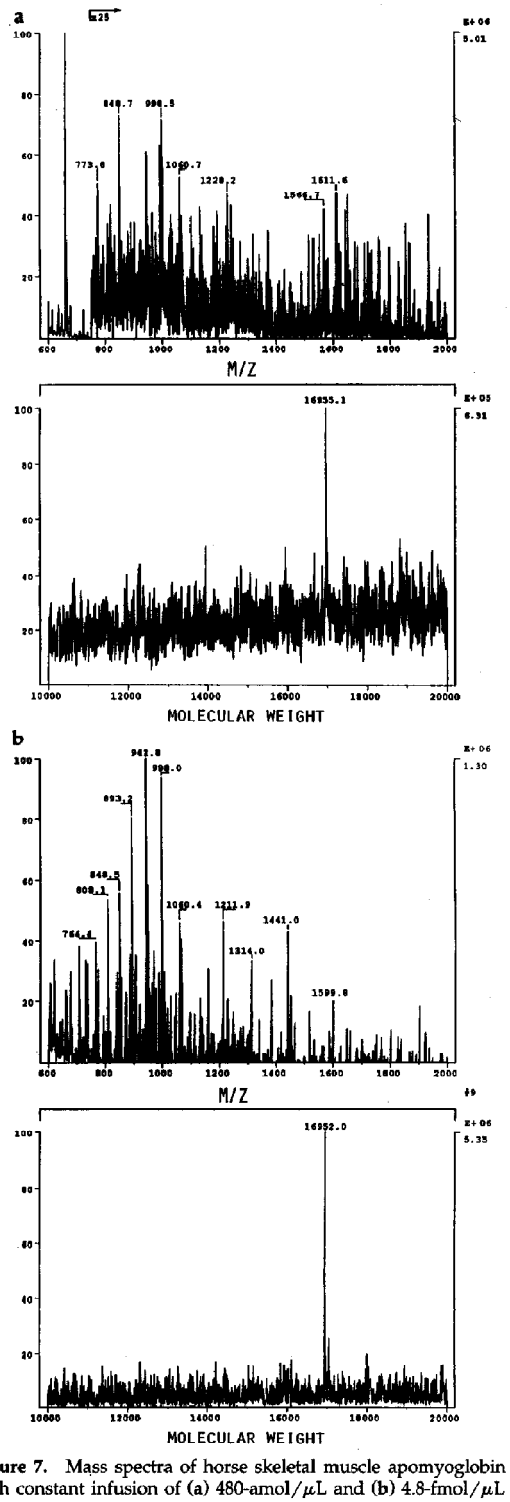

Figure 7. Mass spectra of horse skeletal muscle apomyoglobin with constant infusion of (**a**) 480-amol/μL and (**b**) 4.8-fmol/μL solutions. Upper spectra show raw data and the lower spectra show the deconvoluted data. Spectra were obtained from 20 s/scan (32 scans averaged) for (**a**) and 2 s/scan (32 scans averaged) for (**b**).

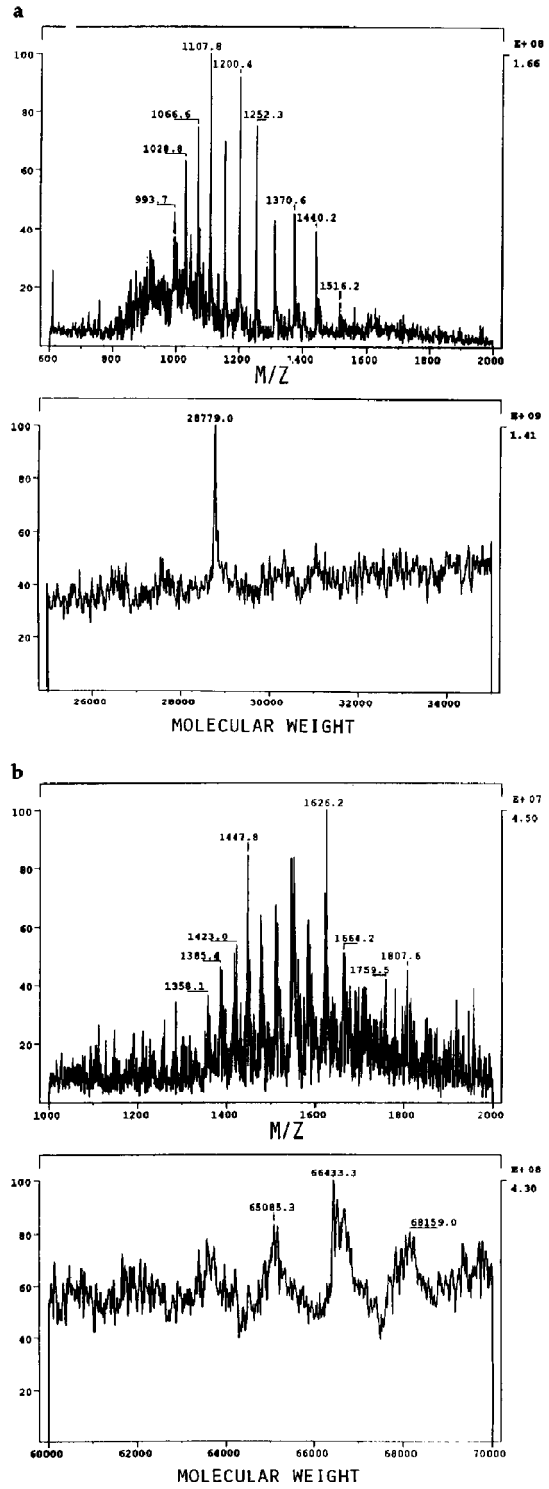

Figure 8. Mass spectra for the direct injection of 2.5 μL of (a) carbonic anhydrase B at 5 fmol/μL and (b) bovine serum albumin at 10 fmol/μL. Raw data (upper spectra) were acquired at 5 s/scan with 16 scans averaged. The deconvoluted data are shown in the lower spectra.

The addition of the dry prepurified N_2 significantly decreased the background when the flat nozzle was used. The effect of the dry prepurified N_2 appeared to be most effective under conditions of high humidity. The increase in sensitivity occurs both with peptides and proteins, and allows measurements on a few femtomoles of compound. Although a quadrupole analyzer was used in the present work, note that the characteristics of the micro-ES source would be advantageous for interfacing electrospray with a high voltage magnetic sector instrument. Low analyte concentrations coupled with low solvent flow rates greatly decrease source contamination and high voltage electrical discharge under normal (low salt) conditions.

Integrating micro-ES with reverse-phase C18 capillary technology facilitates desalting and preconcentration of samples. The elution solvent (50% methanol–0.25% acetic acid) for peptides is also ideal for electrospray conditions, and by using the LC packing in the needle, post-column dead volumes are eliminated. The preconcentration–desalting capability also confers the practical advantage of allowing the source to handle quite dilute solutions. Thus, a 50-amol/μL (5×10^{-11}-M) peptide solution can be loaded in a 5–10-μL loop for direct injection. Other techniques such as capillary electrophoresis may be sensitive under specific conditions, but the low load volumes necessitate the use of much higher sample concentrations (10^{-6}–10^{-8} M). This makes the micro-ES source ideal for certain applications where low flow rates and low concentration samples are produced. Microdialysis is an example of such a technique where the performance capabilities of micro-ES are an excellent match, providing on-line systems for dynamic chemical analysis from specific organs and tissues of live animals.

Acknowledgments

The authors thank Beverly B. DaGue for obtaining the data for the analysis of proteins and NIH grant GM43783-04 for support of this work. Thanks also go to Leesa Deterding for her advice concerning the packing of nanoscale capillary columns. This article is submitted in partial fulfillment of the requirements of M. R. Emmett for the degree of doctor of philosophy in the Graduate School of Biomedical Sciences, University of Texas Health Science Center, Houston, Texas.

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