Optimization of Capillary Zone Electrophoresis/Electrospray Ionization Parameters for the Mass Spectrometry and Tandem Mass Spectrometry Analysis of Peptides

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The solution chemistry conditions necessary for optimum analysis of peptides by capillary zone electrophoresis (CZE)/electrospray ionization mass spectrometry and CZE electrospray ionization tandem mass spectrometry have been studied. To maximize the signal-to-noise ratio of the spectra it was found necessary to use acidic CZE buffers of low ionic strength. This not only increases the total ion current, but it also serves to fully protonate the peptides, minimizing the distribution of ion current across the ensemble of possible charge states.

The use of acidic buffers protonates the peptides, which is advantageous for mass spectrometry and tandem mass spectrometry analysis, but is problematic with CZE when bare fused silica CZE columns are used. These conditions produce positively charged peptides, and negatively charged silanol moieties on the column wall, inducing adsorption of the positively charged peptides, thus causing zone broadening and a loss in separation efficiency. This problem was circumvented by the preparation of chemically modified CZE columns, which, when used with acidic CZE buffers, will have a positively charged inner column wall. The electrostatic repulsion between the positively charged peptides and the positively charged CZE column wall minimizes adsorption problems and facilitates high efficiency separations. Full-scan mass spectra were acquired from injections of as little as 160 fmols of test peptides, with CZE separation efficiencies of up to 250,000 theoretical plates. (*J Am Soc Mass Spectrom* 1992, 3, 289-300)

Revolutionary developments occurred during the 1980s in the fields of separation science and mass spectrometry: capillary zone electrophoresis (CZE) and electrospray ionization (ESI). The development of CZE by Jorgenson and Lukacs [1, 2] yielded a separation system for ionic species based on their differential rates of migration in an electric field, offering a separation mechanism complementary to that

of liquid chromatography (LC). The primary advantage of CZE over LC is a distinctly superior separation efficiency per unit time. CZE has been shown to be capable of generating in excess of 1,000,000 plates in less than 20 min [3], and in excess of 100,000 plates in less than 1 min [4]. While the high separation efficiencies inherent in electrophoresis have been recognized and utilized by biochemists for some time, it was the development of CZE that transformed electrophoresis from a slow, labor intensive, and highly variable separation technique into a rapid, fully automatable, and reproducible instrumental method of analysis [5, 6].

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The first reports of ESI were made more than 15 years ago by Dole and co-workers [7-9]; however, the major developments of ESI were made by the research group of Fenn, whose report of the formation of multiply charged ions using ESI [10-13] first alerted the mass spectrometry community to the ability of ESI to greatly extend the effective mass range of current mass spectrometers. Furthermore, ESI is an extraordinary efficient ionization method, approaching unit ionization efficiency [14]. Because ESI produces gas-phase ions from flowing liquid streams it has proven to be an ideal interface between mass spectrometry and liquid phase separation systems: LC and CZE.

The first report of the use of ESI/MS as a detector for separation systems was made by Whitehouse et al. [15]. The pioneering work in the coupling of CZE with mass spectrometry was performed by Smith and co-workers in 1988 [16-18], who mated CZE with a quadrupole mass spectrometer by using an ESI interface. Their first generation CZE/ESI/MS interface electrosprayed only the CZE buffer, which required that the CZE buffer contain a high concentration of an organic solvent (50/50 methanol/water). The composition of a CZE buffer (typically 10^{-1} to 10^{-3} M buffer in water at a flow rate of 0 to 0.5 $\mu\text{L}/\text{min}$) is significantly different from an electrospray fluid (typically 10^{-4} to 10^{-7} M buffer in an organic solvent at 1 to 5 $\mu\text{L}/\text{min}$). To circumvent this problem Smith et al. [18] incorporated a coaxial sheath flow capillary column around their CZE capillary column in a manner analogous to the method Rose and Jorgenson [19] used in their postcapillary fluorescence reactor for CZE. This sheath flow configuration permits the independent optimization of the ESI buffer and CZE buffer composition and flow rates. An alternate method of coupling CZE with a variant of electrospray known as ionspray (pneumatically assisted electrospray) was developed by Lee, Henion, and co-workers [20, 21] and called a liquid junction coupling. This liquid junction coupling also permits the independent optimization of the ESI buffer and CZE buffer composition and flow rates.

The ability of ESI to generate multiply charged species is, of course, one of its most attractive features because this greatly extends the effective mass range of the ESI/MS system. However, the formation of multiply charged species effectively divides the analyte signal into several different mass-to-charge ratio signals, reducing the signal-to-noise (S/N) ratio of both mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analysis. For CZE/ESI/MS analysis, S/N ratios can be improved by co-adding the single ion electropherograms corresponding to the different charge states of the analyte. While this approach is unfeasible for MS/MS analysis, the electrostatically induced instability of multiply charged ions increases the ease of fragmentation of an ion as the number of charges increases [22, 23]. Therefore, to optimize the CZE/ESI system for MS/MS analysis one must not only increase the total ion current from

the analyte but also maximize the number of charges placed on the analyte while minimizing the distribution of charge states across the ion population. The goal of this present work is the optimization of the CZE/ESI interface for the mass spectrometry and MS/MS of peptides by optimizing the CZE buffer composition and sheath fluid composition.

Experimental

CZE Instrumentation

The CZE columns used in this interface are prepared from 75 μm id, 365 μm od, fused silica capillaries (Polymicro Technologies, Phoenix, AZ). With the exception of the silylated CZE columns, all CZE capillary columns were pretreated by the method of Lauer and McManigill [24].

Aminopropyl silylated (APS) fused silica CZE columns were prepared via a modification of the method of Lukacs [25]. Silylation reagents react with silanol groups on the capillary surface; however, the temperatures used to draw fused silica capillary columns are sufficiently high to dehydrate surface silanols, yielding silyl ether groups. Rehydration of these silyl ethers occurs in the presence of water, but this can be a slow reaction (up to several weeks with neutral water) [26]. The silyl ether groups can be readily opened by using an acid treatment, producing additional surface silanols for silylation [27, 28]. While fused silica APS columns can be prepared without this acid treatment, the resulting columns were notably less durable. Acid treatment was accomplished by rinsing the column with a 6 M solution of HCl for approximately 4 h. The column was then dried by installing in a capillary gas chromatography oven, purging with dry helium gas, and maintaining at a temperature of 300 $^{\circ}\text{C}$ for approximately 12 to 15 h. This temperature is sufficiently high to remove physically adsorbed water, but too low to generate silyl ether groups.

The acid-treated column was rinsed with toluene for approximately 10 min. The column was silylated with a 5% solution of 3-aminopropyl trimethoxy silane in toluene for approximately 4 h at room temperature, followed by a 30-min rinse with toluene. The column was dried by pumping helium through the column for ~ 30 min. Prior to use the column was flushed with the CZE buffer solution for ~ 1 h. APS CZE columns produced by this method were found to be stable for at least 2 to 4 weeks when used with CZE buffers over a pH range 2.5 to 7. The main limitation to the lifetime of these columns is the fouling of the columns with "dirty samples." Unlike bare silica, these columns cannot be "cleaned" by rinsing with 0.1 M NaOH because the Si-O-C bond is hydrolytically unstable with solutions with a pH greater than 7.5. However, these columns can be prepared in 10 to 25

m lengths, and when a column is fouled, it is simply replaced with a new length of APS silylated column. Any changes in migration times, due to changing to a new column, is of minor consequence to CZE/ESI/MS studies because the qualitative identification of an analyte is based upon the mass spectrum rather than upon migration time.

The CZE apparatus used in this work was identical to that used for CZE/coaxial continuous flow fast atom bombardment mass spectrometry (CF-FAB/MS) work by Moseley et al. [29-31].

UV Detector

On-column UV absorption (200 nm) detection of CZE separated analytes was accomplished by using a Model 500 Variable Wavelength Detector (Scientific Systems Inc., State College, PA). CZE/UV data acquisition and processing was performed by using a 12 bit A/D converter (Scientific Solutions, Solon, OH) configured on an IBM PS2/30 computer (Research Triangle Park, NC). Processing of the CZE/UV data was performed by using software written in-house at UNC.

Mass Spectrometry Instrumentation

ESI Interface. The ESI interface used in this work was designed by Whitehouse and Fenn (Analytica of Bradford, Bradford, MA), and marketed specifically for the TSQ-70 by Finnigan (San Jose, CA). This interface has three coaxial flow paths: the fused silica CZE column; a sheath flow stainless steel capillary; and a cooling gas stainless steel capillary. The tip of the CZE column protruded approximately 0.2 mm out of the sheath flow capillary. A syringe pump was used to deliver the sheath fluid at flow rates between 0.5 and 10 $\mu\text{L}/\text{min}$. No cooling gas was used in this work.

A curtain gas of ultrahigh purity nitrogen was used at a flow rate of approximately 6 L/min and a temperature of approximately 60 °C. This gas serves to desolvate the ions as well as to sweep neutral molecules from the ion source.

TSQ-70. A Finnigan TSQ-70 triple sector quadrupole mass spectrometer (Finnigan, San Jose, CA) was used in this work. The standard vacuum pumping system was found to be adequate for use with the ESI interface. To improve ion transmission and, thus, sensitivity of the analysis, the quadrupoles were operated under low resolution conditions (peak width two to three mass-to-charge (M/Z) ratio units at half height for mass spectrometry analysis and three to seven mass-to-charge units at half height for MS/MS analysis). Argon was used as the collision gas, and collision energies were 15 to 20 eV for multiply charged ions, and 20 to 25 eV for singly charged ions.

The improvements in sensitivity obtained by using low resolution MS/MS for peptide sequence analysis more than compensate for the loss in spectral resolu-

tion. An integral part of this methodology for sequencing peptides includes acquiring tandem mass spectra not only of the native peptide, but also the methyl ester and N-acetylated derivatives of the peptide. Separate fractions of the peptide sample are converted to methyl esters (by using 2 N hydrochloric acid in methanol) or are acetylated (by using 0.1% acetic anhydride in 200 mM ammonium acetate). This permits the ready identification of N-terminal and C-terminal peptide fragments as well as the determination of the number of amino and/or carboxyl functional groups present on the fragment ions. The utility of this integrated approach to sequencing peptides and proteins has been well documented [32, 33]. Moreover, it has been demonstrated in such forums as the Mass Spectrometry Workshop held at the 4th Annual Protein Society Symposium [34, 35] and in the Workshop on Peptide and Protein Sequence Analysis at the 38th ASMS Conference on Mass Spectrometry and Allied Topics [36].

Chemicals

All analytes were obtained from Sigma Chemical Co. (St. Louis, MO) and were used as delivered. The water used in the preparation of all sample and buffer solutions had a resistance of $> 15 \text{ M}\Omega$ (Milli-Q System, Millipore, Bedford, MA). Acetic acid, ammonium acetate, and ammonium hydroxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sample and buffer solutions are degassed and filtered (0.2 μm pore size) immediately prior to use.

Results and Discussion

Optimization of Sheath Fluid and Buffer Composition

To determine the optimum conditions for the detection of CZE separated peptides for both mass spectrometry and MS/MS analysis, a series of experiments were conducted using neurotensin as a model peptide. Neurotensin (MW = 1673) has pyroglutamic acid as the N-terminal amino acid, and a C-terminal free acid. Neurotensin contains three basic amino acids (two arginines and one lysine), and, thus, it can carry up to three positive charges. To remove the effects of electromigration and electroosmotic flow from these experiments, flow injection analysis (FIA) was performed. A stainless steel pressure vessel (FIA (M/Z) bomb) was used to pump the analyte/buffer solution through a fused silica capillary column at a known rate in a pulse-free manner. Helium gas was used to pressurize the FIA bomb, forcing the analyte/buffer solution through the fused silica column at a flow similar to that expected for electroosmotic flow in CZE analysis, 0.3 $\mu\text{L}/\text{min}$.

Two sets of experiments were performed, one studying the effect of the sheath fluid composition

and flow rate, and the other studying the effect of the CZE buffer composition (both pH and concentration).

The non-mass spectrometric CZE analysis of peptides is usually performed by using CZE buffers with a pH greater than the pI of the peptides. This is done to minimize adsorption of the analyte onto the walls of the CZE column. Bare fused silica in contact with aqueous solutions with a pH > 1 will have a negatively charged wall due to the deprotonation of surface silanols. Therefore, any positively charged species will be electrostatically attracted to the walls of the CZE column. Theoretical calculations by Walbroehl [37] predict that even very minor adsorption of the analyte onto the wall of the CZE column leads to major reductions in CZE separation efficiency (absorption $k' = 0.05$ reduces CZE separation efficiency by twentyfold). If the pH of the CZE buffer is greater than the pI of the peptide, the peptide will be negatively charged. This induces an electrostatic repulsion between the negatively charged walls of the bare fused silica CZE column and the negatively charged peptides, precluding absorption of the analyte [24]. For these reasons the initial experiments examined the feasibility of separating the analytes as negative ions while using an acidic sheath fluid to induce a charge reversal of the analytes to positive ions for mass spectrometry detection. This approach has proven to be very effective in CZE/coaxial CF-FAB/MS analysis [29–31].

Effect of Sheath Fluid with Basic CZE Buffers

These experiments used 0.005 M ammonium acetate CZE buffers at a pH of 8.5. Neurotensin was spiked into these buffers to give a final concentration of 60 pmol/ μ L. A FIA flow rate of 0.3 μ L/min was used. The first sheath fluid examined was comprised of 1% acetic acid in 80/20 methanol/water at a flow of 1 μ L/min. For comparison purposes a reference spectrum of 60 pmol/ μ L neurotensin in water (also at 0.3 μ L/min) was acquired using an identical sheath fluid composition and flow rate. In this spectrum the ratio of I_{3+}/I_{2+} was 3.3, and the triply charged ion exhibited 1.5×10^6 counts. Analysis of 60 pmol/ μ L neurotensin in pH 8.5 buffer reduced the abundance of the triply charged ion by approximately an order of magnitude and reversed the ratio of I_{3+}/I_{2+} to 0.54, such that the doubly charged ion was the most intense ion. In contrast to the CZE/coaxial CF-FAB/MS work [29–31] the charge reversal from negative to positive ions in the electrospray was inefficient. In an attempt to increase the efficiency of this charge reversal the concentration of the acetic acid in the sheath fluid was increased from 1% of the total sheath fluid to 10%. This reduced both the total ion current and further reduced the abundance of the triply charged ion to approximately 5×10^4 . These changes were correlated with an increase in the ionic strength of the electrosprayed fluid. A variety of acid modifiers in the

sheath fluid were evaluated in an attempt to improve ion current, including trifluoroacetic acid, phosphoric acid, and formic acid. The use of both trifluoroacetic acid and phosphoric acid resulted in a less stable ion beam than either acetic acid or formic acid. Phosphoric acid had a dramatic debilitating effect on the stability of the electrospray ion beam. When phosphoric acid was used in the sheath fluid at a 1% concentration, no neurotensin signal was observed, and the electrospray beam was very erratic. Not until the phosphoric acid concentration was reduced to 0.005% was a stable ion beam obtained. A similar but less pronounced effect on the stability of the ion beam was observed with trifluoroacetic acid. In an attempt to improve the stability of the ion beam, the use of other organic solvents in the sheath fluid were evaluated—acetonitrile, 2-propanol, ethanol, and mixtures of these organics—both with and without the addition of water to the sheath fluid. When non-aqueous sheath fluids (containing varying amounts of acetic acid) were used, the total sheath fluid flow rate had to be increased up to 6 to 10 μ L/min to prevent excessive evaporation of the sheath fluid within the stainless steel capillary. The use of non-aqueous sheath fluids did not enhance the stability of the electrospray or increase the ion current when phosphoric acid or trifluoroacetic acid sheath fluid modifiers were used.

The effects of the different acid modifiers of a sheath fluid comprised of 80/20 acetonitrile/water is summarized in Figure 1. The inorganic acids gave notably lower ion currents than the organic acids. Note also that the use of 1% formic acid resulted in a reduction in the total ion current of approximately a factor of 4 compared to the use of 0.1% formic acid, which gave the best results of this data set. However, the best results here are still a factor of 3 to 4 lower in total ion current than the analysis of neurotensin in

Figure 1. Neurotensin ion abundances as a function of ESI sheath fluid composition. Neurotensin was analyzed at a concentration of 60 pmol/ μ L (0.05 M ammonium acetate pH = 8.5) and a flow rate of 0.3 μ L/min.

water. The resulting ion current is sufficient for the acquisition of CZE/MS data, but it is insufficient for the acquisition of CZE/MS/MS data, thus precluding on-line peptide sequencing experiments.

Attempts to neutralize/acidify the basic buffer by increasing the acid concentration in the sheath fluid were found to reduce the total ion current, which has been attributed to an effect of total ion concentration on the electrospray process. This observation concurs with the work of Ikononou et al. [38] on the effect of total ion concentrations on observed analyte ion current. Ikononou studied a series of organic nitrogen bases and found that the analyte ion sensitivities decreased with increases in analyte ion concentration and with "foreign" ion concentration. The slope of the ion current versus analyte concentration was positive over the range 10^{-8} to 10^{-5} M, and then became negative at higher concentrations. Similarly, analyte ion sensitivity decreased as the concentration of the "foreign" electrolyte increased.

Effects of Acidic CZE Buffers

To increase the total ion current to a level suitable for on-line CZE/MS/MS data acquisition, a series of acidic buffers were evaluated. Again, FIA was used to remove the effects of electroosmosis and electromigration from these experiments. A sheath fluid comprised of 0.1% acetic acid in 80/20 methanol/water was used at a flow rate of 0.5 μ L/min. The results of these experiments are summarized in Figure 2.

Figure 2. Neurotensin ion abundances as a function of CZE buffer and pH.

The first acidic buffer used was 0.01 M citric acid, adjusted to pH 3.2 with ammonium hydroxide. Use of this citric acid buffer caused the abundance of the +3 ion to be greater than the +2 ion; however, the total ion current was approximately a factor of 5 lower than that obtained with neurotensin in water.

The next acidic CZE buffer to be evaluated was formic acid. Two different pH formic acid buffers were evaluated, 3.2 and 3.8. Again, ammonium hydroxide was used to adjust the pH. At pH 3.8 the +2 ion was approximately twice as intense as the +3 ion, and the total ion current was fourfold to fivefold lower than neurotensin in water. However, at pH 3.2 the relative abundances reversed, with the +3 ion becoming approximately twice as intense as the +2 ion. Moreover, the total ion current was improved by approximately threefold over the pH 3.8 buffer, and was almost as intense as observed with neurotensin in water. These changes could be attributed to two different causes: a decrease in pH and in ionic strength. A comparison of the results from the 0.01 M pH 3.2 citric acid buffer with those from the 0.01 M pH 3.2 formic acid buffer suggests that the increased ionic strength of the citric acid buffer (from the use of ammonium hydroxide to raise the pH) is responsible for the fourfold decrease in total ion current observed with the citric acid buffer.

Similar effects were observed with the last acidic CZE buffer studied in these experiments—acetic acid. The use of 0.01 M acetic acid improved the total ion current as compared to 0.05 M acetic acid, and the use of additional ammonium hydroxide to raise the pH from 3.1 to 4.2 caused an approximate fourfold loss in total ion current. The results of this study are summarized along with notable results from the other CZE buffers in Figure 2. The best results, in terms of total ion current and the current of the +3 ion, were obtained with 0.01 M acetic acid buffer at a pH of 3.4.

CZE Analysis of Peptide Mixtures

Basic CZE Buffers

To evaluate the CZE/ESI/MS interface, a standard peptide mixture was prepared and analyzed by CZE/UV. The composition of this mixture was designed to model a variety of peptide classes. This mixture (Table 1) was comprised of peptides with

Table 1. CZE/UV analysis of peptides

Peptide	MW	Sequence	Concentration	Separation efficiency
Neurotensin	1673	p-ELYENKPRRPYIL	80 fm/nL	335,000
β -Casomorphin	790	YPFGPI	126 fm/nL	165,000
Met-enkephalin	573	YGGFM	175 fm/nL	400,000
FLEEI	649	FLEEI	154 fm/nL	620,000
ACTH clip	2465	RPVKVYPNGAED SAEAFPLEF	40 fm/nL	551,000

molecular weights ranging from 574 to 2465, and included representative peptides: an acidic peptide (FLEEI), neutral peptides (met-enkephalin, and β -casmorphin), and two mixed peptides (neurotensin and ACTH clip).

Analysis of this mixture by CZE/UV using an ammonium acetate CZE buffer (pH 8.5) gave good separation of all components in less than 11 min, with separation efficiencies of from 165,000 to 620,000 plates. The observed migration order was that expected based upon the charge/size ratio at this pH. With the exception of neurotensin, all peptides possessed a net negative charge. This is deduced from the observation that neurotensin migrated from the CZE column prior to the electroosmotic flow marker.

As was expected from the FIA/ESI/MS studies of neurotensin, the ESI/MS ion currents from the peptides were very low when basic CZE buffers were used. When injection volumes identical to those used in the CZE/UV experiment were used, positively charged ions could only be observed for the neutral peptides met-enkephalin and β -casmorphin. When long injection times (large injection volumes) were used, all of the peptides could be observed, albeit with poor separation efficiencies. Further increasing the concentration of the analytes in the sample solution would lead to overloading of the CZE column due to perturbation of the electric field homogeneity [1].

Acidic CZE Buffers

From the FIA/ESI/MS studies of neurotensin it was determined that the most abundant ions were observed by using pH 3.4 0.01 M acetic acid buffer. Therefore, this standard peptide mixture was analyzed by using this CZE buffer, and the increases in ion currents that were expected on the basis of the FIA/ESI/MS analysis were observed. When approximately 1 pmol of each peptide was injected onto the CZE column, the peptide ions were sufficiently abundant to permit the acquisition of mass spectra and single ion electropherograms from each peptide. Moreover, the ion currents were sufficiently high to permit the acquisition of MS/MS spectra from each component in this peptide mixture, including both singly charged ions (Figure 3a) and multiply charged ions (Figure 3b). Daughter ions were abundant in these spectra, yielding a large amount of qualitative information regarding amino acid sequence.

The use of the acidic CZE buffer forms positively charged peptides in solution, and this is presumed to be responsible for the observed increases in ion currents. With regard to the CZE separation, the most important consequence of switching from a basic to an acidic buffer is a dramatic change in separation efficiency. At the pH used in this separation (pH 3.4), as with the pH 8.5 buffer, the wall of the fused silica CZE column is populated with deprotonated, negatively charged silanols. At a pH of 3.4 the peptides

Figure 3. CZE/ESI/MS/MS spectra of (a) met-enkephalin and (b) ACTH clip. A bare fused silica CZE column was used with 0.01 M acetic acid buffer (pH = 3.4) and a +30 kV potential.

will be positively charged in solution. The electrostatic attraction between the negatively charged walls and the positively charged peptides induces adsorption of the peptides onto the wall, significantly reducing the separation efficiency. This loss in separation efficiency not only reduces the peak capacity of the separation, but also raises the limits of mass spectrometry detection because the mass flux of the peptide ions is reduced.

Chemically Modified Fused Silica CZE Columns

The deleterious effects of the negatively charged, deprotonated silanols on the separation efficiency and

on the limits of detection of positively charged analytes can be precluded by removal of these deprotonated silanol moieties from the wall of the CZE column. There are two options for this work: (1) to operate at a pH sufficiently low to protonate the silanol groups; or (2) to shield the silanols from the CZE buffer solution by derivation of the silanols or coating the column walls.

The effect of using CZE buffers at a pH sufficiently low to protonate the surface silanols has been investigated by McCormick [39], who used 0.150 M phosphoric acid at a pH of 1.5, which was sufficiently low as to protonate most, but not all, of the silanol moieties. On the basis of our FIA/ESI/MS work, the high concentration of phosphoric acid in McCormick's results in an ionic strength too high to be useful in CZE/ESI/MS studies. Alternatively, the use of high concentrations of potassium salts [40] or zwitterionic species [41] in the CZE buffer has been found to minimize adsorption of proteins. Due to the high ionic strength of these buffer systems this approach was not evaluated with CZE/ESI/MS.

The second approach, covalent derivatization of the surface silanols, has been accomplished by several workers [25, 42-45]. If the silanol is derivatized with a reagent resulting in a neutral surface moiety, such as polyacrylamide [42, 44], the electroosmotic flow will be reduced to zero, leading to long analysis times. While this problem could be circumvented via the use of short CZE columns, this approach can be problematic when using mass spectrometry due to the geometry of the CZE/ESI/MS interface.

In 1983 Lukacs investigated a number of methods to minimize the adsorption of proteins on the silica wall of CZE columns. One of the methods briefly evaluated was the derivatization of the surface silanols with 3-aminopropyl trimethoxy silane [25]. This method was evaluated for its ability to reduce adsorption of lysozyme with a CZE buffer pH of 6.8. At this pH the aminopropyl moiety was neutral, and, while it adequately shielded the protein from the wall of the column, analysis times were long because there was minimal electroosmotic flow at this pH.

Due to the success of 3-aminopropyl silane for minimizing adsorption of lysozyme, it was decided to evaluate APS derivatized columns at the pH used for ESI/MS analysis. At a pH of 3.4 the aminopropyl group will be positively charged, actively repulsing the positively charged peptides from the wall of the silica column. Furthermore, the column will have a strong electroosmotic flow due to the charged wall but, because the wall is positively charged, the flow will be in the opposite direction from that obtained with negatively charged silica walls. This change in direction is compensated by using a negative high voltage at the injection end of the CZE column (-30 kV rather than $+30$ kV), returning the direction of electroosmotic flow back toward the detector end of the CZE column.

Evaluation of APS CZE Columns

CZE/ESI/MS experiments. Application of the APS CZE columns to the CZE/ESI/MS analysis of peptides was found to yield the desired results. A comparison of the analysis of 2 pmols of a basic peptide (neurotensin) and 4 pmols of a neutral peptide (β -casmorphin) by using 0.01 M acetic acid pH 3.4 CZE buffer with a bare fused silica CZE column (Figure 4a) and 160 fmols of each peptide with an APS CZE column (Figure 4b) shows the expected improvement in both peak shape and in mass flux. With the bare fused silica CZE column, the ion intensities for neurotensin and β -casmorphin were 2.8×10^5 and 3.7×10^5 , and when the APS CZE column was used, these values were 9.9×10^5 and 2.9×10^5 . Therefore, when the APS column was used, the ion intensity per mole of peptide injected increased by a factor of 44 for neurotensin and by a factor of 20 for β -casmorphin. To further explore the utility of this approach to the analysis of peptides by CZE/ESI/MS, several differ-

Figure 4. Selected ion electropherograms of a neutral peptide (β -casmorphin) and of a basic peptide (neurotensin) using 0.01 M acetic acid buffer (pH = 3.4): (a) 4 pmols of β -casmorphin (3.7×10^5 ion intensity) and 2 pmols of neurotensin (2.8×10^5 ion intensity); and (b) 160 fmols of each peptide, β -casmorphin (2.9×10^5 ion intensity) and neurotensin (9.9×10^5 ion intensity), with an APS modified CZE column and -30 kV. Note that when the APS CZE columns were used the ratio of the ion intensities to the amount injected increased by a factor of 44 for neurotensin and 20 for β -casmorphin. The ordinate values give the percent relative abundances of the selected ion electropherograms within the two experiments.

ent mixtures of bioactive peptides were prepared and analyzed.

Opioid peptides. A mixture of five opioid peptides (Table 2) was prepared at a concentration of 100 fmol/nL (1×10^{-4} M) and was analyzed by CZE/ESI/MS (Figure 5). With the exception of β -endorphin, which contains five basic and one acidic amino acid, all of these opioid peptides are comprised of neutral amino acids. The mass spectrometer was scanned over the mass-to-charge ratio range 400 to 1500 at a scan rate of 2 s/scan. High separation efficiencies, up to 207,000 plates, were obtained (see Table 2), along with low limits of detection. Three of these peptides—leu-enkephalin, met-enkephalin, and β -casomorphin—migrated from the column closely spaced, but a scale expansion reveals that they were baseline resolved. Approximately 2.6 nL of solution was injected onto the CZE column; thus, 260 fmols of each peptide was injected onto the column.

Angiotensin related peptides. To test the ability of the APS CZE column to analyze a set of structurally similar peptides, a mixture of angiotensin related peptides was prepared at a concentration of 0.05 ng/nL (2.8×10^{-5} to 5.5×10^{-5} M) (Table 3). All of these peptides contain basic amino acid residues and, thus, yield ions with two or more positive charges. It should be noted that in the mass spectra of all of these peptides the most intense ion corresponded to "complete" protonation of all of the available sites—the basic amino acid side chains of lysine and arginine, as well as the N-terminal amine. This indicates the ability of the pH 3.4 CZE buffer to fully protonate the peptide. The SIEs from this separation (Figure 6) exhibit CZE separation efficiencies of up to 200,000 plates. Injection of ≈ 3.9 nL of solution corresponds to injection of from 110 to 220 fmols per analyte.

Neuropeptides. A mixture of six neuropeptides was prepared at a concentration of 40 fmol/nL (4×10^{-5} M) and was analyzed by using the APS CZE column with 0.01 M pH 3.4 acetic acid buffer. Two of these peptides are composed of neutral amino acids, whereas four of these peptides contain two or more basic amino acids. The peptide mixture was injected

Figure 5. Selected ion electropherograms from the CZE/ESI/MS analysis of a mixture of opioid peptides. An APS modified CZE column was used with 0.01 M buffer (pH = 3.4) and a -30 kV potential. A 2.6 nL injection of a 100 fmol/nL (1×10^{-4} M) solution of the peptides was injected onto the column (260 fmol of each peptide). The mass spectrometer was scanned from m/z 400 to 1500 at a rate of 2 s/scan. The ordinate values give the percent relative abundances of the selected ion electropherograms. See Table 2 for additional details.

onto the CZE column at a level corresponding to the injection of 160 fmols per peptide. Good peak symmetry was observed for both singly and multiply charged species (Figure 7). The SIEs from this analysis show separation efficiencies up to 250,000 plates, with separation of all of the peptides except for bradykinin and β -endorphin. The ability of mass spectrometry to provide a separation process (by mass-to-charge ratio) whose mechanism is orthogonal to that of CZE is illustrated by examining the data in a three-dimensional plot (mass-to-charge ratio versus time versus current) (Figure 8). This plot clearly permits the separation of these two peptides by mass-to-charge ratio even though they are not separated in time.

Table 2. CZE/ESI/MS analysis of opioid peptides

Peptide	MW	Sequence	Concentration	Amount injected	Separation efficiency
Met-enkephalin	573	YGGFM	100 fm/nL	260 fm	122,000
β -Casomorphin	790	YPFPGPI	100 fm/nL	260 fm	200,000
Leu-enkephalin	555	YGGFL	100 fm/nL	260 fm	207,000
Morphiceptin	520	YFPF-NH ₂	100 fm/nL	260 fm	86,000
β -Endorphin	3461	YGGFMTSEKSQTPLVTL FKNAIIKNAYKKGE	100 fm/nL	260 fm	33,000

Table 3. CZE/ESI/MS analysis of angiotensin-related peptides

Peptide	MW	Sequence	Concentration	Amount injected	Separation efficiency
Angiotensin II	1045	DRVYIHPF	48 fm/nL	190 fm	99,000
Angiotensinogen	1756	DRVTIHPFHLLVYS	28 fm/nL	110 fm	160,000
Sar ¹ -Gly ⁸ -Angiotensin II	922	Sar-RVYIHPG	56 fm/nL	220 fm	99,000
Substance P(free acid)	1348	RPKPQQFFGLM	37 fm/nL	140 fm	200,000

Figure 6. Selected ion electropherograms from the CZE/ESI/MS analysis of a mixture of angiotensin related peptides. All conditions were identical to those used in Figure 5 except the concentration of each peptide was 0.05 ng/nL (2.8×10^{-5} to 5.5×10^{-5} M). The ordinate values give the percent relative abundances of the selected ion electropherograms. See Table 3 for additional details.

Figure 7. Selected ion electropherograms from the CZE/ESI/MS analysis of a mixture of neuropeptides. All conditions were identical to those of Figure 5 except the concentration of the peptides was 40 fmol/nL. The ordinate values give the percent relative abundances of the selected ion electropherograms. See Table 4 for additional details.

While selected ion electropherograms are the preferred method of data presentation for separation/MS analysis, they require prior knowledge of the analytes present in the sample. The detection of unknown analytes must be made from the total ion electropherogram. Continuous-flow FAB gives an extremely high chemical background from the FAB matrix, which usually dominates the total ion electropherogram. The

lack of a chemical background in ESI/MS (other than from impurities in the CZE buffer/sheath fluid) is a significant advantage in the analysis of unknown samples. The total ion electropherogram (without background subtraction) from the analysis of the neuropeptides at the 160 fmol level (Figure 9) clearly

Table 4. CZE/ESI/MS analysis of Neuropeptides

Peptide	MW	Sequence	Concentration	Amount injected	Separation efficiency
β-Casomorphin	790	YPFPGPI	40 fm/nL	160 fm	130,000
Morphiceptin	520	YPFP-NH ₂	40 fm/nL	160 fm	240,000
Neurotensin	1673	p-ELYENKPRRPYIL	40 fm/nL	160 fm	97,000
Bradykinin	1060	RPPGFSPFR	40 fm/nL	160 fm	120,000
Lys-bradykinin	1188	KRPPGFSPFR	40 fm/nL	160 fm	250,000
β-Endorphin	3461	YGGFMTSEKSTPLVTL FKNAIKNAYKKGE	40 fm/nL	160 fm	86,000

Figure 8. Isometric plot (mass-to-charge versus time versus abundance) from the separation shown in Figure 7.

shows the electrophoretic peak of each of the neuropeptides; thus, these analytes could have been readily identified without prior knowledge of the molecular weight of the analytes.

CZE/ESI/MS/MS analysis. The mixture of neuropeptides was reanalyzed to acquire MS/MS data from each species as it migrates from the CZE col-

umn. To acquire a significant amount of amino acid sequence information the neuropeptides were analyzed by using a CZE injection volume five times larger than that used in the mass spectrometry analysis of this mixture, injecting 780 fmols of each peptide. While the use of a larger injection volume degraded the separation efficiency, it permits the acquisition of tandem mass spectra over a fivefold longer time period. As with the mass spectrometry total ion electropherogram, the MS/MS total ion electropherogram (Figure 10) clearly shows the migration of the analytes, indicating the utility of this methodology for the analysis of unknown samples. MS/MS data were acquired from each peptide, including bradykinin and β -endorphin, due to the selectivity inherent in MS/MS analysis. Representative spectra of singly charged ion MS/MS spectra (β -casomorphin, Figure 11) and of multiply charged ion MS/MS spectra (bradykinin, Figure 12) are shown. Useful sequence information was obtained for all neuropeptides.

CZE/ESI/MS analysis of proteins. While the goal of this work was the acquisition of CZE/ESI/MS and CZE/ESI/MS/MS data from peptides, one brief experiment was performed to study the utility of the APS CZE columns for the ESI/MS analysis of proteins. Two proteins were analyzed by CZE/ESI/MS—myoglobin (MW = 16,951) and cytochrome *c* (MW = 12,384). The analysis of this mixture by CZE/ESI/MS under full-scan conditions (from m/z 600 to 1800 at 2 s/scan) shows only two major peaks

Figure 9. Total ion electropherogram (no background subtraction) from the separation shown in Figure 7. Note the migration of each peptide is clearly evident, indicating the utility of this methodology for the analysis of unknown samples.

Figure 10. Total ion electropherogram (parents and daughters) from the CZE/ESI/MS/MS analysis of the mixture of neuropeptides. A five fold larger injection volume was used for this analysis (780 fmol of each peptide injected onto the column) compared to the CZE/ESI/MS analysis of this peptide mixture shown in Figure 7.

Figure 11. CZE/ESI/MS/MS spectra of (a) β -casomorphin and (b) bradykinin from the separation shown in Figure 10.

in the total ion electropherogram (see Figure 12). Approximately 4 nL of a 3.3×10^{-5} M solution of the proteins was injected onto the column, corresponding to an injection of approximately 133 fmols of each protein. The CZE/ESI mass spectra are given in Figure 13. These preliminary results obtained from the CZE/ESI/MS of proteins with APS columns clearly show promise, and further work is currently underway to expand on this preliminary data.

Figure 12. Total ion electropherogram from the CZE/ESI/MS analysis of a mixture of myoglobin and cytochrome *c* (130 fmol injected onto the column). An APS modified CZE column was used with 0.01 M buffer (pH = 3.4) and a -30 kV potential. The mass spectrometer was scanned from m/z 600 to 1800 at a rate of 2 s/scan.

Conclusions

The requirements for optimum CZE/ESI/MS analysis have been studied. To increase the S/N ratio of CZE/ESI/MS and CZE/ESI/MS/MS analysis, it was necessary to use acidic CZE buffers. This not only increases the total ion current, it also serves to fully protonate the peptides, minimizing the distribution of ion current across the ensemble of possible charge states. A number of different acidic CZE buffers were evaluated, and the most useful buffer studied was 0.01 M pH 3.4 acetic acid.

The use of acidic buffers protonates the peptides, which is advantageous for mass spectrometry analysis but is problematic with CZE when bare fused silica CZE columns are used because negatively charged silanol moieties on the column wall induce adsorption of the positively charged peptides, causing zone broadening and, thus, a loss in separation efficiency. This problem was circumvented by the preparation of APS CZE columns which, when used with acidic CZE buffers, will have a positively charged wall. The electrostatic repulsion between the positively charged peptides and the positively charged CZE column wall minimizes adsorption problems and facilitates high efficiency separations.

The combination of acidic CZE buffers with APS CZE columns resulted in the acquisition of full scan CZE/ESI/MS data from mixtures of bioactive peptides with separation efficiencies up to 250,000 plates from as little as 160 fmol of a peptide. Moreover, the ability to acquire CZE/ESI/MS/MS from less than 1 pmol of

Figure 13. CZE/ESI/MS spectra of (a) myoglobin and (b) cytochrome *c* from the separation shown in Figure 12.

peptide has been demonstrated.

References

- Jorgenson, J. W.; Lukacs, K. D. *Science* **1983**, *222*, 266.
- Jorgenson, J. W.; Lukacs, K. D. *Anal. Chem.* **1981**, *53*, 1298.
- Jorgenson, J. W. *Anal. Chem.* **1986**, *58*, 743A-757A.
- Nickerson, B.; Jorgenson, J. W. *J. High Res. Chromatogr. Chromatogr. Commun.* **1988**, 533.
- Rose, D. J. Jr.; Jorgenson, J. W. *Anal. Chem.* **1988**, *60*, 1840.
- Rose, D. J.; Jorgenson, J. W. *J. Chromatogr.* **1988**, *438*, 23.
- Dole, M.; Cox, H. L. Jr.; Gieniec, J. *Advan. Chem. Ser.* **1973**, *125*, 73.
- Dole, M., et al. *J. Chem. Phys.* **1968**, *49*, 2240.
- Clegg, G. A.; Dole, M. *Biopolymers* **1971**, *10*, 821.
- Meng, C. K.; Mann, M.; Fenn, J. B. *Z. Phys. D—Atoms, Molecules and Clusters* **1988**, *10*, 361.
- Meng, C. K.; Mann, M.; Fenn, J. B. *Proceedings of the 36th ASMS Conference on Mass Spectrometry and Allied Topics*; San Francisco, CA, 1988.
- Wong, S. F.; Meng, C. K.; Fenn, J. B. *J. Phys. Chem.* **1988**, *92*, 546.
- Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.
- Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. D. *Anal. Chem.* **1990**, *62*, 882.
- Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985**, *57*, 675.
- Smith, R. D.; Olivares, J. A.; Nguyen, N. T.; Udseth, H. R. *Anal. Chem.* **1988**, *60*, 436.
- Olivares, J. A.; Nguyen, N. T.; Yonker, C. R.; Smith, R. D. *Anal. Chem.* **1987**, *59*, 1230.
- Smith, R. D.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1988**, *60*, 1948.
- Rose, D. J. Jr.; Jorgenson, J. W. *J. Chromatogr.* **1988**, *447*, 117.
- Lee, E. D.; Mueck, W.; Henion, J. D.; Covey, T. R. *J. Chromatogr.* **1988**, *458*, 313.
- Lee, E. D.; Mueck, W.; Henion, J. D.; Covey, T. R. *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 253.
- Smith, R. D.; Barinaga, C. J.; Udseth, H. D. *J. Chem. Phys.* **1989**, *93*, 5019.
- Smith, R. D.; Loo, J. A.; Barinaga, C. J.; Edmonds, C. G.; Udseth, H. R. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 53.
- Lauer, H. H.; McManigill, D. *Anal. Chem.* **1986**, *58*, 166.
- Lukacs, K. D. *Diss. Abstr. Int.* **1983**, *44*, 3766.
- Iler, R. K. *The Chemistry of Silica*; John Wiley: New York, 1979.
- Moseley, M. A.; Pellizzari, E. D. *J. High Res. Chromatogr. and Chromatogr. Commun.* **1982**, *5*, 488.
- Moseley, M. A.; Pellizzari, E. D. *J. High Res. Chromatogr. and Chromatogr. Commun.* **1982**, *5*, 472.
- Moseley, M. A.; Deterding, L. J.; Tomer, K. B.; Jorgenson, J. W. *Rapid Commun. Spectrom.* **1989**, *3*, 87.
- Mosley, M. A.; Deterding, L. J.; Tomer, K. B.; Jorgenson, J. W. *J. Chromatogr.* **1989**, *480*, 197.
- Mosley, M. A.; Deterding, L. J.; Tomer, K. B.; Jorgenson, J. W. *Anal. Chem.* **1991**, *63*, 109.
- Hunt, D. F., et al. *Proc. Natl. Acad. Sci.* **1987**, *84*, 620.
- Hunt, D. F.; Yates, J. R. I.; Shabanowitz, J.; Winston, S.; Hauer, C. R. *Proc. Natl. Acad. Sci.* **1986**, *83*, 6233.
- Hunt, D. F., et al. *Techniques in Protein Chemistry II*; Villafraña, J. J.; Ed.; Academic: San Diego, 1990; p 441.
- Hunt, D. F., et al. *Techniques in Protein Chemistry II*; Villafraña, J. J.; Ed.; Academic: San Diego, 1990; p 455.
- Martino, P. A.; Alexander, J. E.; McCormack, A. L.; Shabanowitz, J.; Hunt, D. F. *Proceedings of the 38th ASMS Conference on Mass Spectrometry and Allied Topics*; June, 1990.
- Walbroehl, Y. PhD Dissertation, University of North Carolina, 1986.
- Ikononou, M. G.; Blades, A. T.; Kebarle, P. *Anal. Chem.* **1990**, *62*, 957.
- McCormick, R. M. *Anal. Chem.* **1988**, *60*, 2322.
- Green, J. S.; Jorgenson, J. W. *J. Chromatogr.* **1989**, *478*, 63.
- Bushey, M. M.; Jorgenson, J. W. *J. Chromatogr.* **1989**, *480*, 301.
- Hjerten, S. *J. Chromatogr.* **1985**, *347*, 191.
- McCormick, R. M. *Anal. Chem.* **1988**, *60*, 2322.
- Cobb, K. A.; Dolnik, V.; Novotny, M. *Anal. Chem.* **1990**, *62*, 2478.
- Towns, J. K.; Regnier, F. E. *J. Chromatogr.* **1990**, *516*, 69.